

Novel Insights into the Molecular Mechanisms of Human Thyrotropin Action: Structural, Physiological, and Therapeutic Implications for the Glycoprotein Hormone Family

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I. Introduction

A. Historical background

THE scientific history of thyrotropin (thyroid-stimulating hormone, TSH) began in the 1920s with the discovery of thyroid-stimulating activity in the pituitary gland (re-

viewed in Refs. 1 and 2). This was followed in the early 1970s by the determination of the primary amino acid sequence of the TSH subunits (3). In the late 1980s, a detailed description of its carbohydrate structures was accomplished (4, 5). The subsequent cloning of the human (h) α -subunit (6) and hTSH β -subunit gene (7–9) as well as the TSH receptor gene (10–13) set the stage for the ensuing progress in studies on hTSH structure-function relationships and enabled the production of recombinant (r) hTSH (14), now in clinical trials for the follow-up of patients with differentiated thyroid carcinoma (15, 16). From the standpoint of basic science, another major breakthrough occurred in 1994 with the elucidation of the structure of the closely related human chorionic gonadotropin (hCG) (17, 18), which showed that the glycoprotein hormones belong to the superfamily of cystine knot growth factors. In addition, the crystallization of the ribonuclease inhibitor with specific structural elements termed leucine-rich repeats (LRR) (19) paved the way for the modeling of the extracellular domain of glycoprotein hormone receptors, as these receptors also contain such LRR (10, 20, 21).

Since the last excellent review on TSH in this journal (1, 2), there has been considerable progress in the understanding of the molecular features and the clinical applications of TSH. This review will focus on the structure-function relationships of hTSH in the context of the glycoprotein hormone family and present current views of the molecular mechanisms of glycoprotein hormone action. It will also discuss the physiological, pathophysiological, evolutionary, and therapeutic implications emerging from this research. Novel approaches in structure-function studies and their implications for the rational design of glycoprotein hormone analogs will be summarized. The concomitant progress made in the chromosomal localization, structural organization, and regulation of the TSH α - and β -subunit genes will not be dealt with here, as this topic has recently been covered in detail (22–26).

B. TSH and the glycoprotein hormone family

TSH is a 28- to 30-kDa glycoprotein produced in the thyrotrophs of the anterior pituitary gland. Its synthesis and secretion are stimulated by TRH and inhibited by thyroid hormone in a classic endocrine negative feedback loop. Differences in the molecular mass of TSH are primarily due to the heterogeneity of carbohydrate chains. In contrast, heter-

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ogeneity of its subunit termini as well as the different extent of deamidation of glutamine and asparagine residues are presumably isolation artifacts (27). TSH controls thyroid function upon its interaction with the G protein-coupled TSH receptor (28–31). 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-triphosphate and diacylglycerol, ultimately resulting in the modulation of thyroidal gene expression (32).

Physiological roles of TSH include stimulation of differentiated thyroid functions, such as iodine uptake and organification, the release of thyroid hormone from the gland, and promotion of thyroid growth (27). It also acts as a thyrocyte survival factor and protects the cells from apoptosis (33), perhaps, as has been shown for hCG, via regulation of p53 and the bcl-2 gene family (34, 35). A further interesting finding is that TSH plays a critical role in ontogeny. In a mouse model with targeted disruption of the common α -subunit gene and thus devoid of circulating glycoprotein hormones, thyroid development was arrested in late gestation (36).

TSH is a member of the glycoprotein hormone family, which also includes pituitary follitropin (FSH) and lutropin (LH), as well as CG, which is produced predominantly by the placenta. TSH, FSH, and LH are found in all mammalian species as well as in lower vertebrates (3, 37). In contrast, CG is only present in higher primates and in the horse. The CG β gene had probably only recently evolved from the LH β gene by a frame-shift mutation with readthrough into the 3'-untranslated region (38). Structurally, the glycoprotein hormones are related heterodimers comprised of a common α -subunit and a hormone-specific β -subunit (3). The common human α -subunit contains an apoprotein core of 92 amino acids including 10 half-cystine residues, all of which are in disulfide linkage. It is encoded by a single gene, located on chromosome 6 in humans, and thus identical in amino acid sequence within a given species (39). The β -subunits can be aligned according to 12 invariant half-cystine residues forming six disulfide bonds. Despite a 30–80% amino acid sequence identity among the hormones, the β -subunit is sufficiently distinct to direct differential receptor binding with high specificity (less than 0.1% cross-reactivity) (3). The glycoprotein hormone β -subunit genes differ in length, structural organization, and chromosomal localization (22–26) (summarized in Table 1). The human TSH β -subunit gene

predicts a mature protein of 118 amino acid residues and is localized on chromosome 1 (27). The fact that human TSH β -subunit isolated from human pituitaries has an apoprotein core of 112 amino acids is most likely related to carboxyl-terminal truncation during purification. In any case, structure-function studies showed that amino acid residues 113–118 are not required for the activity of hTSH, at least for that *in vitro* (40).

An important structural component of these hormones is their carbohydrate moiety, which constitutes 15–35% by weight. The common α -subunit has two asparagine (N)-linked oligosaccharides, and the β -subunit one (in TSH and LH) or two (in CG and FSH). In addition, the CG β -subunit has a unique 32-residue carboxyl-terminal extension peptide (CTP) with four serine (O)-linked glycosylation sites (5, 41, 42). Similar to LH, the oligosaccharides of TSH have unusual structural features, which are found in few other glycosylated proteins, such as POMC (5, 43): pituitary TSH contains significant amounts of sulfate covalently linked to penultimate *N*-acetylgalactosamine (GalNAc) residues. This was shown to be related to the expression of GalNAc-transferase in the anterior pituitary, which appears to require specific amino acid sequences present in the β -subunits of TSH and LH, but not in that of FSH (44). In contrast, therefore, FSH and placental CG possess the commonly found terminal structure of complex oligosaccharides, where sialic acid is bound to penultimate galactose residues. The carbohydrate structures of TSH in comparison to the other glycoprotein hormones are schematically depicted in Fig. 1.

Recently, the crystal structure of partially deglycosylated hCG has revealed two remarkable features, relevant also for the other glycoprotein hormones, which were not predictable from their primary structures (17). First, both α -subunit and hCG β -subunit have a similar overall topology. Each subunit has two β -hairpin loops (*L1* and *L3*) on one side of a central cystine knot (formed by three disulfide bonds), and a long loop (*L2*) on the other. Thus, glycoprotein hormones are now considered to be a group within the expanding superfamily of cystine knot growth factors, which also includes, among many others, transforming growth factor- β (TGF β), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) (45, 46). Such cystine knot growth factors and their corresponding receptors are listed in Table 2. These structural similarities between glycoprotein hormones and other cystine knot growth

TABLE 1. Human glycoprotein hormone subunits

Subunit	Chromosome location (locus)	Gene length (kb)	No. of Exons (introns)	mRNA Length (kb)	No. of amino acids	No. of glycosylation sites (location) ^a
Common α	6 (p21.1-23)	9.4	4 (3)	0.8	92	2 (N: 52, 78) ^b
TSH β	1 (p22)	4.9	4 (3)	0.7	118 (112) ^c	1 (N:23)
LH β	19 (q13.3)	1.5	4 (3)	0.7	121	1 (N:30)
CG β	19 (q13.3)	1.9 ^d	4 (3)	1.0	145	6 (N: 13, 30; S: 121, 127, 132, 138)
FSH β	11 (p13)	3.9	4 (3)	1.8	111	2 (N: 7, 24)

^a Oligosaccharide chains are attached either to asparagine (N) (N-linked) or to serine (S) (O-linked). N or S residues are numbered according to their position in the respective sequence.

^b Free α -subunit may also contain an additional site of O-glycosylation at threonine (T) 39 (231).

^c 118-Amino acid coding region; six amino acids can be cleaved at the C-terminal end.

^d In contrast to all other glycoprotein hormone subunit genes which exist as a single copy, hCG β is encoded by a cluster of six genes which vary in length (173).

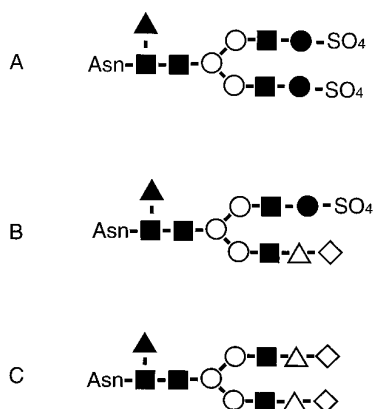


FIG. 1. Asparagine (N)-linked oligosaccharides of TSH. The sulfated biantennary structure (A) represents the typical oligosaccharide chain of pituitary bTSH and bLH. The sulfated and sialylated oligosaccharide (B) is more typical of pituitary hTSH and hLH (4, 5). The sialylated nonsulfated structure (C) represents that of rhTSH expressed in CHO cells. Similarly, pituitary hFSH as well as placental hCG are exclusively sialylated (4, 5). Carbohydrate residues are marked as follows: mannose (○), *N*-acetylglucosamine (■), *N*-acetylgalactosamine (●), fucose (▲), galactose (△), sialic acid (◇).

factors may relate to the recently described nonclassic actions of glycoprotein hormones, or their subunits (47–49). Second, the crystal structure showed that the hCG β -subunit contains an unusual segment, termed the “seat belt” region, that wraps around the α -subunit while remaining covalently linked to the β -subunit through disulfide bonds.

A homology model of hTSH, based on crystallographic data of hCG, indicated similarities in the overall conformation of these two hormones (50). This supported earlier predictions that the members of the glycoprotein hormone family adopt a similar general structure. The basis for this postulate was the observation that all glycoprotein hormone β -subunit cysteine residues, which determine the three-dimensional structure by predicating their folding, are conserved. Moreover, direct experimental evidence comes from a recent study using double alkylation of the TSH β -subunit, which showed that formation of the disulfide bonds in the TSH β -subunit was identical to those in the crystal structure of hCG (51). Although such structural resolution at the molecular level can help to predict protein interactions, the importance of particular structures or amino acid residues in the dynamic multistep process of receptor binding and signal transduction can only be proven in functional studies. Nevertheless, homology models of hTSH and its mutants have shown promise not only for the design of hormone modifications but also for more rational data interpretation (50).

Traditionally, structure-function relationships of human glycoprotein hormones have been predominantly performed with gonadotropins, particularly hCG (41–44, 52–61). This was mostly because hCG purified from urine was readily available and because of the early cloning of the hCG β -subunit genes reflecting their relative abundance in the placenta (24, 38). Studies on hTSH, in contrast, were hampered by the difficulties in isolating sufficient amounts of hTSH from the pituitary and later by limitations of rhTSH expression after the cloning of the 2-kb hTSH β -subunit gene fragment (9, 62). Several recent developments have greatly facilitated hTSH

structure-function analysis: availability of rhTSH (14), construction of a 981-bp hTSH β -minigene from the original 2-kb fragment (63), cloning of the hTSH β -subunit cDNA (M. Grossmann, M. W. Szkudlinski, and B. D. Weintraub, unpublished data), development of suitable hTSH expression systems using eukaryotic cells (14, 50, 63, 64), and the cloning of the TSH receptor cDNA (10–13). The ensuing progress in understanding hTSH action at the molecular level has highlighted unique features of hTSH, which set this hormone apart from other members of the glycoprotein hormone family. In addition, this progress has helped to understand common principles of glycoprotein hormone action.

II. Structure-Function Relationships of TSH in Relation to Studies on Gonadotropins

A. Methodological considerations

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. Structure-function studies on glycoprotein hormones can be categorized into studies on the carbohydrate moiety as well as on the peptide portion, but the two approaches can also be combined (Table 3). In general, each of these methods has its advantages, which are balanced by inherent limitations. Initially, physicochemical and enzymatic studies have identified amino acids, as well as carbohydrate portions, on both subunits that contribute to receptor binding and signal transduction. These approaches, which are summarized in excellent reviews (41, 42, 52–54, 56, 57, 59, 60), 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 (55, 58, 61, 65, 66).

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 classic method of alanine scanning (67, 68) 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 helical 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 (69). Specifically, α -helical structures were found to be strongly kinked and destabilized after the introduction of proline residues (70), in contrast to alanine substitutions, which tend to preserve the α -helix. 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” that al-

TABLE 2. Cystine knot growth factors and their receptors

		Bioactive form	Specific receptor
I.	Glycoprotein Hormones ^a		GPCR ^a
	TSH	α -TSH β heterodimer	TSH-R
	CG	α -CG β heterodimer	CG/LH-R
	LH	α -LH β heterodimer	CG/LH-R
	FSH	α -FSH β heterodimer	FSH-R
	α -Subunit	(?)	(?)
	CG β -Subunit	(?)	(?)
II.	PDGF Family		Trk
	PDGF-AA	Homodimer	PDGF-R α
	PDGF-BB	Homodimer	PDGF-R β
	PDGF-AB	Heterodimer	PDGF-R α
	VEGF ^a	Homodimer	FLT-1, KDR
III.	Neurotrophin Family		Trk
	NGF	Homodimer	A
	BDNF	Homodimer	B
	NT-3	Homodimer	C
	NT-4	Homodimer	B
IV.	TGF- β Family		Ser/Thr rk
	TGF- β 1-5	Homodimer	I, II
	Inhibin A ^a	α - β A Heterodimer	I, II
	Inhibin B ^a	α - β B Heterodimer	I, II
	Activin A ^a	β A- β A Homodimer	I, II
	Activin B ^a	β B- β B Homodimer	I, II
	Activin AB ^a	β A- β B Heterodimer	I, II

Abbreviations: GPCR, G protein-coupled receptor; Trk, receptor tyrosine kinase; Ser/Thr rk, serine/threonine receptor kinase; LRR, leucine-rich repeats; FLT, fms-like tyrosine kinase; KDR, kinase domain receptor; 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. Table derived in part from Sun and Davis, 1995 (45).

^a Glycosylated.

low introduction of nonconservative changes into hormones, thus enabling modulation of function without compromising protein synthesis (46, 50). 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 for by alterations in an unrelated domain (69). Such studies predict that the TSH receptor is capable of tolerating ligands with significant structural modifications, by means of an "analog-induced fit." It may even be possible, therefore, to create alternative contact domains of analog and receptor that are still able to transduce a signal. Such plasticity of ligand-receptor interactions is supported by the observation that the hTSH receptor can be constitutively activated by multiple mutations in various receptor regions (29). Moreover, identification of cooperative, noncooperative, and mutually exclusive hormone domains can provide important leads for further development of therapeutically useful hormone analogs.

It should be pointed out that, as with other approaches, these recombinant techniques are not without 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 re-

sult in distant conformational effects that may alter hormone function in an indirect fashion. This is especially possible if secretion or receptor binding properties of mutated analogs are 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 quantification 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 circular dichroism spectroscopy.

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 is more complex and has so far received only scant attention (71). 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.

B. Structure-function studies of protein domains

Multiple domains of both the α - and β -subunits have been shown to be important for heterodimer assembly, secretion, and bioactivity of the glycoprotein hormones. Among these regions, several segments that are highly conserved among different species have been confirmed to be particularly important for receptor binding and bioactivity of hTSH by a variety of different approaches. Whereas Fig. 2 summarizes

TABLE 3. Structure-function methodology

I. Recombinant DNA Techniques
Alanine scanning
Proline/alanine scanning
Conservative and nonconservative substitutions
Individual residues
Cassette mutagenesis
Multidomain mutagenesis
Parallel ligand-receptor mutagenesis
Evolution-guided mutagenesis
Gene fusion
Addition of carboxyl-terminal extension
Joining of individual subunits
Creation of chimeric hormones
Between different species
Between different subunits
Elimination or addition of glycosylation sites
Expression of hormone analogs in heterologous cell types
Mammalian
Insect
Prokaryotic
Genetic manipulation of host cell machinery
II. Nonmolecular Approaches
Enzymatic digestion (complete or sequential)
Amino-, carboxy- and endopeptidases
Endo- and exoglycosidases
Physicochemical modifications of amino acid or carbohydrate residues
Creation of hormone hybrids by dimerization of individual subunits from different species, or after selective modifications
Immunological studies/epitope mapping
Synthetic peptides

the results from site-directed mutagenesis studies in the linear subunit gene sequences, Fig. 3 shows the topical relations of identified domains in a hTSH ribbon model based on the structure of hCG (17, 18).

These domains are: α 11–20 in the β -hairpin α L1-loop (50), α 33–38 (72), the α -helix α 40–46 (65, 69, 72, 73), the oligosaccharide chain at α -asparagine⁵² (74, 75), the α -carboxyl-terminal residues α 88–92 (64, 65, 76, 77), and, in the TSH β -subunit, TSH β 58–69 in the β -hairpin β L3-loop (77a), and the seat belt TSH β 88–105 consisting of the determinant loop TSH β 88–95 and a carboxyl-terminal segment TSH β 96–105 (78). At the same time, most, but not all, of these domains appear to be also critical for TSH heterodimer formation or secretion. Under otherwise identical conditions, cells transfected with many of these mutant genes secrete lower amounts of hTSH-related immunoreactivity compared with cells secreting wild type hTSH. The underlying mechanisms have not been elucidated in detail and could be related to altered stability of mRNA, effects on subunit folding, subunit assembly, or stability of the heterodimeric protein. Most of these domains have also been recognized to be important for receptor binding and activation of the gonadotropins (79–91). Identification of such functionally similar domains indicates that the underlying mechanisms of signal transduction are common among the glycoprotein hormones, which is to be expected in light of their overall homology as well as their common evolutionary origin.

1. Common α -subunit domains. Despite the general importance of these α -subunit domains (Figs. 2 and 3) in glycoprotein hormone activity, recent studies on hTSH have revealed im-

portant differences in the role of certain domains for hTSH compared with hCG and hFSH. Coexpression of selected mutant α -subunits with the β -subunits of hTSH, hFSH, and hCG showed that specific residues within the α 33–38 domain played strikingly different roles for glycoprotein heterodimer secretion. In light of the high degree of structural and functional homology, these differences were surprising: for example, an α -subunit in which α -alanine³⁶ was replaced by glutamic acid was not able to form a dimer with the hCG β -subunit, whereas this mutated α -subunit combined efficiently with the hTSH β -subunit to give rise to a bioactive heterodimer (72). Alanine scanning showed that residues α -phenylalanine³³ and α -arginine³⁵ were critical for hCG, but not hTSH, receptor binding (72). Conversely, *en bloc* alanine replacement of the surface exposed positively charged α -helical fragment α -arginine⁴²-serine⁴³-lysine⁴⁴ reduced hTSH, but not hCG, activity (72, 86, 92). Similarly, the α -asparagine⁵² oligosaccharide played opposite roles for hCG and hTSH signal transduction, as outlined below (74, 81). In addition, a single amino acid, the ultimate α -serine⁹², was identified to play an important role for heterodimer secretion, receptor binding, and bioactivity of hTSH, but not for that of hCG or hFSH (64, 85, 93). This observation explains the evolutionary constraint to preserve this residue in CG, LH, and FSH, because the α -subunit is encoded by a single gene (39). A study using overlapping α -subunit peptides also showed that α 26–46 and the α -carboxyl-terminus α 81–92 were important receptor-binding domains of hTSH (65), illustrating the validity of both complementary approaches. However, a comprehensive study using alanine-substituted peptides encompassing the α 26–46 region identified specific residues important in receptor binding (73), only some of which were confirmed by creation of the corresponding hTSH mutants with site-directed mutagenesis (72). Thus, such comparisons indicate that the effect of a substitution of an amino acid within a linear, structurally not constrained peptide may not always be comparable to the same substitution within the context of the heterodimeric hormone.

In addition to these differences in the importance of such common α -subunit regions for TSH activity compared with the gonadotropins, there are also similar roles of these domains for the activity of all members of the glycoprotein hormone family. Thus, truncation of three or more residues from the α -carboxyl terminus eliminates the activity of hTSH, hCG, and hFSH almost entirely (64, 76, 77, 84, 85). Moreover, a combination of alanine/proline scanning revealed that several residues of the α 40–51 region were critical for both hTSH and hCG (α -proline³⁸, α -lysine⁵¹), although the role of some residues appeared to be hormone-dependent (α -phenylalanine³³, α -arginine³⁵, α -alanine³⁶, α -arginine⁴²-serine⁴³-lysine⁴⁴, α -leucine⁴⁸) (69, 72, 86).

The α 11–20 region contains a cluster of basic residues in all vertebrates except hominoids and forms a previously unrecognized domain with the ability to potentiate receptor binding and signal transduction, as well as an important motif in the evolution of glycoprotein hormone bioactivity (Table 4 and Ref. 50). In contrast to the above domains, α 11–20 is not highly conserved among the species and is a modification-permissive site. Hence, this region allows amino acid substitutions with no or minimal effect on hor-

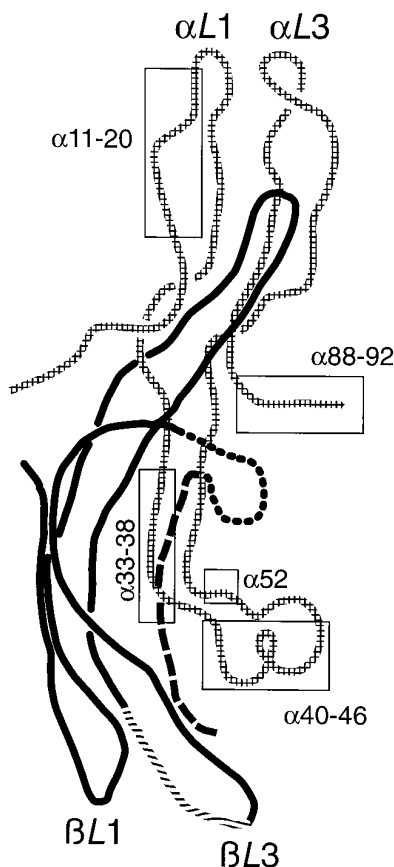


FIG. 3. hTSH ribbon homology model showing domains important for hTSH activity. The schematic drawing of hTSH is based on a molecular homology model of hTSH (50), built on the template of an hCG model derived from crystallographic coordinates obtained from the Brookhaven Data Bank (17). The two β -hairpin loops ($L1$, $L3$) in each subunit are marked. Each subunit also has a long loop ($L2$), which extends from the opposite side of the central cystine knot. The $\beta L2$ loop corresponds to the "Keutmann loop" of the hCG β -subunit, whereas the $\alpha L2$ loop has a peripheral α -helical structure (residues $\alpha 40-46$). Depicted are the topological relationships of regions important for hTSH receptor binding and activity. The α -subunit is shown as a checkered, and the β -subunit as a solid, line. The functionally important α -subunit domains are boxed. Important domains of the β -subunit are marked directly within the line drawing: the TSH β -subunit 58–69 region in the $\beta L3$ -loop is depicted by the crossed line. The seat belt region between the 10th (C88) and 12th (C105) cysteine is highlighted as follows: the N-terminal part of the seat belt, the "determinant loop" (C88–C95 in the hTSH β -subunit), corresponds to the beaded line, and the carboxyl-terminal segment (C95–C105 in the hTSH β -subunit) corresponds to the dashed line. Because the carboxyl terminus beyond hCG β 111 was not traceable in the original electron density map (17), the hTSH β -subunit is only drawn to the corresponding residue 106. Because of deglycosylation of hCG before crystallization, the large and flexible oligosaccharide side chains are not shown. The α -asparagine⁵² carbohydrate (origin marked) is predicted to project away from the protein backbone into the proposed receptor-binding domain (116), which also includes the $\alpha 40-46$ helix and the α -carboxyl-terminus $\alpha 88-92$ (17, 18). In contrast, the $\alpha 11-20$ domain is not located in proximity to the hTSH β -subunit seat belt.

various combinations, increased the potency and efficacy of hTSH and hCG mutants. Most notably, each mutation to a lysine residue in the $\alpha 11-20$ region caused a substantial increase in activity, but alanine mutagenesis of these residues in the hTSH did not significantly alter hormone activity,

indicating that only the selective reconstitution of basic amino acids was functionally significant (50). Moreover, the substitution of α -serine⁴³ to arginine (69) and replacements of α -histidine⁹⁰ and α -lysine⁹¹ (64) either decreased or did not change TSH activity. Thus, introduction of basic residues does not uniformly lead to an increase of hormone activity, but the importance of such basic residues varies depending on their location within the molecule.

2. *TSH β -subunit domains.* In contrast to these well defined α -subunit domains, until recently, little was known about the contribution of the hTSH β -subunit to receptor binding and signal transduction. A synthetic peptide approach spanning the entire TSH β -subunit showed that a TSH β -carboxyl-terminal peptide $\beta 101-112$ possessed the highest TSH receptor-binding activity. Moreover, peptides $\beta 71-85$, $\beta 31-45$, $\beta 41-55$, and $\beta 1-15$ were also active (94). Site-directed mutagenesis indicated that amino acids 113–118 were not important for the *in vitro* activity of hTSH (40). Alanine cassette mutagenesis revealed that the hTSH β -subunit sequence (cysteine⁸⁸-cysteine¹⁰⁵ in hTSH β) was required for high-affinity TSH receptor binding (78). Further, replacing the entire seat belt of hTSH with the corresponding sequence of hCG, conferred full hCG receptor binding affinity and activation to the hTSH/hCG seat belt chimera, whereas TSH receptor binding and activation were abolished (78). This is compatible with earlier findings that the seat belt can determine glycoprotein hormone specificity (83, 90, 95). In contrast, introduction of the hFSH seat belt residues into hTSH did not confer any follitropic activity to the hTSH/hFSH chimera, and its thyrotropic activity was only slightly reduced (78). This may be due to the fact that the net charge of the seat belt is similar in hTSH and hFSH (-2 and -3), but different from hCG ($+1$). Interestingly, however, exchanging other regions of charge divergence between hTSH- β and hFSH- β , $\beta 44-52$ and $\beta 105-112$, did not confer follitropic activity to hTSH (78). It thus appears that charged residues are important for hCG specificity *vs.* hTSH or hFSH, but other as yet unrecognized domains may contribute to the specificity of hTSH and hFSH.

Another functionally important domain in the hTSH β -subunit was recently identified by focusing on regions of nonhomology between the different human β -subunits. In this respect, targeting of residues with charge differences is of particular interest, as basic residues have been implicated to play a role in receptor binding and activation of TSH, as described above (50, 96). Such nonconserved regions of the β -subunits could be involved in regulating glycoprotein hormone specificity or may represent modification-permissive domains generally important for signal transduction, which diverged during evolution of the different β -subunits. If the latter was true, these would constitute regions in which site-directed mutagenesis may be useful to specifically alter hormone activity and therefore would be of primary interest for the generation of hormone analogs. Using this approach, a novel domain within the β -hairpin $\beta L3$ loop of the hTSH- β subunit was identified that appears to modulate hTSH receptor binding and signal transduction (77a). Sequence comparison of hCG and hTSH β -subunits showed a region (residues 58–69 of the TSH- β subunit) that contains a cluster

TABLE 4. Amino acid sequence alignment of various vertebrate α -subunits in the α L1 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	-	-	-	-	-	-	-	-	-	-	-	-
Rhesus	-	K	P	R	-	-	K	-	-	-	K	-	-	-	-	-	Y	-	-
Marmoset	-	K	-	K	-	-	K	Y	-	-	R	L	-	-	-	-	-	-	-
Bovine	-	K	-	K	-	-	K	Y	-	-	K	-	D	-	-	-	Y	-	-
Ovine	-	K	-	K	-	-	K	Y	-	-	K	-	D	-	-	-	Y	-	-
Equine	-	K	-	R	-	-	K	Y	-	F	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	-	-
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	-	-
Salmon	-	K	-	K	-	-	K	V	-	-	N	-	-	-	-	-	-	-	-
Catfish	-	K	-	K	-	-	N	I	-	-	K	-	-	-	-	-	-	-	-
Tuna	-	-	-	K	K	-	N	V	-	-	R	D	R	-	-	-	Y	-	-
Yellowfin	-	-	-	R	K	-	T	V	-	-	R	D	-	-	-	-	-	-	-
Bass	-	-	-	R	K	-	S	V	-	-	R	D	R	-	-	V	Y	-	-
Carp	-	K	-	K	-	-	N	I	-	-	K	-	-	-	-	-	-	-	-
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.* (50).

of basic residues in hCG, but not in hTSH (net charge +2 in hCG *vs.* 0 in hTSH). This domain is located peripherally within the β -hairpin β L3-loop and appears surface-exposed in the crystal structure in hCG. Interestingly, epitope-mapping studies of hCG/hCG receptor complexes had suggested that this region may be in direct contact with the hCG receptor (97, 98). Analogous to previous studies of the α -subunit 11–20 domain, introduction of single and multiple basic residues into this hTSH β -subunit domain led to additive, substantial increases of TSH receptor binding affinity as well as intrinsic activity.

C. Structure-function studies of carbohydrate chains

The oligosaccharide moieties assume importance in every aspect of the life span of TSH, from early translational events during biosynthesis to its removal from the circulation and degradation. The specific functions of the oligosaccharides change as the hormone travels through distinct intracellular compartments during its synthesis, as well as after secretion. Overall, the carbohydrates serve comparable functions among the members of the glycoprotein hormone family (5, 41, 42, 99). However, more recent work has shown that, in certain cases, the oligosaccharides have unique side-chain and residue-dependent roles for hTSH, which are different from those for the gonadotropins. Studies on oligosaccharides of individual hormones are therefore, by analogy to those of the protein component, important to recognize the hormone-specific roles of these structures. Moreover, they can have substantial implications for the design and production of clinically useful glycoprotein hormone analogs. This is especially relevant because an understanding of their function offers the possibility to modify them in a rational fashion using recombinant DNA methodology and heterol-

ogous cell expression (100, 101). Indeed, several studies have demonstrated that bioreactor conditions or cell culture techniques can affect the carbohydrate structures of cell culture-derived glycoproteins including hTSH (100–102).

1. Postranslational modifications and intracellular processing. Various methods have been used to study the functional role of the oligosaccharides for TSH and the other glycoprotein hormones in experimental settings, including physicochemical, enzymatic, and molecular methods (Table 3). Similar to findings for other members of the glycoprotein hormone family, the cotranslational attachment of the oligosaccharides which protects the nascent polypeptide from intracellular degradation is essential for the subunit folding and combination of TSH and is necessary for the secretion of the mature hormone from the cell.

In the endoplasmatic reticulum, high mannose type oligosaccharides are transferred onto an asparagine residue with the recognition sequence asparagine-x-serine/threonine (where x is any amino acid except for proline, and other local structural restrictions that determine enzyme accessibility may apply). Subsequently, the oligosaccharides are partially trimmed by glycosidases, such as Mannosidase I and II (103). In the endoplasmatic reticulum, oligosaccharides are believed to stabilize a conformation that facilitates disulfide bond formation and are hence important for proper subunit folding. Moreover, the carbohydrates are part of a quality control program that ensures correct posttranslational processing. Thus, molecular chaperones have been identified that retain glycoproteins in the endoplasmatic reticulum until proper trimming of the carbohydrates has been accomplished. Only then are the nascent glycoproteins released to the next compartment/chaperone in the postranslational cascade (104). Incubation of mouse pituitary cells

with tunicamycin, an inhibitor of oligosaccharide attachment during translation, led to aggregation and intracellular degradation of TSH (105). Similarly, folding kinetics and disulfide bond formation of the hCG- β subunit lacking carbohydrate consensus sequences were delayed, leading to slow secretion and partial intracellular retention and degradation of the hCG β -subunit (106, 107). Even the selective disruption of single glycosylation sites using site-directed mutagenesis caused significant decreases of hTSH secretion from transiently transfected Chinese hamster ovary (CHO) cells (62, 74).

In the Golgi apparatus, the carbohydrates are further trimmed and subsequently processed to mature complex oligosaccharides by sequential addition of carbohydrate residues catalyzed by various specific glycosyltransferases (5, 103). In this compartment, the oligosaccharides assume a critical role for intracellular translocation and direct the transport of the glycoproteins to specific cell compartments.

2. *Intrinsic activity.* After secretion from the cell, the carbohydrates become important for the intrinsic activity, plasma half-life, and final *in vivo* activity of TSH. Earlier studies on gonadotropins and bovine TSH using chemical and enzymatic deglycosylation as well as hybrid studies had shown that the oligosaccharides, and predominantly those of the α -subunit, are necessary for full *in vitro* activity of these hormones (108–112). In contrast to their critical role in receptor activation, they play a much less important role for high-affinity receptor binding. Thus there is a consensus that carbohydrates affect signal transduction predominantly at a post receptor-binding step. In fact, deglycosylated hCG acted as a competitive antagonist in certain *in vitro* assays (111, 112). By comparison, hTSH was shown to retain higher residual intrinsic activity upon deglycosylation (113–115).

In the absence of structural information on ligand-receptor complexes, the precise molecular basis of how carbohydrates contribute to TSH activity remains unclear. In this respect, it should be emphasized that because of the difficulty in obtaining high quality crystals of intact glycoproteins due to the microheterogeneity and relative flexibility of the oligosaccharide conformations, hCG was partially deglycosylated with hydrogen fluoride before crystallization (17). It is important to bear in mind that deglycosylated hCG acts as a competitive receptor antagonist, and the carbohydrates may be important to stabilize the active conformation of the hormone (see below). Therefore, it is not known how the structure of a fully agonistic hormone compares with the reported crystal structure. Recent structural analysis of the oligosaccharides of ^{13}C , ^{15}N -enriched recombinant hCG by nuclear magnetic resonance suggested that the α -subunit carbohydrates do not interact with the protein backbone, but project outward into solution. Furthermore, the carbohydrates exist in an extended conformation with significant internal motion and have considerable conformational freedom (116).

Whereas one of the more recent models suggests that the carbohydrates appear to affect signal transduction primarily by their bulk (97, 98), other studies indicate that additional features, including specific carbohydrate-receptor interactions, may also be important. For example, sequential enzymatic deglycosylation of hTSH and its expression in glyco-

sylation mutant cell lines, combined with site-directed mutagenesis, suggested that the terminal sugar residues, especially negatively charged sialic acid residues, critically affect the role of a carbohydrate side chain (74, 110, 117–119). Arguments in favor of a direct interaction of the carbohydrates with the receptor stem from the demonstration of oligosaccharide or glycopeptide binding to corpus luteum slices expressing the CG/LH receptor (120). In this respect, it has been pointed out that a segment of the extracellular domain of the LH/CG receptor shares considerable sequence identity with a domain of the *Dolichos biflorus* seed lectin as well as the soybean agglutinin (20). However, at least for TSH, an indirect mechanism involving a conformational change and/or aberrant ligand binding appears more likely as this lectin-like component identified in the hCG receptor is not present in the hTSH receptor (42). A possible role of the carbohydrates in maintaining glycoprotein hormones in a conformation able to activate the receptor is supported by the observation that certain antibodies can convert receptor-bound deglycosylated CG from an antagonist to an agonist (121). Several studies suggested that deglycosylated hormone does not elicit a signal because it binds to the receptor in an aberrant fashion. Thus, it was observed that deglycosylated hCG binds to different domains of the CG/LH receptor from native hCG (122). Further, there is evidence of differences in antibody accessibility of receptor-bound native and deglycosylated hCG (123).

The use of site-directed mutagenesis in combination with expression in glycosylation mutant cell lines (74), as well as the expression of hTSH in insect cells using a baculovirus system (124), have emphasized unique roles of individual side chains for hTSH activity. From these and studies using dimerization of heterologous subunits (110) and sequential enzymatic digestion (119), it appears that the roles of the terminal sialic acids as well as of individual oligosaccharides are different for the *in vitro* activity of hTSH compared with hCG and hFSH. This indicates that conserved structures within the context of a given ligand-receptor complex may contribute to signal transduction in different ways. In hCG, which is exclusively sialylated, sialic acid is required for full expression of *in vitro* activity (111, 125). In hFSH, which is predominantly sialylated, removal of sialic acid residues does not change *in vitro* activity (126). However, if hTSH or LH, which contain significant amounts of sulfated GalNAc termini (4) when produced in the pituitary thyrotroph, are expressed in CHO cells that produce exclusively sialylated termini, *in vitro* activity is attenuated (127, 128). Studies using site-directed mutagenesis of individual glycosylation recognition sites showed that the oligosaccharide at α -asparagine⁵², but not the one at α -asparagine⁷⁸, was necessary for hCG and hFSH action (81, 88, 89, 129). In contrast, the α -asparagine⁵² chain and specifically its terminal sialic acid residues markedly attenuated TSH receptor binding and activation (74). As posttranslational modifications of carbohydrates regulate glycoprotein hormone activity in normal physiology (1, 5, 42, 43), modulation of terminal sialylation of the α -asparagine⁵² oligosaccharide, which appears more heterogeneous than other side chains (56), may thus be important in regulating activity in a hormone-specific manner. Interestingly, deletion of this α -asparagine⁵² side

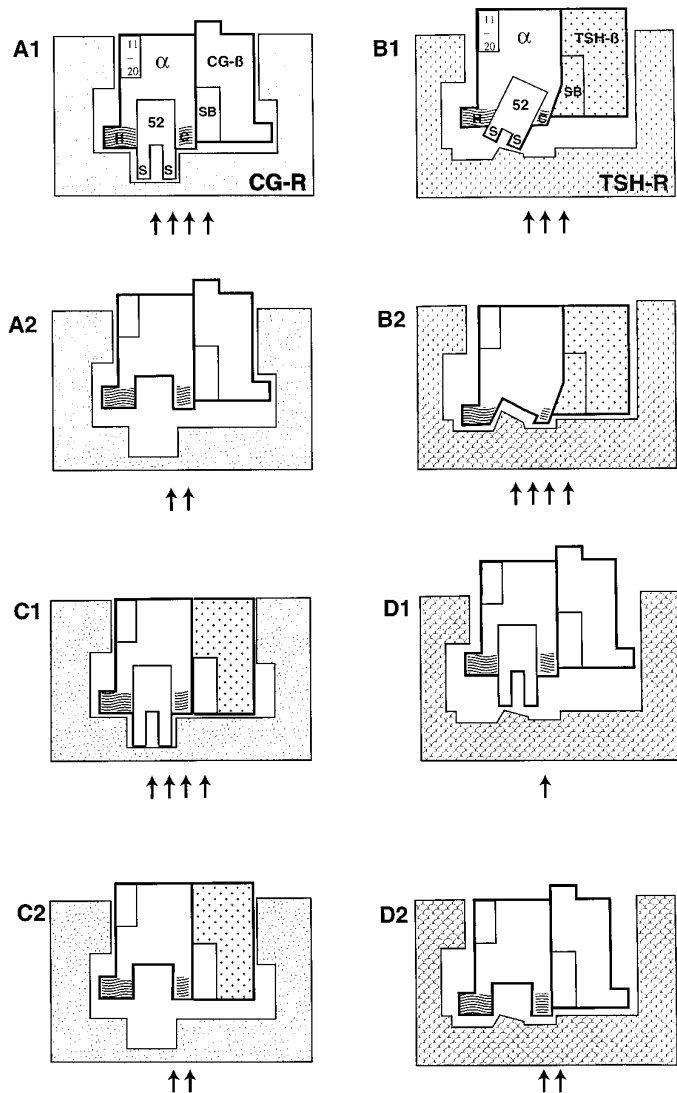


FIG. 4 Mechanistic model depicting cooperation of individual hTSH domains in receptor activation: role of the sialylated α -asparagine⁵² oligosaccharide chain in glycoprotein hormone receptor activation. This mechanistic model of glycoprotein hormone receptor interaction highlights possible explanations for the differential role of the α -asparagine⁵² oligosaccharide and other domains in hTSH receptor binding and activation. This simplified scheme does not reflect the dynamic, multistep interaction between ligand-receptor interaction in three-dimensional space, nor does it predict the nature of subsequent events after ligand receptor interaction that lead to G protein coupling. The model allows for reconciliation of the following findings from site-directed mutagenesis studies: deletion of the α -asparagine⁵² chain decreases hCG activity at the CG/LH receptor (81), increases hTSH activity at the TSH receptor (74), decreases hCG activity at the TSH receptor (74), and decreases the activity of a hTSH/hCG seat belt chimera at the CG/LH receptor (78).

Panel A 1 shows interaction of hCG with the CG/LH receptor. The carbohydrate at α -asparagine⁵², which is important for hCG action, interacts with the receptor either directly or indirectly, *e.g.*, by favorably influencing the spatially related α -helix and the α -carboxyl-terminus, also important for receptor activation. Also shown is a second receptor-binding domain, α 11–20. Upon deletion of the α -asparagine⁵² chain (A 2), activity is reduced (81) either because of the lack of direct interaction of this chain with the CG/LH receptor or by a conformational effect.

chain increased the weak inherent thyrotropic activity of hCG, opposite to the effect at its native receptor (74). Thus, as shown in Fig. 4 (and see below), the differential role of this oligosaccharide chain suggests that its composition, sialic acid/sulfate-dependent negative charge and possibly spatial orientation are critically important not only for signal transduction, but also for the specificity of ligand-receptor interaction, at least for that of hTSH.

3. *Clearance and in vivo activity.* Finally, the oligosaccharides play an important role in tissue targeting and clearance mechanisms and thus modulate circulating hormone levels and biopotency *in vivo*. In general, carbohydrate-mediated effects on clearance are more important than that on intrinsic activity, and even relatively minor changes in clearance can supersede those observed for the *in vitro* activity. In fact, carbohydrates can modify *in vitro* and *in vivo* activity in opposite directions. For example, enzymatically desialylated (asialo)-rhTSH had a 5- to 10-fold higher *in vitro* activity than sialylated rhTSH. However, asialo-rhTSH was cleared significantly faster than rhTSH and exhibited a 5- to 10-fold lower *in vivo* activity (130). Similarly, hTSH expressed in insect cells, which produce glycoproteins with high-mannose chains (131–133), had a higher *in vitro* but lower *in vivo* activity due to rapid clearance compared with hTSH from CHO cells (124). Moreover, a hTSH mutant lacking the α -asparagine⁵² oligosaccharide was more active *in vitro*, but was cleared faster and therefore was less active *in vivo* than the fully glycosylated hormone (74).

Panel B 1 shows the interaction of hTSH with its receptor. In hTSH, the seat belt has a different conformation than in hCG β , which in turn leads to a different spatial orientation of the α -asparagine⁵² chain. In contrast, the orientation of the distant α 11–20 domain, which is equally important for the receptor binding of hCG and hTSH (50), is not affected by the seat belt. Note that the carbohydrate does not allow for optimal interaction of the hormone with the receptor. Rather, it restricts interaction of other activation domains, such as of the α -helix as well as the α -carboxyl-terminus with the receptor. Thus, deletion of α -asparagine⁵² oligosaccharide (B 2) increases receptor activation (74).

Panel C 1 shows how the hTSH/hCG seat belt chimera is able to activate the CG/LH receptor similar to hCG. Since the seat belt residues are identical to those of hCG, the orientation of the α -asparagine⁵² chain is thus similar to its orientation in hCG and allows for efficient receptor interaction. Similar to hCG itself, the deletion of the chain reduces receptor activation (C 2; Ref. 74).

Panel D 1 shows that the α -asparagine⁵² oligosaccharide interferes with the ability of hCG to activate the TSH receptor. Deletion of the chain increases the ability of hCG to bind to the TSH receptor, similar to hTSH (D 2, 74). Deletion of the α -asparagine⁵² chain allows other functionally important domains of the composite receptor interaction site, *e.g.*, α -helix and/or the α -carboxyl terminus to better interact with the receptor. This could be due to a direct role (lack of repulsion of negatively charged sialic residues from negatively charged receptor interface) or an indirect role of the carbohydrate (change of conformation of the composite domain). This repulsion may be more prominent at the TSH receptor, as the hCG receptor possesses an overall higher number of positive charges in its extracellular domain. Thus, the α -asparagine⁵² chain, in addition to attenuating activity of hTSH, acts as a negative specificity determinant, restricting the inappropriate interaction of hCG with the TSH receptor. H, α -helix, C, a carboxyl terminus; 52, α -asparagine⁵² oligosaccharide chain; S, sialic acid; SB, seat belt. The relative degree of receptor activation is indicated by the arrows.

In analogy to what was observed for intrinsic activity, the specific carbohydrate structure at different glycosylation sites may affect hormonal clearance to a different degree. It was shown that the peripherally located single carbohydrate chain of the TSH β -subunit appears to be the most important in determining the MCR of hTSH (110), whereas the α -asparagine⁷⁸ chain is more critical than the α -asparagine⁵² chain in this respect (74). Similar findings for the relative roles of individual carbohydrates for clearance have also been reported for hFSH (129). An important lesson to be learned from such findings is the lack of direct correlation between the effects of carbohydrates on *in vitro* and *in vivo* activities of glycoproteins. This fact is a consequence of the fundamental difference between a hormone-specific interaction with the target organ receptor and carbohydrate-dependent clearance mechanisms determining the circulatory half-life of a given glycoprotein. Such studies highlight the difficulties of translating results obtained using *in vitro* systems into whole organism physiology and illustrate the importance of determining the activity of glycoprotein hormone analogs in adequate animal models.

III. Current Understanding of TSH/Glycoprotein Hormone Action

A. Structural considerations

Proposed models of glycoprotein hormone action have greatly benefitted from the structure of hCG (17, 18), which is also suitable for testing these models prospectively. However, due to the absence of structural data on glycoprotein receptors, the precise molecular mechanisms of glycoprotein hormone signal transduction remain largely unknown. Despite the recent availability of relatively large amounts of pure protein using systems such as baculovirus-based insect cell expression, high resolution structure of any G protein-coupled receptor has not yet been obtained. This is in part due to the difficulties in obtaining crystals that include the transmembrane domain. Furthermore, the large carbohydrate component of the extracellular domain of the glycoprotein hormone receptors makes this task even more difficult. Ultimately, crystal structures of receptor-ligand complexes should directly identify domains of the hormone that contact the receptor. Understanding in molecular detail how glycoprotein hormones interact with their receptor and how the signal is subsequently transmitted to the G proteins will require three-dimensional structures of all the components as well as identification of their conformational changes upon activation.

Even though cocrystallization can map the topography of complementary surfaces of ligand and receptor, functional analysis of such contacts will be necessary. For example, crystallization of the GH-receptor complex showed a large ligand receptor interface. However, solely systematic site-directed mutagenesis of GH revealed that of the residues that contacted the receptor, only a small set was actually important in maintaining high-affinity interaction. The functional relevance of individual residues did not correlate with the extent to which their side chains were buried at the interface of the crystal complex and was therefore not predictable from

the structure (67, 68). Conversely, due to the dynamic nature of ligand-receptor interactions, deletion of protein domains that do not contact the target in cocrystallization studies can, in certain instances, still be important for signal induction (134). In fact, there are many examples in the literature showing that protein functions are influenced by residues far from active sites (135).

In this context, it should be emphasized that glycoprotein hormones range among the largest (28–34 kDa) and most complex naturally occurring ligands. In addition, their receptors are notable for a large extracellular domain that is unusual for G protein-coupled receptors. This extracellular part is encoded by several exons (21, 28, 30, 31, 136). The presence of LRR in their extracellular domains, which appears unique among G protein-coupled receptors, has led to the realization that the glycoprotein hormone receptors belong to the superfamily of LRR proteins (137). This family encompasses a vast variety of molecules with diverse functions and cellular localizations, the common characteristic being that they are involved in protein-protein interaction. Despite their diverse functions, conservation of the LRR indicates similar roles of such modules for these proteins (137). Cocrystallization of the LRR-containing ribonuclease inhibitor complexed with its ligand (19) has inspired recent modeling of the hTSH (138) and hCG receptor (98, 139), and these models have recently begun to be tested using site-directed mutagenesis of the receptor (140). Crystallization of the ribonuclease inhibitor revealed a nonglobular shape with solvent-exposed parallel β -sheets and flexibility of the module, allowing elastic alteration of the entire structure. These aspects support the suitability of LRR for protein-protein interactions. Moreover, the concave surface formed by the repeats allowed for a large interface with the ribonuclease (19, 137). Interestingly, this is compatible with results from epitope mapping, showing that most of the surface of glycoprotein hormones is masked upon interaction with their receptors (98, 123, 141). Thus, these findings predict a similarly large ligand-receptor interface for glycoprotein hormones, which is also supported by the identification of multiple functionally important regions on both subunits. In fact, it was speculated earlier that the extracellular domain of glycoprotein hormone receptors represents a rather flexible entity that wraps around the ligand in a "process-like adaptive manner" (123).

B. Hormone-receptor interaction

There is no general consensus of the specific mechanisms by which the glycoprotein hormone docks into its receptor. It is generally accepted that the $\alpha\beta$ -heterodimer is required for glycoprotein hormone activity, and individual subunits do not possess significant activity at the glycoprotein hormone receptors (3). In fact, multiple contact points of both α -subunit and β -subunit with the receptor, perhaps in a stepwise fashion, appear necessary to induce a conformational change of the receptor, favoring receptor G-protein coupling and subsequent second messenger generation (60). It appears likely that the initial interaction involves specific high-affinity binding of the hormone to the LRR-containing extracellular domain of the receptor. This initial binding

event may control specificity by negative determinants that restrict heterologous ligand-receptor interaction (57, 95). Whether the extracellular domain of the TSH receptor by itself is sufficient for high-affinity ligand binding has not been unequivocally established (28, 30, 31, 142). In addition to interactions with the extracellular domain, secondary contacts between common, possibly α -subunit, domains with the transmembrane portion of the receptor may initiate the signal by analogy to G protein-coupled receptor activation by small ligands, such as for the adrenergic receptors (143). However, it is not known how even parts of the bulky glycoprotein hormones could be accommodated in such a hypothetical pocket. In this respect, modeling of the transmembrane domain of the glycoprotein hormone receptor indicated that, in contrast to the tight hydrophobic pocket of adrenergic receptors, the glycoprotein hormone receptor domain may form a deeper, yet broader, hydrophilic groove (144).

Binding of glycoprotein hormones to additional receptor domains was supported by the identification of a direct interaction between a counterionic pair of residues of the α -carboxyl terminus of hCG and the first exoloop of the CG/LH receptor (71). Subsequently, specific binding of an α -carboxyl-terminal peptide to the CG/LH receptor was demonstrated (145). Further, binding of hCG to the extracellular domain of the receptor unmasked an immunoreactive site on the α -subunit, which was not accessible if the hormone bound to the full-length receptor (141), supporting the notion that some α -subunit regions may contact the carboxyl-terminal half of the receptor. Moreover, coexpression of the extracellular domain of the CG/LH receptor with the transmembrane domain restored efficient hCG-mediated signal transduction (146). However, in a recently proposed model of hCG action, binding to the extracellular domain alone could account for G protein activation without the need for secondary contact points (98). Even though it was reported that hCG can bind with low affinity to, and activate a truncated form of the CG/LH receptor lacking the extracellular domain (147), this was not observed with similar studies of the TSH receptor (148). In fact, several N-terminally truncated TSH receptor constructs were not stimulated by either TSH or hCG (148). These findings again underscore the need for structural data on hormone-receptor complexes to understand potential causes for such discrepancies. In any case, ligand binding is believed to modulate interactions between the transmembrane helices, effecting conformational changes in the intracellular loops and thus altering G protein coupling (149), ultimately activating the second messenger systems (150). Figure 5 shows a potential orientation of hTSH within the hormone-receptor complex, by analogy to models that have been proposed by others (30, 98, 138–140).

Interestingly, the TSH receptor possesses significant constitutive activity in the unliganded state, which is considerably higher than that of the LH/CG receptor (29, 151, 152). Further, the TSH receptor is readily activated by a multiplicity of different experimental as well as naturally occurring (the latter causing hyperfunctioning thyroid adenomas) mutations (29, 151). This suggests that ligand binding activates the TSH receptor, by analogy to other G protein-coupled receptors (141, 153), by the release of a negative con-

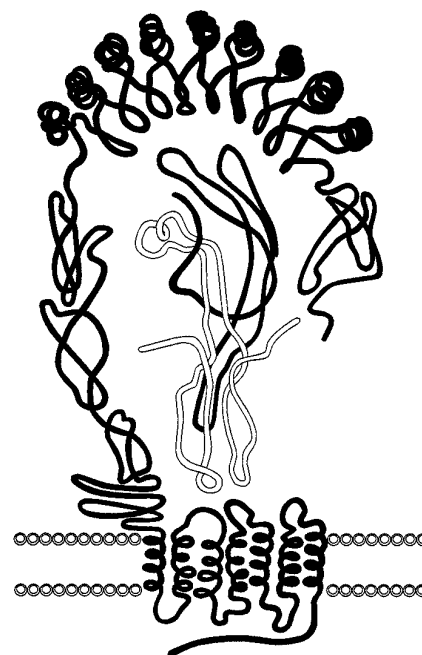


FIG. 5. Structural model of the hTSH-hTSH receptor complex. This schematic drawing is intended to reflect a potential initial interaction of hTSH with its receptor. The receptor is depicted in accordance to models of the hTSH receptor (138) based on the LRR-containing ribonuclease inhibitor (19). Actual spatial relationships will require cocrystallization of hormone receptor complexes. The two parallel β -hairpin loops of the α -subunit ($\alpha L1$, $\alpha L3$) face toward the receptor and may participate in the interaction of the hormone with the extracellular loops of the receptor transmembrane domain (139). The equivalent loops of the TSH β -subunit ($\beta L1$, $\beta L3$) are shown projecting to a proposed binding site within the concave surface of the nine LRR of the extracellular domain of the TSH receptor (138). However, site-directed mutagenesis studies have implicated additional domains to contact the receptor. It is conceivable that conformational changes in receptor and/or ligand upon high-affinity binding would allow for secondary interactions.

straint that normally maintains the unliganded receptor in an inactive state. In an interesting hypothesis, it was proposed that this "noisy" character of the TSH receptor may be related to the unique propensity of the TSH receptor to be activated by the autoantibodies of Graves' disease (151). In contrast, inactivating mutations of the TSH receptor, which lead to resistance to TSH, first identified in the *hyt/hyt* hypothyroid mouse (154), appear very rare in man (155).

C. Cooperation of individual hTSH domains in receptor activation

This paragraph attempts to integrate the results of individual site-directed mutagenesis studies into a model highlighting several aspects of hTSH action. A hypothesis of how individual hTSH domains may interplay in receptor activation is summarized in Fig. 4. As stated earlier, the importance of several highly conserved domains in the common α -subunit for the signal transduction of all glycoprotein hormones emphasizes that these hormones elicit their biological responses in a similar fashion. Yet, as described above, the α -asparagine⁵² oligosaccharide, and in particular its negatively charged sialic acid moieties, play an opposite role for

hTSH activity compared with hCG or hFSH (74). Further, as discussed above, the relative contributions of the α -helix and the α -carboxyl-terminus to signal transduction are, at least in part, different for each glycoprotein hormone (64, 69, 72, 85, 86, 93). This implies that these α -subunit activity domains may, to a certain degree, function in a β -subunit-dependent fashion.

As mentioned earlier, chimeric studies have shown that the β -subunit seat belt appears to direct, at least in part, glycoprotein hormone specificity (78, 83, 90, 95). Accordingly, the seat belt may achieve this by influencing common α -subunit domains important for signal transduction, such as the α -asparagine⁵² oligosaccharide, to function in a hormone-dependent fashion. This was shown by deleting the α -asparagine⁵² oligosaccharide in a hTSH chimera in which the native seat belt sequence had been replaced with the corresponding residues of hCG (M. Grossmann, M. W. Szkudlinski, and B. D. Weintraub, unpublished data). This oligosaccharide was chosen because of its differential effect on glycoprotein hormone activity: absence of this oligosaccharide, if sialylated, decreased hCG activity (81), but increased hTSH activity (74). Remarkably, the hCG-like activity of this hTSH/hCG seat belt chimera decreased upon deletion of the α -asparagine⁵² oligosaccharide. Thus, the function of this domain in the chimera was similar to its function in hCG, but different from that in hTSH. This suggests that the seat belt may indirectly modulate hormonal specificity by orienting α -subunit domains that are in close proximity (see Fig. 4). This is consistent with the hormone-dependent differences in the contribution of these domains for receptor activation.

In contrast, an 11- to 20- α -subunit domain engineered for increased binding, located within the β -hairpin α L1 loop, appears to be important for all glycoprotein hormones (50). This relative absence of specificity of the engineered α 11-20 domain may be associated with its distance from the seat belt and other regions of the β -subunit. In a recent model of hCG bound to its receptor, the α 11-20 region may contact the transmembrane portion of the receptor, further supporting its possible direct involvement in receptor binding (139). Accordingly, the potential orientation of hTSH within the hormone-receptor complex is depicted in Fig. 5.

IV. Physiological and Pathophysiological Implications

A. Carbohydrate heterogeneity

1. *Regulation of physiological microheterogeneity.* Different degrees in the terminal processing of oligosaccharides give rise to a mixture of circulating glycoforms, which in turn are responsible for the physiological microheterogeneity of the glycoprotein hormones. The processed hormone N-linked oligosaccharides are typically bi- or multiantennary structures displaying notable hormone-dependent differences in their terminal residues (Fig. 1). Pituitary TSH and LH are unique in that they contain predominantly sulfated oligosaccharides, due to the presence of GalNAc-transferase and GalNAc-4-sulfotransferase in the pituitary thyrotrophs and luteotrophs, and terminate mostly in $\text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha$.

On the other hand, CG and FSH, like almost all other serum glycoproteins, terminate in $\text{Sia}\alpha\text{2-3(6)Gal}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha$ (4, 5). Such selective glycosylation may have primarily evolved as a means to preserve the pulsatile pattern of TSH and LH levels in the circulation and thus avoid receptor desensitization of the target organ. In fact, a separate hepatic receptor specific for oligosaccharides terminating with sulfated GalNAc residues has been implicated in the rapid clearance of LH and TSH (156). By contrast, terminal sialylation enables the glycoprotein hormone to escape such specific receptor-mediated hepatic clearance mechanisms, and the kidney becomes the major organ of (less efficient) clearance. For example, rhTSH is produced in Chinese hamster ovary cells that lack GalNAc-transferase and GalNAc-4-sulfotransferase and TSH produced in these cells terminates exclusively in sialic acids (14, 102, 127). This appears to be the main reason why its circulatory half-life is prolonged compared with its predominantly sulfated pituitary counterpart (110, 127, 130).

The concept of how the carbohydrates affect clearance and hence *in vivo* bioactivity is also exemplified by hTSH produced in insect cells using a baculovirus system. Insect cell-expressed hTSH, which lacks sialic acids but contains predominantly high-mannose residues, was cleared very rapidly compared with rhTSH, presumably via the hepatic mannose receptor (157, 158), and had a lower bioactivity than sialylated rhTSH (124). Such observations emphasize that the main physiological role of carbohydrate moieties and their terminal residues lies in the differential targeting and clearance of the hormones. Therefore, the glycosylation state and specifically the degree of terminal sialylation have a powerful impact on plasma levels and thus the final *in vivo* bioactivity of the hTSH and other glycoprotein hormones. It is tempting to speculate that the unusual efficiency of high-mannose- and asialo-glycoprotein clearance mechanisms may have evolved in vertebrates to reduce or eliminate the *in vivo* activity of glycoproteins with incompletely processed oligosaccharide chains.

2. *Alteration of carbohydrate structures in thyroid dysfunction.* It is not surprising, therefore, that the distribution of hTSH glycoforms is under endocrine control and altered in various states of thyroid dysfunction (1, 2, 159). For example, hTSH is more highly sialylated in patients with primary hypothyroidism (160, 161) and similarly, hTSH glycosylation isoforms with higher bioactivity have been reported in patients with resistance to thyroid hormone (162). 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 (161-164). Such regulation of hTSH glycosylation may be viewed largely as an adaptive response, thus contributing to the classic negative T_3/T_4 -TSH-TRH feedback loop. In primary hypothyroidism, pituitary compensation would not only consist of increased production and release of the hormone, but the secreted 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 hor-

mone, as, for example, thyroid hormone status has been shown to modulate α -2,3- and α -2,6-sialyltransferase mRNA levels in mouse thyrotrophs (165, 166). Similarly, estrogen appears to regulate expression of specific glycosyltransferases essential for synthesis of sulfated oligosaccharides of LH (167). This ensures that secreted LH is fully sulfated and thus rapidly cleared, maintaining its pulsatility and thereby preventing receptor desensitization (43, 167).

B. Naturally occurring glycoprotein hormone mutations

In contrast to the physiological heterogeneity of the carbohydrate component of the glycoprotein hormones, functionally relevant alterations of the amino acid sequence appear rare in man. This is to be expected given the profound effects glycoprotein hormone dysfunction has in the whole organism. Mutations leading to disturbances of glycoprotein hormone function in humans have thus far only been described in the genes coding for the β -subunits of hTSH (168–170), hFSH (171), and hLH (172), but not in the hCG β -subunit or the common α -subunit gene (see Table 5). hCG- β subunit gene mutations are not expected to be phenotypically apparent, as the hCG β -subunit is encoded by a cluster of six genes, all of which appear to be transcribed *in vivo* (173).

Two families with secondary hypothyroidism were reported in which mutations in the TSH β -subunit gene compromised TSH synthesis in such a way that affected family members had undetectable TSH serum levels. One family had a β -glycine²⁹ to arginine point mutation in the TSH β -subunit ²⁷CAGY³⁰ region (168), shown to be essential for heterodimer formation in structure-function studies (174). In the second family, a nonsense mutation gave rise to a truncated peptide coding only the first 11 amino acids of the hTSH β -subunit (169). In contrast, a recent report of a Brazilian family with secondary hypothyroidism described the interesting finding of circulating hTSH heterodimer. However, due to a frameshift mutation with the loss of β -cysteine¹⁰⁵, a residue of known importance for the structural integrity of the seat belt, the circulating hTSH was biologically inactive (170).

Mutations have also been detected in the gonadotropin β -subunits. A frameshift deletion altering the FSH β -subunit beyond β -threonine⁶⁰ led to amenorrhea and infertility (171), and a point mutation of arginine⁵⁴ to glutamine resulted in hypogonadism (172). An interesting polymorphism in the LH β -subunit gene with a frequency of 28% has been described in the Finnish population (175) and was also recognized in Japanese individuals (176): carriers have a double mutation, changing β -tryptophan⁸ to arginine and β -isoleucine¹⁵ to threonine. Remarkably, the latter creates an N-linked carbohydrate consensus sequence, but it has not yet been established whether this site is indeed glycosylated. Carriers may present with falsely low serum LH levels because certain antibodies in clinically used LH immunoassays do not recognize this LH mutant. The effect of this polymorphism on LH function is less clear. Whereas infertility was described in some of the Japanese patients (176), the Finnish subjects examined had, apart from a slight delay in the onset of puberty, no clinical features of hypogonadism (175). Taken together, such studies illustrate how structure-function studies can explain different mechanisms of secondary hypothyroidism or gonadal dysfunction at the molecular level.

In contrast to the β -subunit mutations, mutations in the common α -subunit gene have not been described, to our knowledge, in humans. Common α -subunit gene mutations may be lethal in man, although a mouse with a targeted disruption of the common α -subunit gene was viable with hypothyroidism and hypogonadism (36). α -Subunit mutations would be expected to result in combined glycoprotein hormone deficiencies. However, site-directed mutagenesis studies have identified specific artificial mutations in the α 33–38 domain that were differentially important for glycoprotein hormone heterodimerization. For example, an α -subunit in which α -alanine³⁶ was mutated to glutamic acid was not able to form a dimer with the hCG β -subunit, whereas this mutated subunit combined efficiently with the hTSH β -subunit giving rise to a bioactive heterodimer (72). These findings suggest that mutations in the α -subunit cannot a

TABLE 5. Natural human β -subunit mutations

Subunit	Mutation type	Nucleotide codon change	Amino acid change	Hormone domain affected	Clinical presentation	Reference
TSH β	Missense	<u>GGA</u> → <u>AGA</u>	G29R	CAGYC region	Familial hypothyroidism	Hayashizaki <i>et al.</i> (168)
	Nonsense	<u>GAA</u> → <u>TAA</u> (stop)	truncation 12-112	β L1 loop	Familial hypothyroidism	Dacou-Voutetakis <i>et al.</i> (169)
	Frameshift	<u>TGTA</u> → GTA	C105V ^a	Seat belt C Terminus	Familial hypothyroidism	Medeiros-Neto <i>et al.</i> (170)
LH β	Missense	<u>CGG</u> → <u>CAG</u>	R54Q	β L2 “Keutmann loop”	Familial hypogonadism	Weiss <i>et al.</i> (172)
	Double missense	<u>TGG</u> → <u>CGG</u> <u>ATC</u> → <u>ACC</u>	W8R I15T	β L1 loop	Familial hypogonadism(?) ^b	Raivio <i>et al.</i> (175) Suganuma <i>et al.</i> (176)
FSH β	Frameshift	<u>GTGAG</u> → GAG	V61E truncation 87-111	β L3 loop Seat belt	Sporadic hypogonadism	Matthews <i>et al.</i> (171)

^a Disulfide bond C19-C105 disrupted.

^b In contrast to all the other mutations in this table, which were only reported in a few individuals, the double missense mutation may represent a common variant with a prevalence of 28% (4% homo- and 24% heterozygotes) in one study of the Finnish population (175). Whereas in Finnish subjects these mutations appeared to be asymptomatic in adults except for a slight delay in the onset of puberty, infertility has been described in Japanese homozygous for this variant (see text).

priori be ruled out as a cause of a deficiency of a specific glycoprotein hormone.

C. "Specificity spillover" syndromes

Because of the high degree of sequence identity among the glycoprotein hormones even in their specific β -subunits (30%–80%) (3), as well as in the extracellular domains of their respective receptors (39%–46%) (28), glycoprotein hormones can interact with heterologous receptors, albeit with low cross-reactivity. At physiologically occurring hormone levels, this degree of specificity prevents stimulation of heterologous receptors, but cross-activation of heterologous receptors can be observed with high hormone levels. The resulting clinical manifestations have been termed "specificity spillover" syndromes (177). The point to be emphasized is that hCG in the first trimester of pregnancy or in patients with trophoblastic neoplasms circulates at such high concentrations that even a cross-reactivity of less than 0.1% at the TSH receptor may become physiologically significant. In contrast, neither TSH nor pituitary gonadotropins are elevated to such high concentrations even in target organ failure, so that cross-reactivity at a heterologous receptor would have to be quantitatively much higher to be physiologically relevant. For the glycoprotein hormones, the prominent clinical example is the increased function of the thyroid gland, *i.e.*, goiter or occasionally frank thyrotoxicosis, in individuals with high circulating hCG levels, such as occurs in early pregnancy or trophoblastic tumors (178, 179). Accordingly, a weak thyrotropic activity of hCG (estimated to be less than 0.1% that of TSH on a molar basis) has been demonstrated in a variety of experimental settings, and recent studies using the rhTSH receptor have confirmed direct interaction of hCG with the hTSH receptor (180–182). hLH has considerably higher thyrotropic activity than hCG, which may be related to the lack of the β -subunit carboxyl-terminal peptide, and to similarities in its carbohydrate structure with hTSH (181, 183). Thus it has been speculated that one of the reasons for the evolution of a placental hormone distinct from LH was to prevent the development of overt hyperthyroidism in early pregnancy (183).

Similarly, it had been known since the initial case description in 1905 (184) that severe juvenile hypothyroidism can cause a distinct form of precocious puberty. Evidence that these symptoms were responsive to thyroid hormone treatment implicated the elevated TSH as a gonadal stimulator (185). Indeed, recent data showed that rhTSH can bind to and stimulate cells transfected with the rFSH receptor (186). Whether TSH can stimulate the CG/LH receptor, however, is more controversial. Even though pituitary bovine TSH was able to activate the CG/LH receptor in one study (187), others have suggested that this could be related to LH contamination, particularly since rhTSH did not show significant activity at the CG/LH receptor (188). The clinical picture as well as histological findings in precocious puberty associated with juvenile primary hypothyroidism suggested that hTSH stimulation of the FSH receptor may be responsible for the observed phenotype (186). Affected girls present with breast development, uterine bleeding, and polycystic ovaries and boys with macroorchidism and relatively little virilization

(185). In contrast, precocious puberty associated with high levels of hCG, such as in hCG-producing tumors, affects only boys with marked virilization due to increases in testosterone levels, but not macroorchidism (189). Histological testicular examination from hypothyroid patients shows predominance of tubular elements without Leydig cell hyperplasia. In contrast, high hCG levels commonly induce pure Leydig cell hyperplasia (186). In part, the observed alterations of testicular morphology and differentiation may also be related to a direct effect of thyroid hormone deficiency on the immature seminiferous epithelium (190).

V. Evolutionary Considerations

A. Glycoprotein hormone specificity

The relatively high overall structural homology (30–80% primary structure identity) among the different β -subunits suggests that they were derived from a common ancestor gene (3). Similarly, the extracellular domains of the receptors appear to have evolved by exon duplication and shuffling from a single prototypic LRR domain, as they display a high degree of homology in their intron-exon junctions (95). In less developed organisms, a single primordial hormone, dimeric or monomeric, and a corresponding primordial receptor were likely sufficient for the necessary endocrine functions. Chimeric studies on the gonadotropins hCG and hFSH and their receptors indicated that specificity evolved independently from signal transduction by the introduction of domains that block inappropriate ligand-receptor interactions (57, 95). This concept of "negative specificity" is not without precedent among G protein-coupled receptor-ligand interactions. Recently, the finding of unpredicted high affinities of a variety of natural tachykinins for chimeric neurokinin receptors implies that inhibitory domains that could restrict inappropriate ligand-receptor interactions may exist in such receptors (191).

From an evolutionary standpoint, it is justifiable to assume that diversification and ligand selectivity did not evolve by development of new mechanisms of receptor activation, but rather by the emergence of inhibitory domains that impose steric hindrances thereby allowing only the intended ligand to interact with the common activation domain. Such negative specificity determinants have been identified in both the extracellular domain of the gonadotropin receptors as well as their β -subunits, such as the seat belt region (83, 90, 95). Studies on the seat belt region of the hTSH β -subunit using chimeric substitutions with gonadotropic sequences (78) suggested that, during this evolutionary diversification of the glycoprotein hormones from a common ancestor gene, determinants of ligand specificity appear to have evolved independently and in a selective fashion. Specifically, replacing the seat belt domain of the TSH β -subunit with the corresponding residues of hCG conferred hCG specificity to the chimera. However, analogous replacement of the TSH β -subunit seat belt with FSH residues did not confer hFSH specificity (78). Thus, hTSH/hFSH specificity must be located in an unknown domain, distinct from the one mediating hCG specificity.

In addition to such β -subunit determinants, hormone-de-

pendent roles of specific α -subunit residues within domains of general importance for all glycoprotein hormones may contribute to the specificity of glycoprotein hormone-receptor interactions. As described above, the hormone-specific β -subunit may regulate involvement of such domains in signal generation. In fact, several studies have indicated that the conformation of the common α -subunit can differ depending on the β -subunit with which it associates (192–194). Again, modulation of heterodimer activity and hence specificity can be achieved without fundamental changes in the underlying molecular activation mechanisms. When combined, the differences in several domains would be additive or synergistic and could result in a relatively high level of specificity. In this respect, the oligosaccharide at α -asparagine⁵² appears to play a role as a negative specificity determinant. Perhaps this carbohydrate masks α -domains required for efficient interaction with the TSH receptor, thus reducing hTSH activity as well as the thyrotropic activity of hCG (see Fig. 4). Accordingly, deglycosylated glycoprotein hormones bind to the receptor differently from the native hormone (121–123). This attenuating effect of the α -asparagine⁵² carbohydrate on the thyrotropic activity of hCG is reminiscent of the observation that the carboxyl-terminal peptide of the hCG β -subunit containing several O-linked oligosaccharide chains inhibits TSH receptor binding of hCG (181, 183).

B. Evolutionary changes in TSH activity

In addition to providing specificity, evolution of hormone activity is likely to reflect adaptation of endocrine processes to changes in environment or other outside factors. In this respect, bovine and rat TSH are known to have higher intrinsic activity than hTSH (110, 127, 195, 196). In addition to species-dependent differences in ligand potency, such differences can also vary with the species of TSH receptor (195). Using site-directed mutagenesis, selective introduction of basic residues present in the α 11–20 domain of the nonhominoid α -subunit into the human α -subunit increased the activity of hTSH in a variety of systems from different species (50). 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). Since this selective elimination of basic residues in the α 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 (197). In man, 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 man may be to conserve iodine for thyroid hormone synthesis during periods of fasting in nomadic life. Perhaps, modulation of gonadotropic activity by these evolutionary changes in the α -subunit sequence is re-

lated to concomitant adaptation to slower reproductive turnover (198). It should be pointed out in this context that man's 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) (199).

Coevolution of glycoprotein hormones and their respective receptors likely controlled spillover of hormone activity to nonhomologous receptors (such as the above discussed thyrotropic activity of hCG) by attenuation of hormone activity. Significant interspecies variations in biopotency were identified not only between glycoprotein hormones, but also for GH and GnRH. Increases of activity during evolution observed in primate GHs were correlated with the gain of lactogenic (PRL-like) properties not seen in the GHs of nonprimates (200). 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 (37). 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, the 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 (77a), suggest that non-conservative 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 (50).

VI. Strategies in the Design of Novel TSH Analogs and Therapeutic Implications

A. Clinical use of *rhTSH*

The incidence of thyroid carcinoma in the United States is approximately 14,000 cases per year (15, 16). Most of these are differentiated, and papillary or follicular cancers are the most common subtypes. As the 10- and 20-yr survival rate of such differentiated thyroid carcinomas is 90% and 60%, respectively, long-term monitoring to detect local recurrence and distant metastases becomes essential in the management of such patients, especially since tumor can recur even decades after primary therapy. The principal methods used for follow-up are whole-body radioiodine scanning and serum thyroglobulin (Tg) measurements. For optimal sensitivity of these diagnostic procedures, stimulation of residual thyroid tissue by TSH to increase ¹³¹Iodine uptake or Tg secretion, respectively, is required. However, postthyroidectomy thyroid cancer patients are treated with thyroid hormone to suppress endogenous TSH to avoid potential stimulatory effects of TSH on residual thyroid tissue, as well as to main-

tain euthyroidism. Usually therefore, levo-T₄ or, less commonly used, T₃ is withdrawn 4–6 and 2 weeks before radioiodine scanning and Tg determination to stimulate endogenous TSH secretion. The accompanying transient, but severe, hypothyroidism leads to considerable impairment of the quality of life of such patients and may interfere with their ability to work. Further, since TSH can act as a growth factor for malignant thyroid tissue, prolonged periods of increased endogenous TSH secretion may pose a potential risk for such patients.

In the 1960s, bovine TSH (bTSH) was used to stimulate residual thyroid tissue to overcome the need for elevating endogenous TSH (201). However, several disadvantages soon became apparent, which led to the discontinuation of its use in clinical practice. Compared with hormone withdrawal, bTSH proved to be less efficacious in detecting residual malignant thyroid tissue and metastases. In addition, allergic reactions as well as the development of neutralizing antibodies that can further limit the effect of subsequent bTSH administration as well as interference with endogenous TSH determinations were frequently recognized (202). The use of hTSH from pituitary sources is prohibitive because of the potential risk of copurification of prions and subsequent transmission of Creutzfeld-Jacob disease. After the cloning of the hTSH β gene, it was possible to obtain sufficient amounts of highly purified rhTSH from CHO cells (14). Although the amino acid sequence is preserved upon production in heterologous cells, host-specific glycosylation leads to terminal sialylation of the rhTSH, whereas pituitary TSH is partially sulfated. Initial characterization of rhTSH in a variety of *in vitro* systems showed that its intrinsic activity was slightly lower than that of the pituitary hormone (14, 102, 110, 127). However, studies in rodents (130, 196) and monkeys (202) demonstrated that, due to its decreased clearance rate, the *in vivo* activity of rhTSH was higher compared with pituitary hTSH. As discussed above, these disparate effects on the *in vitro* and *in vivo* activity are related to the different glycosylation patterns and predominantly to the degree of terminal sialylation of both hTSH preparations. Since erythropoietin and tissue type plasminogen activator, both also produced in CHO cells, are associated with an extremely low prevalence of antibody formation after administration to man (203), it is unlikely that the differences in carbohydrate structures would induce an immunological response.

A small initial phase I/II study showed rhTSH to be safe and demonstrated preliminary efficacy in stimulating ¹³¹I uptake and Tg secretion in the diagnosis and follow-up of 19 patients with differentiated thyroid carcinoma, thus avoiding the side effects of thyroid hormone withdrawal (15). This phase I/II trial was followed by two multicenter phase III studies, which compared the effect of rhTSH administration to thyroid hormone withdrawal for radioactive iodine scanning and Tg secretion in a much larger number of patients. Although the data from these two studies are still being analyzed, preliminary results from the first trial are highly encouraging (16). However, further studies will be required to optimize the dosage as well as mode and timing of rhTSH administration, and rhTSH analogs with higher activity than rhTSH may be valuable for some patients. In addition, rhTSH and its analogs should prove useful in the stimulation of

thyroidal and metastatic tissue before therapeutic ablation with radioactive iodine. rhTSH may also help in the detection of suppressed, but functional, thyroid tissue in patients with autonomous hyperfunctioning thyroid nodules or exogenous thyroid hormone therapy. Additional possible uses of rhTSH relate to the diagnosis of central and combined primary and central hypothyroidism, hemiatrophy of the thyroid, resistance to TSH action, as well as stimulation of benign multinodular goiters before radioablation.

By analogy to rhTSH, clinical trials are also conducted with other recombinant glycoprotein hormones. For example, rhFSH produced in CHO cells has been shown to be as effective as urinary hFSH in stimulating ovarian follicular development in women before *in vitro* fertilization (204).

B. Design of novel glycoprotein hormone analogs

From a therapeutic perspective, there is considerable interest for the use of novel hTSH analogs. From a basic science standpoint, engineering of glycoprotein hormone analogs may be directed at an alteration of existing functions, such as modulation of bioactivity or introduction of novel features, *e.g.*, altered specificity. Hormone activity, in general, can be augmented by the prolongation of hormonal half-life (long acting analogs) or by increasing its intrinsic activity (superactive analogs). Better understanding of structure-function relationships should also help in the development of competitive receptor antagonists and pathway-selective analogs (*i.e.*, analogs specific for certain biological effects mediated by TSH).

1. Long acting analogs. In gene fusion experiments, the carboxyl-terminal extension peptide of the hCG β -subunit, which contains several O-linked carbohydrates, was added to the hFSH or the hTSH β -subunit, as well as to the α -subunit (63, 205–207). Although the *in vitro* activity of these chimeras was not altered, their circulatory half-lives were prolonged, resulting in enhanced *in vivo* bioactivity. Additional approaches aimed at decreasing the clearance rate of glycoprotein hormone analogs, an area of active investigation, include the introduction of new glycosylation recognition sequences into the subunits. Further, it is possible to regulate the carbohydrate composition by modification of tissue culture conditions during the expression of the recombinant hormones, or by modification of the glycosylation machinery of host cells, *e.g.*, by transfection of glycosyltransferases with desired properties (74, 100–102). Also, selective chemical conjugation of the nontoxic amphipathic polymer polyethylene glycol to hTSH prolonged its half-life significantly without changing its intrinsic activity (M. W. Szkudlinski, N. R. Thotakura, M. Grossmann, and B. D. Weintraub, unpublished data). Another recent approach pioneered for the gonadotropins consisted of expressing the gonadotropic β - and α -subunits genetically fused as a single chain (208–210). By analogy to what was observed with the hCG or hFSH fusion products, similar experiments with hTSH showed that fused hTSH had a similar *in vitro* activity to heterodimeric hTSH. Interestingly, the single fused hTSH $\alpha\beta$ -chain displayed enhanced stability and a prolonged plasma half-life compared with heterodimeric hTSH (210a).

2. *Superactive analogs.* Although the prolongation of the half-life of recombinant glycoproteins may be promising, the design of superactive analogs may, in certain instances, be more desirable, as they may limit the potential for receptor desensitization associated with analogs that remain in the circulation for prolonged periods. This consideration may be more relevant for TSH and LH, which are physiologically secreted in a pulsatile fashion. In this respect, there is experimental evidence in rodents that pulsatile administration of hTSH may be superior to continuous infusion (211) for thyroid stimulation. Moreover, several studies had suggested that natural hTSH is not an optimal stimulator at its receptor, as superior agonists could be designed by modifications of the primary structure of the hormone as well as of selected peptides (64, 96).

The generation of the first superactive analogs of human glycoprotein hormones with major increases in receptor binding affinity, as well as enhanced *in vitro* and *in vivo* activity, has been reported recently (50). Selective replacement of certain critical residues in homologous nonhuman hormones was based on structural and evolutionary considerations, as discussed above. A hTSH analog with a quadruple mutation in the α 11–20 domain (α -glutamine¹³ + glutamic acid¹⁴ + proline¹⁶ + glutamine²⁰ were substituted with lysine) and an additional β -leucine⁶⁹ to arginine replacement in the hTSH β -subunit possessed a 95-fold increase of the *in vitro* potency and a significant increase in the *in vivo* activity without a change of serum half-life. Similarly, introduction of basic residues into the β -hairpin β L3-loop of the TSH β -subunit increased hTSH *in vitro* potency more than 50-fold (77a). Ultimately, the most suitable *in vivo* agonist may be designed by a combination of different approaches (Table 3). However, the final therapeutic utility of such analogs cannot be predicted without carefully designed and conducted clinical trials.

3. *Competitive antagonists.* The hTSH receptor 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 (212–214). However, it should be pointed out that the majority of studies investigating TSH receptor expression in orbital or other extrathyroidal tissues have relied on detection of its mRNA, sometimes requiring prior RT-PCR amplification. Thus, whether significant amounts of TSH receptor protein or TSH receptor variant proteins are present in such tissues is still controversial (214). hTSH receptor antagonists that block the actions of TSH receptor-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 (212). Thus far, except for deglycosylated glycoprotein hormone analogs, which are cleared rapidly from the circulation (41, 42), a major dissociation of high-affinity binding from signal transduction has not been achieved for any member of the glycoprotein hormone family. Thus, the prototype of a hTSH receptor antagonist with proven *in vivo* activity, albeit at very high doses, is asialoagalacto-hCG (215).

Certain peptides encompassing domains of the hTSH subunits show specific TSH receptor binding at very high concentrations (millimolar range), but do not generate a signal (65, 94). More recently, competitive hTSH receptor antagonists were developed by fusing such peptides to overcome low-affinity interactions and are likely to be further improved (216). An additional approach, which may be of promise, is the use of multiple domain mutagenesis. Such recent studies of the 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 (69). This could provide a basis for dissociation of signal transduction and receptor binding and thereby overcome the problems associated with the low receptor affinity of existing experimental TSH receptor antagonists.

4. *Pathway-selective analogs.* As hTSH has a variety of different biological effects, selective hTSH analogs would be potentially useful. The most obvious example would be a hTSH agonist with reduced thyroid growth-stimulating effects, which should be advantageous to stimulate radioiodine uptake in patients with differentiated thyroid carcinoma. In this respect, mutation of individual residues in the α 33–44 region of the TSH α -subunit revealed that subtle, but significant, dissociation of postreceptor events, such as receptor binding, second messenger production, and thyrocyte growth, could be achieved with mutagenesis of single amino acid residues (72). For example, replacing α -alanine³⁶ with glutamic acid led to a reduction of growth promotion, but not cAMP stimulation. However, these differences were small, and it is not clear whether a combination of individual substitutions would achieve a synergistic effect. Thus, it should be clearly pointed out that it has not been established whether such different biological effects can indeed be separated by mutagenesis of the ligand. Therefore, whether such a strategy could ultimately be useful for the development of clinically useful pathway-selective hTSH analogs is presently unknown.

VII. Nonclassic Actions of TSH and Gonadotropins

A. Extrathyroidal/extragenadal glycoprotein hormone actions

Several lines of evidence have led to speculations of possible extrathyroidal actions of TSH. Thus, the presence of TSH-binding sites in a variety of extrathyroidal tissues such as lymphocytes (217), adipocytes (218), or testicular and adrenal tissue (219) has long been known. More recently, the expression of hTSH receptor or its splicing variants (220) in nonthyroidal tissues, including adipocytes and lymphocytes, was demonstrated (213, 214), as well as the extrapituitary expression of the TSH β -subunit gene (221). Evidence for both hTSH receptor and hTSH β -subunit expression in the same tissue, such as lymphocytes (221), may indicate that such tissues are under paracrine or autocrine TSH regulation. According to one study, proliferative capacity and natural killer cell activity of murine spleen lymphocytes improved upon stimulation with TSH (222). The presence

of TSH receptor in fat (223) may explain the lipolytic effects of TSH, which have been implicated in promoting physiologically occurring lipolysis during the neonatal period (224). Perhaps immunoglobulin interaction with the TSH receptor on adipocytes or lymphocytes may contribute to the weight loss and impaired immune function observed in Graves' patients. As mentioned above, however, it has not been unequivocally established whether functional hTSH receptor or hTSH protein is expressed in such tissues and whether the amount expressed, if any, would be physiologically relevant.

There is analogous evidence for extragonadal LH/CG receptor expression in a variety of tissues, such as skin (225) and the testicular microvasculature (226). In fact, the LH/CG receptor in these endothelial cells has been proposed to mediate hCG transcytosis through the vessel wall and thus enhance hormone delivery to the interstitial space (226). Low levels of hCG, presumably of pituitary origin, are detectable in men and nonpregnant women (227). hCG is also produced by malignant cells and serves as a tumor marker in certain types of human neoplasms of trophoblastic and nontrophoblastic origin, such as gynecological or gastrointestinal malignancies (228, 229). A gene encoding an hCG β -subunit-like protein has been cloned from the prokaryote *Xanthomonas maltophilia*, and hCG has been claimed to influence the growth of these bacteria under certain culture conditions (230). Further, it has recently been shown that the free α -subunit, but not dimeric hCG, can stimulate PRL secretion from human decidual cells, raising the possibility of an independent endocrine function of the α -subunit, which appears not to be mediated through a classic glycoprotein hormone receptor (231). However, it must be emphasized again that, in general, the physiological relevance of these nonclassic actions of the glycoprotein hormones needs to be clarified in future studies.

B. Relationship to the cystine knot growth factor superfamily

Recently, commercial preparations of hCG have been shown to possess antineoplastic activity in a cell line derived from AIDS-related Kaposi sarcoma (49). Subsequently, in a small preliminary clinical study, local treatment of small cutaneous Kaposi sarcoma lesions with certain preparations of hCG in AIDS patients led to a decrease in the size of such lesions (232). The variability observed with different commercial preparations of hCG could suggest that the active component may be an as yet unidentified contaminant. It is tempting to speculate, however, that such components may function on the basis of certain structural similarities of the glycoprotein hormones with other members of the cystine knot superfamily implicated in regulating tumor growth, such as PDGF or TGF β (17, 18, 45). Indeed, the hCG degradation product, hCG- β core, normally present in the urine, bears a higher structural resemblance to PDGF than the intact hCG β -subunit (17, 18).

It must be emphasized, however, that there are only approximately 10% primary structure identities between the glycoprotein hormones and other cystine knot growth factors (17, 18), which is of borderline evolutionary sig-

nificance. This diversity is even higher in the peripheral loops of these proteins, which appear especially important for the specific biological function of these molecules (45, 233). However, it has been recognized that in the course of evolution, the tertiary structure of various proteins changed less rapidly than its amino acid sequence (234). Therefore, in addition to conventional amino acid sequence alignments, structural and/or functional comparisons may be important in determining the extent of evolutionary relationships between glycoprotein hormones and other cystine knot growth factors. In this respect, there may be general similarities in the functional role of the peripheral loop segments within the cystine knot growth factor family. Accordingly, a cluster of positive charges involved in receptor binding has recently been localized in the peripheral loop regions of the neurotrophins, TGF β (235) and osteogenic protein 1 (OP-1) (236), as well as of hTSH analogs (50, 77a). Further, in both hTSH and OP-1, such functionally important loop segments were localized to opposite sides of the central cystine knot (50, 236). The structural and functional similarities between the loops of glycoprotein hormones and cystine knot growth factors thus indicate that these proteins may share common mechanisms of receptor binding and activation, which may involve, at least in part, charged residues at certain positions. However, most other cystine knot growth factors bind to receptor tyrosine kinases (Trk) or receptor serine/threonine kinases and thus signal through different pathways than glycoprotein hormone receptors. Interestingly, LRR are also found in the Trk receptor tyrosine kinases, which are receptors for neurotrophins such as NGF, brain-derived growth factor and neurotrophin-3, further suggesting similarities in ligand-receptor interactions between glycoprotein hormones and other cystine knot growth factors (Table 2).

VIII. Perspectives on Structure-Function Studies of TSH

The development of improved recombinant expression techniques as well as of novel concepts in glycoprotein hormone structure-function relationships has recently led to a more detailed understanding of hTSH action at the molecular level with implications for the other members of the glycoprotein hormone family. hTSH molecular modeling on the basis of the crystal structure of hCG is an important tool to provide prospective, testable models of hTSH action and to guide the rational design of hTSH analogs with predicted activities. The combination of modeling with refined site-directed mutagenesis techniques guided by evolutionary considerations and comparison to other cystine knot growth factors will provide a promising paradigm for protein structure-function studies and should be instrumental in further understanding of hTSH action.

In addition to site-directed mutagenesis of the entire TSH molecule, improved peptide-mimetic approaches using combinations of several active fragments, with structural modifications using mutagenesis or cyclization, is an attractive concept. Alternative strategies to develop smaller

TSH receptor ligands may consist of reducing binding sites by successive rounds of deletion mutagenesis by analogy to protein A (134). Such minimizing may not only be useful as lead compounds for synthetic chemistry, but also to facilitate further probing of structure-function relationships. Moreover, usage of random but structurally constrained peptide libraries should constitute promising avenues to the design of novel TSH receptor ligands. However, in view of the large ligand-receptor interface of glycoprotein hormones and their receptors, it is not clear whether a limited number of interactions will be sufficient to result in specific high-affinity binding, especially since optimal binding requires several discontinuous domains in a defined spatial arrangement. In conjunction with model-based glycoprotein hormone receptor mutagenesis, it should be possible to identify functionally relevant ligand-receptor interactions and to refine existing models in the absence of structural information on ligand-receptor complexes. Such research not only broadens our understanding of classic endocrine glycoprotein hormone physiology but will also shed light on the relevance of less well defined nonclassic actions of such hormones. Thus, strategies outlined here should lead to the design of novel glycoprotein hormone analogs with a broad potential range of applications from basic research to clinical therapy.

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