MINIREVIEW

The Thyrotropin Receptor 25 Years after Its Discovery: New Insight after Its Molecular Cloning

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The molecular cloning and functional expression of the TSH receptor has led to rapid advances in understanding the structure and function of the molecule. Knowledge of its genomic structure provides information on the evolutionary origin of the TSH receptor as well as on the functional organization of its extracellular domain, which is responsible for ligand binding. A beginning has been made in defining the discontinuous contact points for TSH in this extracellular region, but determination of all of the amino acids involved will be difficult. The binding sites of TSH receptor autoantibodies do not appear to be identical to the TSH binding site. Two of the six potential glycosylation sites in the extracellular domain are important in the expression of a functional receptor. Disulfide bonding contributes toward maintenance of the three-dimensional structure of the receptor. Recent evidence suggests that the TSH receptor exists as a single polypeptide chain without subunits. Significant progress has been made in understanding the intracellular regions of the TSH receptor that are involved in signal transduction.

Although still in the distant future, we are closer to the goal of understanding precisely how TSH interacts with and activates its receptor. More importantly from the clinical perspective, we are closer to defining the B cell, and ultimately T cell, epitopes on the TSH receptor that are recognized by the immune system. This information may ultimately facilitate the development of immunological approaches to treating Graves' disease, which will be an improvement over thyroid gland destruction and consequent hypothyroidism, the most common form of therapy at the present time. (Molecular Endocrinology 6: 145–156, 1992)

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INTRODUCTION

TSH exerts its biological effects by binding to the TSH receptor (TSH-R) on the thyroid cell plasma membrane (1, 2) followed by activation of the adenylate cyclase-cAMP (3, 4) and, less well defined, phosphatidyl inositol (5) pathways. The serum component responsible for thyrotoxicosis in Graves' disease (6), identified to be an immunoglobulin (7, 8), is a TSH-R autoantibody (TSH-R Ab) (9, 10). In a smaller number of patients, blocking TSH-R Ab cause thyroid underactivity and hypothyroidism (11, 12). For all these reasons, elucidation of the structure and function of the TSH-R has been an obsessive goal for many laboratories. However, the very low abundance of the TSH-R in thyroid cells (10^3–10^4 receptors per cell) (13), as well as the instability of the protein (14), made its purification a daunting task. Despite many attempts, the TSH-R was never purified sufficiently to provide amino acid sequence information.

In contrast, workers with the related LH/CG receptor were more fortunate because its greater stability and abundance in gonadal tissue permitted its purification (15). Also of importance, unlike with TSH binding to thyroid tissue, there is no low affinity, nonspecific human CG (hCG) binding to gonadal tissue. These qualities facilitated the molecular cloning of the cDNAs for the rat and pig LH/CG receptors (16, 17). Subsequently, the TSH-R cDNA was cloned by the strategies mentioned below. The goal of this review is to describe new insight into the structure, function, and pathophysiology of the TSH-R obtained since its molecular cloning.

MOLECULAR CLONING OF THE TSH-R

Expression Screening

Failing the purification of the TSH-R, initial attempts at its molecular cloning involved the expression screening

of thyroid cDNA libraries with TSH-R Ab. A number of cDNA clones were isolated by screening with Graves' disease immunoglobulin G, either before (18) or after partial affinity purification on thyroid plasma membranes (Hirayu, H., K. Kaufman, and B. Rapoport, unpublished observations). None of these clones coded for the TSH-R. Despite initial optimism, the same result occurred when putative TSH-R monoclonal antibodies (mAb) (19) were used for screening.

Nucleic Acid Screening

With the recognition of the superfamily of guanine nucleotide regulatory (G) protein-coupled receptors with seven transmembrane regions, a reasonable assumption was that the transmembrane and intracellular components of the TSH-R would bear some homology to other members of this family. Because of the functional similarities of the TSH, LH/CG, and FSH receptors, as well as the similar structures of their glycoprotein ligands, these receptors were also thought likely to be closely related.

Before the molecular cloning of the LH/CG receptor cDNA, an attempt was made to clone the TSH-R by low stringency polymerase chain reaction (PCR) using a thyroid cDNA library as template and degenerate oligonucleotide primers based on transmembrane regions III and VI of known members of the receptor superfamily. Unfortunately, although four new G protein-coupled receptor cDNAs were obtained, the TSH-R cDNA was not among them (20). Four months after the molecular cloning of the LH/CG receptor, the nucleotide sequences of full-length dog (21) and human (22–24) TSH-R cDNA clones were reported, as described below.

Parmentier *et al.* (21), in a continuation of their previous approach (20; see above), used a new set of undefined degenerate oligonucleotide primers complementary to transmembrane regions II and VII of G protein-coupled receptors and genomic DNA as template to obtain a putative FSH receptor. This DNA clone was then used as a probe to obtain the dog TSH-R from a thyroid cDNA library. Simultaneously (22), a full-length human TSH-R cDNA clone was isolated and characterized after screening a thyroid cDNA library with a mixture of oligonucleotide probes based on transmembrane regions II and III of the LH/CG receptor (16, 17) and a putative TSH-R fragment (25). Also at the same time, dog TSH-R cDNA (23) and pig LH/CG receptor cDNA (24) probes were used to obtain human TSH-R cDNA clones. Confirmation of the human TSH-R cDNA nucleotide sequence (26), as well as information on the rat TSH-R cDNA sequence (27), followed.

It is interesting to contemplate that all successful attempts to clone the TSH-R cDNA used probes based on transmembrane region sequences. In contrast, previous attempts involving the screening of thyroid cDNA libraries with LH/CG receptor extracellular region cDNA probes failed (22, 26). In retrospect, this latter approach was unlikely to be successful in view of the relatively low homology in the extracellular regions of the glycoprotein receptors (see below).

Functional Expression of the TSH-R

The expression of a functional recombinant TSH-R is an important step in understanding the structure and function of the molecule. The human TSH-R has been stably expressed in Chinese hamster ovary (CHO) cells (22, 28). Transient expression of the dog (21) and human (23, 24, 26) TSH-Rs has been reported in *Xenopus* oocytes, COS 7, and human 293 cells. The affinity for TSH of the recombinant human TSH-R [dissociation constant (K_d) of ~3×10^{-10} M] (23, 29) is similar to that of the TSH-R in thyroid tissue. One particularly responsive clone has been isolated with a K_d of 5×10^{-11} M (28). Stably transfected CHO cells can express approximately 10^5 TSH-Rs per cell (29), 10- to 100-fold the number estimated to be present on human thyroid cells (13). Functionally, the recombinant TSH-R responds to TSH stimulation, both in terms of the adenylate cyclase (21, 22, 24, 26, 27) and phosphotidyl inositol (30) pathways. Recombinant human TSH-R has been used to assay TSH-R autoantibodies (31, 32).

Two forms of TSH-R have been reported to exist in thyroid tissue, one of high affinity and one of low affinity (33). While the high affinity site was regarded as physiologically important, there was disagreement as to the physiological relevance of the low affinity TSH binding site. Detection of low affinity TSH binding to bacteria led to the suggestion that antigenic cross-reactivity between bacteria and thyroid cells played a role in the pathogenesis of Graves' disease (34). Data with the recombinant TSH-R effectively settles this debate. The low affinity TSH binding site is an artifact. This is because both transfected and untransfected CHO cells bind TSH with low affinity (K_d of ~7×10^{-8} M). In retrospect, the presence of low affinity TSH binding sites on plastic dishes (35) provided the same information.

GENOMIC STRUCTURE OF THE TSH-R

The human TSH-R gene spans more than 60 kilobases (kb) (36) and is located on chromosome 14q31 (37, 38). The rat TSH-R is on chromosome 12 (27). Information on the exonic/intronic structure of the TSH and LH/CG receptors provides insight into their evolutionary development and structural function. Before the molecular cloning of the glycoprotein hormone receptors, all known members of the superfamily of G protein-coupled receptors with seven transmembrane domains had very small extracellular regions (subgroup A). The genes for many of these receptors, including the adrenergic and muscarinic receptors, contain single exons and are, therefore, variants of a prototypic receptor gene. The glycoprotein hormone receptors with very large extracellular domains (subgroup B) have a more

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complex genomic structure. These receptors appear to have evolved from the prototypic subgroup A receptors by the integration of a multi-exon component at the 5′-end of the gene. This component codes for a series of leucine-rich repeats that are important in protein-protein interaction, including ligand binding (see below).

The extracellular region of the TSH-R is coded for by 9 exons in addition to the single, much larger, prototypic exon coding for the transmembrane and cytoplasmic regions (36) (Fig. 1). Despite the smaller size of the LH/CG receptor extracellular region, this region is coded for by 10 exons (39, 40). All introns in the TSH and LH/CG receptors are in phase 2, meaning that splice junctions occur between the second and third bases of each codon. A variant dog TSH-R cDNA has been described with deletion of amino acids 61–86 (41), corresponding to the leucine-rich repeat coded for by exon 3 (36).

TISSUE DISTRIBUTION AND REGULATION OF TSH-R GENE EXPRESSION

Thyroid cells contain one or two major TSH-R mRNA transcripts; 4.9 kb in dog (21), 3.9–4.6 kb in human (23, 24, 26), and 4.4–3.0 kb (26) and 5.6–3.3 kb (27) in FRTL-5 cells. Several minor, smaller transcripts (1.7 and 1.1 kb) are also present in human thyroid cells (24). Variations in these estimates of size may, in part, relate to the use of different molecular weight markers. The longer mRNA transcript in FRTL-5 cells may be immature mRNA because it contains a long 3′-noncoding region (27). The smaller bands in human thyroid cells may be created by alternative splicing, as with the LH/CG receptor (17).

Hormonal regulation of TSH-R mRNA levels appears to differ depending on species. In human thyroid cells, there is evidence that physiological concentrations of TSH increase TSH-R mRNA levels (42). In contrast, TSH and Ca^{++} decrease TSH-R mRNA levels in FRTL-5 cells (27, 43). Also in FRTL-5 cells, blocking and stimulatory TSH-R Ab are reported to increase and decrease TSH-R mRNA levels, respectively (27). Information on autoantibody regulation of human TSH-R mRNA levels (presently lacking) would be of interest because of the opposite effect of TSH on receptor mRNA levels in human and rat thyroid cells.

There are reports that TSH-R mRNA levels are decreased in thyroid cancer cells (42, 44), although the number of samples examined is low. Experimentally transformed thyroid cells and an anaplastic thyroid cancer cell line express no or very low levels of TSH-R mRNA (45–47). Overall, TSH-R mRNA levels appear to correlate with the degree of differentiation of each cancer.

Besides thyroid cells, specific, high affinity TSH binding has been demonstrated in lymphocytes (48) and in the guinea pig epididymal fat pad (49). Recent studies using PCR have confirmed the expression of TSH-R mRNA in lymphocytes (50), although the level of expression is extremely low, as evident by the absence of detectable transcripts on Northern blot analysis.

THE EXTRACELLULAR REGION OF THE TSH-R

Primary Amino Acid Structure

The human, dog, and rat TSH-R cDNAs code for a protein of 764 amino acids, including a 20-amino acid signal peptide (Fig. 1). The calculated mol wt of the TSH-R is approximately 84.5K after removal of the signal peptide and excluding any posttranslational glycosylation. The amino-terminal half of the mature TSH-R (398 amino acids) is generally hydrophilic and encodes the large extracellular region with homology to the leucine-rich glycoprotein (LRG) family (51). Consistent with its genomic structure, the structure of the carboxyl-terminal half of the TSH-R (346 amino acids) is quite different to that of the amino terminus and contains the characteristic 7 hydrophobic membrane-spanning segments.

In the extracellular region, amino acid homology between the human, dog, and rat TSH-Rs is high (85–90%). Homology in this region among the TSH, LH/CG, and FSH receptors is less (35–45%) (Fig. 1), being lowest at the extreme ends (TSH-R amino acids 1–57 and 287–404). These relatively nonconserved regions of the TSH-R contain two additional segments, or insertions (amino acid residues 38–45 and 317–366), when compared to the LH/CG receptor. The functional significance of these regions is discussed below. The more homologous region of the receptor extracellular regions (TSH-R amino acids 58–286) corresponds approximately to the leucine-rich repeats (see below). The extracellular loops linking the seven membrane-spanning regions of the TSH-R are relatively conserved among the glycoprotein hormone receptors.

Leucine-Rich Repeats There is amino acid sequence homology between the extracellular region of the TSH-R and many proteins in the LRG family (51). The presence of proteins belonging to this family in species as widely diverse as yeast and primates speaks for their evolutionary importance in protein structure and function. The principal characteristic of this family is the very high content of leucine and the periodic pattern of the consensus sequence, the definition of which, however, is controversial (16, 51). The leucine-rich repeats are likely to form amphipathic β-sheet structures (52) which are thought to interact with both hydrophilic and hydrophobic surfaces.

Based on the genomic DNA structure, we propose that there are nine consensus leucine-rich repeats in the TSH-R between amino acid residues 58–277. These 9 repeated sequences show strong intersegment homology. The consensus sequence proposed is X-Leu-X-X-Thr-X-X-Leu-Thr-X-Leu-Pro-X-X-Ala-Phe-X-X-Leu-X-X-Leu-X-X-X-Leu. Leucine-rich repeats 1–7 cor-

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        1 EXON                                  38    45           2
TSHR  mrpadllqlvllldlprdlg GMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPSTQT.LKLIETHLRTIP
LHR                        ...-PE-.-N-VPDGAL........-C-GPTAGLTR.-S-AYLPVKV--
FSHR                       -S--HHRI-H-SNRVFICQES-VTE.---DL-RNAIE-RFVL-K--V-Q

      69       3                    4                        5
TSHR  SHAFSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNLTYIDPDALKELPLLKFLGIFNTGL
LHR   -Q--RG-NEVIK-EI-QIDS-ERI-ANA-D--INLSE-L-Q--K--R--E-G-FIN--G--Y-S-C---I
FSHR  KG---GFGDLEK-EI-QNDV-EVI-ADV-S--P-LHE-R-EKAN--L--T-E-FQN--N-QY-L-S---I

      139             6                           7                    8
TSHR  KMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQGLCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLN
LHR   RK---V---F-SESN-----C--LHI-T--G-----MN--SV-----G---EE--SH-----T-TSLE-K
FSHR  -HL--VH-IH-LQ.KVL-D-Q--INIHT-ER-S-V--SF-SVI-W-NK--IQEIHNC-----Q---VN-S

      209                    9
TSHR  KNKYLTVIDKDAFGGVYSGPSLLDVSQTSVTALPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLS
LHR   E-VH-EKMHNG--RGA.T--KT--I-S-KLQ----Y---SIQR---TSSYS-----SRET-VN-LE-T-T
FSHR  D-NN-EELPN-V-H-A.---VI--I-R-RIHS---Y---N--K-R--S-YN-----TLEKLVA-ME-S-T

      279          10                            317
TSHR  YPSHCCAFKNQKKIRGILESL........MCNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEK
LHR   -------R-LPTKEQNFSHS.........ISENFSKQCESTVRKVS........................
FSHR  -------A-WRRQISE-HPICNKSILRQEVDYMTQARGQRSSLAE........................

      341                      366
TSHR  SKFQDTHNNAHYYVFFEEQEDEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCED
LHR   .........................NKT-YSSMLAESELSGWD-E-GF-.LPKTPR-A-EP-A------
FSHR  .........................DN-SSYS..RGFDMTYTEF--DL-NEVV-VT-S--P-A------

      411                  I                          II
TSHR  IMGYKFLRIVVWFVSLLALLGNVFVLLILLTSHYKLNVPRFLMCNLAFADFCMGMYLLLIASVDLYTHSE
LHR   ----D---VLI-LINI--IM--MT--FV----R---T---------S-------L---------SQ-KGQ
FSHR  ----NI--VLI--I-I--IT--II--V--T--Q---T---------A---L-I-I---------IH-KSQ

      481                    III                                        IV
TSHR  YYNHAIDWQTGPGCNTAGFFTVFASELSVYTLTVITLERWYAITFAMRLDRKIRLRHACAIMVGGWVCCF
LHR   -----------S--S--------------------------HT--Y-IH--Q-L-----IL--L---LFSS
FSHR  -H-Y-------A--DA-----------------A--------HT--H--Q--C-VQ----ASV--M--IFA-

      551                                    V
TSHR  LLALLPLVGISSYAKVSICLPMDTETPLALAYIVFVLTLNIVAFVIVCCCHVKIYITVRNPQYNPGDKDT
LHR   -I-M-----V-N-M-----F---V--T-SQV--LTI-I--V---F-I-A-YI---FA----ELMATN---
FSHR  AA--F-IF-----M---------IDS--SQL-VMSL-V--VL--VVI-G-YIH--LT----NIVSSSS--

      621            VI                                 VII
TSHR  KIAKRMAVLIFTDFICMAPISFYALSAILNKPLITVSNSKILLVLFYPLNSCANPFLYAIFTKAFQRDVF
LHR   ----K--I------T-------F-I--AFKV-----T---V-------I-------------T----F-
FSHR  R------M------L-------F-I--S-KV------KA-I-----H-I-------------N-R--F-

      691
TSHR  ILLSKFGICKRQAQAYRGQRVPPKNSTDIQVQKVTHDMRQGLHNMEDVYELIENSHLTPKKQGQISEEYM
LHR   L------C---R-EL--RKDFSAYT-NCKNGFTGSNKPS-STLKLSTLHCQGTALLDKTRYTEC
FSHR  -----C-CYEM---I--TETSSTVHN-HPRNGHCSSAP-VTSGSTYILVP-SHLAQN

      761
TSHR  QTVL
```

Fig. 1. Amino Acid Sequence of the Human TSH-R (22)

Amino acid numbering includes the 20-residue signal peptide. Homology to the human LH/CG and FSH receptors (97, 98) is shown. Amino acids are shown in the single amino acid code. - - -, Identical amino acids; · · ·, gap. The exons are indicated as **1–10**. Membrane-spanning regions are *boxed* and are designated I-VII. The entire transmembrane region of the receptor is shown within the *large box*. A number of features in the extracellular region of the receptor are boxed. These are the cysteine residues (C), the potential N-linked glycosylation sites (N-X-S/T), and the two unique tracts in the TSH-R (amino acid residues 38–45 and 317–366).

respond to the amino acids in exons 2–8 (36). Leucine-rich repeats 8 and 9 are situated in exon 9 (36). Thirty five of 220 amino acids (16%) are leucine and 59 of 220 (27%) are aliphatic (isoleucine, leucine, or valine). In contrast, in the bordering segments of the extracellular domain (amino acids 21–57 and 278–418), only 6% (11 of 178) of the amino acids are leucine and 14% (25 of 178) are aliphatic.

Glycoprotein Nature of the TSH-R Studies after the

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molecular cloning of the TSH-R support the concept (53) that it is a glycoprotein. The extracellular domain of the human TSH-R contains six potential N-linked glycosylation sites (consensus sequence: Asn-X-Ser/Thr, where X is any amino acid except Pro). The dog and rat TSH-Rs contain only five potential N-linked glycosylation sites (21, 27). Site-directed mutagenesis studies suggest that two of the six potential N-linked glycosylation sites are important in the expression of a functional TSH-R (54). Thus, the conservative replacement (with Gln) of Asn_{77} abolishes high affinity TSH binding. Substitution of Asn_{113} reduces TSH binding by 10-fold. In contrast, substitution of Asn at the other four potential glycosylation sites (positions 99, 177, 198, and 302) has no effect on TSH binding. Therefore, these four sites are either not glycosylated or do not play a functional role in TSH binding. Although nonglycosylated rat TSH-R produced by *in vitro* transcription and translation was reported to bind TSH, the affinity of this binding was not determined (55). Further studies are necessary to resolve these issues, as well as to determine the importance of glycosylation on TSH-R processing, intracellular trafficking, and expression.

Disulfide Bonds There are 13 Cys residues in the extracellular regions of the TSH-R, including 2 in the first and second extracellular loops. Ten Cys are clearly conserved among the glycoprotein hormone receptors and are likely to be involved in disulfide bonding. These 10 Cys include 2 in the extracellular loops which are conserved among all members of the G protein-coupled receptor family and are known to form disulfide bonds in the adrenergic receptor and rhodopsin. Of the other three Cys in the TSH-R, one is, presumably, an orphan. Conservation of TSH-R Cys_{41} and Cys_{301} is uncertain. Although in the vicinity of Cys residues present in the other glycoprotein hormone receptors, they cannot be aligned directly (Fig. 1). However, Cys_{176} is the most isolated and may well prove to be the orphan.

The nine leucine-rich repeats are flanked at their amino- and carboxyl-termini by conserved, Cys-rich regions. This grouping is a common characteristic of the LRG family. In particular, many leucine-rich proteins have a Cys-rich motif of four Cys residues at their amino-termini (56). This observation suggests that TSH-R Cys_{41} may, indeed, be conserved.

TSH Binding Sites on the TSH-R

There is a large and confusing body of literature regarding putative subunits, protein and nonprotein components of the TSH-R. Much of these data are included in a previous review (13). The most convincing studies, involving chemical or photoaffinity cross-linking of radiolabeled TSH to thyroid membrane preparation (13), suggested that the extracellular region of the TSH-R plays an important role in TSH binding.

The extracellular domain of the TSH-R is extremely large (398 amino acid residues), making it difficult to define the segments which interact with TSH. Not surprisingly, many studies attempting to define the TSH binding sites have focused on the regions of TSH-R least homologous with the LH/CG and FSH receptors. First, at the amino terminus, present evidence suggests that a segment between amino acids 32–50 is either involved in TSH binding or is critically related to maintaining the correct conformation of the molecule. Thus, TSH does not bind CHO cells transfected with a TSH-R cDNA in which amino acids 38–45 are deleted or substituted (57). This study is not definitive, however, because of the possibility of destabilizing conformational changes in the mutant proteins. The 8 amino acids mutated include Cys_{41} (see above). On an individual basis, none of the substitutions in this area, except Cys_{41}, affect TSH binding (57a). Studies of TSH binding to synthetic peptides do (58, 59) or do not (60) support the notion that the TSH-R amino-terminus contains a specific binding site for TSH.

Turning to the carboxyl-terminus of the TSH-R extracellular region, the most striking feature is the unique 50 amino acid insertion (residues 317–366) (Fig. 1). It was, therefore, very surprising that the deletion of this segment had no effect on TSH binding or signal transduction (57). Despite this apparent lack of importance in ligand binding, there is a report of a peptide in this region (amino acids 344–364) binding TSH (58), while a much shorter peptide (amino acids 353–364) does not (60). Recently, an extensive deletion and nonhomologous substitution study of residues flanking the 50 amino acid insertion in the rat TSH-R has suggested the involvement of many amino acids in the TSH binding site, Cys_{390}, Cys_{301}, and Tyr_{385} being particularly interesting (61, 62).

Homologous amino acid substitutions are less likely to cause conformational changes than the deletions and nonhomologous mutations performed in the studies described above. Data obtained with a series of TSH-LH/CG chimeric receptors with homologous substitutions provide provocative information on the TSH binding sites (63, 64). Although (as anticipated) the extracellular region of the TSH-R is critical for high affinity TSH binding, there is remarkable tolerance for homologous substitution in all areas of the TSH-R extracellular domain, indicating that the TSH binding sites span the entire region. These findings have been confirmed in another study (65). However, the middle region (domain C; amino acids 171–260) appears to be particularly important in TSH binding.

Finer mapping indicates that 11 amino acids (segment C3, Lys_{201}–Lys_{211}) and the homologous region of the LH/CG receptor (Thr_{202}–Ile_{212}) are important for TSH and hCG binding, respectively. In addition, 9 amino acids in the TSH-R (segment C5; Gly_{222}–Leu_{230}) are also involved in the hormone binding, though to a lesser degree. Thus the TSH and hCG binding sites coincide for at least one of the hormone-receptor contact points (segment C3) in the respective receptors.

Binding Sites for TSH-R Ab

The molecular mechanism by which TSH-R Ab can cause either hyperthyroidism or hypothyroidism is an

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unanswered question of clinical importance. Studies with the recombinant human TSH-R provide new insight into the interaction between the receptor and TSH-R Ab. Deletional and nonhomologous substitutions of amino acids 38–45 in the TSH-R suggest that this segment may be important in stimulatory TSH-R Ab action (57), although these data are not definitive (see above). Data obtained with synthetic peptides suggest that TSH-R amino acids 32–56, 29–57 (which include the 8-amino acid region), 123–131, 172–202, 309–337, 341–358, 353–364, 352–366, and 372–397 may contribute to Ab epitopes (60, 66–70). There are, however, also contradictory data (59, 60). In addition, failure to recognize polypeptides generated in TSH-R cDNA fragment libraries (71; Nagayama. Y., and B. Rapoport, unpublished data) suggest that TSH-R Abs do not recognize linear epitopes. However, the concentration of TSH-R Ab in serum is unknown and may be too low to allow a detectable signal on screening.

Data obtained with extracellular domain chimeric receptors support the concept that the epitopes for TSH-R Ab are not linear. Thus, as for TSH, the epitopes for autoantibodies span the entire TSH-R extracellular domain (72). Within TSH-R amino acids 261–418 (domains D and E), the TSH binding site and the epitopes for both stimulatory and blocking Ab appear to overlap closely. In contrast, within amino acids 1–260 (domains A, B, and C) there is less overlap between the epitopes for both types of TSH-R Ab and the TSH binding site. Alternatively, TSH and TSH-R Ab may bind to the same regions, but with differing affinities. Fine mapping of these regions implicates amino acids 25–30 as a binding site for some, but not all, stimulatory TSH-R Ab. This segment is not a TSH binding site (Nagayama, Y., and B. Rapoport, submitted). Determination of the precise epitopes and affinities of the different TSH-R Abs is very important, but will require the availability of human, disease-associated mAbs. Previous reports of the generation of human TSH-R mAb by conventional cell-fusion methods (19, 73) have not been substantiated. The bacteriophage combinatorial library approach, recently used to isolate human autoantibodies against thyroid peroxidase (74), may be an important new tool to attain this long-standing goal.

The TSH-R Extracellular Region in Signal Transduction

The extracellular region of the TSH-R is important in signal transduction (63) as well as ligand binding. Thus, substitution with the homologous region of the LH/CG receptor of TSH-R amino acids 261–360 (domain D) or 361–418 (domain E) reduces signal transduction without affecting TSH binding. In addition, substitution of amino acids 1–260 (domains A, B, and C) leads to complete dissociation between ligand binding and signal transduction. Therefore, domains D and E, but especially domain C, appear to play an important role in signal transduction.

The foregoing observations are not surprising because the primordial receptor coded for by exon 10, which contains the transmembrane and intracellular components of the TSH-R, must be informed in some manner that TSH is occupying the extracellular domain. In contrast, in other members of the G protein-coupled receptor family (subgroup A), small ligands bind directly to a spatially defined site within the seven transmembrane segments. These chimeric receptor studies also support the concept that the amino and carboxyl-termini of the TSH-R extracellular domain are in contact with one another during the process of signal transduction. Thus the concurrent substitution of domains A and B and domain E restores, and even enhances, signal transduction (63). As discussed above, the conserved Cys residues in the A and E regions are likely to be involved in disulfide bonding.

THE TRANSMEMBRANE/INTRACELLULAR REGION OF THE TSH-R

Primary Amino Acid Structure

The transmembrane region of the TSH-R consists of 346 amino acids with 7 transmembrane segments, three extracellular loops (discussed above), three cytoplasmic loops, and a carboxyl-terminal cytoplasmic tail. The transmembrane region of the human TSH-R is approximately 85–90% homologous with these regions in the dog and rat TSH-Rs, 70–75% homologous with the LH/CG and FSH receptors, and only 20–25% homologous with other members of the G protein-coupled receptor family (subgroup A).

In the glycoprotein hormone receptors, the second, third, and seventh membrane-spanning regions are highly conserved (Fig. 1). There is much lower conservation in the other four transmembrane regions. A similar relationship is observed among the different subtypes of the β-adrenergic receptors. The members of subgroup A, unlike the TSH-R, contain an aspartic acid in the third transmembrane segment and two serine residues in the fifth transmembrane segment. These residues are reported to be important for the intramembrane ligand binding in the adrenergic receptors (75). Prolines, which may be necessary for proper insertion of the protein into the membrane, are seen in the fourth, sixth, and seventh transmembrane segments of all glycoprotein hormone receptors.

Among the glycoprotein hormone receptors, there is greatest amino acid homology in the first cytoplasmic loop and the amino-terminal one-third of the cytoplasmic tail. When comparing the glycoprotein hormone receptor sequences with those of subgroup A, the most conserved cytoplasmic regions are the first and second cytoplasmic loops (76), suggesting divergent functions for the third cytoplasmic loop and the cytoplasmic tail. Even in the adrenergic receptors, the third cytoplasmic loops are also dissimilar, probably contributing to the unique functions of each receptor subtype (α-1 receptor with the PI pathway; α-2 receptor with the G_i pathway,

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and β-receptor with the G_s pathway). The third cytoplasmic loop in the glycoprotein hormone receptors is much shorter than the corresponding region in the other G protein-coupled receptors.

The cytoplasmic regions of the TSH-R do not have the typical consensus recognition site for cAMP-dependent protein kinase (Arg-Arg-X-*Ser/Thr*). Potential phosphorylation sites for protein kinase C include Thr_{607}-Val-Arg, Lys-Asp-Thr_{620}-Lys, Ser_{694}-Lys, Lys-Asn-Ser_{716}-Thr_{717}, Lys-Val-Thr_{725} and Thr_{748}-Pro-Lys-Lys (77). Further, some Ser and Thr residues with acidic amino acids (Asp and Glu) at their carboxyl- or amino-terminal ends (Asp-Thr_{620}, Ser_{716}-*Thr*-Asp, Thr_{725}-His-Asp, Glu-Asn-Ser_{745}, and Ser_{756}-Glu-Glu) may be targets for phosphorylation by an enzyme similar to rhodopsin kinase/β-adrenergic receptor kinase, if this type of kinase is present in thyroid cells. The amino-half of the third cytoplasmic loop is homologous to the carboxyl-terminal 12-amino acid motif present in all the protein tyrosine kinases of the nonreceptor type (24). However, no consensus sequence of ATP binding site in tyrosine kinase, Gly-Gly-X-X-Gly, is observed.

Mapping Regions of Importance in Signal Transduction (cAMP Production)

Because of their more recent cloning, there are only limited data on mapping the intracellular regions of the subgroup B receptors (TSH, LH/CG, and FSH) receptors that are involved in signal transduction. These data, involving site-directed mutagenesis of the TSH-R cytoplasmic regions (78), indicate some similarities, but also substantial differences, with the subgroup A receptors. The first cytoplasmic loop (the smallest of the three; amino acids 441–450) is important in TSH-R signal transduction (cAMP generation) because conservative amino acid substitutions in this region completely abolish signal transduction without affecting high affinity ligand binding. Definitive data for this region are not available for subgroup A receptors because the mutations performed greatly reduced ligand binding (79). In contrast to the β-adrenergic receptor, the TSH-R second cytoplasmic loop carboxyl-terminus (amino acids 528–537) is also vital for signal transduction. As in studies with the β-adrenergic receptor, mutations in the TSH-R second cytoplasmic loop amino-terminus were not informative because of loss of ligand binding (78, 79). Mutagenesis of the TSH-R third cytoplasmic loop amino-terminus had no effect on receptor function, suggesting that it is not involved in coupling to Gs. Data on the functional importance of this segment in the β-adrenergic receptor are contradictory (79).

The greatest similarity between the A and B receptor subgroups was observed for the carboxyl-terminus of the third cytoplasmic loop (TSH-R amino acids 603–614), in which mutations interrupt signal transduction (78, 79). Individual amino acid substitutions in this area of the TSH-R do not change signal transduction, indicating the coordinate involvement of multiple amino acids (80). Regarding the TSH-R cytoplasmic tail, deletion of two-thirds of the 82 amino acids has no effect on cAMP production (78). Similar data were obtained in the β-adrenergic receptor (79). Positively charged residues and a tyrosine in the amino terminus of the TSH-R cytoplasmic tail (between residues 699–707) are not important in signal transduction (78).

TSH Receptor Desensitization

Homologous (hormone-specific) desensitization is a general phenomenon involving many hormone-responsive tissues. In the case of the thyroid follicular cell, prior TSH stimulation leads to a 30–70% decrease in the subsequent cAMP response to TSH stimulation (81, 82). As with homologous desensitization to a variety of hormones, TSH desensitization involves decreased coupling of the TSH-R with G_s (83). Both the functional activity of G_s and the adenylate cyclase catalytic unit remain unaffected (83).

The mechanism underlying homologous TSH desensitization has not been determined. TSH-R down-regulation can be excluded (81, 84). There is evidence that a putative desensitization protein in thyroid cells plays a role in this process (81, 85), as may TSH-mediated ADP ribosylation of an unknown substrate (86, 87). However, for nearly a decade there has been little progress in this area of investigation, primarily because the TSH-R could not be studied at a molecular level. Surprisingly, when stably expressed in CHO cells, the TSH-R does not undergo desensitization to TSH stimulation (29). Knowledge of the molecular structure of the TSH-R, particularly its cytoplasmic regions, may permit further elucidation of the TSH desensitization mechanism, in particular the identification of a putative desensitization protein which may be thyroid-specific and absent in CHO cells. One candidate is a thyroid arrestin-like protein, as has been demonstrated for the β-adrenergic receptor.

SUBUNIT STRUCTURE OF THE TSH-R AND ASSOCIATED MOLECULES

TSH Receptor Subunits

There have been many contradictory reports on the TSH-R subunit structure. Before its molecular cloning, the most widely held model for the TSH-R was that of a 80K heterodimer linked by disulfide bonds (13). Evidence suggested that TSH, primarily through its β-subunit, bound to a hydrophilic approximately 50K A subunit linked noncovalently to a membrane-spanning approximately 30K B subunit. The B subunit could not be visualized, but its size was estimated on the basis of the size of the holoreceptor and receptor A subunit. Other studies too numerous to list suggested mol wts for the TSH-R, or its subunits, of between 15–200K.

With the molecular cloning of the TSH-R, it became difficult to reconcile previous models with the primary amino acid sequence of the receptor. Thus, the calcu-

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lated mol wt of the 744 amino acid polypeptide backbone of the TSH-R (after deletion of the signal peptide) is approximately 84K. To this should be added an unknown molecular mass depending on which of the six potential glycosylation sites are, in fact, glycosylated. As mentioned above, there is evidence for glycosylation at, at least, two of these sites (54). Therefore, rather than approximately 80K, the predicted size of the TSH holoreceptor is closer to approximately 100K.

Recent studies with the recombinant TSH-R stably expressed in CHO cells have resolved the size paradox and suggest further that the two-subunit model for the TSH-R may not be correct. Like the LH/CG receptor (15), it appears that the TSH-R exists, in large part, as a single subunit (88). The evidence for this conclusion is that when radiolabeled TSH is cross-linked to the human TSH-R expressed in cells *in vivo*, rather than to plasma membranes (as used in previous studies), a single polypeptide chain of approximately 100K (as well as a ~54K fragment) is visible even under reducing conditions (88). The holoreceptor appears to be highly susceptible to proteolytic digestion during membrane preparation despite the use of multiple proteolytic inhibitors. The reason for the estimation of a lesser size for the TSH-R after radiolabeled TSH cross-linking to thyroid membranes may be that, under these conditions, the holoreceptor can only be visualized in the nonreduced state. The molecular mass of a protein is more accurately estimated after complete reduction.

Based, in part, on the present data, we propose a new model for the human TSH-R (Fig. 2). The mature TSH-R appears to be a glycoprotein with a single polypeptide chain. Intramolecular disulfide bonds are likely to occur between the clusters of highly conserved Cys residues located at the amino- and carboxyl-termini of the extracellular domain. Support for this model is provided by the larger form of the TSH-R observed under reducing conditions after TSH cross-linking to intact rat FRTL-5 cells (89, 90). This form of the TSH-R was, however, considered to be a precursor and not the mature molecule (13).

The new model predicts proteolytic cleavage of the TSH-R during membrane preparation at a site(s) between the disulfide bridges. Cross-linking of radiolabeled TSH to intact cells expressing a mutant TSH-R (TSHR-D1) lacking amino acids 317–366 localizes the proteolytic cleavage site to just upstream of amino acid residue 317 (88). The molecular mass of a polypeptide of approximately 297 amino acid residues (after deletion of the signal peptide) would be approximately 34.5K, smaller than the 54K TSH-binding fragment released by proteolysis (88). Glycosylation of at least two of the six potential glycosylation sites in this fragment (54) is a likely explanation for this discrepancy. It must be recognized that although proteolytic cleavage of the TSH-R may be an artifact of membrane preparation, the *in vivo* physiological significance of this phenomenon cannot be excluded. The extracellular region of the LH/CG receptor is released by proteolysis after hormone binding (91).

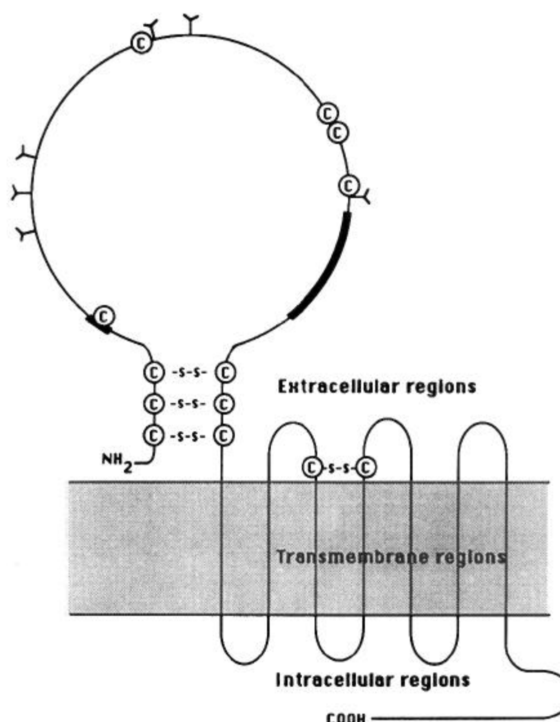

Fig. 2. Proposed Model of the Human TSH-R

Disulfide bonds are shown between highly conserved cysteine (C) residues at the amino and carboxyl termini of the extracellular region. The *thick black segments* represent the regions unique to the TSH-R between amino acid residues 38–45 and 317–366. The Y symbols depict potential N-linked glycosylation sites.

Other Molecules Associated with the TSH-R

There is an extensive literature contending that, besides a glycoprotein component, the TSH-R also contains a ganglioside component of functional significance (92). Supporting evidence for a ganglioside component in the TSH-R included: 1) glycoprotein and ganglioside components could be separated from one another after membrane solubilization; 2) gangliosides were reported to specifically inhibit TSH binding to the TSH-R; 3) reconstitution of liposomes with mixed gangliosides were reported to create a high affinity TSH binding site similar to the TSH-R; 4) a thyroid cell-specific ganglioside was absent in the plasma membranes of a thyroid tumor unable to bind TSH; 5) reconstitution of appropriate gangliosides in thyroid cells from this tumor was reported to restore specific TSH binding and signal transduction; and 6) putative mAbs against the TSH-R were used to support the two-component (glycoprotein and ganglioside) model of the TSH-R. In sum, these studies concluded that the glycoprotein component of the TSH-R was responsible for specific binding by the β-subunit of the ligand (TSH). The ganglioside component of the TSH-R was believed to be responsible for signal transduction after interacting with the ligand α-subunit.

Some investigators on the original reports on the

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importance of gangliosides in the TSH-R re-examined this thesis in carefully controlled studies. Manipulation of ganglioside composition in rat thyroid cells altered cholera toxin, but not TSH, binding, and function (93). They concluded that their studies "rule out any role for gangliosides in the binding of TSH to thyroid cells" (93). Despite this retraction, as well as other data suggesting a postreceptor effect for gangliosides (94), a ganglioside in the GM1 family has been reported very recently to be an integral component of the purified TSH-R (95).

We suggest that it is time to discard the hypothesis of a ganglioside component to the TSH-R for the following reasons. First, the contradictory data described above should be recognized. Second, there is no definitive evidence that the purified thyroid protein reported to contain a ganglioside is, in fact, the TSH-R. Third, there is no confirmation by independent laboratories of the specificity of the TSH-R mAb antibodies reported to react preferentially with gangliosides. Finally, the expression of a functional, recombinant TSH-R in nonthyroidal cells excludes the requirement for a thyroid-specific ganglioside. Although gangliosides may play a role in postreceptor events involved in signal transduction, such a role would not be as an integral part of the TSH-R molecule.

Recently, a 70K heat shock protein and 43K τ-actin have been reported to copurify with a protein believed to be the TSH-R (96). These proteins, particularly the hydrophobic heat shock protein, are recognized to interact nonspecifically with many proteins (96). Nevertheless, the data are interpreted as indicating that the association between the heat shock protein 70 and the TSH-R appears to be important to the development or expression of Graves' disease. Further evidence is required to substantiate this concept.

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