Thyroid-Stimulating Hormone and Thyroid-Stimulating Hormone Receptor Structure-Function Relationships

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Szkudlinski, Mariusz W., Valerie Fremont, Catherine Ronin, and Bruce D. Weintraub. Thyroid-Stimulating Hormone and Thyroid-Stimulating Hormone Receptor Structure-Function Relationships. *Physiol Rev* 82: 473–502, 2002; 10.1152/physrev.00031.2001.—This review focuses on recent advances in the structure-function relationships of thyroid-stimulating hormone (TSH) and its receptor. TSH is a member of the glycoprotein hormone family constituting a subset of the cystine-knot growth factor superfamily. TSH is produced by the pituitary thyrotrophs and released to the circulation in a pulsatile manner. It stimulates thyroid functions using specific membrane TSH receptor (TSHR) that belongs to the superfamily of G protein-coupled receptors (GPCRs). New insights into the structure-function relationships of TSH permitted better understanding of the role of specific protein and carbohydrate domains in the synthesis, bioactivity, and clearance of this hormone. Recent progress in studies on TSHR as well as studies on the other GPCRs provided new clues regarding the molecular mechanisms of receptor activation. Such advances are a result of extensive site-directed mutagenesis, peptide and antibody approaches, detailed sequence analyses, and molecular modeling as well as studies on naturally occurring gain- and loss-of-function mutations. This review integrates expanding information on TSH and TSHR structure-function relationships and summarizes current concepts on ligand-dependent and -independent TSHR activation. Special emphasis has been

placed on TSH domains involved in receptor recognition, constitutive activity of TSHR, new insights into the evolution of TSH bioactivity, and the development of high-affinity TSH analogs. Such structural, physiological, pathophysiological, evolutionary, and therapeutic implications of TSH-TSHR structure-function studies are frequently discussed in relation to concomitant progress made in studies on gonadotropins and their receptors.

I. INTRODUCTION

Thyroid-stimulating hormone (TSH; thyrotropin) and TSH receptor (TSHR) are key proteins in the control of thyroid function. TSH synthesis in the anterior pituitary is stimulated by thyrotropin-releasing hormone (TRH) and inhibited by thyroid hormone in a classical endocrine negative-feedback loop. TSH controls thyroid function upon its interaction with the G protein-coupled TSHR (215). TSH binding to its receptor on thyroid cells leads to the stimulation of second messenger pathways involving predominantly cAMP and, in high concentrations, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), ultimately resulting in the modulation of thyroidal gene expression. Physiological roles of TSH include stimulation of differentiated thyroid functions, such as iodine uptake and organification, production and release of iodothyronines from the gland, and promotion of thyroid growth. TSH also acts as a factor protecting thyroid cells from apoptosis and plays a critical role in ontogeny. In a mouse model with targeted disruption of the common α-subunit gene and thus devoid circulating glycoprotein hormones, thyroid development was arrested in late gestation (93).

II. HISTORICAL BACKGROUND OF THYROID-STIMULATING HORMONE AND THYROID-STIMULATING HORMONE RECEPTOR RESEARCH

The history of TSH began with the discovery of thyroid-stimulating activity in the pituitary gland. In 1926 Eduard Uhlenhuth from the University of Maryland Medical School was the first to demonstrate that the anterior lobe of the pituitary gland secreted a thyroid stimulator (209). Using several species of salamanders (amphibians) he showed that injections of bovine pituitary extracts caused a clear histological stimulation of the thyroid gland. In 1929, Leo Loeb and Max Aaron working independently confirmed Uhlenhuth's results using guinea pigs (mammals). These initial findings were followed in the 1960s by the purification and in the early 1970s by the determination of the primary structure of the TSH subunits (114). In the 1980s, the cloning of the human α-subunit (52) and TSH β-subunit genes (72, 77, 223) were important milestones in studying TSH expression, regulation, and action. From the basic science standpoint, another major breakthrough occurred in 1994 with the elucidation of the crystal structure of the closely related human chorionic gonadotropin (hCG) (103, 226), which

indicated that the glycoprotein hormones belong to the superfamily of cystine-knot growth factors (CKGF). It also enabled the generation of homology models of human TSH (hTSH) that combined with homology comparisons and identification of modification permissive residues in the peripheral loops resulted in 1994 in the generation of the first hTSH superactive analogs (191).

Thirty-five years ago it was found that TSH exerts its biological effects by binding to a protein on the thyroid cell plasma membrane (152). Subsequent milestones included studies showing that *1*) TSHR is a major thyroid autoantigen, *2*) TSHR can be stimulated or inhibited by specific antibodies, and *3*) TSHR is composed of two subunits as a result of its proteolytic cleavage (for reviews see Refs. 135, 159). Following the molecular cloning and sequencing of luteinizing hormone (LH) receptor cDNA (111, 122), several groups reported cloning and functional expression of TSHR cDNA (2, 55, 85, 108, 109, 127, 134, 149). These advances initiated a period of rapid progress in studies on TSHR.

III. THYROID-STIMULATING HORMONE CHEMISTRY AND MOLECULAR BIOLOGY

TSH is a 28- to 30-kDa glycoprotein synthesized and secreted from thyrotrophs (basophile cells) of the anterior pituitary gland. It is a member of the glycoprotein hormone family that includes follicle-stimulating hormone (FSH), LH, and hCG. Glycoprotein hormones are among the largest and most complicated endocrine ligands known to date. They are heterodimeric cystine-knot glycoproteins consisting of a common α-subunit and a unique β-subunit, which confers biological specificity to each hormone (103, 155).

A. TSH Subunit Genes

The common human α-subunit and TSH β-subunit are encoded by genes located on chromosomes 6 and 1, respectively (42). The organization of the human α-subunit and TSH β-subunit genes is shown in Figure 1. The α-subunit gene contains four exons and three introns, whereas the TSH β-subunit gene contains three exons and two introns. The α-subunit gene is almost two times larger (9.4 kb) than the TSH β-subunit gene (4.9 kb). The first exon is short in both cases, untranslated, and separated from the coding region by a large first intron. Each gene contains a single transcription start site with an upstream
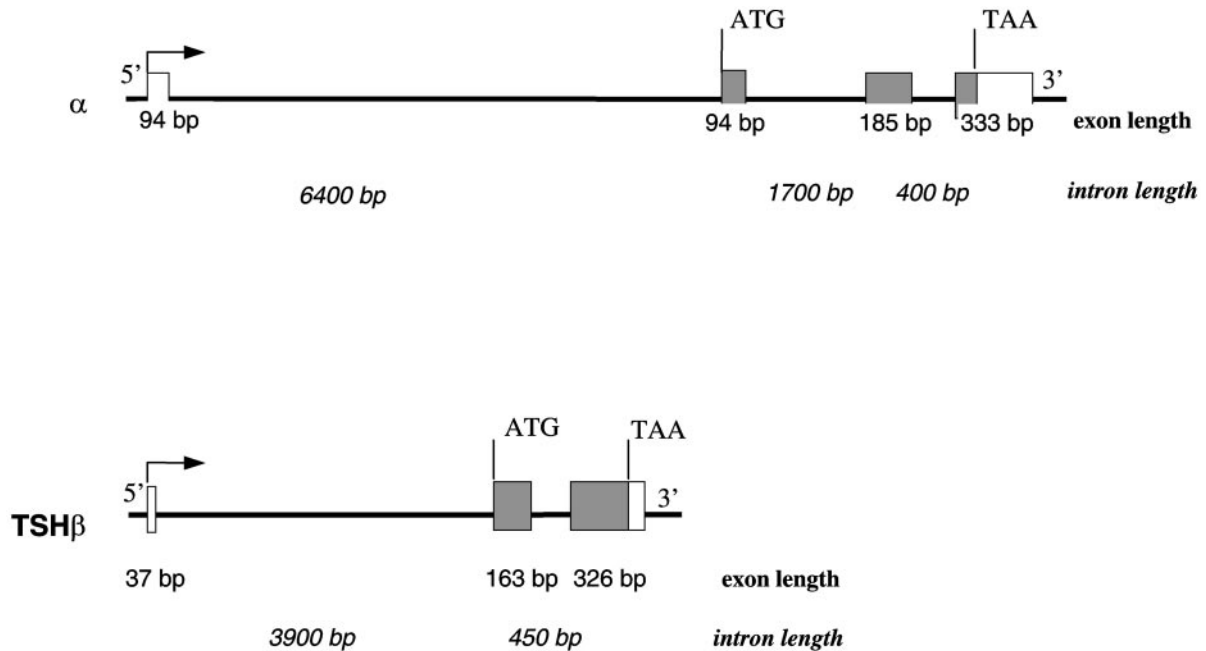
FIG. 1. Structure of the common α- and thyroid-stimulating hormone (TSH) β-subunit genes. Exons are denoted by the boxes, and introns or flanking DNA sequences are denoted by lines. The length of exons and introns are shown in base pairs. Coding regions of exons are shaded; noncoding regions are white. Transcription start site is shown by a bent arrow. This diagram serves to illustrate the general structure of each gene and is drawn to scale.

TATA box that binds RNA polymerase II. In contrast to more general expression of α-subunit gene, TSH β-subunit gene expression in the anterior pituitary is restricted to thyrotroph cells, which constitute ~5% of all adenohypophysial cells. The concomitant progress made in understanding the regulation of the α- and TSH β-subunit genes is not discussed here, because this topic has been covered in detail in many papers and several excellent review articles and chapters (25, 177, 221, 225).

B. TSH Structure

1. Protein structure

hTSH consists of two noncovalently linked subunits, α-subunit (92 amino acids; common for other human glycoprotein hormones) and TSH β-subunit. The primary structures (see Fig. 2) of TSH subunits are species specific. hTSH, for example, differs from bovine TSH by 28 amino acids in the α-subunit and by 12 amino acids in TSH β-subunit. The coding sequence of the TSH β-subunit gene predicts a 118-amino acid protein (223). However, β-subunit of TSH isolated from cadaver pituitary is composed of 112 amino acids (155), most likely due to proteolytic cleavage during purification. A 113- to 118-amino acid deletion does not affect bioactivity of recombinant hTSH, indicating that the COOH-terminal amino acid residues are not important in hormone function (198).

TSH is a member of the glycoprotein hormone family (155), structurally classified as part of the CKGF superfamily of structurally related proteins with important biological activities (103, 226). The crystal structure of homologous hCG has revealed that each subunit contains a central cystine-knot and three loops, two β-hairpin loops (L1 and L3) on one side of a cystine-knot, and a long loop (L2) on the other side (see Fig. 3). The long loop in the α-subunit (αL2) contains a two-turn α-helix. The cystine-knot is made up of three central disulfide bridges, where one threads through a ring formed by two other disulfide bridges and backbone atoms. This structure has previously been found in several growth factors including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), and nerve growth factor (NGF) (185) (Table 1). In contrast to the other CKGFs that exist as homo- or heterodimers with interchain disulfide bridges, glycoprotein hormones are noncovalently linked heterodimers stabilized by a unique segment of the β-subunit termed "seatbelt," because it wraps around the α-subunit long loop (αL2). This additional stabilization by the seat-belt results in doubling of the subunit interface compared with the other CKGFs. This may be necessary because of the extensive glycosylation of glycoprotein hormones, which constitutes up to one-third of their molecular weight (Table 2). The common α-subunit and 38% sequence identity between the hCGβ- and hTSH β-subunit permitted homol-

A

```
1   A  P  D  V  Q  D  C  P  E  C  T  L  Q  E  N  P  F  F  S  Q  P  G  A  P  I  L  Q  C  M  G        30
```

```
                                                                    52
31  C  C  F  S  R  A  Y  P  T  P  L  R  S  K  K  T  M  L  V  Q  K  N  V  T  S  E  S  T  C  C        60
```

```
                                                     78
61  V  A  K  S  Y  N  R  V  T  V  M  G  G  F  K  V  E  N  H  T  A  C  H  C  S  T  C  Y  Y  H  K  S     92
```

B

```
                                                  23
1   F  C  I  P  T  E  Y  T  M  H  I  E  R  R  E  C  A  Y  C  L  T  I  N  T  T  I  C  A  G  Y        30
```

```
31  C  M  T  R  D  I  N  G  K  L  F  L  P  K  Y  A  L  S  Q  D  V  C  T  Y  R  D  F  I  Y  R        60
```

```
61  T  V  E  I  P  G  C  P  L  H  V  A  P  Y  F  S  Y  P  V  A  L  S  C  K  C  G  K  C  N  T        90
```

```
91  D  Y  S  D  C  I  H  E  A  I  K  T  N  Y  C  T  K  P  Q  K  S  Y  L  V  G  F  S  V        118
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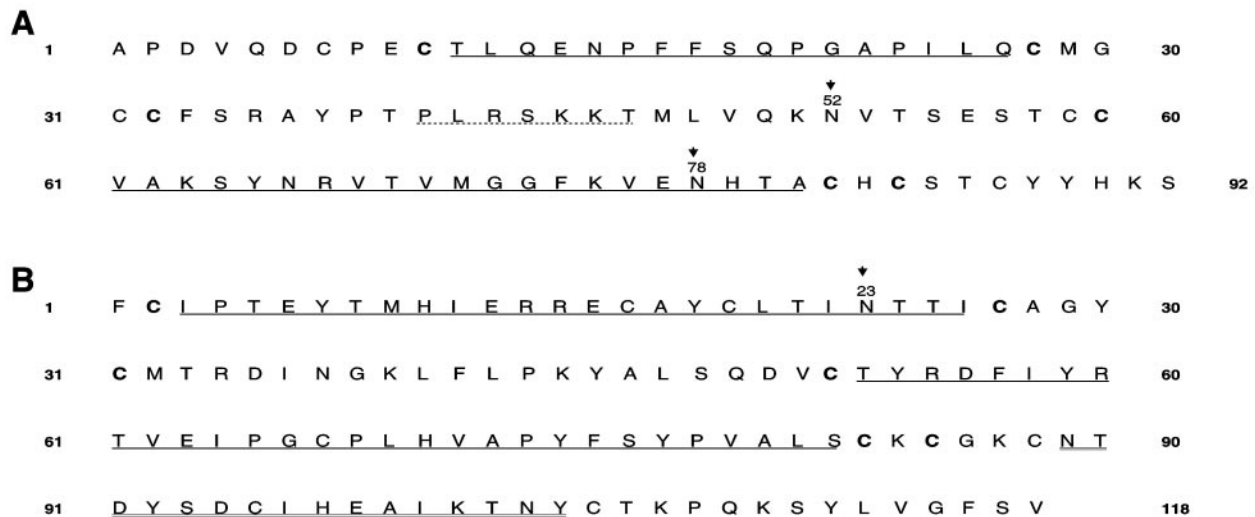
FIG. 2. The primary structure of the human α-subunit (*A*) and the human TSH (hTSH) β-subunit (*B*). Functionally important residues are numbered and highlighted in boldface in the wild-type sequence. Specific mutations are shown either above the corresponding wild-type residue (if the mutation resulted in an increase of hTSH activity) or below (if mutation caused a decrease of activity). Mutations $\alpha N^{78}Q$ and $\beta N^{23}Q$, which disrupt a glycosylation recognition sequence (highlighted by the outlined font) decrease in vivo, but not in vitro activity (see text), whereas disruption of the glycosylation recognition sequence at αN^{52} ($\alpha N^{52}Q$, $\alpha N^{52}D$) increases in vitro, but slightly decreases in vivo activity (67). Multiple residue mutations are underlined. Also depicted is the location of selected structural features. The β-hairpin loops correspond to the continuous lines above the primary structure flanked by arrows. The bold part of these lines indicates β-sheet, and the thin part of the line indicates the actual loops. α-Helix (between $\alpha 40–46$) is marked with an interrupted line below the sequence (—). The seat-belt between $\beta C88$ and $\beta C105$ is marked by a double line (=). Chimeric substitutions in this not only decreased hTSH activity, but also changed receptor specificity (see text). See also Figure 3 for structural features.

ogy modeling of hTSH. Such homology models confirmed expected similarities in the conformation of glycoprotein hormones (191). Accordingly, assignment of disulfide bridges in bovine TSH β-subunit using a double alkylation strategy revealed bonding analogous to hCG (48). Thus, in hTSH β-subunit, three disulfide bridges (Cys2-Cys52, Cys27-Cys83, and Cys31-Cys85) form cystine-knot motif that determines the core structure, two disulfide bridges (Cys19-Cys105, Cys88-Cys95) are involved in seat-belt formation, and one (Cys17-Cys67) links two β-hairpin loops.

2. Carbohydrate chains

TSH, similar to other glycoprotein hormones, is a glycosylated protein. The carbohydrate chains constitute 15–25% of its weight and include three asparagine (Asn; *N*)-linked carbohydrate chains. The human α-subunit has two carbohydrate chains linked to Asn-52 and Asn-78, respectively, and the human TSH β-subunit has one carbohydrate chain attached at the Asn-23. Such *N*-linked oligosaccharides are complex-type structures displaying notable hormone- and species-dependent differences in their terminal residues. In TSH like in most glycoproteins, the inner core fucosylation and variable glycan branching are two common forms of *N*-glycan variability. Figure 4 shows typical biantennary structures of pituitary bovine TSH (bTSH) terminated almost exclusively with SO_4-4Gal-

NAcβ1–4GlcNAcβ1–2R, pituitary human TSH (phTSH) oligosaccharides terminated with SO_4-4GalNAcβ1–4Glc-NAcβ1–2R, but also with NeuAcα2–3/6Galβ1–4Glc-NAcβ1–2R and recombinant human TSH (rhTSH) expressed in Chinese hamster ovary (CHO) cells containing only NeuAcα2–3Galβ1–4GlcNAcβ1–2R terminal sequences (188). Pituitary TSH and LH are unique in that they contain a terminal sulfated GalNAc, due to the expression of both GalNAc-transferase and sulfotransferase in the pituitary thyrotrophs and gonadotrophs.

Several studies have assigned to each carbohydrate chain a different role in the uncombined and combined subunits. Differently deglycosylated variants of the common α-subunit were shown to differ in thermal stability. Glycosylation at Asn-78 appeared to be required for the stability of the protein with the inner core interacting with hydrophobic amino acids and reducing the mobility of the glycan not only at the attachment site but also around the $\alpha 1,6$ arm (213). This glycan has been proposed to stabilize the hydrophobic packing of the β-sheets within the subunit and as a consequence to affect the interaction of the modified L1 and L3 loops with the extracellular domain of the TSHR (107, 191). Because both modified loops contribute to TSH bioactivity, it is conceivable that such effect may influence receptor binding and activation. In this regard, it should be noted that Asn-78 glycan is sul-
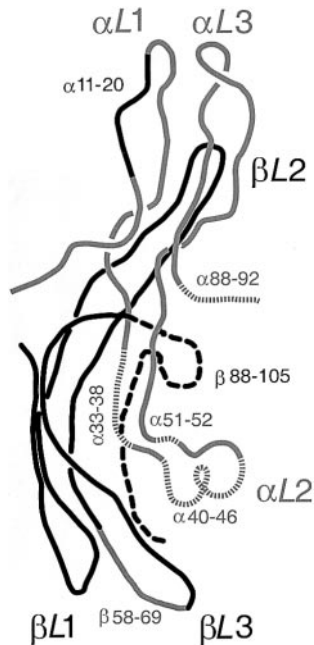
FIG. 3. The schematic drawing of hTSH showing domains important for bioactivity. For clarity, the carbohydrate chains are not shown. The α-subunit backbone is shown as a gray line, and the β-subunit chain is shown as a black line. The functionally critical domains are marked directly within the line drawings. The peripheral β-hairpin loops are marked as follows: $\alpha L1$, $\alpha L3$ in the α-subunit; $\beta L1$, $\beta L3$ in the β-subunit. Two long loops are $\alpha L2$ with α-helical structure and $\beta L2$, a loop analogous to the "Keutmann loop" in the human chorionic gonadotropin β-subunit.

fated in TSH and contains a unique NeuAc $\alpha2,6$ linkage in LH. In contrast to Asn-78, the glycan at Asn-52 in the α-subunit L2 loop was found to be highly mobile in the free subunit, but its mobility in the heterodimer is restricted by the seat-belt (80). It is known that the region $\alpha33–57$ is largely involved in signal transduction and significantly altered by subunit dissociation. According to Erbel et al. (47), comparison of X-ray structures of intact deglycosylated hCG and NMR structure of α-subunit in solution revealed that the dissociation of the hormone dimer induces large structural changes in the α-subunit, resulting in increased mobility of the glycan at Asn-52. Recently also, the structure of intact and deglycosylated hCG in complex with two Fv fragments of high-affinity anti-α and anti-β monoclonal antibodies has been solved. Fv fragment of an antibody is the smallest unit that contains complete antigen-binding site. The structure of hCG in such complexes is very similar to that of the partially deglycosylated hormone, suggesting that neither the Fv antibodies nor the whole oligosaccharides have substantial influence on hormone structure (201). However, these data do not entirely agree with NMR analysis in solution; overall, the structural data indicate that the heterodimer backbone is highly flexible to undergo local conformational changes to acquire various active (carbohydrate-

dependent) and inactive (carbohydrate-independent) states.

C. TSH Heterogeneity

hTSH, purified from the cadaver pituitary, has been shown to be heterogeneous at the NH_2 terminus of each subunit due to variable terminal truncation of both subunit polypeptide chains occurring physiologically or during purification. Presence of shortened isoforms of circulating TSH may affect interaction with antibodies binding at the COOH terminus and influence immunoassays using them. In addition, variable amidation of glutamic acid and aspartic acid residues, known to occur during prolonged storage of purified protein preparations, may introduce additional artifacts that can cause discordance in assaying TSH.

Differences in oligosaccharide structure generate a mixture of circulating isoforms (glycoforms), which represent the majority of physiological heterogeneity of hTSH. Heterogeneity of carbohydrate chains is a property shared by all glycoproteins, which appeared to be remarkably specific among glycoprotein hormones. It is based on alternate inner core fucosylation, variable oligosaccharide branching, and specific terminal GalNAc sulfation in TSH and LH. As a result, each hormone of the family has been shown to exist as a distinct set of glycoforms differing in oligosaccharide structure and bioactivity. Alterations of the bioactivity to immunoreactivity (B/I) ratio of glycoforms have been extensively studied in several pathophysiological conditions, indicating that glycosylation-dependent polymorphism of glycoprotein hormones has important physiological implications.

The detection of glycoprotein hormone isoforms is based on their respective isoelectric points using isoelectric focusing or chromatofocusing. Specific isoforms are separated on the basis of their negative charge content that for TSH is determined by a presence of both sulfated and sialic acid residues. Glycoforms of pituitary TSH ranged over pH $6.8–8.3$, indicating that modification of the underlying peptide backbone should occur upon changes in glycosylation (176). Since neuraminidase treatment did not significantly affect alkaline forms, the shift observed for more acidic forms was explained by a presence or absence of terminal sialic acid residues. Human pituitary standard TSH (2ndIRP 80558) has been recently separated according to its glycan core structure. Various glycoforms selectively activating different signal transduction pathways were found (170). Desialylated (sulfated) TSH forms showed an increased B/I ratio for cAMP production while core-fucosylated glycoforms were more potent in the IP_3 pathway activation. These data are in agreement with previous observations showing that alkaline TSH glycoforms are more active in vitro than acidic forms (124).

TABLE 1. *Cystine-knot growth factors and their receptors*

	Bioactive Form		Specific Receptors	
Glycoprotein hormones*		GPCR*		
TSH	α-TSHβ heterodimer		TSHR	
CG	α-CGβ heterodimer		LH-R	Containing LRR
LH	α-LHβ heterodimer		LH-R	
FSH	α-FSHβ heterodimer		FSH-R	
α-Subunit	(?)		(?)	
CG β-Subunit	(?)		(?)	
PDGF family		Trk		
PDGF-AA	Homodimer		PDGFR-α	
PDGF-BB	Homodimer		PDGFR-β	
PDGF-AB	Heterodimer		PDGFR-α	
VEGF-A	Homodimer		FLT-1, KDR	
PIGF	Homodimer		FLT-1	
VEGF-B	Homodimer		FLT-1	
VEGF-C	Homodimer		FLT-4, KDR	
VEGF-D	Homodimer		KDR, FLT-4	
VEGF-E	Homodimer		KDR	
Neurotrophin family		Trk		
NGF	Homodimer		A	
BDNF	Homodimer		B	Containing LRR
NT-3	Homodimer		C	
NT-4	Homodimer		B	
TGF-β family		Ser/Thr rk		
TGF-β 1–5	Homodimers		I, II	
Inhibin A*	α-βA heterodimer		I, II	
Inhibin B*	α-βB heterodimer		I, II	
Activin A*	βA-βA homodimer		I, II	Type I (ActR-I, ActR-IB)
Activin B*	βB-βB homodimer		I, II	Type II (ActR-II, ActR-IIB)
Activin AB*	βA-βB heterodimer		I, II	

Other proteins in this family
  GDF-1, GDF-5, GDF-6/BMP-13, GDF-7/BMP-12, GDF-8, GDF-9, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6/Vgrl, BMP-7/OP-1, BMP-8/OP-2, GDNF, Neurturin, PTTH, MIS, NDP,
Toxins [>30; conotoxins, atracotoxins, serine protease inhibitors (CMTI-I)]

GPCR, G protein-coupled receptor(s); Trk, receptor tyrosine kinase(s); Ser/Thr rk, serine/threonine receptor kinase; LRR, leucine-rich repeats; PDGF, platelet-derived growth factor; PDGF-B/*v-sis*, PDGF-B-like factor; VEGF, vascular endothelial growth factor; NGF, nerve growth factor; BDNF, brain-derived growth factor; NT, neurotrophin; TGF, transforming growth factor; * glycoprotein(s). [Adapted in part from Sun and Davies (185).]

Various preparations of recombinant human TSH (rhTSH) have been produced in CHO cells under various cell culture conditions (193) and were found to differ essentially in sialic acid content, providing enough mate-rial to correlate polymorphism with biological activity. rhTSH produced in CHO cells is more highly branched than the pituitary native hormone and terminated in sialic acid. As shown in Table 3, several rhTSH glycoforms

TABLE 2. *Structural features of hTSH compared with other CKGFs*

Dimer	hTSH	hCG	PDGF-BB	VEGF	TGF-β2	NGF
Molecular weight	28,000	38,000	35,000	45,000	25,000	35,000
Composition	Heterodimer	Heterodimer	Homodimer	Homodimer	Homodimer	Homodimer
Number of α-helices	2*	2*	0	0	6	0
Mode of dimerization	Noncovalent seat-belt	Noncovalent seat-belt	2 S-S bridges	2 S-S bridges	1 S-S bridge	Noncovalent
Subunit orientation	Head to tail	Head to tail	Head to tail	Head to tail	Head to tail	Head to head
Interface, Å^2	~4,000	~4,000	~2,200	~2,000	~2,600	~2,300
Identical residues, %†	100	68	19	19	18	10
Oligosaccharides						
N-linked	3	4	0	2	0	0
O-linked	0	4	0	0	0	0
Specific receptor	TSHR	LHR	PDGFR	flt-1, KDR	TGFβR	TrkA
Receptor family	GPCR	GPCR	Trk	Trk	Ser/Thr rk	Trk

S-S, interchain disulfide; flt, fms-like tyrosine kinase; KDR, kinase insert domain-containing receptor; GPCR, G protein-coupled receptor; Trk, tyrosine kinase receptor; Ser/Thr rk, serine/threonine receptor kinase; hCG, human chorionic gonadotropin. Other definitions are as in Table 1. * α-Helical structures in the α-subunit (residues 40–46 and 16–19).   † Compared with hTSH (100%).
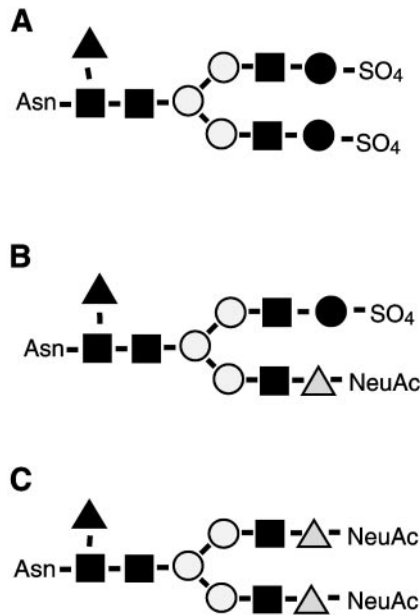
FIG. 4. *N*-linked oligosaccharides of TSH. The sulfated biantennary structure (*A*) represents that of bovine TSH and bovine luteinizing hormone (LH). The sulfated and sialylated oligosaccharide (*B*) is more typical of pituitary-derived hTSH as well as human LH. The sialylated nonsulfated structure (*C*) represents that of recombinant hTSH expressed in Chinese hamster ovary cells, pituitary human follicle-stimulating hormone, as well as placental human chorionic gonadotropin. Carbohydrate residues are marked as follows: mannose (○), *N*-acetylglucosamine (■), *N*-acetylgalactosamine (●), fucose (▲), galactose (△), and sialic acid (NeuAc).

could be detected ranging from pI 6.2 to pI 8.8 depending on the amount of sialic acid in the preparation, but none of them proved to overlap with those of pitTSH. The fact that asialo-rhTSH is homogeneous at pI 8.8 confirms that sialic acid residues play a key role in determining rhTSH polymorphism.

Gonadotropin microheterogeneity has been also extensively studied. Pituitary LH was solved as 24 highly purified isoforms within the pI range of 7.03 to 8.98 that exhibited a wide variability in their respective capacity to stimulate rat testicular steroidogenesis (222). FSH was separated as nine isoforms within pI 4.1–7.1 (232), showing variable receptor binding activity dependent on sialic acid content. In agreement with previous studies on rhTSH isoforms (193), less acidic variants of urinary FSH exhibited higher bioactivity as tested using androgen aromatization in heterologous bioassays. In contrast, several previous studies indicated that *N*-linked carbohydrates are not necessary for binding to the receptor but are needed to stabilize the active conformation of glycoprotein hormone-receptor complex resulting in signal transduction (80, 169). Altogether, these findings delineate a different role of sialic acid in glycoprotein hormones with an enhancement of in vitro activity for hCG and a lowering for hTSH, hLH, and hFSH. Glycoforms are predicted to stabilize distinct receptor conformations resulting in

different degrees of activation/inhibition of signal transduction pathway(s). Thus it appears that terminal glycosylation of glycoprotein hormones provides additional structural versatility to modulate signal transduction without modifying ligand recognition of the underlying polypeptide backbone and thus ligand specificity.

IV. THYROID-STIMULATING HORMONE STRUCTURE-FUNCTION STUDIES

A. Methodology

Structure-function studies on glycoprotein hormones can be categorized into studies on the carbohydrate moiety as well as on the peptide portion, but these two approaches can be combined and studied simultaneously. Structure-function studies involve alteration of coding sequences using molecular biology techniques, various chemical and biochemical modifications, as well as utilization of antibodies or synthetic peptides (70). In general, each of these methods has its advantages that are balanced by inherent limitations. Initially, physicochemical and enzymatic studies have identified amino acids as well as carbohydrate portions on both subunits which contribute to dimerization of two subunits, receptor binding, and signal transduction. These approaches, which are summarized in excellent reviews (5, 14, 39, 140, 171, 187), have been instrumental in gaining an initial understanding of glycoprotein hormone structure-function relationships and continue to provide valuable information to the present time. Other valuable strategies rely primarily on epitope mapping or the use of synthetic peptides (39, 88, 94, 129).

The advent of recombinant DNA technology provided new and unique opportunities to recognize functional domains of glycoprotein hormones. In particular, site-directed mutagenesis has recently gained a predominant role in such analyses. The now classical method of alanine scanning relies on the fact that alanine is generally considered to be the least disruptive mutation that can be made in the absence of any specific knowledge about protein interactions. The high helical propensity of alanine makes it especially favorable for substitution at he-

TABLE 3. *Isoelectric points (pls) of different hTSH preparations based on isoelectric focusing studies*

Pituitary hTSH	8.3, 8.1, 7.8, 7.6, 7.1, 6.8
RhTSH-A (1 NeuAc/glycan)	8.5, 8.4, 8.2, 8.0, 7.7, 7.3, 7.0, 6.7, 6.5, 6.2
RhTSH-B (2.1 NeuAc/glycan)	8.5, 8.4, 8.2, 8.0, 7.7, 7.3, 7.0
RecTSH-H (produced in hollow fiber bioreactor: 0.4 NeuAc/glycan)	8.8, 8.5, 8.4, 8.2
Asialo-rhTSH	8.8

lical residues. This technique was recently expanded to a proline/alanine scanning approach, taking additional advantage of the tendency of proline to introduce bending into the polypeptide chain (192). Specifically, α-helical structures are found to be kinked and destabilized following the introduction of proline residues (231), in contrast to alanine substitutions, which tend to preserve the α-helical conformation. Therefore, in addition to conventional alanine scanning, selective introduction of proline constitutes a test for conformational stringency in different areas. This approach may thus help to quickly differentiate the effect of peptide backbone perturbations from the role of specific amino acid side chains in protein function. In addition, such combined techniques can lead to the recognition of "modification permissive domains," which allow introduction of nonconservative changes into hormones, thus enabling modulation of function without compromising protein synthesis, folding, and function (191). Further development of such strategies including multiple residue replacement should be helpful to elucidate cooperative effects of individual residues, and this can be extended to the simultaneous mutagenesis of multiple, topically unrelated hormone regions. With such an approach, it should ultimately be possible to individually modulate and dissociate defined biological properties of complex molecules such as hTSH. In fact, this strategy led to the finding that a partial or complete loss of hTSH activity caused by modifications in one domain may in certain instances be completely compensated by alterations in an unrelated domain (192). Such studies predict that the TSHR is capable of tolerating ligands with significant structural modifications, by means of an "analog-induced fit." It may therefore even be possible to create alternative contact domains of analog and receptor resulting in signal transduction. Such plasticity of ligand-receptor interactions is supported by the observation that the TSHR can be constitutively activated by multiple mutations in various receptor regions (44). Moreover, identification of cooperative, noncooperative, and mutually exclusive hormone domains can provide important leads for further development of therapeutically useful hormone analogs.

Restoration of the activity of a mutant hormone analog by appropriate modifications of the receptor can also demonstrate that a mutation causes a site-specific decrease of hormone activity. Such parallel mutagenesis of ligand and receptor is a promising approach that received only scant attention so far (86). This combined strategy should allow identification of cooperative interactions of specific domains of ligand and receptor and therefore be highly informative in understanding mechanistic aspects of glycoprotein hormone signal transduction. Another newly developed powerful technique, linker scanning mutagenesis, is a method for introducing codons (peptides) into cloned DNA to select modification permissive and nonpermissive sites in the proteins they encode (6, 73).

It should be pointed out that, as with other approaches, these recombinant techniques are not free of limitations. For adequate interpretation of mutagenesis studies, possible effects of a mutation caused by aberrant subunit folding and dimerization should be considered. Such changes could result in distant conformational effects that may alter hormone function in an indirect fashion. This is especially possible if secretion, immunoreactivity, receptor binding, or bioactivity of mutated analogs is profoundly impaired. In contrast, "gain-of-function" changes, such as enhanced receptor binding or switch of hormonal specificity, are more likely to be the result of direct residue/domain-specific effects. Nevertheless, it is prudent to ascertain accurate quantitation and to rule out the possibility of global conformational changes of analogs with multiple mutations by testing them against a panel of different antibodies or by circular dichroic (CD) spectrometry.

B. Studies of Protein Domains

TSH expression and biological activity requires a noncovalent association of α-subunit and TSH β-subunit. Free TSH β-subunit, similarly to free LH β-subunit, is degraded intracellularly, and <10% produced is secreted into the culture medium. Therefore, simultaneous coexpression of α-subunit prevents intracellular degradation of TSH β-subunit (118). The most important domains in TSH expression and bioactivity recognized thus far are depicted in Figure 3. Certain domains are tightly conserved among different species or homologous hormones, and even minor modifications of such areas result in decreased expression and/or receptor binding. Most of these domains are located in close proximity and within the "composite binding domain" as described in hCG (103). Particularly important domains/residues in receptor activation include α-helix (α40–46), αLys51, αAsn52-linked oligosaccharide, the α-COOH terminus (α88–92), α33–38, "the Keutmann's loop" (TSHβ 31–52), and the "seat-belt" in the β-subunit (TSHβ 88–105) (70, 188). The seat-belt region is critical in conferring glycoprotein hormone specificity, probably by restricting heterologous ligand-receptor interactions and/or influencing the conformation of the composite binding domain. Studies involving β-subunit chimeras indicated that the seat-belt is also critical for heterodimer expression and stability (69). Functionally important residues were also identified in studies of patients with familial hypothyroidism and natural mutations in the TSH β-subunit gene (see below).

Several additional regions and residues have been recently recognized to be involved in the modulation of TSH and gonadotropin function. Studies employing the

combination of alanine- and proline-scanning mutagenesis have revealed the importance of α-helical conformation (α40–46) in TSH bioactivity. Furthermore, the α11–20 region with a cluster of basic residues (K-K/R-K—K/R), present in all vertebrates except hominoids (apes and humans), has been recognized as an important motif in the evolution of TSH and gonadotropin bioactivity in primates (191). Importantly, the elimination of basic residues in this region resulted in a decrease of TSH intrinsic activity and coincided with the divergence of apes from Old World monkeys (Table 4, Fig. 5). Identification of such nonconservative amino acid changes during hormone evolution suggested that rapid adaptive mechanisms directed by natural selection were involved. In addition to these evolutionary insights (see below), this study (191) provided the first evidence that selective alteration of residues in the loop domains to charged residues may permit design of analogs with enhanced bioactivity. Further studies suggested that the presence of basic amino acids in the β-subunit sequence of L3 loop modulated intrinsic activity of TSH and gonadotropins (Fig. 5) (66).

With regard to the role of cystine-knot motif, it is quite surprising that an intact cystine-knot structure is important only for intracellular stability and hetrodimerization of subunits, but not essential for receptor binding and bioactivity of glycoprotein hormones (82). However, as observed for several other proteins, the evolutionary pressure to maintain or optimize receptor binding sites and activation seems to be much stronger than relatively low pressure to enhance stability, which is dependent on two cystine-knot motifs and can be maintained just above a certain threshold to preserve heterodimer function. Remarkable flexibility of the glycoprotein hormones in maintaining receptor binding was supported by our "restoration of activity" strategy. We have observed that certain gain-of-function mutations can compensate for al-

TABLE 4. *Amino acid sequence alignment of various vertebrate α-subunits in the αL1 β-hairpin loop*

	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Human	C	T	L	Q	E	N	P	F	F	S	Q	P	G	A	P	I	L	Q	C
Chimpanzee	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Orangutan	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gibbon	-	Q	-	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Baboon	-	**K**	P	**R**	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-
Macaque	-	**K**	P	**R**	-	-	**K**	-	-	-	**K**	-	-	-	-	-	Y	-	-
Marmoset	-	**K**	-	**K**	-	-	**K**	Y	-	-	**R**	L	-	S	-	-	Y	-	-
Bovine	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	-	D	-	-	-	Y	-	-
Ovine	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	-	D	-	-	-	Y	-	-
Dog	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Horse	-	**K**	-	**R**	-	-	**K**	Y	-	F	**K**	L	-	V	-	-	Y	-	-
Zebra	-	**K**	-	**K**	V	-	**K**	Y	-	-	**K**	L	-	V	-	-	Y	-	-
Donkey	-	**K**	-	**K**	**K**	-	**K**	Y	-	-	**K**	L	-	V	-	-	Y	-	-
Porcine	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Rabbit	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Mouse	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Rat	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Gerbil	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	G	-	-	-	-	Y	-	-
Hamster	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Guinea pig	-	**K**	-	**K**	-	-	**K**	L	-	-	M	L	-	-	-	-	Y	-	-
Kangaroo	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Whale	-	**K**	-	**K**	Q	-	**K**	Y	-	-	**K**	L	-	-	-	-	Y	-	-
Quail	-	**K**	-	G	-	-	**R**	-	-	-	**K**	-	-	-	-	-	Y	-	-
Chicken	-	**K**	-	G	-	-	**R**	-	-	-	**K**	-	-	-	-	-	Y	-	-
Turkey	-	**K**	-	G	-	-	**R**	-	-	-	**K**	-	-	-	-	-	Y	-	-
Ostrich	-	**K**	-	**G**	-	-	**R**	-	-	-	**K**	-	-	-	-	V	Y	-	-
Salmon	-	**K**	-	**K**	-	-	**K**	V	-	-	N	-	-	-	-	V	Y	-	-
Lungfish	-	**K**	-	**K**	-	-	**K**	Y	-	-	**K**	-	-	-	-	-	Y	-	-
Goldfish	-	**K**	-	**K**	-	-	N	I	-	-	**K**	-	-	-	-	V	Y	-	-
Catfish	-	**K**	-	**K**	-	-	N	I	-	-	**K**	-	-	-	-	V	Y	-	-
Carp	-	**K**	-	**K**	-	-	N	I	-	-	**K**	-	-	-	-	V	Y	-	-
Bass	-	-	-	**R**	**K**	-	S	V	-	-	**R**	D	**R**	-	-	V	Y	-	-
Tuna	-	-	-	**K**	**K**	-	N	V	-	-	**R**	D	**R**	-	-	-	Y	-	-
Tilapia	-	-	-	**R**	**K**	-	N	L	-	-	**R**	D	**R**	-	-	V	Y	-	-
Yellowfin	-	-	-	**R**	**K**	-	T	V	-	-	**R**	D	**R**	-	-	-	Y	-	-
Seabream	-	-	-	**R**	**K**	-	T	V	-	-	**R**	D	**R**	-	-	-	Y	-	-
Pike eel	-	**R**	-	**K**	D	-	**K**	-	-	-	**K**	-	S	-	-	-	F	-	-
Eur. eel	-	**R**	-	-	-	-	**K**	I	-	-	**K**	-	S	-	-	-	F	-	-

Dashes (-) indicate amino acids identical to the human α-subunit sequence. Basic amino acids (K, R, H) are bold. [Derived in part from Szkudlinski et al. (191).]
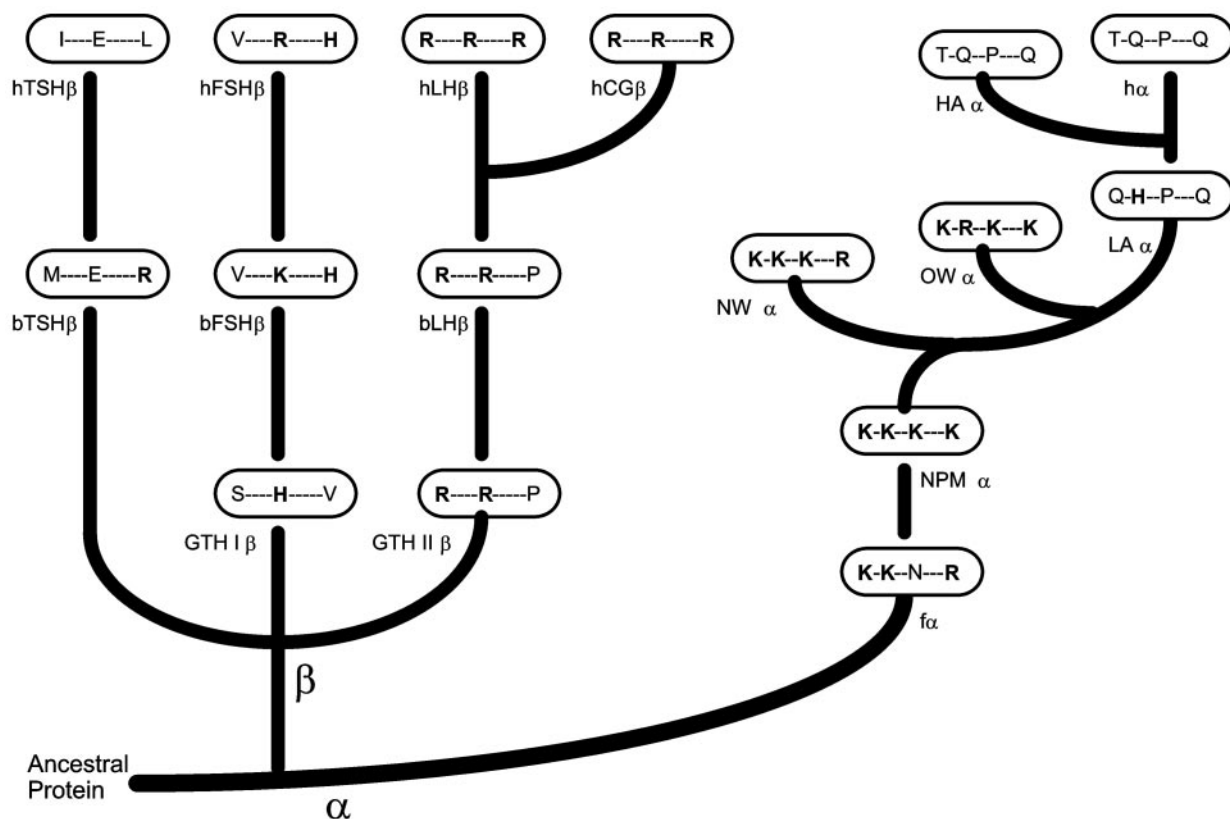
FIG. 5. Sequence evolution of two basic motifs in the $\alpha L1$ and $\beta L3$ loop of glycoprotein hormones. Whereas intervening amino acid residues (depicted as dashes) remained identical or underwent conservative changes, modulation of the TSH $\beta L3$ loop resulted in a progressive substitution of basic residues (R—R——R) with hydrophobic or acidic residues. In the α-subunit $\alpha L1$ loop, such progressive loss of basic residues (K-K-K—K) occurred late in evolution and is restricted to apes and humans. The concept of "bipolar electrostatic binding" (192) where specificity was maintained by "negative specificity determinants" may explain the large differences in the intrinsic activity of glycoprotein hormones at their respective receptors. GTH, gonadotropin; NPM, nonprimate mammals; NW, New World monkeys; OW, Old World monkeys; LA, lower apes; HA, higher apes; h, human; f, fish. [Derived from Grossmann et al. (66).]

most all "loss-of-function" mutations in hTSH and hCG (69, 192).

C. Naturally Occurring hTSH β-Subunit Mutations

Familial TSH deficiency has been described in several families with point mutations in the TSH β-subunit gene (Table 5). In a Greek kindred described by Dacou-Voutetakis et al. (35), a point mutation converted a glutamic acid codon (GAA, codon 12) to a premature stop codon (TAA). No functional TSH β-subunit was produced in patients with two defective alleles, and TSH was not detectable in the serum. Hayashizaki et al. (76) described several related Japanese families with a point mutation in the CAGY region of the TSH β-subunit gene. The CAGY region (named based on the one-letter amino acid code) is a conserved region of glycoprotein hormone β-subunit essential for α-β subunit interactions. In these kindred, codon 29 was converted from a GGA (Gly) to AGA (Arg). This alteration prevents the mutant TSH β-subunit from

interacting with the common α-subunit, and intact TSH is not secreted from the thyrotroph in patients containing two defective alleles. Next kindred with a TSH β-gene point mutation was described by Medeiros-Neto et al. (123). This family had a frameshift deletion in codon 105 resulting in premature truncation of the TSH β-subunit. TSH was detectable at low levels in the serum, but radioactive iodine scans clearly demonstrated thyroid hypofunction in affected family members. Hypothyroidism in this family may be due either to impaired TSH secretion or secretion of TSH with reduced or absent biological activity. Other authors (12, 41) described similar autosomal recessive TSH defects. In all cases, sequencing of the entire coding region of the human TSH β-subunit gene revealed a homozygous single base pair deletion in codon 105, resulting in a change of a highly conserved Cys to Val followed by eight altered amino acids and a premature stop codon due to the frame-shift. It is likely that elimination of highly conserved Cys-105 causes a major conformational change in the TSH molecule. However, unlike

TABLE 5. *Natural mutations of human TSH β-subunit*

Gene Alteration	Amino Acid Alteration	Specific Structural Change	TSH Heterodimer Formation	Bioactivity	Clinical Presentation
Point mutation	Gly29Arg	Arginine introduced to the cystine-knot region (CAGYC)	Undetected		Familial central hypothyroidism (76)
Stop at codon 12	Deletion of residues 12–112	TSH β-subunit 11 amino acid NH_2-terminal peptide produced	Undetected		Familial central hypothyroidism (35)
Frameshift mutation	Cys105Val, change in the amino acid sequence	Disulfide bond in the seat-belt region disrupted	Reduced	Undetected	Familial central hypothyroidism (123)
Frameshift mutation and stop at codon 114	Cys105Val, change in the amino acid sequence at positions 106–114	Disulfide bond in the seat-belt region disrupted	Undetected		Congenital central hypothyroidism (41)

the previous TSH β-subunit gene mutations, immunoreactive TSH is detectable in these patients' circulation. Because of the early development of severe symptoms in neonatal period, it was proposed, but not proven, that such altered TSH may suppress the physiological constitutive activity of the unliganded TSHR (12).

D. Function of Carbohydrate Chains

The presence of alternative $GalNAc4-SO_4$ (and/or $Gal-3-SO_4$) termini in LH and TSH, but not in FSH and hCG, indicates that outer glycosylation is hormone specific and raises questions regarding the role of carbohydrate termini in hormone biosynthesis and their biological significance. Deglycosylation of hCG and bovine TSH has long been known to result in increased receptor binding but loss in efficient signal transduction. Many reviews have covered the role of carbohydrate chains in glycoprotein hormone assembly, secretion, and bioactivity, and the reader is invited to refer to them for more detailed information (4, 14, 146, 188, 222). It appears that glycosylation can play a role in the life span of the hormone through different glycan structure. More recently, the work of several laboratories focused on two aspects: *1*) subunit folding and *2*) site-directed mutagenesis of individual glycosylation sites and their distinct roles in hormone function. We will emphasize these new developments describing studies on subunit folding and assembly, biological activity, and metabolic clearance.

1. Subunit assembly

Subunit folding and assembly are crucial intracellular events for hormone formation, especially in view of the heterologous expression of these hormones in host cells. Subunit folding relies on the action of many accessory proteins including chaperone-like glucose-specific calnexin/calreticulin and disulfide isomerase, all proteins present in the CHO cells during biosynthesis of recombi-

nant proteins. Site-directed mutagenesis of cysteine residues in the hCG β-subunit revealed that disulfide bonding is a prerequisite for subunit association and oligosaccharide assembly. It has been noticed that folding intermediates differ in disulfide pairing from the native dimer and that elimination of an earlier forming disulfide bond (Cys34-Cys88, Cys9-Cys57, or Cys38-Cys90) interferes with the completion of glycosylation. Elimination of a later disulfide bond (Cys23-Cys72, Cys93-Cys100, or Cys26-Cys110) does not influence glycan completion (128). These findings suggest that early conformation of the hormone dimer is crucial for oligosaccharide processing and may affect final polymorphism. Accordingly, it can be expected that glycan structure at each glycosylation site may indeed be governed by polypeptide folding of the respective subunit as well as of the heterodimer since the β-subunit folds in the presence of the α-subunit that exerts a chaperone-like function (180). As a result, the final glycan structure can be different among dimers of the glycoprotein hormone family. In hCG, the COOH-terminal peptide (CTP) has been also shown to affect the core mannosyl unit of the β-subunit, resulting in complex forms at both *N*-glycosylation sites (133). Noteworthy, outer glycosylation was achieved through polylactosamine units rather than sialic acid when the heterodimer lacking CTP was expressed in CHO cells. This and other studies (116) indicate that polypeptide folding affects carbohydrate processing resulting in distinct oligosaccharide patterns among closely related glycoprotein hormones.

2. Biological activity

Over the past years, extensive work of several groups explored the glycosylation patterns at individual glycosylation sites using site-directed mutagenesis and different host cells. Recombinant TSH produced in CHO cells is sialylated and not sulfated because these cells lack the two enzymes (GalNAc- and sulfo-transferase) involved in

addition of the terminal sulfated GalNAc residues. Expressing the wild-type hormone in CHO lectin-resistant cells (CHO-LEC2 cells) deficient in sialyltransferases (181–183) showed a five- to eightfold higher activity than in parent cells able to sialylate the heterodimer, and this was not further augmented by site-specific deglycosylation (67). TSH with incomplete glycans retained full receptor binding while exhibiting increased signal transduction, demonstrating that sialic acid attenuates receptor activation. Differences could also been noticed upon expression of the wild-type dimer in different host cells, suggesting again that TSH glycoforms can further differ in activity probably on the basis of the underlying glycan structure. Deleting the glycosylation sites by Asn to Gln mutation revealed that the hormone lacking Asn-52 displayed a sixfold increase in the in vitro activity, whereas activities of αN78Q/TSHβ and α/TSHβN23Q were only increased by a factor of 2–3 (67). These findings show that the presence of each glycan is crucial to attenuate intrinsic TSH activity, probably through a conformational effect on functional domains. These observations suggest that modulation of TSH bioactivity is dependent on the glycosylation site, the extent of oligosaccharide branching, and the oligosaccharide termini. Interestingly, it was noted that sialylation of the oligosaccharide at Asn-52 specifically attenuated intrinsic TSH activity in contrast to the previously reported stimulatory role in hCG (158) and FSH (210). Altogether, these studies further emphasize the role of individual glycans in modulating signal transduction and hormone-specific effects of sialic acid residues.

3. Metabolic clearance

As rhTSH has been recently used for thyroid cancer imaging, it was essential to estimate the effect of glycosylation on in vivo biological activity of the drug. Structure-function studies were performed to delineate the molecular determinants of metabolic clearance of rhTSH to select optimal preparations in vivo. An initial finding was that carbohydrate-mediated effect on rhTSH clearance is largely based on sialic acid capping (202). rhTSH with decreased sialylation is rapidly cleared from the circulation by the hepatic asialoglycoprotein receptor while highly sialylated hormone displayed prolonged plasma half-life (193). Sulfated forms of pituitary TSH can be cleared through a specific hepatic lectin (53) while the presence of sialic acid increases their duration in blood and limit clearance by the kidneys (194). Therefore, a variable sulfate-to-sialic acid ratio may be considered as a structural fine-tuning of the amount of TSH that can reach the thyroid under physiological conditions. Importantly, it was demonstrated that glycan structure affects in vitro and in vivo TSH bioactivity in opposite directions (193, 195). Although asialo-rhTSH has a 5- to 10-fold increased

in vitro activity, it has also the shortest plasma half-life in vivo. Similarly, rhTSH produced in insect cells containing mannose-rich glycans was found to be of high activity but displayed a rapid clearance because it was eliminated through a mannose-specific receptor. Studies by Simpson et al. (178) indicated that SO_4-4GalNAc-containing LH and TSH bind to the NH_2-terminal cysteine-rich domain of the macrophage mannose receptor, distinct from the mannose binding site. Thus sialic acid and its derivatives thus appeared optimal terminal sugar candidates to create long-lasting glycoforms of rhTSH drug. To this end, sialylation at different sites may affect hormonal clearance to a different degree. It was shown that the peripherally located carbohydrate chain of the TSH β-subunit at the Asn-23 appears the most important in determining the clearance rate of TSH (195). In the α-subunit, the Asn-78 glycan is more critical than that attached to Asn-52 (67). Again, variable terminal residues at each position may determine individual clearance parameters of each TSH glycoform.

E. Alterations of TSH Carbohydrates Structures in Pathophysiology

Circulating TSH should be considered as being composed of a panel of forms differing in glycan structure, released by the pituitary under hypothalamic stimulation and/or thyroid status, and cleared according to their individual metabolic rate. As a result, it is now widely admitted that structural microheterogeneity of plasma TSH can reflect the status of the whole endocrine axis. This mechanism provides to the pituitary-thyroid balance an enormous potential to adapt to physiological needs by regulating both the quality and the amount of TSH with a combinatorial flexibility, which has not been easy to decipher experimentally. It was first observed that distribution of pituitary rat TSH was modified in primary hypothyroidism, suggesting a selective release of hormonal forms with altered glycosylation (74). In hypothyroid patients, plasma TSH glycoforms appeared mostly acidic with an increased sialic acid content due to a shift to higher glycan branching (148). This observation has been confirmed more recently since differences could be measured between subclinical and overt hypothyroidism. TSH from patients with subclinical hypothyroidism displayed increased core-fucosylation and a moderately augmented content in sialic acid compared with euthyroid TSH (206). More overt hypothyroidism gradually results in a more pronounced increase in sialic acid (increased branching or shift in terminal capping), suggesting that as thyroid dysfunction is accentuated, low active glycoforms with prolonged half-time are selectively released by the pituitary to stimulate the TSHR.

Indeed, TRH was found capable of releasing such acidic forms from TSH-secreting adenomas (175), indicating a selective discrimination of the pituitary stock upon secretion. Very recently, sera obtained from euthyroid volunteers before and 30, 60, 120, 180, and 240 min after intravenous, nasal, and oral administration of TSH were found to contain increased acidic forms as a function of time (171). Lectin analysis confirmed a concomitant increase in core-fucosylated glycans. Overall, these findings point to a tight endocrine regulation of TSH polymorphism based on structural changes in core branching as well as outer glycosylation that probably accounts for altered B/I ratio. It is expected that the understanding of such molecular changes will help to design new TSH immunoassays to better assess plasma levels especially in detecting early subclinical hypothyroidism.

As described above, terminal carbohydrate residues can affect plasma half-life and in vivo bioactivity of TSH through an interaction with specific hepatic carbohydrate receptors. It is therefore not surprising that the distribution of hTSH glycoforms is under endocrine control and is altered in various states of thyroid dysfunction. hTSH glycosylation isoforms with higher bioactivity have been reported in patients with resistance to thyroid hormone (9). Variable carbohydrate structures of circulating TSH have also been described in TSH-secreting pituitary adenomas and central (hypothalamic) hypothyroidism and have also been associated with the euthyroid sick syndrome, chronic uremia, TRH/octreotide administration, cranial irradiation, intrauterine stage, and aging (8, 9, 70). Such regulation of hTSH glycosylation may be viewed largely as an adaptive response, thus contributing to the classical negative triiodothyronine (T_3)/thyroxine (T_4)-TSH-TRH feedback loop. The evidence exists that TRH enhances the biologic activity of TSH by modifying its glycosylation pattern (199, 200, 228). In primary hypothyroidism, pituitary compensation would not only result in an increased hormone production and secretion, but the released TSH would have an altered carbohydrate structure that prolongs its plasma half-life. At the molecular level, this may involve a direct regulation of the transcription of glycosyltransferases by thyroid hormone, as, for example, thyroid hormone status has been shown to modulate $\alpha 2,3$- and $\alpha 2,6$-sialyltransferase mRNA levels in mouse thyrotrophs (81). It has been shown that in patients with central hypothyroidism and "abnormally normal" or slightly elevated TSH levels, TSH lacks its bioactivity owing to an altered glycosylation pattern. Such patients thought to suffer from hypothalamic TRH deficiency are shown to benefit from chronic TRH administration, which was found to increase TSH bioactivity and subsequently circulating thyroid hormone level in some patients (7, 115).

V. THYROID-STIMULATING HORMONE RECEPTOR AND THYROID-STIMULATING HORMONE-THYROID-STIMULATING HORMONE RECEPTOR INTERACTION

The TSHR together with LH and FSH receptors are related members of rhodopsin/β-adrenergic receptor family in the seven-transmembrane domain, GPCR superfamily. The TSHR is critical in the development, growth, and function of the thyroid. In contrast to many other subfamilies of GPCRs, the TSHR and other glycoprotein hormone receptors contain a large 300- to 400-amino acid-long extracellular domain with at least 8 highly conserved Cys residues, involved in formation of extracellular domain tertiary structure that appears important in both ligand binding and inactive receptor conformation. Only selected particularly new aspects of TSHR are discussed here. Several other aspects of TSHR studies, including its relation to Graves' disease, are summarized in several comprehensive reviews (63, 97, 156, 159, 214, 229).

A. TSHR: Gene and Expression

The human TSHR gene is located on chromosome 14q31 (163). The extracellular domain is encoded by the first nine exons and part of the last exon, whereas the transmembrane and intracellular domains are encoded entirely by the last exon. In addition to the expression of the gene encoding this receptor in thyroid tissue, the presence of TSHR (mRNA transcripts and/or protein) has been described in several other sites including lymphocytes, adipocytes, retroocular fibroblasts, neuronal cells, and astrocytes (32, 63, 126, 150). The low-affinity TSH binding site, detected previously in bacteria, is considered an artifact (135). An initial indication of potential extrathyroidal action of TSH derived from early studies demonstrating the presence of TSHR in human lymphocytes (154). Expression of TSHR in lymphocytes has been supported by more recent studies and indicated a possibility of paracrine or autocrine regulation by TSH (36). However, the physiological or pathophysiological relevance of such extrapituitary TSH secretion is not yet clear. Recently, additional data have supported possible functions of locally secreted hTSH. Specifically, there has been a description of the TRH-TSH network in the intestinal mucosa, suggesting that TSH produced by enterocytes may regulate function of intraepithelial lymphocytes as well as enterocytes by the TSHR-mediated mechanism (218). Interestingly, mice bearing a natural inactivating mutation in the TSHR display signs of impaired gastrointestinal immunity (184, 218). In addition, TSH has been recognized to mediate immunopotentializing effects of TRH and for the possible role of TSH-dependent interleukin-2 activation of cytotoxic lymphocytes in the course of

thyroid autoimmune disorders (153). Recently, studies using TSHR knock-out mice indicated that TSH may serve as a negative regulator of osteoblast and osteoclast formation (117). Finally, the lipolytic effects of TSH have long been known and explained by the presence of low level of TSHR in adipocytes (33, 46). However, it must be emphasized that it has not been unequivocally established whether functional TSHR protein is expressed in such tissues and/or whether the number of receptors expressed would be physiologically relevant.

There are two recent reports of isolation of TSHR cDNAs from nonmammalian vertebrates. Two thyrotropin receptor cDNAs (sTSHRa and sTSHRb) were recently cloned from thyroid tissue of the amago salmon (143). sTSHRa and sTSHRb showed a high degree of sequence homology to mammalian TSHRs. Functional characterization in COS-7 cells transiently transfected with sTSHRa or sTSHRb showed the largest increase in cAMP when exposed to bovine TSH, suggesting that the cloned cDNAs encode functional TSHR proteins. RT-PCR analysis demonstrated that sTSHRa and sTSHRb were expressed in the basibranchial region, but not in the ovary, testis, liver, kidney, or brain. In situ hybridization revealed that sTSHRa and sTSHRb were exclusively expressed in thyroid follicular epithelial cells (143). TSHR was also recently cloned from the gonads of a nonmammalian vertebrate, a bony fish [striped bass (stb)] (101). The striped bass TSHR (stbTSHR) transcripts were abundant in both the thyroid and gonads and detectable in skeletal muscle, heart, and brain tissues. The stbTSHR cDNA encoded a 779-amino acid glycoprotein hormone receptor with much higher homology (57–59%) to the mammalian TSHRs than to the gonadotropin receptors. It contains a TSHR-specific insertion in the extracellular domain as seen in mammalian TSHRs. Recombinant stbTSHR expressed in COS-1 cells activated reporter genes (luciferase) driven by either a cAMP response element or the c-*fos* promoter in response to bovine TSH, stbLH, or hCG, but not human FSH. In situ hybridization studies revealed the presence of stbTSHR transcripts in the gametes but not in the follicular cells (101). This pattern of expression suggested a direct, although unknown, role for TSH in fish gamete physiology.

B. TSHR Structure-Function Studies

The TSHR, lutropin receptor (LHR), and follitropin receptor (FSHR) are related members of the superfamily of GPCR. Together with newly discovered receptors (141) they are now classified in the leucine-rich repeats-containing GPCR (LGR) proteins (see below). Several recent reviews addressed various aspects of their structure-function relationships (44, 99, 156). We focus here only on selected aspects, including common and unique features of TSHR, constitutive activity, and naturally occurring TSHR mutations.

Similar to other glycoprotein hormone receptors, TSHR has a large extracellular domain, accounting for about one-half the molecular size of the receptor (96, 97, 159, 174). Studies using isolated extracellular domain of TSHR and LHR have confirmed that extracellular domain is sufficient for high-affinity hormone binding (22, 34, 145, 173). In addition, the crystallization of the porcine ribonuclease inhibitor, the first structurally known protein with specific structural elements termed leucine-rich repeats (LRRs) (95) paved the way for the modeling of the extracellular domain of glycoprotein hormone receptors, as these receptors also contain such LRRs (10, 90). The LRRs are encoded by separate exons, suggesting that the TSHR gene has arisen by insertion of a DNA sequence encoding repeated LRRs between the regions encoding the extracellular and the transmembrane domains of a protoreceptor gene resembling the intronless β-adrenergic receptor genes (65). However, different modeling studies suggested six to nine LRRs; each repeat contains β-sheet oriented toward the interior circumference of the horseshoe-like tertiary structure, which is now considered to bind the hormone molecule. In addition to the three glycoprotein hormone receptors, such LRRs have been found in other homologous receptors, including mammalian LGR-4, LGR-5, LGR-6, LGR-7, and LGRs in sea anemone, fly, and snail (84, 100, 141).

There are several important functional differences between TSHR and other glycoprotein hormone receptors. First, TSHR is more frequently activated by gain-of-function mutations than gonadotropin receptors (212, 214). Second, TSHR is unique among glycoprotein hormone receptors in that some mature receptors on the cell surface are cleaved into two subunits (156, 159). Third, unlike the single-chain gonadotropin receptors, TSHR is considered to be "noisy," transducing a signal via adenylate cyclase even in the absence of ligand (17). Fourth, similar to the thrombin receptor, proteolytic degradation or small deletion in the extracellular domain of TSHR can lead to its activation (211, 234). Although the LH receptor seems to be activated by proteolytic enzymes as well (162), TSHR seems to be much more prone to such activation. Fifth, unlike many other GPCRs, including the FSHR where guanine nucleotides reduce agonist binding (235), TSH binding to TSHR did not change in the presence of a nonhydrolyzable GTP analog (3). Finally, our studies on hTSH superactive analogs, including analogs with multiple mutations, demonstrate that the increase in receptor binding activity and in vitro bioactivity is generally parallel (70, 107, 191), suggesting that there is no clear distinction between recognition and activation binding sites in the TSHR.

In contrast to the LH and FSH receptor, there are two unique insertions in the TSH primary structure. The first

8-amino acid insertion near the NH_2 terminus (residues 38–45) was shown to be important for TSH and stimulating antibody binding (156, 216), whereas a second 50-amino acid residue insertion (residues 317–366) has no apparent effect on TSHR function. The cluster of four cysteine residues at positions 24, 29, 31, and 41 was recently shown to be involved in formation of highly conformational epitope for thyroid stimulating antibodies (23). Two highly conserved cysteines in extracellular loops 1 and 2 of TSHR are also predicted to form a disulfide bond as identified in bovine rhodopsin structure (147).

Posttranslational modifications of TSHR include glycosylation of six asparagine residues. However, substitution of Asn at positions 99, 177, 198, and 302 did not appreciably affect the affinity of the TSHR for TSH or its ability to mediate an increase in intracellular cAMP levels (166). In contrast, *N*-linked glycosylation of Asn-77 and Asn-113 does play a role in the expression of a biologically active TSHR on the cell surface. Other posttranslational modifications of TSHR may include amidation and tyrosine sulfation (see below). However, clearly unique is the existence of natural processing mechanism leading to the two-subunit structure with not yet clear functional significance (63, 156). Further characterization of a soluble bioactive NH_2-terminal extracellular domain (29, 34) should ultimately result in the elucidation of structural basis of TSH recognition.

C. Naturally Occurring TSHR Mutations

Numerous mutations in the TSHR gene have been identified and associated with specific thyroid diseases (139, 151). Resistance to TSH is a syndrome due to reduced responsiveness of the thyroid gland to biologically active TSH. Inactivating mutations of the TSHR have been detected in several cases of resistance to TSH, both partial and complete, sporadic and familial. More than 10 different inactivating mutations have been described. Germline mutations inactivating TSHR may cause primary hypothyroidism due to TSH unresponsiveness.

There are more than 30 different activating mutations causing nonautoimmune hyperthyroidism. Toxic nodules are quite frequently caused by somatic mutations constitutively activating TSHR. Germline mutations activating TSHR explain pathophysiology of autosomal dominant nonautoimmune hyperthyroidism (50).

Constitutive receptor activity, i.e., signaling by receptors in the absence of ligand binding, has been described for numerous GPCRs. Constitutively active glycoprotein hormone receptors showed increased ligand affinity compared with the wild-type receptors (136). This may suggest that the receptor in an open constitutively active conformation may have exposed additional binding domain not present in the closed conformation (see below). Extracellular loops of transmembrane domain may form such a domain, fully accessible to the ligand only in certain active conformations. Although functional characterization of different activating mutations contributed to the identification of TSHR domain important for receptor binding and signal transduction, new insights into the mechanism of TSHR activation have been also provided using experimental site-directed mutagenesis, molecular modeling, synthetic peptides, and enzymatic and immunological approaches.

Recently, a family was described with a resistance to TSH responsible for euthyroid hyperthyrotropinemia in two siblings (165). A new mutation responsible for the Arg to Cys substitution at position 310, in the extracellular domain, has been described. When stably transfected in CHO cells, the Cys-310 TSHR mutant showed loss of response to TSH. However, increased constitutive activity assessed based on cAMP production explained the presence of TSH resistance with the clinical euthyroidism detected in this family (165).

D. Suppressive Effect of Extracellular Domain: The "Two-State" Model

As described above, the TSHR is much more susceptible than other glycoprotein hormone receptors to constitutive activation by mutations, deletions, or even mild trypsin digestion (211). In our study, constitutive activity has been demonstrated for the first time for truncated TSHR lacking 98% of the extracellular domain (233). The active state of truncated receptor with respect to G_s protein coupling and adenylate cyclase activation can be suppressed at least in part by the presence of α-subunit linked to the truncated receptor. In this study, the constitutive activity of the TSHR missing 386 NH_2-terminal amino acid residues was normalized based on cell surface expression. Such normalized activity of this deletion mutant was four to seven times higher than the normalized constitutive activity of the wild-type TSHR, suggesting that the extracellular domain of TSHR inhibits constitutive activity of the transmembrane domain. These findings provided important evidence supporting a two-state model of TSHR activation and suggest a potential role of proteolytic cleavage in receptor activation and indicated new strategies for design of TSHR antagonists. The two-state model of receptor activation (92, 106) is illustrated with important modifications in Figure 6. According to this model adapted to the TSHR (43), receptors are in equilibrium between the inactive ("closed") conformation and a constitutively active ("opened") conformation that can associate with G_s in the absence of hormone and trigger the intracellular signal. TSH preferentially binds to opened receptors and stabilizes them. This model is dif-
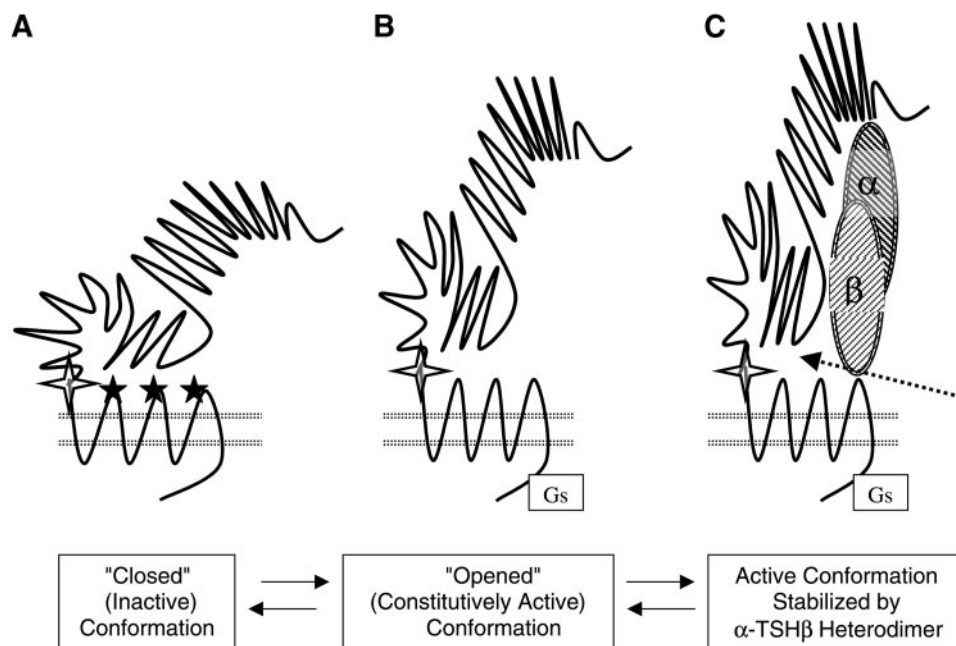
FIG. 6. The two-state model of TSH receptor (TSHR) activation. *A*: "closed" (inactive) TSHR conformation; three five-point stars in the closed (inactive) conformation illustrate putative interactions between the extracellular domain and extracellular loops of transmembrane domain; these interactions are critical for an inactive state and may include electrostatic interactions. Four-point star depicts the location of TSHR 409–418 fragment, which may contribute to receptor activation, when the constraints of extracellular domain-transmembrane domain interaction are removed. *B*: "opened" (unliganded) TSHR conformation. *C*: "opened" (hormone-activated) TSHR conformation. [Schematic drawing derived in part from Duprez et al. (43).]

ferent from earlier models based on the assumption that receptors require a hormone-induced conformational change. A major prediction based on this model is that an agonist has a higher affinity for the constitutively active (opened) conformation and that such an active receptor conformation is stabilized by hormone binding. Although the essential concept of these models has not changed, there is now more evidence suggesting that the equilibrium between active and inactive states is not limited to two receptor states but include multiple equilibria with a number of partially or full activated states. Unlike previous models, our two-state model of TSHR activation includes a possibility of the activating 409–418 region present in the TSHR (see below). As depicted in Figure 6, ligand binding will always lead to an opened conformation and receptor activation. This can be prevented only by antagonists (inverse agonists) capable of binding to the constitutively active ("opened") conformation and transforming it into an inactive conformation. Because hormone binding is inherently associated with an opened conformation, resembling the constitutively active unliganded conformation, simple site-specific modification of the ligand cannot result in a receptor antagonist. According to our model, the mechanism of hormone-induced receptor activation is not only dependent on the elimination of inhibitory interactions between the extracellular domain and extracellular loops of transmembrane domain, recently supported by studies using LH receptor chimeras (142) and TSHR mutations in the hinge region (83), but also may include an "activating region" located in the extracellular domain near the first transmembrane helix, which may function similar to the tethered ligand sequence in the protease-activated receptors (PARs) (31).

Similar inhibitory constraints of extracellular domain released after ligand binding have previously been described for insulin and other CKGF receptors (236). Furthermore, our studies on glycoprotein hormone superagonists highlight the concept that glycoprotein hormone receptor activation is based, at least in part, on electrostatic interactions between the hormone and the extracellular domain of the receptor leading to the disruption of charge-based silencing effect of extracellular domain. According to such a model, activity of the unbound TSHR is kept at relatively low level by an inhibitory interaction between the extracellular domain and the transmembrane domain (43). A similar concept was proposed for the thrombin receptor (137) based on findings that alterations of extracellular domain and extracellular loops of transmembrane domain may cause constitutive signaling. This model is also supported by several other observations. First, constitutively active receptors with mutations in different parts of extracellular domain and extracellular loops of the transmembrane domain of human TSHR are among the strongest activating mutations identified (43); both domains were previously found to contribute to TSHR ligand binding and signaling. In addition, numerous TSH antibodies binding to different parts of extracellular domains may lead to TSHR activation (156) or inhibit TSH binding (144). Second, proteolytic degradation of TSHR (211) and thrombin receptors (137) leads to activation of these receptors. Third, a small 339–367 deletion within the extracellular domain activates TSHR (234). Fourth, the hormone binding affinity of the isolated LH receptor extracellular domain was shown to be slightly, but consistently, higher than that of the entire LH receptor (168), suggesting that interaction of extracellular domain with

transmembrane domain may limit ligand binding affinity of extracellular domain. Finally, the general parallelism between receptor binding and activation observed for numerous TSH mutants (66, 69, 191) and no guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) shift in TSH binding (3) suggest that, at least for TSHR, there is no clear distinction between recognition and activation binding sites.

We have previously observed that a partial or complete loss of ligand activity caused by modification of one domain may in certain instances be completely compensated by additional charged residues in spatially distant TSH domain (69, 192). These and other studies indicate that TSHR and other glycoprotein hormone receptors are capable of tolerating ligands with significant structural modifications (15, 59, 196, 197, 227). Moreover, conformational flexibility is not limited to the ligand but holds also true for the hTSHR and is supported by the following findings. First, the mutations in the constitutively active hTSHR are located in various portions of this molecule, including extracellular loops (204). The large diversity of activating mutations suggests that constitutively activated TSHR may assume different active conformations. Such constitutively active TSHRs have presumably rearranged or eliminated certain intrareceptor interactions in a manner similar to those resulting from ligand binding (Figs. 6 and 7). Second, different activating mutations or deletions in the receptor lead to variable increase of basal and TSH-induced activation of cAMP or inositol phosphate (IP) cascades (58, 203). Such diversity suggests that activated receptor may assume different energetically almost equal conformations and that TSH presumably releases electrostatic constraints of the unliganded state. Third, receptor activation can be induced by specific changes such as certain mutations, localized proteolytic cleavage, or specific antibodies, but not by nonspecific destabilizing factors such as varying pH or temperature (17, 211). Accordingly, mild trypsin treatment resulted in a site-specific TSHR autoactivation but only 25% decrease of TSH-stimulated cAMP production (211). However, studies using TSHR/LHR chimera indicated that receptor cleavage into two subunits is not a prerequisite for TSH action (21).

To further investigate the role of the extracellular domain in the TSHR function, we have recently designed deletion mutants lacking 66–100% of the extracellular domain. In this study, we analyzed constitutive activity as measured by cAMP and IP production since TSH can activate both G_s and G_q protein in human thyroid cells (58). The TSHR lacking the entire extracellular domain showed no detectable cell surface expression, suggesting that the presence of at least a 10-amino acid fragment of the extracellular domain is crucial for expression. The expression levels of the other deletion mutants ranged from 10 to 40% of the wild-type TSHR. Basal levels of
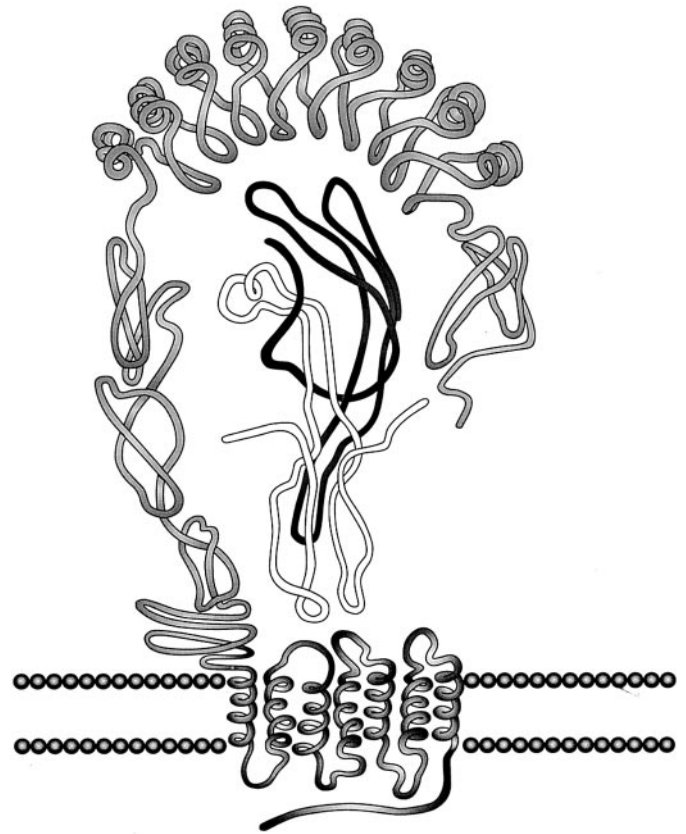

FIG. 7. Schematic configuration of TSH-TSHR complex. Two parallel β-hairpin loops of the α-subunit (αL1, αL3) are located in the bottom part of the model and may participate in the interaction with the extracellular loops of the receptor transmembrane domain [compatible with model of Jiang et al. (89)]. Two analogous loops of the β-subunit (βL1, βL3) are shown in the top part with proposed binding site within the concave of leucine-rich repeats (LRR) modeled by Kajava et al. (90).

cAMP and IPs attained for each deletion mutant were normalized relative to their expression levels. They showed a 4- to 6-fold higher constitutive activity as measured by cAMP production and a 4- to 10-fold increase in IP levels compared with wild-type TSHR. Remarkably, the effect of the specific deletions revealed a differential activation of cAMP and IP signaling pathways by the respective truncated TSHR constructs (58). These results suggested that after deletion or mutation in the TSHR extracellular domain, an activated receptor exists in various conformations preferentially coupling to G_s or G_q proteins.

More recently, we have used an additional approach to explore the role of the extracellular domain in TSHR function (57). Previously described extracellular domain construct linked to the cell membrane by means of a glycosylphosphatidylinositol (GPI) anchor (29, 34) was used to establish a stable cell line (CHO-ECD-GPI). We have transiently transfected this cell line with either the wild-type TSHR or the constitutively activated truncated TSHR to investigate the suppressive effect of the extra-

cellular domain. The basal activity of the wild-type TSHR was higher in CHO-ECD-GPI cells than in COS-7 cells, indicating that an additional activation mechanism may involve dimerization of the extracellular domains, present as a GPI construct and in wild-type TSHR. A recently published study on oligomerization of the human TSHR provided evidence for the close proximity of individual receptor molecules (105). The authors demonstrated that cleavage of the extracellular domain was required for the formation of TSHR dimers and higher order complexes. It is then likely that the overexpression of extracellular domain by means of a GPI anchor increases the dimerization and cleavage process leading to a higher basal activity of a wild-type TSHR expressed in our CHO-ECD-GPI cell line. Our study showed also that the overexpression of extracellular domain by means of a GPI anchor did not inhibit the constitutive activity of the truncated TSHR, suggesting that the silencing effect of the extracellular domain on the transmembrane domain is effective only when the receptor is expressed as a full-length polypeptide chain, and it is not reproduced by independently folded extracellular domain (57). Occurring only in the folding process, intramolecular interactions between the extracellular domain and the transmembrane domain may be responsible for maintaining the receptor in a constrained and closed conformation. Any disruption of the TSHR constrained conformation by mutation or deletion may lead to an increased basal activity.

The two-state model of TSHR activation provides also an explanation of why all previous attempts to develop antagonists based on hormone mutagenesis have been generally unsuccessful. Because the binding of hormone to the receptor is inherently associated with an "opened" receptor conformation, any TSH analog binding to the TSHR extracellular domain will result in signal transduction (Fig. 6). Such an opened conformation can also be stabilized by interaction between the single-chain hormone and its covalently linked receptor that results in a constitutively active complex (138). A similar effect could be exerted by TSHR stimulatory antibodies that may disrupt the interaction between extracellular domain and exoloops of transmembrane domain. According to the two-state model, it is possible to envision TSHR blocking antibodies, TSH analogs, or chimeras with properties characteristic of inverse agonist binding to both extracellular domain and exoloops of transmembrane domain. Such antibodies may reduce constitutive activity of TSHR by enhancing interactions between the extracellular and the transmembrane domain. In contrast to TSH, TSHR blocking antibodies may bind TSHR at sites other than those occupied by TSH and promote self-inhibition of TSHR resulting in the closed (inactive) conformation. With the understanding that binding of TSH to its receptor is inherently associated with a certain degree of receptor activation, a new class of antagonists (inverse agonists),

capable of mimicking unliganded extracellular domain action in silencing constitutive activity of transmembrane domain, can be designed.

The constitutive activity of TSHR and the TSH binding to TSHR are both markedly influenced by ionic strength and selective introduction or elimination of basic residues in the TSH molecule (17, 68, 159). Since the removal of NaCl clearly enhanced the constitutive activity of TSHR while unmasking constitutive activity of LH receptors (17), activation of unliganded TSHR may involve generation of intra- and/or intermolecular ionic bonds composed of charged residues normally involved in hormone-receptor interaction (189). Moreover, the noncovalent interaction between the TSHR A and B subunits may also involve ionic forces (159). Thus specific electrostatic interactions are predicted not only to help prealign TSH with the complementary interface of TSHR and increase receptor binding affinity as demonstrated using TSH superagonists (191) (see below), but also to contribute to the mechanism of unliganded TSHR activation. In general, glycoprotein hormone receptor activation is predicted to be based at least in part on electrostatic interactions (11, 189).

E. Other Concepts of TSH-TSHR Interaction

Despite more than 20 years of intensive studies, the nature of TSH interaction with TSHR and the mechanism of transmembrane signaling remain largely unknown in part due to the absence of information regarding receptor structure. It is anticipated from multiple studies that both subunits of the TSH heterodimer interact with several portions of the TSHR and that receptor binding sites of TSH and TSHR antibodies may be identical or in part different (144, 220). We and others envision receptor activation to be a multistep process involving primary high-affinity interactions with the internal concave of LRRs located in the NH_2-terminal portion of the extracellular domain, as well as secondary interactions with extracellular loops and/or transmembrane regions that are of lower affinity and may involve common α-subunit domains (70, 88) (Fig. 7). However, the exact spatial relationships of ligand and receptor are still unknown, and different models have been proposed (97). Similarly, there is no consensus regarding whether the extracellular loops of the receptor participate in direct hormone-receptor interaction (89, 132).

The localization of many natural and engineered constitutively activating substitutions in GPCRs transmembrane domains led to the concept that interhelical contacts are most important in regulating isomerization of these receptors between the active and inactive states. The process of disrupting interhelical contacts necessary to maintain an inactive state may be spontaneous, in-

duced by mutations or ligand binding (62). In contrast to the role of transmembrane domain in activation, many loss-of-function mutations were localized in the TSHR extracellular domain. Studies showing that TSHR activation can be induced by mutations or deletions in various receptor domains support the model of different active conformations of the TSHR. Moreover, multiple active receptor states underlie coupling of a GPCR to different G proteins (13, 224). In certain cases mutation appears to cause an altered receptor conformation that affects TSH binding, but not constitutive activity (98).

Several recent studies have indicated that clusters of charged residues within the peripheral loops of neurotrophins, PDGF, VEGF, TGF-β, osteogenic protein-1, TSH, and hCG are important in receptor binding (64, 104, 120, 167, 190, 191). Consequently, we hypothesize that glycoprotein hormones may bind their respective receptors in a similar fashion to other CKGFs, which also show a head-to-tail arrangement of their monomers in the bioactive dimer. Accordingly, models for PDGF and VEGF binding propose that two regions located within the opposite loops participate in receptor binding and subsequent dimerization required for signal transduction (120). Analogously, two hormone-specific binding domains may be located at opposite poles of TSH molecule ($\alpha L1$-$\alpha L3$-$\beta L2$ and $\beta L1$-$\beta L3$-$\alpha L2$). Two Fv fragments from monoclonal antibodies were shown to interact with the tips and sides of hCG β-hairpin loops (201). In particular, residues 14–21 in the αL1 loop and 74–81 in the βL3 loop of hCG are involved in the interaction. These epitopes are distant from a putative receptor binding site postulated in the central part of hCG molecule (103). Although the relevance of TSHR dimerization for signaling has not yet been demonstrated (105), interestingly a putative dimerization motif in the cytoplasmic end of sixth transmembrane domain of the β_2-adrenergic receptor (79) corresponds to a hot spot for constitutively activating mutations in the TSHR.

Thus, despite considerable efforts in many laboratories, the binding site(s) for TSH on the TSHR has not been unequivocally determined yet, and lessons learned from structure-function studies confirm the complexity of the hormone-receptor interaction involving various molecular forces.

F. Electrostatic Forces in the TSH-TSHR Interaction

In general, protein-protein complexes can be stabilized by hydrophobic and Van der Waals' interactions, ionic bonds (salt linkages), as well as by the formation of hydrogen bonds at the contact surface. However, the contribution of each particular interaction to the total binding energy is difficult to estimate and can be different for different proteins. Several analyses of protein-protein interactions, including interleukin-4 binding to its receptor, indicated that binding energy is provided primarily by charged groups, and ionic forces may become dominant in ligand-receptor complex formation (56, 172, 219). However, based on the recent studies of human growth hormone-receptor complex where the majority of the binding energy is provided by hydrophobic interaction (112), it was proposed that a similar type of interaction may be critical for glycoprotein hormone-receptor binding (40). In contrast, clusters of charged residues localized in the loops of CKGFs such as PDGF, VEGF, TGF-β, and NGF appear essential for high-affinity receptor binding (64, 104, 120, 167). Similarly, a long-standing postulate held that charge-charge interactions are of major importance in the TSH-TSHR interaction (159). Accordingly, basic residues in different regions of hTSH and hCG β-subunits have been shown to be important in hormone binding activity and specificity (16, 24, 69, 191). The role of positively charged Lys residues was also recognized as an important factor in the receptor recognition ability of ovine LH (179) and hTSH. Hormone analogs synthesized with the use of reagents modifying ϵ-NH$_2$ groups on Lys residues resulted in progressive decrease in the receptor binding activity of each hormone, including hTSH (Szkudlinski and Thotakura, unpublished data).

Development of superagonists of hTSH and hCG (68, 191) has suggested that it is possible to increase the electrostatic components of hormone-receptor interaction. The receptor binding data of TSH analogs, including experiments performed in various salt concentrations, have suggested that specific ionic bonds between basic amino acid side chains of the ligand with specific acidic residues in the receptor, yet to be identified, are essential in guiding TSH-TSHR interaction. Accordingly, introduction of additional basic residues in the αL1 and βL3 loop of hTSH increased TSHR binding and activation (66, 191). For example, the introduction of three Lys residues at positions 13, 16, and 20 in the α-subunit resulted in hTSH analogs with increased affinity, comparable to the affinity of highly active bovine or rat TSH. Introduction of Ala or other nonbasic residues at positions 13, 16, and 20 did not affect receptor binding or bioactivity, indicating that a site-specific charge-charge interaction is causing superagonistic effect. In addition to parallel increases in both receptor binding affinity and biopotency, there were also observed significant increases in the analog efficacy (V_{max}). Thus the electrostatic forces are predicted not only to help to prealign the glycoprotein hormone with the complementary interface of receptor, but also to determine their association and dissociation rates.

TSHR has increased constitutive activity in salt-free conditions (17), indicating that activation of unliganded receptor may also involve generation of intra- and/or intermolecular ionic bonds composed of charged residues

normally involved in hormone-receptor interaction. Intriguingly, progressively lower affinities for TSH binding were associated with higher levels of TSHR expression in CHO cells (20), supporting a possibility of receptor-receptor interaction and multimerization.

Recent studies indicated that tyrosine sulfation occurs in the extracellular domain of GPCRs, including many chemokine receptors, and may contribute to the ligand-receptor interactions (51). Preliminary studies using tyrosine sulfation inhibitor suggested that the extracellular domain of the TSHR could be sulfated (30). Such negatively charged Tyr in the TSHR may contribute not only to the interaction of TSHR with autoantibodies, but also to the association with positively charged residues of the TSH ligand (57).

G. Evolutionary Adaptation of TSH Bioactivity

In addition to modifying specificity, evolution of hormone activity is likely to reflect adaptation of endocrine processes to environmental changes. In this respect, bovine and rat TSH are known to have higher intrinsic activity than hTSH (45, 157). With the use of site-directed mutagenesis, selective introduction of basic residues present in the $\alpha 11$–20 domain of the nonhominoid α-subunit into the human α-subunit increased the activity of hTSH (191). Sequence determination of this domain in several species of lesser apes, Old and New World monkeys, indicated a gradual loss of such residues during evolution (Table 4, Fig. 5). Because this selective elimination of basic residues in the $\alpha 11$–20 domain coincided with the divergence of hominoids from Old World monkeys, this could have caused a decrease of glycoprotein hormone activity occurring relatively late in primate evolution. Thus the attenuation of TSH bioactivity in early hominoids may be related to the adaptation of new functions for glycoprotein hormones. In rodents and other lower mammals, exposure to cold is a potent stimulus for TSH secretion, resulting in increased production of thyroid hormones and thermogenesis (28). In humans, however, cold is a relatively ineffective stimulus for TSH secretion as other more sophisticated mechanisms have developed for conserving body heat and promoting thermogenesis. A major new function of TSH in humans may be to conserve iodine for thyroid hormone synthesis during period of fasting in nomadic life. Perhaps modulation of gonadotropic activity by these evolutionary changes in the α-subunit sequence is related to concomitant adaptation to slower reproductive turnover (207). It should be pointed out in this context that adjustment to nomadic life and intermittent feeding has likewise resulted in mutations of certain other genes, such as those causing obesity and type 2 diabetes mellitus (the "thrifty genotype" hypothesis) (208).

Progressive elimination of basic residues in the α-subunit during evolution of mammalian TSH was correlated with decreased intrinsic activity of hTSH (191). Similar modifications of CG and LH intrinsic activities in apes and humans compared with New World and Old World monkeys may be related to slower rates of reproduction and maturation in hominoids (i.e., all apes and humans) as well as enhanced protection against hyperthyroidism in pregnancy (191, 230). However, the hypothesis on the evolution of TSH in primates may not simply apply for modulation of gonadotropin activity by charged residues. Such alterations of hormones critical for reproduction may be more tightly controlled and associated more frequently with parallel compensatory changes in other sites of the ligand. In addition, it is possible that gonadotropins underwent several modifications increasing and decreasing bioactivity at various stages of hormone and receptor evolution. Interestingly, a decrease in hormone bioactivity may be related to the doubling of cAMP response elements (CREs) in the promoter region of α-subunit gene (60, 140). Remarkably, it was shown that the presence of a second copy of CRE increased the level of basal expression by fivefold (38). Such tandem CREs are unique to hominoids, and the α-subunit genes of other monkeys contain a single functional CRE. This may explain in part why the levels of CG in pregnant women and apes greatly exceed those found in pregnant Old World monkeys (207).

We further speculate that changes in gonadotropin potency during evolution were not always paralleled by changes in the receptor. Accordingly, the evolutionary rate of gonadotropin receptors is considerably slower than that of their ligands, and coevolution of receptors is primarily restricting specificity of hormone-receptor interaction (27, 87, 131). The discovery of human glycoprotein hormone superagonists (191) raises the question why mutations enhancing receptor binding affinity were not selected during evolution. Certain mutations, particularly located in the α-subunit, may not be desirable because they increase binding to all glycoprotein hormone receptors, rather than optimize binding to one specific receptor. In addition, the engineering of "ancient binding domains" into modern gonadotropins has to take into consideration the possibility that in evolution optimization of one binding domain could be followed by changes in other domains to stabilize hormone conformation, which in part may diminish the effect of such optimization. Therefore, the optimized superagonists will involve a combination of domains selected at a different stage of evolution together with the gain-of-function mutations recognized by charge-scanning mutagenesis (107).

Coevolution of glycoprotein hormones and their respective receptors likely controlled spillover of hormone activity to nonhomologous receptors by attenuation of hormone activity. Significant interspecies variations in

biopotency were identified not only between glycoprotein hormones, but also for growth hormone (GH) and gonadotropin-releasing hormone (GnRH). Increases of activity during evolution observed in primate GHs were correlated with the gain of lactogenic (prolactin-like) properties not seen in the GHs of nonprimates (217). There is general agreement that the glycoprotein hormones diversified as a result of positive selection related to the need for the adaptation of new functions (110). However, little is known about the role of such adaptive mechanisms with regard to diversity of sequences between different species. Identification of amino acid substitutions significantly affecting biological activity of the hormone may support rapid adaptive mechanisms of molecular evolution, as opposed to functionally neutral amino acid replacements resulting from nonselective genetic drift. Finally, an increase of hTSH bioactivity upon selective introduction of basic residues based on their locations in hCG into a modification-permissive domain of its β-subunit (66, 68) suggests that nonconservative amino acid changes in certain regions could have occurred after evolutionary diversion of the individual β-subunits from a common ancestor gene and hence have led to modulation of specific activities of individual members of the glycoprotein hormone family. Thus such unifying evolutionary hypotheses combined with molecular modeling may not only guide site-directed mutagenesis of ligand-receptor interactions but may also provide insights into the basis of molecular evolution.

To further study evolutionary aspects, we envision generation of transgenic mice using a "double-replacement" strategy to test the elimination (neutralization) of the basic motif in the αL1 loop of glycoprotein hormone α-subunit (Table 4, Fig. 5). With a major progress achieved in the generation of transgenic Rhesus monkey (18), similar neutralization of basic motif can be achieved in Old World monkeys to study their reproductive and metabolic functions compared with apes. This should result in new insight into the relative contribution of such changes in primate evolution.

H. Relationship to Other CKGF

Because many CKGFs are dimeric and induce receptor dimerization, it is tempting to speculate that similar interaction may occur for glycoprotein hormones and their receptors. In recent years there has been an increasing number of reports describing spontaneous and ligand induced homo- or heterodimerization of GPCR (61, 78). However, there is no evidence suggesting that dimerization or higher order receptor oligomerization of glycoprotein hormone receptors has specific role in receptor signaling; recent studies suggested that cleavage of the TSHR into its two-subunit structure is required for the

formation of TSHR dimers and higher order complexes (105).

VI. RECOMBINANT THYROID-STIMULATING HORMONE AND ITS ANALOGS

A. Applications of rhTSH

RhTSH (Thyrogen, Genzyme) has been produced in large-scale bioreactor using CHO cells stably transfected with TSH genes (26). Because CHO cells, unlike the pituitary thyrotroph cells, have no capacity to add penultimate GalNAc and terminal sulfate, rhTSH is predominantly composed of oligosaccharide chains terminating in sialic acid. This pattern is more similar to the sialylated form of hTSH that circulates in primary hypothyroidism (see below), and rhTSH also has a slower metabolic clearance rate compared with normal pituitary hTSH (188). However, rhTSH can fully replace pituitary hTSH as both standard and tracer in diagnostic in vitro immunoassays (161).

Although the glycosylation pattern of rhTSH is similar but not identical to hTSH found in humans, several in vitro bioassays indicated that the activity of rhTSH is similar to that of pituitary hTSH. An initial phase I/II study showed rhTSH to be safe and demonstrated preliminary efficacy in stimulating ^{131}I uptake and thyroglobulin secretion in the diagnosis and follow-up of 19 patients with differentiated thyroid carcinoma, thus avoiding the side effects of thyroid hormone withdrawal (125). Subsequent phase III and confirmatory phase III trials with more than 100 patients have shown that rhTSH is virtually equivalent to conventional hormone withdrawal, but leads to considerable improvement of the quality of life because it avoids the symptoms of hypothyroidism (102). These major studies as well as case reports, including description of a patient with papillary thyroid carcinoma and hypopituitarism who had metastasis detected only after administration of rhTSH, but not thyroid hormone withdrawal, exemplifies the diagnostic potential of rhTSH. Thyrogen has been approved for use in conducting thyroid scanning and thyroglobulin testing in the follow-up of patients with well-differentiated thyroid cancer (75) (Table 6).

B. Design of TSH Analogs

1. Superagonists

Protein engineering and design started in 1982, after the first results of oligonucleotide-directed mutagenesis had been published. However, despite the numerous site-directed mutagenesis studies, it is still difficult to produce a protein with desirable or improved properties. Successful examples of protein engineering are quite rare (1, 164).

TABLE 6. *Current and potential rhTSH uses*

Clinical	Differentiated thyroid cancer follow up (rhTSH-stimulated thyroglobulin testing and whole body scanning), FDA approved indication
	TSH stimulation test (for example, testing thyroid reserve, identifying "warm" thyroid nodules, detecting thyroid hemiagenesis, etc.)
	Nonthyroidal illness syndrome
	Differentiated thyroid cancer treatment (rhTSH-stimulated radioiodine ablation)
	Large euthyroid goiter treatment (rhTSH-stimulated radioiodine ablation)
Laboratory	Standards and ^{125}I-labeled hTSH in TSH immunoassays (161)
	TSH-stimulated thyroglobulin mRNA testing in thyroglobulin antibody positive thyroid cancer patients
	TSH binding inhibition assay for autoantibodies to the TSH receptor (91)
	TSH bioactivity testing

rhTSH, recombinant human thyroid-stimulating hormone; FDA, Food and Drug Administration.

Based on evolutionary considerations, primary structure comparisons, as well as computer-assisted homology modeling, we were able to define a design strategy for bioengineering superactive analogs ("superagonists") of human thyrotropin with major increases in both receptor binding affinity as well as signal transduction that were the first to be described for any glycoprotein hormone or any member of the CKGF superfamily and that by far exceeded the increases in biopotency obtained for other protein ligands such as GH (112) or interleukin-6 (205) using empirical design approaches. In contrast to the characterization of loss-of-function mutations, which could result in distant conformational effects leading to an indirect alteration of hormone function, the analysis of gain-of-function mutations is expected to be much more specific with respect to their location and the underlying mechanism and therefore more informative in glycoprotein structure-function studies. hTSH with quadruple mutations in the α-subunit (Q13K + E14K + P16K + Q20K) and an additional replacement in the hTSH β-subunit (L69R) showed 95-fold higher potency and >1.5-fold increase in efficacy compared with the in vitro bioactivity of the wild-type hormone (191). These experiments were widely recognized as breaking new grounds concerning the development of glycoprotein hormone agonists with increased activity (121, 164). Subsequent studies involving the combination of these four mutations in the α-subunit with three mutations in the β-subunit (I58R + E63R + L69R) resulted in an analog with greater than 1,000-fold increase in receptor binding and in vitro bioactivity and 100-fold increase in in vivo activity (66). Furthermore, the recently found new "gain-of-activity" mutations (4 in the αL3 loop and 3 in βL1 loop) permit us to select the most optimal combinations of hTSH mutations (107, 186). Such novel TSH analogs are significantly more potent than any known species of TSH and hold great promise as second generation therapeutic forms of rhTSH. They should prove useful in the study of TSH action and may be of therapeutic benefit in stimulating ^{131}I uptake and thyroglobulin secretion inpatients with thyroid cancer. Ultimately, the most suitable in vivo agonist may be designed by a combination of different approaches. However, the final therapeutic utility of such analogs cannot be predicted without carefully designed and conducted clinical trials.

The novel superactive analogs of hTSH (both superagonists and long-acting agonists described below) may be advantageous to stimulate ^{131}I uptake and thyroglobulin secretion in patients with differentiated thyroid carcinoma. In addition, rhTSH and its analogs should prove useful in the stimulation of thyroidal and metastatic tissue before therapeutic ablation with radioactive iodine. RhTSH and its analogs may also help in the detection of suppressed, but functional, thyroid tissue in patients with autonomous hyperfunctioning thyroid nodules or patients on thyroid hormone therapy. Development of superactive analogs of hTSH is expected to result in a new generation of TSH binding inhibition (TBI) assays utilizing both human TSHR and human high-affinity TSH (91). Such assays may avoid a caveat related to the possibility that different species of radiolabled TSH (bovine vs. human) can affect the TBI values. The superagonists of hTSH described above provide a useful tool to study TSH binding and cellular effects in various populations of human lymphocytes, enterocytes, and adipocytes. Such future studies may permit design of analogs with different effects on thyroidal versus nonthyroidal TSHRs.

2. Antagonists

The human TSHR is considered to be the predominant autoantigen in autoimmune thyroid hyperfunction of Graves' disease. Its expression in retroorbital and connective tissue may be, at least in part, responsible for the extrathyroidal manifestations of this disease, such as ophthalmopathy and pretibial myxedema. Moreover, a proposed strategy of blocking thyroid receptor stimulating antibodies in the treatment of Graves' disease justifies the development of potent hTSH antagonists. TSHR antagonists that block the actions of TSHR-stimulating immunoglobulins should be thus of interest for the study and treatment of Graves' disease including its associated ophthalmopathy, for which no satisfactory therapies currently exist. Previous attempts to dissociate binding from signal transduction have been summarized previously (70). More recent efforts using deglycosylation of linked TSH subunits resulted in engineering a potential antagonist with inhibitory effects in the in vitro bioassay (49). Certain peptides encompassing domains of the hTSH subunits show specific TSHR binding at very high concentra-

tions (mM range) but do not generate a signal (130). Recent studies on hTSH α-and β-subunits have shown that mutations in one domain that cause loss of function can be functionally rescued by simultaneous mutations in a spatially unrelated domain (192). This could provide a basis for dissociation of signal transduction and receptor binding and thus overcome the problems associated with the low receptor affinity of existing experimental TSHR antagonists.

3. Inverse agonists (negative antagonists)

Considering the role of constitutive activity in TSHR function, it is clear that the inverse agonist may have several basic and clinical applications. Such an analog with the ability to decrease the number of constitutively active TSHRs should also limit spontaneous fluctuations between the active and inactive states. It may be useful in Graves' disease and serve as a important tool to study receptor function.

C. Perspectives in the Engineering of TSH, Gonadotropins, and Other CKGFs

Proteins such as TSH are engineered with the goal of better understanding the molecular mechanisms of their function as well as creating novel analogs for practical purposes. Such analogs with major increases in receptor binding affinity and signal transduction are unique tools to study both thyroidal and extrathyroidal actions of TSH. Moreover, similar strategies may be employed to design analogs of other members of the CKGF superfamily. From knowledge of the relationship between the structure and function of specific TSH and TSHR domains, predictions and design principles can be derived and used to develop plans for further hormone modifications. Several laboratories are trying to identify small-molecular-weight gonadotropin agonistic molecules (19). To achieve this goal they develop high throughput assays and create small molecules predicted to induce signal transduction without binding to the extracellular domain of the membrane protein. It is expected that such small mimetics could be administered orally and will exert receptor specific effects.

Design of hTSH analogs with specific pharmacological properties provides better understanding of hormone biological activity and specificity, creates useful tools for further studies, and generates potentially valuable pharmaceuticals. Moreover, these studies should provide a rational strategy for the design of similar analogs not only for other glycoprotein hormones but also for other members of the CKGF superfamily.

VII. NEW CONCEPTS AND TECHNOLOGIES

Because the GPCRs are implicated in an estimated 60% of all pharmaceutical interactions, they are obvious targets for development of new drugs. The use of computational analysis to understand how these receptors work should provide new more effective compounds with fewer deleterious effects. Current strategies in pharmaceutical research comprise two methodologically different but complementary approaches for lead finding purposes, namely, the random screening of combinatorial compound libraries and the structure-based effort, commonly termed rational drug design.

An important new development is the determination of the structure of bovine rhododpsin, the first known structure in GPCR superfamily (147). It is expected that structures of other GPCR will follow, and the modeling of receptors with short extracellular domains will be much improved. However, receptors with large extracellular domain containing multiple carbohydrate chains are still posing special challenge for structural determination (160). Therefore, the experimental approaches reviewed in this paper will certainly remain critical in studies of receptor function and analog design.

The structure-based approach is aimed to exploit three-dimensional structure data of the molecular components involved in the molecular recognition event that underlies the attempt to therapeutically modulate the biological function of a macromolecular target with proven pathophysiological relevance for a disease state. In this context, GPCRs constitute the most prominent family of validated drug targets within biomedical research, since ~60% of approved drugs elicit their therapeutic effects by selectively addressing members of that target family. From a three-dimensional structure point of view, these transmembrane signal transduction systems represent the most challenging task for structure determination, which is due to the heterogeneous and fine-balanced environment conditions that are necessary for structural and functional integrity of the receptor protein. Structural studies will require the multidisciplinarity of the applied methodologies. Techniques from bioinformatics and homology-related molecular modeling in combination with tailor-made protein simulation methodologies complement the experimentally derived data should also facilitate the three-dimensional structure generation and structure validation process. Studies on protein function will be largely facilitated by using protein microarrays permitting simultaneous testing of thousands of protein mutants and screening for protein-protein interactions (113).

Future challenges of engineering therapeutic proteins will involve improvements of affinity and target specificity, engineering new functions, alterations of pharmacokinetic properties, and control or reduction of the immunogenicity of modified protein drugs. In particular,

engineering of new functions or specific design of new proteins may evolve from current approaches using chimeras, mutants, and fusion proteins. It is rather unlikely that further progress in protein drugs will be limited by development of many low-molecular-weight chemical compounds. However, synthetic compounds could complement or be incorporated into the analogs of natural or artificial protein drugs. Several recent studies indicate that it is possible to design small molecules or domains of proteins to recover activity associated with specific mutations (54, 71). Analogously to GH receptor, small organic compounds could be designed for TSHR with inactivating mutations that restore binding of hTSH or its analogs.

In recent years, the chemical ligation of unprotected peptide segments in aqueous solution has been established as the most practical method for the total synthesis of native proteins (37). A wide range of proteins has been prepared. The facile access to novel analogs provided by chemical protein synthesis has led to new insights into the molecular basis of protein function in a number of systems. Chemical protein synthesis has also enabled the development of proteins with enhanced potency and specificity as candidate therapeutic agents. However, due to the presence of cystine-knot motif and carbohydrate chains, chemical synthesis of TSH and gonadotropins may not be easily achieved using this technology.

VIII. PERSPECTIVES AND CLOSING COMMENTARY

According to a recent survey, ~100 drugs derived from biotechnology have been approved so far, and several hundred drugs are under development. Lessons learned in clinical trials suggested that many drugs failed because of their low specificity/affinity, short half-lives, or high immunogenicity (119). Combination of high-throughput screening and structure-function guided rational approaches should result in increasing number of engineered protein therapeutics, including engineered protein hormones. Our studies have shown that traditional, hypothesis-driven design of glycoprotein analogs can be even more effective than purely empiric high-throughput methods. At the very least we feel that such rational, structure-based methods should be considered complementary to purely empiric approaches.

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