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The Thyrotropin (TSH) Receptor: Interaction with TSH and Autoantibodies*

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I. Introduction

THE 9 yr since the molecular cloning of the TSH receptor (TSHR) (1–6) have seen major advances in our understanding of this protein, a long sought after molecule of vital importance in the physiological and pathological regulation of thyroid function. Initial studies focused on oligonucleotide-directed mutagenesis and on the creation of chimeric TSH-gonadotropin receptor molecules as approaches to understanding TSHR structure and function. Other avenues of investigation involved the use of synthetic TSHR peptides and antisera generated by immunizing animals with recombinant TSHR preparations. Information rapidly accumulated, primarily on the extracellular portion of the TSHR (ectodomain). Unfortunately, the sheer volume, complexity, and often contradictory nature of this information made the field very difficult to follow.

In 1992, during this logarithmic expansion of data on the TSHR, Lefkowitz and co-workers (7) made the fundamental observation that mutations in the cytoplasmic region of the

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α1B-adrenergic receptor, a member of the same G protein-coupled receptor (GPCR) family, led to constitutive activation of the receptor in the absence of ligand. Spontaneous mutations in the serpentine portion of the TSHR were soon sought and found to be the cause of a significant number of toxic thyroid adenomata in 1993 (8) and familial, nonautoimmune hyperthyroidism in 1994 (9). Suddenly, the molecular biology of the TSHR became logical and understandable. Spontaneously occurring TSHR mutants provided exciting new insights into receptor structure and function (reviewed in Refs. 10 and 11).

The great contrast between the comprehensibility and clinical importance of experiments of nature (primarily in the TSHR transmembrane and cytoplasmic regions) and the experimental mutants (largely in the ectodomain) led many observers to despair of trying to understand the latter or, worse, to be so awed by the complexity of the field as to accept speculation or misinterpretation of data as fact. The present review is an attempt to correct this imbalance. This goal cannot be achieved by simple repetition of a mass of data. We, therefore, provide our interpretation of the subject, for which reason some bias is unavoidable. In particular, we wish to distinguish between conclusions that, at least in our view, are unequivocal and those that remain hypotheses or can be discarded. We have tried to be fair but accept that some of our interpretations may prove to be wrong in the future.

In some respects, investigation of the TSHR ectodomain is more difficult than that of the serpentine segment of the receptor. Thus, mechanistically informative, spontaneous mutations causing thyroid dysfunction are less likely to occur in the ectodomain. With some exceptions, major changes in TSHR binding and function are unlikely to be caused by single amino acid alterations in the ectodomain. Moreover, the lesser conservation of the TSHR ectodomain than of the serpentine region reduces the lessons that can be learned from other members of the GPCR superfamily. With the exception of its leucine-rich repeats (LRRs), the conformational structure of the TSHR ectodomain is an enigma. Nevertheless, despite these disadvantages, studies on the ectodomain cannot be avoided if we are to understand the actions of TSH and TSHR autoantibodies.

Even since its molecular cloning, the TSHR has lived up to its reputation of being a very difficult molecule to study. Expression of significant quantities of recombinant receptor in a form capable of binding TSH and autoantibodies has been an exceptionally difficult, frustrating, and time-consuming undertaking. Because the expression of many other recombinant proteins is a straightforward, mundane, and technically uninteresting subject, difficulties experienced with the TSHR are commonly attributed to lack of experience or suitable facilities. The conceptual hurdles in TSHR expression, including the highly conformational nature of the protein or the extremely high glycan content of the ectodomain, are not generally appreciated. It is also not well understood how great a handicap is the very low concentration of TSHR autoantibodies in patients' sera, why the cloning of human autoantibodies from patients' B cells has been far more difficult for the TSHR than for thyroid peroxidase (TPO), why it is difficult to generate antibodies in experimental animals that mimic the actions of autoantibodies and, finally, why it has been so difficult to develop an animal model of Graves' disease by simply injecting recombinant antigen. In the future, determination of the three-dimensional structure of the TSHR ectodomain will also be exceptionally difficult and success is by no means assured.

On the other hand, it is precisely these difficulties and challenges that make the TSHR, especially the ectodomain, such a rewarding molecule to study. We are fortunate to have the opportunity to study the role of the ectodomain in TSHR activation by the immune system in Graves' disease, a common clinical phenomenon only rarely observed with other members of the GPCR family. Recent evidence also now reveals that the TSHR subunit structure is unique to presently known members of the superfamily. Further improvements in autoantigen generation will no doubt lead to advances in the diagnosis of Graves' disease and, possibly, to immunotherapy that will replace the present options of surgery, radioiodine ablation, or long-term antithyroid drug therapy.

II. TSHR Ectodomain Structure

A. Primary amino acid structure

The polypeptide backbone of the TSHR, deduced from its cDNA sequence, is 764 amino acids in length (84.5 kDa) (1–4). The N terminus of the protein is orientated toward the exterior of the cell and is estimated to first enter the membrane after amino acid residue 418. On this basis, after deletion of a 21-amino acid signal peptide, the TSHR ectodomain contains 397 residues (45.2 kDa) (Fig. 1). Even excluding its carbohydrate component (see below), the TSHR ectodomain represents nearly half the mass of the holoreceptor, a very large size compared with the vast majority of members of the GPCR superfamily with seven membrane-spanning regions. The complexity and size of the TSHR ectodomain derives from the evolutionary addition of nine exons (12) to the single prototypic exon encoding receptors, such as for rhodopsin and epinephrine, that have small ectodomains. This disparity in ectodomain size is consistent with the difference in size of the ligands for their cognate receptors, although some interesting disparities exist (the Ca^{++} receptor has a very small ligand and a large ectodomain). All introns in the TSHR are in phase 2, meaning that splice junctions occur between the second and third bases of each codon.

As would be expected from the fact that the glycoprotein hormone receptors (for TSH, LH/CG, and FSH) have different ligands yet similar signaling pathways, homology among their ectodomains (35–45%) is lower than among their transmembrane and cytoplasmic regions. Within the ectodomains themselves, homology is greatest in the midregions (TSHR amino acid residues ~58–288), corresponding primarily to the LRRs (see below). The less conserved N-terminal and C-terminal regions of the TSHR are interesting in that they contain additional residues in comparison to the LH/CG or FSH receptors. This difference is greatest between the TSH and LH/CG receptors (LH/CGR). Thus, alignment of their amino acid sequences reveals that these additional residues are clustered in two approximate regions, giving the

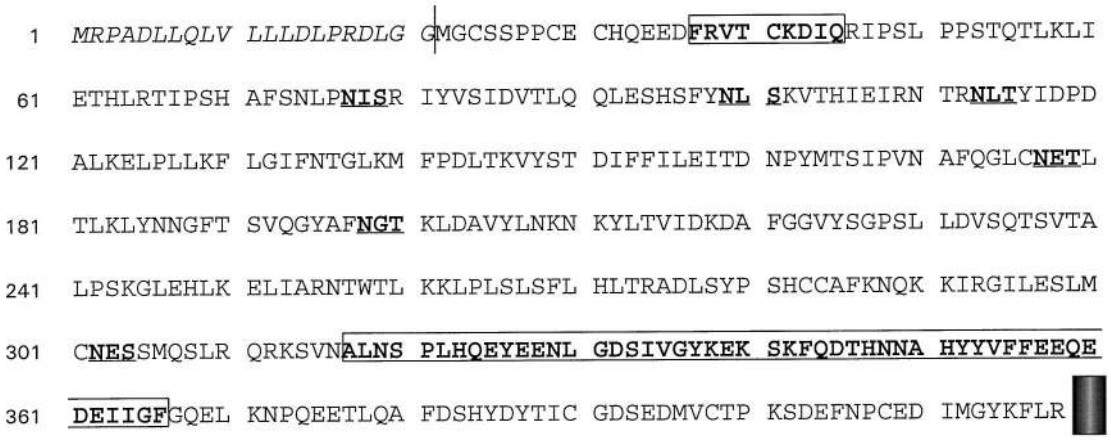

FIG. 1. TSHR ectodomain amino acid sequence. The signal peptide, deduced to be residues 1–21, is shown in *italics*. The six putative sites of N-linked glycosylation are in *bold and underlined*. The 8 and 50 amino acid residues that are "inserted" into the TSHR ectodomain relative to that of the LH/CGR are *boxed*. The location of these residues must be regarded as only approximate because of low homology between the TSHR and LH/CGR in these regions.

appearance of "insertions" of 8 amino acids (residues 38–45) and 50 amino acids (residues 317–366) (Fig. 1). It must be emphasized that, given the low homology between these regions of the TSHR and LH/CGR ectodomains, the designated boundaries of the two insertions must be regarded as approximate.

The genomic structure of the TSHR ectodomain indicated that the division into exons corresponded to the limits of monomers or multimers of LRRs (12). Analysis of the amino acid sequence of this region suggested nine LRRs between amino acid residues 58–277 (13, 14). These nine repeated sequences show strong intersegment homology. The consensus sequence proposed was xLxxTxxLTxLPxxAFxxLxx-LxxxL. LRRs 1–7 corresponded to the amino acids in exons 2–8 (12). LRRs 8 and 9 are situated in exon 9 (12). A variant dog TSHR cDNA has been described with deletion of amino acids 61–86 (15), corresponding to the LRRs encoded by exon 3. More recently, computer modeling based on ribonuclease inhibitor (16), the first molecule with LRRs to have its three-dimensional structure solved, has suggested a slightly different structure with eight LRRs between amino acid residues 54–254 corresponding to exons (17).

B. Subunit structure

1. Single and two-subunit forms of the TSHR. Clarification of the subunit structure of the TSHR is fundamental for understanding the mechanism of action of TSH and TSHR autoantibodies. Before its molecular cloning, studies too numerous to mention described a TSHR with a variety of subunits with molecular masses ranging from 15 kDa to 200 kDa. The most credible data, obtained by [^{125}I]TSH cross-linking to thyroid plasma membrane preparations or to cultures of FRTL-5 rat thyroid cells, provided two schools of thought regarding TSHR subunit structure (reviewed in Ref. 18). On the one hand, the TSHR was suggested to be a 80-kDa heterodimer with a 50-kDa hormone-binding A subunit linked by disulfide bonds to a membrane-spanning 30-kDa B subunit (19, 20). Surprisingly, using similar reagents, other investigators suggested that the TSHR contained two subunits of 53 and 40 kDa (18), or three subunits of 31, 17, and 63 kDa (21), none of which were linked by disulfide bonds.

The molecular cloning of the TSHR in 1989 resolved this controversy but also generated puzzling and apparently contradictory observations that have led to considerable misunderstanding or skepticism. Very recently, important pieces of the puzzle have fallen into place and reveal a receptor more complex and fascinating than previously imagined. The following is our perspective of the series of advances and controversies in recent years.

1. Covalent cross-linking of radiolabeled TSH to membranes prepared from nonthyroidal mammalian cells expressing the recombinant human TSHR (22, 23) essentially confirmed the prior observations of Rees Smith and associates (19, 20), although these investigators slightly underestimated the sizes of the A and B subunits (see below).

2. The fact that the TSHR was encoded by a single mRNA (1–6) established that TSHR subunits are formed by intramolecular cleavage of a single polypeptide chain. This situation contrasts with the subunits of its cognate ligand, TSH, which are encoded by separate genes.

3. The most surprising initial finding with the recombinant human TSHR was that cross-linking of [^{125}I]TSH to intact cells in monolayer culture, rather than to membranes from cell homogenates, revealed two types of receptors on the cell surface. In addition to the two-subunit form previously observed in broken cell preparations, a considerable proportion of the receptors on the cell surface were present as a single, uncleaved chain without subunits (22). Both the single chain and the two-subunit forms of the TSHR bound TSH with similar high affinity. These data raised the possibility that the monomeric TSHR was the physiological form and that the two-subunit TSHR was present to a greater extent after cell homogenization because of lysosomal enzyme release (22). In retrospect, a single-chain TSHR had been observed on TSH cross-linking to intact, cultured rat (FRTL5) cells but had been considered a precursor (24). Support for the concept of a single-chain TSHR was also obtained in immunoblot studies of cell extracts in which only monomeric (25) or predom-

inantly monomeric (26) forms of the TSHR were detected. As discussed below, however, it is difficult to interpret studies involving the immunodetection of the TSHR without analysis of the carbohydrate composition of the TSHR and without knowing whether the monomeric receptors detected are on the cell surface.

4. The development of murine monoclonal antibodies (mAbs) to the TSHR greatly facilitated investigation of its subunit structure. Data obtained with these reagents challenged the concept of a physiological single-chain form of the TSHR, for the following reasons (27, 28): (i) Only A and B subunits (renamed α and β), and not even a trace of monomeric receptor, could be detected after TSHR affinity purification from homogenized human thyroid tissue. (ii) TSHR cleavage into A and B subunits (original terminology retained for reasons stated below) occurred late in the synthetic process, either in the Golgi complex or at the cell surface. Thus, kinetic studies of precursor-labeled recombinant TSHR expressed in mouse L cells revealed that TSHR intramolecular cleavage occurred only after maturation of N-linked glycan moieties from the high-mannose to the complex form (28). Glycan trimming and maturation to complex forms occur in the Golgi complex and are required for protein trafficking to the cell surface (29). These data suggested that TSHR A and B subunit formation was an ordered physiological event rather than a proteolytic artifact consequent to cell homogenization. (iii) Unlike in human thyroid tissue extracts, homogenates of transfected mouse L cells contained large amounts of TSHR monomers with immature, high-mannose carbohydrate (28). This material was suggested to reflect the abnormal intracellular accumulation of TSHR precursors consequent to receptor overexpression overwhelming the protein-processing capacity of the cells.

Based on all of these findings, the perception has arisen that the monomeric TSHR is an unphysiological artifact of transfected nonthyroidal cells, unrelated to the situation in thyroid tissue *in vivo*. A reconciliation of these apparently contradictory data is provided below.

2. Loss of a C peptide during intramolecular cleavage of the TSHR into two subunits. A major surprise in the past year has been the realization that intramolecular cleavage of the TSHR into disulfide-linked A and B subunits does not occur at a single site as previously suspected, but at *two* sites (30). Consequently, although it has not yet been directly identified, a putative C peptide is released, analagous to the derivation of insulin from proinsulin (Fig. 2). This remarkable phenomenon is unique to presently known members of the GPCR superfamily. Three lines of evidence suggested the presence of two cleavage sites in the TSHR:

1. For a number of years, we were puzzled by an observation in TSH cross-linking studies with certain chimeric TSH-LH/CGRs. These receptors are valuable in investigating intramolecular cleavage because the LH/CGR, unlike the TSH, does not undergo cleavage. Neither substitution of TSHR residues 261–362 nor residues 363–418 with the homologous regions of the noncleaving LH/CGR prevented TSHR cleavage into two subunits (23). Only the simultaneous substitution of both segments abrogated cleavage. In retrospect, these findings are consistent with each segment containing a cleavage site.

2. The sum of the TSHR A and B subunit polypeptide chains is ~5–7 kDa smaller than that of the uncleaved, monomeric TSHR. The predicted and observed size of the single chain polypeptide backbone is 84.5 kDa. Yet, the enzymatically deglycosylated A subunit polypeptide and the nonglycosylated B subunit are no more than ~35 kDa and ~42 kDa in size, respectively (27, 28, 30, 31). Although size estimations cannot be very precise, they are sufficiently reproducible among different laboratories using different methodologies for TSHR detection to suggest that a piece of the TSHR has been lost during intramolecular cleavage. In addition to the predominant subunit forms, lesser amounts of A and B subunits of slightly larger size exist, suggesting that there is a minor degree of incomplete cleavage at both sites.

3. A c-*myc* epitope strategically inserted within the putative C peptide region (Fig. 2) was lost in the cleaved, but not in the uncleaved, monomeric receptor (30). Thus, despite the selective inability of a c-*myc* mAb to detect the two-subunit TSHR, this receptor was readily detected with another mAb to the N terminus of the A subunit. Loss of the c-*myc* epitope

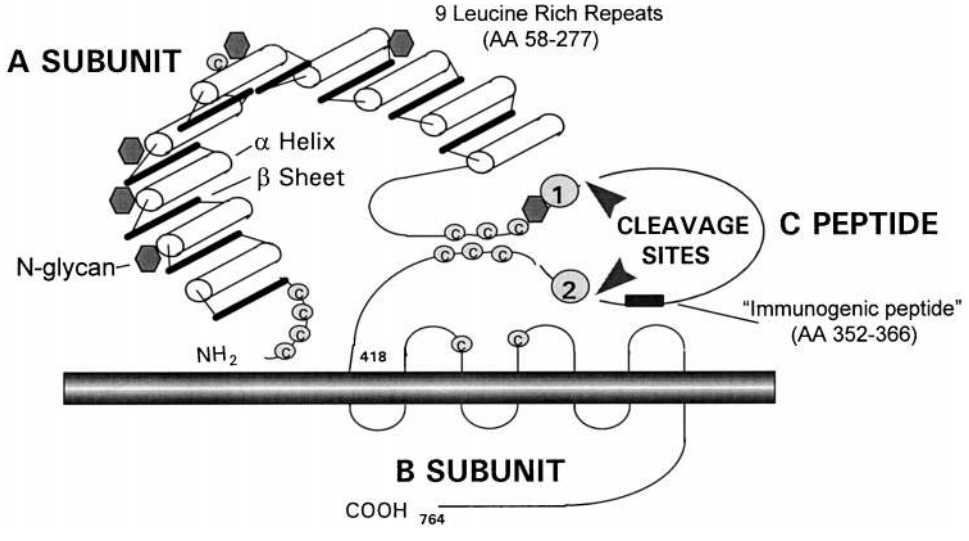

FIG. 2. Schematic representation of the TSHR with two cleavage sites.

by cleavage at a single site within this epitope was unlikely for a number of reasons. Thus (i) single cleavage within amino acid residues 338–349 (the c-*myc* epitope) would generate a B subunit of 46–47 kDa, clearly larger than the actual size (33–42 kDa) observed experimentally (27, 28, 32). (ii) Chimeric receptor TSH-LHR-4 (33) and a deletion mutant (residues 317–366) of the wild-type TSHR (34) lack the region in which the c-*myc* epitope was inserted, yet still cleave into two subunits (22, 23). (iii) The c-*myc* epitope substitution substantially alters the sequence of the wild-type receptor. Cleavage within the c-*myc* epitope would, therefore, imply nonspecificity in the amino acid sequence at a TSHR cleavage site.

Purification and characterization of the putative TSHR C peptide would provide proof for the two-cleavage site hypothesis. However, it is not presently feasible to detect the release of a small TSHR polypeptide fragment into the culture medium from the TSHRmyc cell line that is not overexpressing the TSHR. Very recently, the concept of two cleavage sites has been confirmed by mutagenesis of the TSHR (see *Section II.B.5*). Because there has been misunderstanding on this issue, the existence of two cleavage sites should not be construed as evidence for three TSHR subunits or evidence against a physiological monomeric receptor. Thus, a C peptide would not be a subunit, but a fragment released during the formation of the two-subunit (A and B) TSHR. Further, the release of a C peptide during intramolecular cleavage does not negate evidence for the simultaneous presence of other single-chain, monomeric TSHR on the cell surface.

3. TSHR subunit terminology. Because there is an evolving change in the literature on the nomenclature of the TSHR subunits, it is necessary to comment on our choice for describing the TSHR subunits as A and B, rather than α and β. We suggest that the former is preferable for three reasons. First, use of A and B gives credit to the original discoverers of the two TSHR subunits (19). Second, a C peptide (rather than a γ-peptide) conveys the concept well established for conversion of proinsulin to insulin. Finally, TSH, the ligand of the TSHR, contains α- and β-subunits, and use of a different terminology will avoid confusion in future ligand-receptor structural studies.

4. Shedding of the TSHR A subunit. There is evidence to suggest that the majority of TSHR on the cell surface shed their A subunits into the circulation (27). This hypothesis, which could have important implications in the pathogenesis of Graves' disease, arose from the observation that 3-fold fewer A subunits than B subunits were recovered from human thyroid homogenates using TSHR-specific mAbs (27). Consistent with the disproportionate number of B subunits in human thyroid tissue, shed A subunits in the anticipated number were recovered from the culture medium of human thyrocytes, as well as from mouse L cells and Chinese hamster ovary (CHO) cells expressing the recombinant human TSHR (35). Moreover, A subunits were also reported to be detectable in human serum (35–37). Further evidence suggests that shedding of the A subunit is caused by cleavage by a matrix metalloprotease (35) accompanied by cell surface protein disulfide isomerase disruption of the disulfide bond(s) linking the A and B subunits (38).

Although a clearly established *in vitro* phenomenon, questions remain about the physiological nature of TSHR ectodomain shedding. Thus:

1. TSHR shedding is particularly evident when cells are "starved" in serum-poor tissue culture medium (35), conditions associated with decreased cell viability and autophagy of self-proteins (39).

2. The shed TSHR A subunit is smaller than the non-shed A subunit because of a reduction in its carbohydrate content. The protein backbone is of similar size.

3. No B subunit excess was apparent in CHO cells expressing a sufficient number of TSHR to permit direct subunit detection without the need for prior affinity purification (30).

5. Localization of the two cleavage sites in the TSHR ectodomain. Much progress has been made in characterizing the upstream and downstream cleavage sites, hereafter referred to as site 1 and site 2, respectively.

a. Site 1. This site delineates the C terminus of the glycosylated A subunit (Fig. 2). Initial efforts to deduce the location of site 1 focused on the size of the A subunit. Because the size of the TSH-binding A subunit was the same in the wild-type TSHR as in a mutant receptor lacking amino acid residues 317–366, cleavage at site 1 was likely to be closely upstream of residue 317 (22, 34). Subsequent data, however, raised doubts as to this localization for site 1. Thus (i) It was reported that the C terminus of the A subunit must be downstream of residue 366 (26), rather than closely upstream of residue 317. The basis for this conclusion was that the glycosylated, N-terminal portion of the TSHR (the A subunit) was recognized by an antiserum to amino acids 352–366, a region termed the "immunodominant peptide" (40, 41). (ii) It was suggested that the TSH cross-linking data were potentially misleading because TSH bound with high affinity to the B subunit (26). (iii) Mutagenesis of two Arg/Lys-rich regions closely upstream to amino acid 317 failed to prevent TSHR intramolecular cleavage and the formation of a normally sized A subunit (42). These regions had been considered likely cleavage sites because of their striking homology to subtilisin-related proprotein convertase motifs. (iv) The A subunit polypeptide backbone, after enzymatic deglycosylation, was observed to be 35 kDa (27, 28, 30) or 42 kDa (31) in size, which would place site 1 in the region between amino acid residues 330–390.

Surprisingly, despite all this evidence that site 1 (the C terminus of the A subunit) was not upstream of residue 317, extensive mutagenesis of the entire region downstream of residue 317 as far as cleavage site 2 (see below) failed to eliminate site 1 (43). To be informative, these mutations were performed on a background of a TSHR with a mutation ($GQE_{367-369}NET$) that abolished cleavage at site 2. Fortuitously, the clue to the location and nature of site 1 was obtained in studies on the effect of the proteolytic enzyme, trypsin, on TSHR structure. Thus, light trypsinization was observed to delete a small (~1–2 kDa) polypeptide fragment containing an N-linked glycan moiety from the C terminus of the A subunit (44). This glycan moiety could only be the

most downstream (sixth) N-linked glycosylation motif in the TSHR ectodomain (residue 302), the more proximal (fifth) potential N-linked glycosylation site being far upstream at amino acid 198 (Fig. 3). Therefore, site 1 could be localized to the small region between amino acids 303 and the vicinity of residue 317, as originally suggested by the TSH cross-linking data (22). Once again, however, extensive mutagenesis in this region failed to prevent cleavage at site 1 (44).

The perplexing situation, therefore, existed that every amino acid between residues 303 and 362 could be mutated without abolishing cleavage at site 1. Moreover, as mentioned above, it had been observed soon after the cloning of the TSHR that the entire 50-amino acid "insertion" between residues 317–366 could be deleted without abolishing TSHR cleavage (22). However, when this deletion had been performed, the existence of two cleavage sites had not been discovered. The enigma was solved by cross-linking TSH to a receptor in which both residues 317–366 were deleted and site 2 (see below) was eliminated. For the first time, a TSHR was generated that largely remained uncleaved on the surface of the cell (44). Previously, cleavage could only be abrogated in chimeric receptors containing large segments of the noncleaving LH/CGR (23).

b. Site 2. Chimeric TSH-LH/CGRs were the key to localizing this cleavage site. Indeed, chimeric receptors had also provided the first clue for the presence of two separate cleavage sites in the TSHR (see *Section II.B.2.a*). Obviously, mutations at one cleavage site would only be informative (prevention of subunit formation) when performed on a background of a receptor in which the other site was lacking. The search for site 2 in one domain, therefore, involved mutagenesis of this region using a chimeric receptor in which cleavage at site 1 was prevented by replacement with the corresponding region of the noncleaving LH/CGR.

Fortunately, mutations within the site 2 region involved a 'mini' chimeric receptor approach in which TSHR residues were replaced with the corresponding residues of the LH/CGR. This procedure rapidly identified TSHR amino acid residues 367–369 to be critical for cleavage at site 2. Remarkably, replacement of the same amino acid residues with Ala did not prevent cleavage at site 2. The simultaneous substitution of three LH/CGR residues ($NET_{291–293}$) (single letter amino acid code) for the corresponding TSHR residues $GQE_{367–369}$ were required to abolish TSHR cleavage at site 2. This mutation ($GQE_{367–369}NET$) introduced a motif for an N-linked glycosylation site (N-X-S/T; where X can be a wide range of amino acids with variable efficiency for glycosylation) (45). Additional mutagenesis of these three residues provided further support for the likelihood that it was the lack of a glycosylation site in the TSHR, rather than the presence of a particular amino acid motif, that permitted cleavage at site 2. Thus, replacement of TSHR residues GQE with NQT (glycosylation motif), but not with NQE (incorrect motif), largely prevented cleavage at site 2. These mutations also confirmed the existence of the upstream site 1. Thus, when the glycosylation sequons NET or NQT were introduced into the wild-type TSHR, intramolecular cleavage was restored.

c. Summary. From the above data, the following conclusions can be made regarding the sites of TSHR intramolecular cleavage: (i) Cleavage at site 1 occurs downstream of TSHR residue Asn302 and (probably) upstream of residue 317. (ii) TSHR amino acids GQE at positions 367–369 are critically related to cleavage at site 2. Thus, cleavage is prevented by the introduction at this site of an N-linked glycan motif, known to be glycosylated at the same site in the noncleaving LH/CGR (46). (iii) Neither cleavage at site 1 nor at site 2 involve a specific amino acid motif. (iv) The TSHR 50-amino acid insertion (residues 317–366) is required for cleavage at site 1, but not at site 2.

6. Mechanism of TSHR cleavage into subunits. The thrombin receptor, another member of the GPCR family, is activated by the ability of its own ligand to proteolytically remove a fragment of the receptor ectodomain, exposing a "tethered ligand" (47). However, the attractive hypothesis that TSH

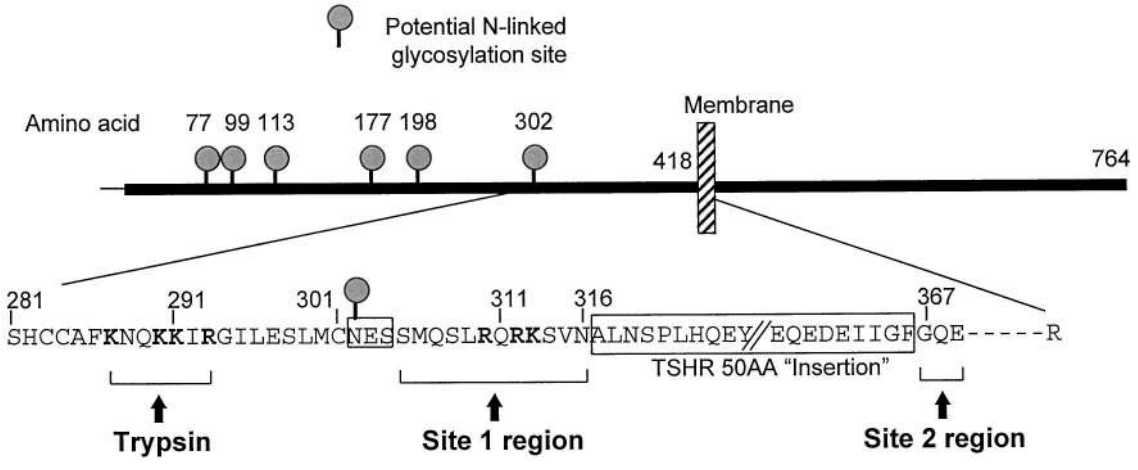

FIG. 3. Schematic representation of the TSHR, with emphasis on the regions of spontaneous and trypsin-induced cleavage. Site 1 indicates the region involved at the TSHR upstream cleavage site. Based on the reduction in A subunit size with trypsin, clipping by trypsin upstream of the glycan at residue 302 must occur at the indicated cluster of R and K residues. Site 2 refers to the amino acid triplet that, when substituted with the corresponding N-linked glycosylation motif at the homologous region of the LH/CGR ($GQE_{367–369}NET$), abrogates cleavage. [Reproduced with permission from K. Tanaka *et al.*: *J Biol Chem* 273:1959–1963, 1998 (44).]

could be the protease responsible for cleavage of its cognate receptor could be discarded because two-subunit TSHR was detected in transfected cells cultured in the absence of TSH (22, 28).

A second potential mechanism for TSHR intramolecular cleavage was involvement of one of a series of subtilisin-like endoproteases that convert many mammalian proproteins and prohormones into the active form by clipping at a pair of basic amino acid residues (48). Indeed, as mentioned above, such motifs were noted to be present in the vicinity of TSHR cleavage site 1 (22, 27). However, mutagenesis of these motifs fails to prevent TSHR cleavage, suggesting the absence of a role for classic subtilisin-related proprotein convertases (42).

More recently, matrix metalloproteinases (MMPs) have attracted much attention for their role in releasing from the cell surface a large number of membrane-anchored growth factors, receptors, adhesion molecules, and proinflammatory cytokines, such as tumor necrosis factor-α (reviewed in Refs. 49 and 50). This mechanism is important in view of the potential pharmacological ability to influence disease processes. Of interest from the point of view of the TSHR, ligand-induced cleavage of the V_2 vasopressin receptor (also a member of the GPCR family) appears to be mediated by a plasma membrane MMP (51). One MMP inhibitor, BB-2116, strongly inhibits TSHR cleavage into A and B subunits (35), suggesting a role for MMPs in this process. Unfortunately, MMPs do not interact with a specific amino acid motif, precluding the ability to predict the TSHR residues with which these enzymes may interact. The concept of MMP-mediated cleavage of the TSHR is supported, but not proven, by the absence of specific amino acid motifs for cleavage at sites 1 and 2 (43, 44). Another difficulty regarding the MMP concept is that inhibitors of these enzymes are not necessarily specific and may inhibit other types of proteases that are active at the cell surface (52). At present, therefore, although involvement of MMPs represents the most credible theory for TSHR intramolecular cleavage, confirmatory data for this mechanism are awaited.

Regardless of the actual enzyme involved in TSHR cleavage, the mutagenesis studies described above provide interesting new insight into, and generate new hypotheses regarding, the mechanism of cleavage. Thus (i) the presence or absence of glycosylation at cleavage site 2 represents a novel mechanism by which two closely related receptors have evolved to have a different subunit structure. (ii) The lack of a specific amino acid motif at TSHR cleavage site 2, together with the abrogation of this site by an N-linked glycan, suggests that carbohydrate may prevent the binding or action of a proteolytic enzyme such as a MMP. TSHR residues 367–369 may, therefore, not necessarily be the exact cleavage site. Precise identification of this site may require the purification of the TSHR B subunit and determination of its N-terminal sequence. (iii) Because the 50-amino acid insertion (residues 317–366) is necessary for cleavage at site 1 but does not, itself, appear to harbor the cleavage site, this region may also be a binding site or 'anchor' for a protease. Alternatively, amino acid residues 317–366 may, themselves, function as a protease and clip an adjacent region of the TSHR. (iv) Different proteases may be involved at sites 1 and 2. This possibility is raised by the observation that deletion of the 50-amino acid insertion (a potential protease or protease-binding site) abrogates cleavage at site 1 but not at site 2.

7. Role of the TSHR 50 amino acid 'insertion'. Although the precise boundaries of these additional 50 amino acids in the TSHR are uncertain (because of low homology in adjacent regions of the glycoprotein hormone receptors), this segment has been the subject of intense study. The very hydrophilic nature of residues 317–366 led us to speculate that it was a projection on the exterior of the TSHR molecule, perhaps important in ligand specificity (34). Surprisingly, however, its deletion had no effect on TSH binding or on TSH-mediated signal transduction (34). The deduced superficial topography of TSHR residues 317–366 was also the reason for selection of this region for c-*myc* epitope tagging (53). The critical role of amino acids 367–369 at TSHR cleavage site 2, as well as the localization of site 1 to the proximate vicinity of residues 303–316, support the original demarcation of the 50-amino acid insertion (2). Cleavage sites at either end of residues ~317–366, together with the absence of Cys residues within this region, support the concept that this insertion is, indeed, the putative C peptide lost during intramolecular cleavage.

An interesting finding regarding the 50-amino acid insertion is that a synthetic peptide corresponding to its C-terminal region (residues 352–367) is highly immunogenic when injected into rabbits with adjuvant and is also reported to be recognized by TSHR autoantibodies in the majority of Graves' sera (41, 54). Further, an antiserum to a closely related synthetic peptide (residues 352–366) recognizes the TSHR A subunit in FRTL-5 rat thyroid cells, but very poorly in transfected COS cells (26). These reports must now be reevaluated in the light of the discovery that the immunogenic peptide is deleted from the TSHR A subunit after intramolecular cleavage at site 1 (Fig. 2). There is evidence that cleavage at sites 1 and 2 may not be complete (30). Residues 352–366 could, therefore, be present on an incompletely cleaved "large" A subunit resulting from cleavage at site 2 alone.

The 50-amino acid insertion also contains the epitope (residues 354–359) for a murine mAb to the TSHR (2C11) that recognizes the native receptor on the cell surface (55). Light trypsinization of intact CHO or COS-7 cells destroys antibody recognition of this epitope and also activates the TSHR (56). The potential role of intramolecular cleavage in constitutive TSHR activity is discussed below (*Section II.B.9*). Because its epitope appears to be on the TSHR C peptide, it is likely that mAb 2C11 is recognizing the single-chain TSHR and not the cleaved TSHR on the cell surface. Further, these data indicate that trypsin is removing at least part of the C peptide from the monomeric receptor. Support for this conclusion is the direct observation that trypsinization of intact cells converts all single-chain TSHR on the cell surface into cleaved forms with A subunits equal in size to, or smaller than, the A subunit formed by spontaneous cleavage (44).

8. Reconciliation and interpretation of a confusing literature. Strong opinions and prejudices exist as to the subunit structure of the physiological TSHR. The most widely accepted

view is that only two-subunit TSHRs exist on thyroid cells *in vivo* and that single-chain TSHRs are, in large part, an artifact of transfected nonthyroidal cells (27, 28). Another camp argues that the TSHR subunits are an *in vitro* proteolytic artifact (26, 57). A third view (our opinion) is that both single-chain and two-subunit TSHRs are present and functional on the surface of thyroid cells.

To reconcile these diametrically opposing views, it is necessary to appreciate that the type of TSHR "seen" experimentally depends greatly on the methodology used. Thus, the TSHR or its subunits have been detected by (i) [^{125}I]TSH covalent cross-linking; (ii) Western (immuno) blotting of TSHR forms separated on PAGE; and (iii) immunoprecipitation of [^{35}S]methionine/cysteine precursor-labeled TSHR. In our view, TSH cross-linking is the most powerful (surprisingly underused) tool because it is the only one that specifically detects mature TSHR on the *surface* of intact cells. On the other hand, characterization of the TSHR by immunoblotting or immunoprecipitation using antibodies must be performed on broken cell preparations. Antibodies (unlike TSH) may recognize many forms of the TSHR including intracellular precursors and degradation products incapable of binding TSH. Finally, even using the *same* antibody for detection of the TSHR in homogenates from the *same* cell line, the TSHR can be seen as either predominantly single chain or predominantly two subunit, depending on the methodology employed (30).

Other important procedural differences that can influence the data obtained should be considered. For example (i) the homogenization procedure of cultured cells and very firm thyroid tissue can vary markedly in duration and vigor; and (ii) the need for prior affinity purification to detect a signal (27, 28) has the potential to introduce bias toward detection of one or another form of TSHR or TSHR subunit. This potential handicap is overcome by TSHR overexpression which, by providing a strong signal, eliminates the requirement for prior affinity purification (32).

Bearing in mind these methodological differences, we suggest the following: (i) Regardless of how much immature, monomeric TSHR is detected by immunological means in transfected, nonthyroidal cells, when assessed by TSH cross-linking, there is no doubt that both single-chain and two-subunit TSHRs are present on the surface of intact thyrocytes (24) and transfected nonthyroidal cells (22), at least in culture. (ii) When detected by TSH cross-linking, the proportion of single-chain *vs.* two-subunit TSHRs detected on the cell surface is remarkably similar (~1:1) over a very wide range of receptor numbers (~15,000 or 2 million TSHRs per cell) (22, 32), and irrespective of whether thyrocytes (24) or transfected CHO cells are examined. These observations do not support the hypothesis that single-chain TSHRs represent an unphysiological accumulation in transfected cells. Taking all these data together, we believe that it is very difficult to assess the physiological role of TSHR subunit structure in broken cell preparations without consideration of the methodology used and without focusing on the forms of TSHR expressed on the cell surface, such as by analysis of their glycan composition (see below).

We are also drawn to comment on the common perception (31, 35, 57) that we presently support the viewpoint that the two-subunit TSHR is a proteolytic artifact. In our original cross-linking data, we detected only two-subunit (and no monomeric) TSHR on TSH cross-linking to membrane preparations from homogenized, transfected CHO cells (22). In contrast, both two-subunit and single-chain TSHRs were detected on the surface of intact cells (22). On this basis, we raised the logical (in our view) hypothesis that the single-chain TSHR could be a physiological receptor *in vivo*, rather than being an immature precursor (24, 58), and that the two subunit TSHRs could be a degradation product, either artifactual or physiological. However, subsequent data on TSHR subunit structure reported by ourselves (23, 30, 32, 42, 43, 59) and others (27, 28, 31, 35) have clearly indicated that TSHR intramolecular cleavage is not an artifact. On the other hand, we stand by our original observation that a single-chain TSHR with high affinity for TSH also exists on the surface of intact cells (22). In our view, the single-chain *vs.* two-subunit controversy is moot; both receptor forms are present and functional. Rather, the outstanding question at present is whether or not the dual expression of single-chain and two-subunit TSHRs on the surface of cultured cells also occurs in thyroid cells *in vivo*. Obviously, this will be a very difficult question to answer.

9. The functional importance of TSHR cleavage. The evolutionary divergence of the TSHR into a receptor that cleaves into two subunits is unique and enigmatic. As mentioned above, TSH binds to both cleaved and uncleaved forms of the TSHR with similar high affinity (22). Moreover, TSH action does not require a cleaved receptor. Thus, TSH can activate chimeric TSH-LH/CGRs that do not cleave into two subunits (59). On the other hand, circumstantial evidence suggests that cleavage into subunits may influence the basal, as opposed to the TSH-induced, level of TSHR activity. Thus:

1. The TSHR ectodomain, particularly the region involved in intramolecular cleavage, plays a role in signal transduction (33, 60). Moreover, the spontaneous mutation of Ser281 in the TSHR ectodomain leads to increased constitutive activity (61, 62). This residue is very close to the C terminus of the A subunit that may be more exposed after cleavage at site 1 (44).

2. Unlike the noncleaving gonadotropin receptors, the TSHR is "noisy" in that it has significant constitutive (ligand-independent) activity (10, 63–65).

3. Light trypsinization of cells expressing the TSHR leads to receptor activation with the loss of an epitope at amino acid residues 354–359 (56), an epitope unlikely to be present in the two-subunit receptor (30, 43, 44). Trypsin also converts monomeric TSHR on the cell surface into two-subunit forms, as well as clipping the C terminus of the A subunit (44).

4. Deletion by mutagenesis of residues 339–367 is also reported to increase TSHR constitutive activity (66). However, the effect of this deletion on TSHR subunit structure is unknown.

Given the rapid progress in this field, an answer to the question of whether or not TSHR constitutive activity is related to intramolecular cleavage is likely to be available in the near future. Another possible consequence of TSHR intramolecular cleavage that will require future investigation relates to the pathogenesis of Graves' disease. Thus, the release of a C peptide from the TSHR may be associated with

the very common occurrence of disease-causing autoantibodies, a phenomenon rarely (if ever) encountered with other known members of the GPCR family that do not have a similar subunit structure.

C. Disulfide bonds in the TSHR ectodomain

As for any large protein, disulfide bonding is vital for the correct folding of the TSHR and the maintenance of its tertiary conformation. In addition, disulfide bonds are involved in the quaternary structure of the TSHR A and B subunits (19, 20). Determination of which Cys residues form pairs would provide important information on the TSHR pending the solution of its three-dimensional structure by crystal x-ray diffraction, a task that may never be possible (see below). The most important present question, on which there has been much speculation, is which Cys residues are involved in the association of A and B subunits in the two-subunit form of the TSHR.

There are 11 Cys residues in the TSHR ectodomain that could form five pairs, with one residue remaining as the orphan. An additional two Cys in the first and second extracellular loops are conserved in the GPCR family, and, like other known members of the family, are likely to be paired with each other. The 11 Cys in the TSHR ectodomain can be classified into four groups (Fig. 4).

1. Group I. As is characteristic of glycoproteins with LRRs (67), there is a cluster of four Cys residues (between residues 24–41) at the N terminus of the LRR region. This region is the extreme N terminus of the ectodomain, residues 1–21 being the signal peptide.

2. Group II. Also characteristic of the LRR family is a Cys cluster between residues 283–301 at the C terminus of the LRR region.

3. Group III. Three Cys are clustered between residues 390–408 at the extreme C terminus of the TSHR ectodomain, close to its entry into the plasma membrane.

4. Group IV. A solo Cys is present at residue 176, within the LRR region. Because of its isolation and the relative rigidity of the LRR structure, Cys176 is a good candidate to be the orphan.

At present, there are no direct data to indicate which Cys form the five pairs. However, a number of indirect lines of evidence permit informed speculation. Thus, (i) localization of the approximate sites of TSHR cleavage (see above) establishes Cys residues 390, 398, and 408 (group 3) to be the only ones on the B subunit. One or more of these residues must, therefore, be responsible for linkage to the A subunit. (ii) Of the residues in group 3 on the B subunit, Cys390 cannot be the sole link to the A subunit because its mutation reduces, but does not abolish, TSH binding (60, 68). Loss of the A subunit would be incompatible with TSH binding. TSH binding, on the other hand, is completely abrogated by mutation of Cys398 (60, 68) and Cys408 (68), making one or both of these residues the prime candidates for tethering of the A subunit. (iii) Two clusters of Cys residues on the A subunit could link with the B subunit; namely, the four Cys in group 1 or the three Cys in group 2. The three-dimensional model of the TSHR LRR region (17) makes Cys pairing between groups 1 and 2 very unlikely. (iv) All of the Cys residues in group 1 (68), except for Cys41 (68, 69), can be mutated without loss of high-affinity TSH binding. Again, because dis-

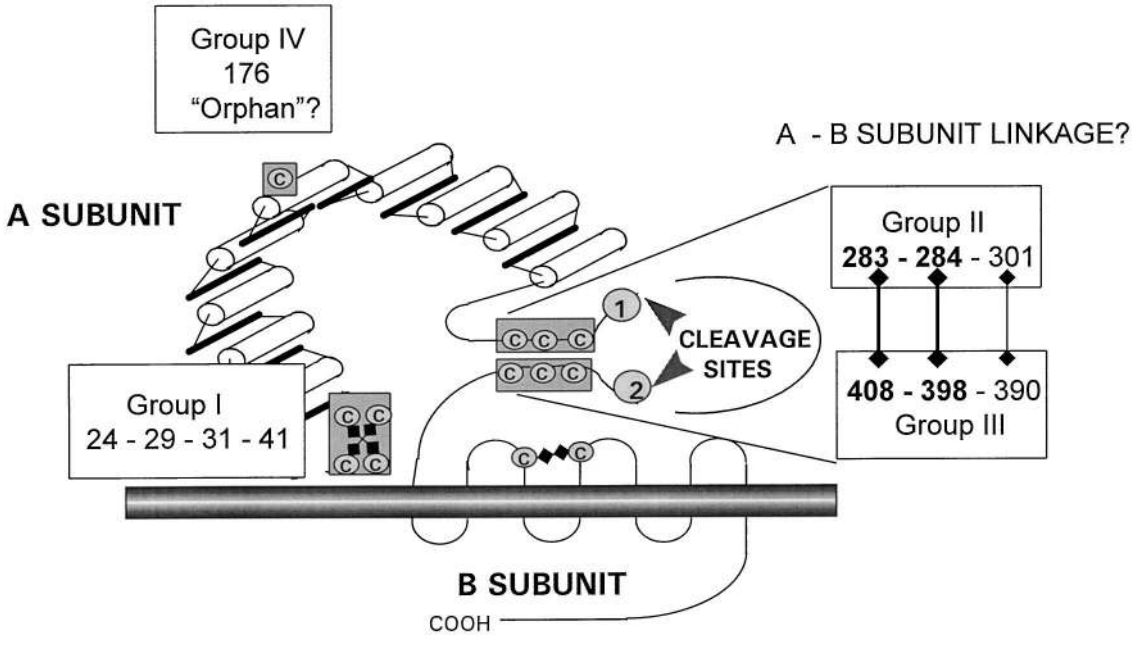

FIG. 4. Cysteines in the TSHR ectodomain. The 11 cysteine residues in the TSHR ectodomain can be assigned to four groups. This hypothetical model for cysteine pairing is based on presently available evidence. The essential feature of this model is that Cys283,284 and Cys398,408 link the A and B subunits. Contrary to our previous view, the four cysteines in group 1 pair among themselves and are unlikely to pair with cysteines near the C terminus of the ectodomain. Disulfide bonding between residues 301 and 390, in the vicinity of the two cleavage sites, may stabilize the TSHR but are not in themselves essential for subunit linkage.

ruption of A and B subunit linkage is likely to be 'fatal' to high-affinity TSH binding, only Cys41 remains a candidate for this function. (v) Mutation of group 2 Cys residues, in particular Cys283 and Cys284, have a devastating effect on TSH binding (68), making these two residues (in our view) the most likely to be pairing with Cys398 and Cys408 in group 3. (vi) The hypothesis that Cys301 (group 2) and Cys390 (group 3) are paired (68) is supported by the recent evidence that intramolecular cleavage of the TSHR releases a C peptide approximately between these two residues (30, 43, 44). Further evidence for this likelihood is that mutation of either Cys301 or Cys390 produces the identical effect of reducing the affinity of TSH binding (60, 68). Disruption of a disulfide bond through either of its two residues would be expected to produce a similar phenotype. However, the fact that TSH binding persists despite these mutations indicates that a Cys301–390 bond cannot be the sole link between the A and B subunits. This conclusion is supported by the deletion of Cys301 by trypsin without the abolition of TSH binding (44).

Taking all these data together, our working hypothesis is depicted in Fig. 4. The essential feature is that Cys283,284 and Cys398,408 link the A and B subunits. If correct, Cys41 remains the enigma. Thus, in this schema, the four Cys in group 1 remain isolated from the remainder of the ectodomain. Yet, only mutation of Cys41 abolishes TSH binding. If disulfide bonding among these four Cys residues is important in the three-dimensional structure of the TSHR, then mutation of the unknown Cys paired to Cys41 should produce an identical effect. If Cys41 is not paired within group 1, then it is likely to be paired with a Cys in group 3. Therefore, our hypothesis that Cys41 does not link with the B subunit is wrong, or Cys41 is, indeed, an important component in the TSH-binding site (34).

D. Carbohydrate moieties in the TSHR ectodomain

Early reports on affinity enrichment using lectins of TSH-binding activity from thyroid tissue revealed that the TSHR, like most other cell surface proteins, was a glycoprotein (70–73). TSH-binding activity could be adsorbed by a wide selection of lectins and varied depending on the TSHR species, making it very difficult to define the glycan moieties involved other than that they were N linked (73). Modest progress has been made since the molecular cloning of the TSHR. The human TSHR ectodomain contains six potential N-linked glycosylation sites (Fig. 1), but evidence on which of these sites actually contains glycan is limited. All sites are on the A subunit of the cleaved TSHR, the sixth and last being at Asn302.

Perhaps the biggest surprise has been the unexpectedly high ectodomain glycan content of the TSHR. Estimates of 10–14 kDa (31, 57) or even 18 kDa (27, 28) may be too low. In transfected mouse L cells (28) and CHO cells (30), 25–27 kDa of N-linked glycan has been detected. Even a truncated TSHR A subunit lacking N302 has 20 kDa of carbohydrate (74). Based on these estimates, the recombinant TSHR A subunit expressed in mammalian cells contains more than 40% glycan, a level nearly consistent with a proteoglycan rather than a glycoprotein! The suggestion that TSHR glycosylation is greater in transfected cells than in thyroid tissue (28) is a distinct possibility and needs further evaluation. Another possible explanation for the apparent discrepancy between the glycan content of thyroid tissue (19, 20, 27, 28) and cultured cells (28, 30) is that the glycan on Asn302 is removed during thyroid tissue homogenization, as it is with trypsin (44).

Regarding which of the N-linked glycosylation sequons contain glycan, estimates of only 12–14 kDa of TSHR glycan do not support a conclusion that all six sites are glycosylated (57). Only Asn302 has been determined directly to contain glycan (44). Mutagenesis studies on the TSHR have only provided limited information. Thus, four N-linked glycosylation motifs on the human TSHR (Asn residues 99, 177, 198, and 302) could be individually eliminated by mutagenesis without affecting trafficking of a functional receptor to the cell surface (75). Mutation of Asn77 and Asn113 completely abolished TSHR expression on the cell surface or generated a receptor with reduced affinity for TSH, respectively (75). Whether or not these effects are consequent to loss of glycan moieties or secondary to folding abnormalities in the polypeptide chain are unknown. However, the presence of more than 25 kDa of glycan suggests that most, if not all, potential sites contain glycan, at least in transfected cells. Surprisingly, mutation of the five potential N-linked glycosylation sites in the rat TSHR (Asn113 is absent) produced effects different from those observed with the human TSHR (41). Thus, mutation of only Asn198 reduced TSH binding affinity, apparently without alteration in the biological response to TSH stimulation.

The precise composition of the TSHR ectodomain glycan will be difficult to determine. Potential heterogeneity among glycans at different sites will require purification of polypeptide fragments containing the individual glycan moieties, a daunting task. In generic terms, however, it is clear that, as with most glycoproteins that reach the cell surface (29), the carbohydrate on the mature TSHR (both single chain and cleaved) has been transformed from the high-mannose variety in the endoplasmic reticulum to complex form. The possible role of TSHR ectodomain glycan in ligand and autoantibody binding will be discussed separately below.

E. TSHR dimerization

Dimerization of ligand-coupled receptors is a well recognized phenomenon that may also apply to the TSHR. For example, at a functional level, there is evidence that 'blocking' TSHR autoantibodies may become stimulatory when linked by divalent anti-IgG (76). It has also been reported that the recombinant TSHR ectodomain generated in bacteria and in insect cells (77, 78) can form multimers when detected by PAGE. Mammalian cell B subunits have also been reported to form dimers (31). Further, high molecular weight complexes consistent with receptor dimerization are typically detected on TSH cross-linking studies. However, the sizes of these bands vary widely in different experiments (22, 23, 30, 32, 79), and we are uncertain whether these bands are physiological multimers or artifactual aggregates. It is clear that the TSHR, even when fully glycosylated, is a particularly 'sticky' protein that is prone to aggregate. In particular, we

confirm the observation (26) that heating TSHR to more than 50 C before PAGE leads to marked aggregation. Such heating may induce 'caramelization' of the very high TSHR glycan content.

III. Recombinant TSHR Expression

The very small number of TSHRs in thyroid tissue, estimated as ≤5,000 receptors per thyrocyte (58), and instability during the purification process have prevented receptor purification from this source. Despite valiant efforts (73, 80, 81), thyroidal TSHRs have never been purified sufficiently to be visualized as an unequivocal and discrete electrophoretic band on direct protein staining. Recombinant TSHR is, therefore, the only practical source for purification.

Recombinant TSHRs expressed in nonthyroidal cells have been of value in the assay of TSHR autoantibodies (see below). However, full exploitation of this material for understanding the structure-function relationship of the TSHR, as well as for exploring the pathogenesis and possible immunotherapy of Graves' disease, will require its high level expression and purification. A number of groups have devoted years of effort to this endeavor, admittedly only a means to an end and not, in itself, of direct value.

A. Approaches to TSHR expression

Two major decisions are required of investigators expressing the recombinant TSHR.

1. The expression system to be used. Systems used to date have involved the transfer of TSHR cDNA in numerous vectors (plasmid and viral) into different types of cells including: (i) prokaryotic bacteria, (ii) eukaryotic insect cells, (iii) eukaryotic mammalian cells, such as CHO cells, mouse L fibroblasts, mouse SP2/0 myeloma cells, human embryonal kidney (HEK), and simian virus-40-transformed African green monkey kidney (COS) cells.

In addition, TSHR protein, or fragments thereof, can be generated as (i) short, synthetic peptides (typically 16–20 amino acids in length); (ii) cell-free translation of TSHR mRNA in an *in vitro* system.

2. Expression of the TSH holoreceptor, the TSHR ectodomain, or ectodomain fragments. The holoreceptor (743 amino acids after signal peptide deletion) includes the hydrophobic membrane-spanning segments. The smaller ectodomain (397 amino acids after signal peptide deletion) contains more hydrophilic amino acid residues. The decision on whether to express the holoreceptor or the ectodomain depends heavily on the intended use of the recombinant TSHR. Obviously, the membrane-spanning segments are necessary for studying TSHR signal transduction. On the other hand, if the goal is to study hormone or autoantibody binding independent of signal transduction, or to generate antisera to the TSHR, the ectodomain (or parts of the ectodomain) may suffice and may be advantageous. Expression of only the ectodomain carries the theoretical advantage of generating a more water-soluble product. In addition, the absence of a membrane-anchoring region offers the potential for generating a protein that is not retained in the membrane but is secreted into culture medium. Under these conditions, as has been accomplished with the ectodomain of TPO (82, 83), TSHR could theoretically be extracted from spent medium, harvested repeatedly from long-term mammalian cell cultures.

B. TSHR expression in prokaryotes

Much effort has been made to express the recombinant TSHR in bacteria, probably because this system is technically the easiest to apply and, at least for some proteins, a high level of expression can be attained. Unfortunately, however, major difficulties have been encountered, most likely explaining the lack of reports on TSH holoreceptor expression in bacteria. Numerous groups have reported on the prokaryotic expression of the more soluble TSHR ectodomain (27, 40, 77, 84–87). In some systems, such as with the pMAL vector (Ref. 86 and G. D. Chazenbalk and B. Rapoport, unpublished data) and with a GST-expressing fusion vector (88), the TSHR ectodomain has been generated in a (at least in part) soluble form, rather than as insoluble inclusion bodies that require dissolving in high concentrations of chaotropic agents such as urea or guanidine.

There is debate as to whether or not the nonglycosylated TSHR ectodomain of prokaryotic origin specifically binds TSH and/or TSHR autoantibodies. There are some reports that TSHR autoantibodies (77, 78, 88) and TSH (88) bind to this material, although the latter is "inefficient" (88). It is noteworthy that, on immunoblotting, 60% of patients' sera containing high titers of TSHR autoantibodies recognize nonglycosylated, bacterial TSHR ectodomain (78). Others (85, 86), including ourselves (G. D. Chazenbalk and B. Rapoport, unpublished data) have not detected specific TSH or TSHR autoantibody binding to the TSHR ectodomain generated in bacteria.

C. TSHR expression as cell-free translates or as synthetic peptides

As for TSHR expression in prokaryotes, cell-free translation of TSHR mRNA using microsomal preparations (89) is technically straightforward and generates products lacking carbohydrate moieties. Modification of the system can generate glycosylated material, but not with the quantity and quality of glycans present on glycoproteins expressed on the surface of mammalian cells. TSHR holoreceptor (90) or ectodomain (91–93) expression by cell-free translation has been accomplished, however, with conflicting findings. Remarkably, TSH is reported to bind to the holoreceptor (90), a finding that awaits confirmation. TSHR autoantibodies either did (91, 92), or did not (93), interact with material produced in similar systems. The role of conformational integrity and glycan content in autoantibody recognition of the TSHR ectodomain (discussed below), as well as other factors, may explain these discrepant results.

Synthetic TSHR polypeptide synthesis is a technically demanding but routine procedure performed by commercial or specialized laboratories. The practical advantages of generating peptides is counterbalanced by their limited ability to reconstitute the binding sites on a native, conformational, and glycosylated protein. Nevertheless, if one or more pep-

tides do interact specifically with a ligand or antibody, valuable information is immediately available because the sequence of the peptide is known. Data on TSH and autoantibody interactions with the TSHR are, therefore, deferred to the section on epitopes and binding sites (*Section IV*).

D. TSHR expression in eukaryotic insect cells

Extremely high levels of proteins can be expressed in insect cells infected with a baculovirus transfer vector modified to contain a gene of interest (94). This positive feature, together with improved vectors and the fact that proteins produced in these cells, unlike in prokaryotic cells, are glycosylated, has made the baculovirus system a major focus for TSHR expression. Attempts to express the active TSH holoreceptor in insect cells failed (Refs. 95–97 and B. Rapoport, unpublished observations). Only the TSHR ectodomain has been expressed in this system. Initial reports (96, 97) on the generation of milligram quantities of TSHR ectodomain using conventional baculovirus vectors were optimistic, even when this material was largely insoluble, having been expressed without a signal peptide (97). Thus, after solubilization, the signal peptide-lacking TSHR ectodomain bound TSH with moderately high affinity (10^{-9} M K_d) (98) and was believed to be glycosylated (97). Most important, solubilized insect cell TSHR ectodomain expressed with (96) or without (99) a signal peptide was recognized on enzyme-linked immunosorbent assay (ELISA) by autoantibodies in patients' sera (96, 99). This material was, therefore, reported to provide a simple, specific, and highly sensitive assay for TSHR autoantibodies (99).

Unfortunately, enthusiasm for TSHR ectodomain expression in insect cells using conventional vectors soon tempered. TSH binding to this material, even when generated with a signal peptide and when directly determined to contain 14 kDa of glycan, was not confirmed (96). Subsequently, it was determined that the signal peptide-lacking ectodomain, although previously reported to be recognized by autoantibodies (97), did not contain carbohydrate and did not interact with TSHR autoantibodies (100). Three modifications have so far been attempted to improve the quality of the TSHR ectodomain produced in insect cells.

1. Use of an earlier baculovirus promoter to enhance TSHR ectodomain glycosylation. The final stage after baculovirus infection of insect cells is host cell death with release of infective viral particles encapsulated in polyhedrin protein (94). In the few days before the host cell dies, the baculovirus usurps the host protein synthesis mechanism to make large quantities of polyhedrin protein. This diversion greatly reduces synthesis of posttranslational modification enzymes, such as glycosylases. To improve recombinant protein glycosylation, new baculovirus transfer vectors were introduced with the cDNA being driven by a promoter that functions at an earlier phase of the infective cycle than the classic polyhedrin promoter.

TSHR ectodomain generated using one of these vectors (pAcMP3) did, indeed, increase the extent of TSHR glycosylation compared with that generated with the conventional polyhedrin promoter (~14 kDa *vs.* ~9 kDa of glycan) (79). However, the level of TSHR expression with this system was very low, consistent with the early stage of the viral cycle. Further, most of the TSHR ectodomain remained within the cell in particulate form. Unfortunately, only trace amounts of soluble TSHR ectodomain were secreted or were present in the cytosol fraction of infected insect cells, too little to be of practical value. Qualitatively, however, this material provided important information. Thus, it completely neutralized TSHR autoantibodies in patients' sera but only marginally inhibited TSH binding. These data suggested that TSHR glycosylation was important for autoantibody recognition and supported the viewpoint (96, 101) that autoantibodies bind better to the isolated TSHR ectodomain than does TSH.

2. Denaturation of insoluble material followed by refolding. Another approach taken to overcome the deficiencies of the baculovirus system with respect to the TSHR expression was to accept the largely insoluble nature of the protein and its lesser degree of glycosylation, but to attempt refolding of the protein into its correct conformation (78, 98). As with refolding of prokaryotic TSHR ectodomain (77), this process generated electrophoretic bands interpreted to represent folded and unfolded monomers and tetramers. Remarkably, glycosylated insect cell TSHR material was recognized less well than nonglycosylated bacterial ectodomain by the same panel of sera with high titers of TSHR autoantibodies (78). Moreover, these data suggested that, for the majority of sera, the native, folded state was not an important factor in autoantibody binding (78).

Important and provocative conclusions were made from these studies (77, 78). First, TSHR conformation and glycosylation were not major factors in autoantibody recognition of the TSHR, and linear epitopes were clearly recognized by many TSHR autoantibodies. Second, the apparent polyclonality of autoantibody recognition of different forms of TSHR ectodomain (folded *vs.* unfolded; glycosylated *vs.* nonglycosylated) implied that T cells, not autoantibodies, are of primary importance in Graves' disease. We suggest some caution in making these conclusions because of the very high concentration at which sera were studied (1:20), the extremely low titer in serum of TSHR autoantibodies (102, 103), and the known oligoclonality of TSHR autoantibodies in serum (104–106). In addition, as pointed out for TPO autoantibodies (107), qualitative recognition by a serum of a particular form of antigen (or epitope) does not indicate what proportion of the antibodies in the serum interact with this antigenic form (or epitope). Adsorption studies or quantitative competition with mAbs to different epitopes or antigenic forms are required to address this issue.

3. Replacement of the mammalian TSHR signal peptide with an insect cell signal peptide. Very recently, to overcome the handicap of protein expression without a signal peptide, or the potential deleterious effect of a mammalian signal peptide, the human (100) and mouse (108) TSHR ectodomains have been expressed in insect cells using an insect cell signal peptide (part of the baculovirus envelope glycoprotein 67). Although such a strategy frequently does not work for other proteins (109), the insect cell gp signal peptide enhanced TSHR ectodomain expression relative to use of the TSHR signal peptide (100) and produced a more heavily glycosy-

lated protein (100, 108). However, as occurred with TSHR expression in mammalian cells (see below), the TSHR ectomain was still not secreted by insect cells and was largely insoluble, requiring solubilization and refolding (100, 108).

As observed previously with the more heavily glycosylated TSHR ectodomain generated in insect cells using an earlier promoter (79), as well as with the glycosylated ectodomain retained within mammalian cells (see below) (110), the new form of insect cell TSHR ectodomain neutralized Graves' patients' TSHR autoantibodies (100). However, the amount of TSHR required for autoantibody neutralization and the nature of the glycan on the neutralizing material were not determined and remain important unanswered questions with respect to the role of carbohydrate in autoantibody recognition (discussed below). In contrast to these neutralization studies with crude insect cell homogenates, purified mouse TSHR ectodomain produced using an insect cell signal peptide was recognized on ELISA by only a minority of Graves' sera (108). All studies on more heavily glycosylated TSHR ectodomains produced in insect cells agree on one point. TSH binds very poorly (79, 108) or not at all (100) to this material. The specific and relatively high-affinity binding of TSH to nonglycosylated TSHR ectodomain (98), recently confirmed (100), remains an enigma.

E. TSHR expression in mammalian cells

Theoretically, as described above, expression of the TSHR ectodomain may be sufficient for studying interaction with ligand or autoantibodies, as opposed to studying the functional activity of the TSHR. In practice, however, until recently, it was easier to study TSH and autoantibody binding to the TSHR ectodomain, not as an isolated fragment, but as part of the holoreceptor. For the purpose of generating large amounts of recombinant TSHR in mammalian cells, stable integration of cDNA into the genome is preferable to transient transfection, although improvements in the latter, particularly the application of new viral vectors (111–113), may alter this balance in the future. This review will, therefore, focus on TSHR expression in stably transfected mammalian cells.

1. Stable TSH holoreceptor expression.

a. Mammalian cells are most effective for generating conformationally intact TSHR. There has been no question since its molecular cloning that the TSHR expressed in mammalian cells, unlike in other systems (see above), is completely functional. Stable expression of the human TSH holoreceptor in CHO mammalian cells has been reported by many investigators (2, 95, 114–118). In addition to CHO cells, stable TSHR expression has also been achieved in SP2/0 mouse myeloma cells (116). The latter have the advantage of growing well in suspension culture in a fermentor, permitting the propagation of large numbers of cells. Cells stably expressing the TSHR transduce a signal after TSH (2, 114) and TSHR autoantibody (34, 114, 119, 120) stimulation. Intact cells (121) or membranes produced from these cells (119, 122) can be used in a TSH binding inhibition assay for TSHR autoantibodies. A typical level of TSHR expression in stably transfected cell is ~90,000/cell, as in the commonly used JPO9 line (122). Unfortunately, although this level of expression is far higher than estimated in thyroid tissue (2,000–5,000 receptors per cell) (58), it is still too low to make TSHR purification practical.

b. Role of TSHR mRNA 5′- and 3′-untranslated regions. Parenthetically, an interesting story emerged from studies performed years later to explore the inferior sensitivity of the original CHO cell line developed in our laboratory compared with cell lines such as JPO9. We noted that whereas we had transfected the full-length (4-kb) TSHR cDNA, other cell lines such as JP26 (114) and JPO9 (122) had been transfected with only the coding region of the cDNA (2.3 kb). Subsequent studies revealed that the 5′- and 3′-untranslated regions of the TSHR cDNA reduced by nearly 10-fold the level of TSHR expression in transfected CHO cells, from ~150,000 to ~16,000 receptors per cell (123). This number of TSHR, far lower than we originally estimated (124), explained the lesser sensitivity of our original cell line. This observation is also of potential pathophysiological importance. Thus, the TSHR untranslated ends are obviously present *in vivo* and may possibly explain the low level of TSHR expression reported in thyroid tissue (58). It remains to be seen whether mutations in the TSHR untranslated regions can derepress this suppressive effect and lead to increased TSHR expression. The TSHR is "noisy" in the absence of ligand (10, 63–65). Consequently, as reported in mice transgenic for the β2-adrenergic receptor (125), constitutive expression of enhanced numbers of TSHR could be a cause of goiter, or even hyperthyroidism. Such a mechanism could complement the already demonstrated effect of activating mutations in the coding region of the TSHR that occur without a change in TSHR number (8, 9).

c. TSHR overexpression. Even with as many as 100,000 TSHRs per cell, a level readily achieved in CHO and SP2/0 cells (see above), purification of significant amounts of TSHR would be a formidable task. For example, at this level of expression, about six confluent 10-cm dishes of cells would yield only 1 μg of TSHR, assuming 100% recovery. Clearly, overexpression of greater numbers of TSHR on each cell would be desirable. Such amplification has recently been attained using a dihydrofolate reductase minigene system. Thus, CHO cells expressing approximately 2 million TSHRs have been generated (32). Without such a high level of expression and even with the availability of mouse mAbs to the TSHR (27, 55, 126, 127), the recombinant TSHR expressed in mammalian cells has required prior affinity purification for efficient immunological detection (28). In addition to being an excellent source of antigen, these cells (dubbed "TSHR-10,000") are also of value, or potential value, for (i) flow cytometric analysis of TSHR autoantibodies in patients' sera (103); (ii) isolation of human mAbs from Ig gene combinatorial libraries derived from patients' B cells; (iii) study of subunit structure, carbohydrate composition, and intracellular trafficking.

However, it should be recognized that, even with overexpression, the task of purifying milligram amounts of TSHR will be a major endeavor, and it is uncertain that crystals of purified TSHR will ever be attained in view of the holo-

receptor being a membrane-associated, heavily glycosylated protein.

The TSHR overexpressing CHO cells have a number of other interesting features.

1. Functional data obtained with these cells confirmed previous transient transfection data (8, 63) that the TSHR maintains a moderate level of activity in the absence of ligand. Such spontaneous activity was first observed with the α1B-adrenergic receptor (7). The high basal cAMP levels in TSHR-10,000 cells reduce their sensitivity and make them suboptimal for the bioassay of stimulatory ligands and autoantibodies.

2. The proportion of single-chain and two-subunit TSHRs on the surface of CHO cells was independent of the number of receptors expressed, ruling out the suggestion (28) that the single-chain receptor is observed because overexpression overwhelms the synthetic capacity of transfected cells.

3. Expression of an increasing number of TSHRs on the cells was associated with a progressive reduction in receptor affinity for ligand (32). Possible explanations for the phenomenon include negative cooperativity between TSHRs. Thus, it is possible that the TSHR aggregate or "patch" in the fluid plasma membrane, especially at high density. Alternatively, conformational changes in the TSHR itself or steric hindrance to TSH binding could also lead to an apparent reduction in affinity, as could adducts between holoreceptors and excess free TSHR B subunits, previously reported to exist (27, 35). Further studies are required to explore which, if any, of these alternatives is the underlying mechanism and whether this phenomenon exists in the thyroid. If so, TSHR-negative cooperativity could represent another type of autoregulation so typical of the thyroid.

2. TSHR ectodomain expression.

a. Nonsecretion of the TSHR ectodomain. Unfortunately, and for unclear reasons, the TSHR is not as cooperative as TPO in becoming a secreted protein when translation is terminated by a stop codon introduced at the point of entry of the ectodomain into the plasma membrane. Instead, in mammalian cells (101, 110, 112), as in infected insect cells (see above), the TSHR ectodomain is retained within the cell in a form containing immature, high-mannose carbohydrate (110). Unlike in insect cells, however, the retained ectodomain in mammalian cells remains largely soluble, even when overexpressed by transgenome amplification (110). Glycoproteins contain immature, high-mannose carbohydrate when they leave the endoplasmic reticulum. Transfer to the Golgi complex, where the glycan matures to complex form, only occurs after folding is satisfactorily completed (29). Therefore, it is likely that the immature glycan on the TSHR ectodomain within the endoplasmic reticulum is the consequence, rather than the cause, of abnormal folding and trafficking. Some soluble TSHR ectodomain in the 100,000 $\times$ *g* supernatant ('cytosol') of overexpressing CHO cells does neutralize TSHR autoantibodies in Graves' sera (although it does not bind TSH) (110). However, this autoantibody neutralizing activity is not within the dominant, high-mannose TSHR component (*Section IV.C.2*).

b. Conversion of the TSHR ectodomain into a secreted form recognized by autoantibodies. Recently, it has been possible to overcome the previous inability to generate in mammalian cells a secreted, soluble form of the TSHR lacking the hydrophobic, membrane-spanning portion of the holoreceptor (74). The strategy was based on the hypothesis that synthesis of the A subunit rather than the whole ectodomain would lead to correct folding and hence secretion. Remarkably, carboxyl-terminal truncations of the TSHR ectodomain at three potential cleavage sites, characterized by clusters of Arg and Lys residues, led to progressive increases in receptor secretion into the culture medium by CHO cells. These data contrasted with previous experience with TPO, in which progressive C-terminal truncations led to reduced secretion by CHO cells but were consistent with observations of variable secretion of naturally occurring truncated forms of the LH/CGR (128, 129). Alternately spliced truncated forms of TSHR mRNA have been detected in thyroid tissue (130, 131); however, it is unknown whether these transcripts are actually expressed and the protein secreted by thyrocytes.

The secreted TSHR ectodomain variants contain complex carbohydrate, like the holoreceptor expressed on the cell surface (28, 32). Although they do not represent the entire TSHR molecule, they are extremely potent in neutralizing functional autoantibodies in Graves' sera, but do not bind TSH (74). By epitope-tagging the antigen, together with amplification of the transgenome and sequential concanavalin A and Ni-chelate chromatography, one of these variants (TSHR-261; amino acids 21–260 after signal peptide removal) was purified to 20–40% of homogeneity. This study on the secreted TSHR (74), and another on the nonsecreted TSHR (112), provide the first direct visualization and quantitation of either thyroid or recombinant mammalian cell TSHR. Antigenically active TSHR will provide a major impetus for future studies on the diagnosis, pathogenesis, and (possibly) immunotherapy of Graves' disease.

c. Expression of ectodomain with a single-transmembrane segment or membrane anchor. In 1993, it was reported that the TSHR ectomain linked to a single hydrophobic transmembrane segment was expressed on the surface of transfected cells (132). This report appeared to contradict the inability of the nonanchored ectodomain to traffic to the cell surface and be secreted (see above), although unpublished data [referred to in a publication (55) from other investigators] suggested that the TSHR ectodomain attached to the CD4 or CD8 transmembrane regions did traffic normally. Very recently, a number of laboratories have confirmed these findings. Thus, the TSHR anchored to the membrane by either the CD8 transmembrane region (133) or by a glycosylphosphatidylinositol tag (133a, 133b) is indeed expressed on the cell surface and is recognized by patients' autoantibodies. Insertion of a thrombin cleavage site at the junction of the TSHR ectodomain and the CD8 transmembrane region permits the enzymatic release and purification of soluble ectodomain (133c). Taken together, these studies indicate that a membrane anchor at the C terminus of the TSHR ectodomain markedly influences, and is necessary for, normal folding and trafficking of the molecule.

IV. TSH and Autoantibody Binding to the TSHR Ectodomain

A. Holoreceptor vs. ectodomain

1. Holoreceptor. Very small ligands, such as the adrenergic hormones and acetylcholine, interact with receptors in the GPCR family with very small ectodomains. Receptor engagement and activation by these ligands is believed to involve direct contact with amino acid residues within the membrane-spanning region. The glycoprotein hormones, on the other hand, are very large glycosylated molecules with more than 200 amino acid residues. While it is quite possible that small parts of these hormones dip into the plasma membrane, it is intuitive that the major component of their binding sites is in the very large (350–400 amino acids) ectodomains of their cognate receptors. Autoantibodies, too, are very large. Even the monomeric Fab component of an antibody contains approximately 300 amino acid residues.

It is, therefore, not surprising that studies involving the recombinant TSHR have confirmed the role of the ectodomain in ligand and autoantibody binding. The most direct proof that TSH interacts primarily with the TSHR ectodomain is its binding to the A subunit, as detected by covalent cross-linking to the surface of intact cells (22, 24). Additional proof is that when the TSHR ectodomain is exchanged with that of the LH/CGR (the remainder of the TSHR being unchanged), the chimeric receptor so formed becomes an LH/CGR in terms of specificity, high-affinity binding, and receptor activation (134). Moreover, in the converse experiment, the TSHR ectodomain spliced to the LH/CGR membrane-spanning segment binds TSH with high affinity (135).

TSHR autoantibody interaction with the TSH binding site has been recognized for more than 20 yr (136, 137). In the molecular biology era, support for the concept that autoantibodies interact with the TSHR ectodomain was obtained using chimeric receptors in which parts of the TSHR ectodomain were replaced with the corresponding region(s) of the LH/CGR (138, 139). However, a novel (although not unanticipated) observation from these studies was that the TSH- and autoantibody-binding sites in the ectodomain were overlapping but nonidentical (138).

2. Isolated TSHR ectodomain. A point that is frequently misunderstood when citing the TSHR literature, particularly by groups studying the other glycoprotein hormone receptors, is whether or not TSH and TSHR autoantibodies can bind to the isolated ectodomain, *i.e.*, in the absence of the membrane-spanning segments of the TSHR. Such misunderstandings are not surprising in view of the strong evidence that LH/CG does bind to the isolated LH/CGR ectodomain (reviewed in Ref. 140), together with the confusion on this subject in the TSHR literature. It must also be recognized that binding to the isolated TSHR ectodomain does not exclude concurrent binding to some elements in the membrane-spanning segment of the receptor, in keeping with the discontinuous nature of the TSH-binding site (see below). Moreover, although specific binding of TSH to a TSHR ectodomain-LH/CGR membrane-spanning segment chimera indicates that the TSHR ectodomain is necessary for TSH binding (135), it does not prove that the ectodomain is sufficient for such binding.

a. TSH. Numerous studies describe TSH binding to the TSHR ectodomain generated without the membrane-spanning segments in eukaryotic insect cells (79, 98, 141), mammalian cells (132, 133), as well as in the form of synthetic peptides (142, 143). On the other hand, other investigators have not detected TSH binding to the TSHR ectodomain generated in mammalian cells (101), insect cells (96), or to a TSHR variant truncated at its carboxyl terminus (residue 261) (74).

In interpreting these findings, it is important to appreciate that TSH binding to a TSHR ectodomian product does not, in itself, imply that this molecule contains the full TSH-binding site. If only a part of the binding site is present, if posttranslational modifications are incomplete, or if the folding of the molecule is abnormal, TSH binding would not be expected to be of the same high affinity observed with the intact, native molecule. Therefore, information on the affinity of interaction between TSH and TSHR ectodomain products is essential to interpret whether or not the TSHR ectodomain is sufficient for TSH binding.

In this light, when TSH binding to the isolated ectodomain has been observed, some studies have reported a high (132) or moderately high ($\sim 10^{-9}$ M) (98) affinity interaction. Other investigators have been unable to determine this affinity (133) or have observed very weak or low-affinity binding to the isolated TSHR ectodomain (79, 141). TSHR peptides would not be expected to form the entire TSH-binding region, and their low-affinity interaction with TSH is anticipated. Consequently, studies with peptides (or other ectodomain fragments) do not address the question of whether or not the TSHR ectodomain is sufficient for physiological TSH binding.

If the TSHR ectodomain (residues 22–418) does not constitute the entire, discontinuous TSH-binding site, one or more of the three exo-loops in the membrane-spanning region of the TSHR are likely contributors to this site. In early studies to test this hypothesis, mutations introduced into all three exo-loops abolished TSH binding to stably transfected cells (D. Russo and B. Rapoport, unpublished data). Similar findings have been reported for the first and second exo-loops (144, 145). However, the inability to determine whether or not these mutated receptors were expressed on the cell surface precluded definitive conclusions from these studies. Data on the third exo-loop are more definitive. Reduced signal transduction despite retention of TSH binding suggests the involvement of this loop in the former activity (146).

In summary, numerous observations of low-affinity (or absent) binding, as well as studies on the exo-loops, would support the notion that TSH binds to the TSHR ectodomain as well as to other regions downstream of residue 418. However, the most important positive observation, requiring confirmation, was that of Shi *et al.* (132), who reported high-affinity TSH binding to the TSHR ectodomain expressed on the surface of mammalian cells by means of a single, hydrophobic membrane-spanning segment. It is of interest that, unlike for the TSHR, evidence that hCG does bind to the isolated LH/CGR ectodomain is clearcut, possibly because carbohydrate maturity is unimportant for binding of this

ligand (147, 148). Very recently, as mentioned above (*Section III.E.2.c*), anchoring the TSHR ectodomain to the CD8 transmembrane region (133, 133c), or to a glycosylphosphatidylinositol tail (133a, 133b) has indeed established that the ectodomain, at least when expressed in mammalian cells, is sufficient for high-affinity TSH binding, thereby confirming the data of Shi *et al.*

b. TSHR autoantibodies. The question of whether or not TSHR autoantibodies bind to the isolated TSHR ectodomain in the absence of the membrane-spanning segments (residues 22–418) can be answered unambiguously in the affirmative. Adsorption of autoantibody activity by recombinant TSHR ectodomain (79, 100, 101, 110) or ectodomain truncated at residue 261 (74) provides the most convincing data. More recently, similar observations have been made using the TSHR ectodomain attached to the CD8 transmembrane region (133c), or to a glycosylphosphatidylinositol tail (133a, 133b). For reasons discussed below, studies involving the direct binding of Graves' sera to TSHR preparations are more difficult to interpret. Affinity purification of autoantibody activity with TSHR peptides suggests that autoantibodies bind to the exo-loops as well as to the ectodomain (149). These potentially important data require confirmation with additional control peptides.

Again, it is necessary to remember that many of the phenomena presently being studied by molecular biology approaches were observed previously, albeit without knowing the TSHR segments involved. Thus, autoantibody reactivity with the recombinant TSHR ectodomain is reminiscent of the observation nearly three decades ago that freezing and thawing thyroid tissue releases a water-soluble factor that neutralizes TSHR autoantibodies (150–152). At that time, TSHR autoantibodies were referred to as "LATS" (long acting thyroid stimulator), hence the term "LAA" (LATS absorbing activity) for the soluble factor. Remarkably, LAA (like autoantibody-neutralizing TSHR-261) was estimated to be ~50 kDa in size (sedimentation coefficient of 4S) (151). More recently, a fragment of the TSHR released by trypsin has been observed to neutralize TSHR autoantibodies (153). The portion of the TSHR with LAA, produced either by freeze-thawing or with trypsin, is unknown.

B. Conformational nature of binding sites

1. Nature of the debate. As for TPO autoantibodies (reviewed in Ref. 107), there has been vigorous debate as to whether the binding sites for TSH and TSHR autoantibodies can be defined using linear peptides or are, instead, highly conformational. There is a vast literature on the TSH binding site and autoantibody epitopes using linear peptides or polypeptide fragments (discussed below). This approach is attractive. These molecules can readily be generated in abundance. Moreover, recognition of a defined peptide provides precise information on the amino acids involved in binding. In contrast, it is extraordinarily difficult (or impossible) to determine precise contact points in the conformationally intact, native molecule using techniques such as mutagenesis.

Investigators using peptides and polypeptide fragments in their studies are, understandably, defensive in response to evidence that binding sites or epitopes are conformational because such data may be perceived to undermine the validity of their data. Gradually, the concept of conformational epitopes has become more widespread, but it is countered that specific interactions with linear peptides, even when of lower affinity, contribute to the overall picture of the binding site or epitope. Moreover, it is argued that autoantibodies to linear epitopes are present in the great majority of Graves' patients' sera and are hence an important component of the immune response.

2. TSH-binding site. Given the complexity of other molecules of similar size, it is not surprising that the TSH-binding site appears to be highly conformational. The most dramatic support for this conclusion is that when the TSHR ectodomain is divided into five arbitrary segments, every one of these segments can be replaced with the corresponding segments of the LH/CGR without compromising high affinity TSH binding (33, 134). Such redundancy indicates that multiple, discontinuous elements contribute to the TSH-binding site. The very low affinity interaction between TSH and TSHR peptides (see below) supports the notion that the peptides comprise discontinuous elements in a highly conformational binding site.

3. TSHR autoantibody epitopes. This topic is more controversial than that of the TSH-binding site.

a. Principles. There are a number of principles that are sometimes overlooked in the heat of debate.

1. The majority, not the minority, of antibodies to large polypeptide molecules (unlike to small haptens) recognize conformational epitopes (154).

2. The same antibody may recognize both a discontinuous (by definition conformational) epitope and a linear component of this epitope. In other words, recognition of linear and conformational epitopes is not mutually exclusive. Thus, a typical antibody to a polypeptide makes contact with about 20 amino acid residues (155). The closeness of fit, as well as charge, hydrophobic interactions, and van der Waal forces, between individual residues on the antibody and antigen contribute to the overall affinity of the interaction. If, for example, three discontinuous segments contribute 10, 6, and 4 residues each, it is possible that a linear peptide of 10 residues (representing the first segment) can bind with sufficient affinity to be detected in a particular assay, such as an ELISA. However, the affinity of this interaction is likely to be lower than with the entire antigen.

3. Distinction must be made between (i) the proportion, or percentage, of sera from different patients that recognize a particular epitope, and (ii) the proportion of the polyclonal antibodies within an individual serum that recognize this epitope. Thus, it is possible that all (100%) sera recognize epitope A even though only 2% of the antibodies within the sera are to epitope A on the antigen. The other 98% of antibodies may be to epitope B, hence an "immunodominant region." By the same token, the detection of a linear epitope by 50%, 70%, or even 100% of a panel of patients' sera does not indicate that the majority of antibodies in the individual sera are to linear epitopes, or that this epitope represents an immunodominant region.

4. The inability of an antibody to detect polypeptide frag-

ments of an antigen generated in a particular expression system does not indicate that the antibody's epitope is conformational unless the antibody is shown to interact with the whole molecule generated in the same system.

5. It is now evident that TSHR autoantibodies in patients' sera are typically present at a ~100-fold lower concentration that TPO autoantibodies (103).

b. Evidence for conformational TSHR autoantibody epitopes.

1. The most frequently cited evidence for this thesis is that sera from patients with autoimmune thyroid disease recognize linear epitopes in TPO, but *not* in TSHR, polypeptide fragments expressed by TPO and TSHR cDNA fragment libraries, respectively (156). Although the conclusion of this study may be correct, the basis for this conclusion requires reassessment. Thus, the very low concentration of TSHR autoantibodies was not appreciated at the time of the study and the appropriate positive control, recognition by patients' sera of the full-length TSHR in the same expression system, was not shown.

2. As for the TSH-binding site, studies with chimeric TSH-LH/CGRs indicate that autoantibodies interact with multiple regions of the TSHR ectodomain (138, 139). The autoantibody epitopes are, therefore, discontinuous and conformational epitopes.

3. Recognition by TSHR autoantibodies in patients' sera of the recombinant TSHRs expressed in bacteria (77) and insect cells (108), or in a cell-free translation system (92), is reported to be influenced by the state of folding or naturation of the polypeptide. These studies used patients' sera to detect purified material by immunoblotting, ELISA, or immunoprecipitation and, in our view, should be interpreted with caution. Unlike with mAb, it is very difficult to detect the TSHR by these approaches using polyclonal patients' sera, especially in view of their very low TSHR autoantibody levels. Moreover, in our experience, recombinant TSHR material is very "sticky" and is readily bound by irrelevant Ig molecules. Therefore, it will be important to confirm these findings in adsorption studies to specifically remove TSHR autoantibodies in the polyclonal sera. Such adsorption studies will also reveal whether or not antibodies recognizing this material represent the majority or only a small fraction of TSHR autoantibodies in patients' sera.

4. Evidence for linear, sequential TSHR autoantibody epitopes. The large literature describing autoantibody recognition of linear peptides is discussed below in regard to the precise epitopes that they define. Although linear determinants on the TSHR may well be defined with peptides, these are likely to be components of larger, conformational epitopes (see *Principles* above). The affinity of TSHR autoantibodies for the TSHR and for TSHR peptides is unknown. On the other hand, bovine (b)TSH binds to the TSHR with an affinity of $\sim 2 \times 10^{-10}$ M K_d, to plastic or untransfected CHO cells with an affinity of $\sim 10^{-7}$ M K_d (124, 157), and to synthetic peptides with affinities in the range of 10^{-4} M to 10^{-5} M K_d (142, 143). If autoantibodies recognize TSHR peptides with a similar low affinity, the specificity of polyclonal serum interaction with peptides is less conclusive than for TSH, a purified, homogeneous ligand. Autoantibody enrichment with immobilized peptides (158) is an important step toward establishing specificity. However, we have seen similar autoantibody enrichment by adsorption to liver membranes and to bacteria (H. Hirayu and B. Rapoport, unpublished observations). Most important, on the likely presumption that TSHR autoantibodies that preferentially recognize linear peptides or denatured antigen do exist, it will be necessary to determine whether such autoantibodies reflect the dominant population of TSHR autoantibodies in an individual serum. Studies for most TPO autoantibodies indicate that this is not the case (reviewed in Ref. 107).

C. TSHR carbohydrate: part of the binding site?

1. TSH binding site. As described above (*Section II.D*), ~40% of the mass of the TSH binding A subunit is carbohydrate. Whether these glycan moieties contribute to the TSH-binding site is an interesting, unanswered question. Also as mentioned above (*Section IV.A.2.a*), it is possible that TSH interaction with glycan may explain the puzzling observation that hCG, but not TSH, binds to its cognate ectodomain, when isolated from the holoreceptor. It is also possible that the lack of glycan in prokaryotic TSHR polypeptide fragments and synthetic peptides, as well as the immaturity of the glycan on TSHR generated in insect cells, may contribute to very low, or absent, TSH binding to this material.

2. Autoantibody epitope(s). The role of glycan moieties in autoantibody binding is a subject of intense current debate. Nonglycosylated TSHR ectodomain was reported to be recognized by autoantibodies in patients' sera (99). Moreover, in a direct comparison, more patients' sera recognized nonglycosylated (bacterial) than glycosylated (insect cell) TSHR ectodomain (78). Quantitatively, recognition was also greater for the former. It was suggested, therefore, that glycan was not a major factor in autoantibody recognition (78).

Contrary data obtained with the insect cell TSHR ectodomain (79), recently confirmed (100), suggested that TSHR glycosylation may be a factor in autoantibody recognition. In addition, using mammalian cell TSHR material, evidence was obtained for a critical relationship between autoantibody recognition and TSHR maturation as reflected in the acquisition of complex carbohydrate. Thus, a TSHR ectodomain variant with complex glycan secreted by mammalian cells (74), but not the full TSHR ectodomain with immature, high-mannose glycan retained within the cell (110), neutralized autoantibody binding to the TSHR. Consistent with these observations, the TSHR ectodomain purified from transfected HeLa cells was relatively ineffective in neutralizing autoantibody binding to the TSHR (112). Although the nature of the glycan in this intracellular material was not determined, it is likely to be high mannose.

Two additional points require emphasis.

1. Studies noting a relationship between TSHR glycan and autoantibody binding do not establish whether or not the glycan moieties actually comprise part of the epitope, even though this has recently been implied (100, 159). Moreover, it is premature on the basis of current evidence to state that glycosylation is an important determinant of TSHR autoantigenicity (100, 159). Thus, incomplete glycosylation can be secondary to abnormal folding (29). That is, lack of autoan-

tibody binding to nonglycosylated TSHR preparations may be explained by incorrect polypeptide folding rather than because of lack of glycan, as previously discussed (110).

2. The issue of TSHR glycan is particularly important in regard to the debate on linear TSHR epitopes. Synthetic peptides and polypeptides generated in bacteria are not glycosylated. This difficulty has resulted in a dramatic conceptual change among some investigators who previously reported extensively on the importance of TSHR peptide and polypeptide fragments in TSHR autoantibody recognition (40, 41, 97, 99, 149). Thus, many previous peptide studies are now considered "nondefinitive" (159).

D. TSHR amino acid residues in TSH-binding site

Given the discontinuous and conformational nature of the TSH binding site, it is not surprising that it has been very difficult to determine the precise TSHR amino acids in contact with ligand. Different approaches have been used for this purpose, all having serious problems.

1. Synthetic peptides. As mentioned above, this approach is technically the most straightforward and has the potential to provide precise information on the TSH-binding sites. Peptides have been used in four general manners (Table 1): (i) direct binding of TSH to peptide; (ii) competition by peptide for TSH binding to the native TSHR; (iii) competition by antiserum to a peptide for TSH binding to the native receptor; and (iv) inhibition by peptide of thyrocyte stimulation by an antiserum generated by immunization of an animal with a TSHR polypeptide or peptide.

There are a number of difficulties in the interpretation of the voluminous data obtained by these approaches. (i) The binding affinity of TSHR peptides is very low. (ii) Even though hormone specificity for this interaction can be demonstrated, ligand specificity exists for low-affinity binding to plastic and to cells not expressing the TSHR (124, 157). Indeed, before the molecular cloning of the TSHR, this phenomenon was interpreted as a second, low-affinity TSHR (160, 161). (iii) Competition for TSH binding by Ig molecules (150 kDa in size) cannot give precise information because of steric hindrance. (iv) Data reported using peptides have implicated more than 300 of the 397 amino acids in the TSHR ectodomain as TSH binding residues (Table 1). TSH cannot make contact with such a large number of residues. Some of these data must, therefore, be artifactual. Conversely, some of these residues are, indeed, likely to be TSH contact residues regardless of the validity of the data. (v) Remarkably, the approximately 300 TSHR residues deduced to be contact points from peptide studies do not include amino acids 201–211, definitively established to be part of the TSH-binding site using the chimeric receptor approach (see below).

2. Mutagenesis of the TSHR. From the practical standpoint, mutagenesis of the TSHR is a more difficult approach. The principle of these studies is that alteration of the amino acid residues in the TSH-binding site will affect (typically reduce) TSH binding. Three mutagenic variations have been used: (i) deletion of TSHR residues; (ii) replacement of TSHR residues with irrelevant residues (nonhomologous substitutions); and (iii) replacement of TSHR residues with the corresponding residues from a closely related receptor, such as the LH/CGR (homologous substitutions). Interchange of blocks of homologous residues generate "chimeric" receptors.

Deletion or substitution of many residues has resulted in altered TSH binding to the TSHR (33, 34, 54, 60, 68, 69, 134, 139, 162–166). It is unfortunate that the complexity and mass of detail in some of these reports have made the subject difficult to follow. We suggest a number of principles that should be considered in interpreting these studies.

1. Because of the discontinuous and highly conformational nature of the TSHR, many deletions and mutations in the TSHR that reduce TSH binding are noninformative. Thus, mutations can affect the level of TSHR expression and/or can lead to allosteric changes in the TSH-binding site. Immuno-

TABLE 1. Studies using synthetic peptides to determine the TSH receptor amino acids making contact with TSH ligand

TSHR residues	Description of study	Ref.
16–35	Competion by peptide for ^{125}I-TSH binding to native TSHR	142
29–57	Competition for ^{125}I-TSH binding to native TSHR by serum of rabbits immunized with peptide	173
32–50	Direct ^{125}I-TSH binding to peptide	386
35–50	Competion by peptide for TSH stimulation of cAMP generation	387
106–125	Competion by peptide for ^{125}I-TSH binding to native TSHR	142
132–150	Generation of TSI by peptide immunization	324
145–163	Generation of TSI by peptide immunization	324
172–186	Generation of TSI by peptide immunization	324
226–245	Competion by peptide for ^{125}I-TSH binding to native TSHR	142
256–275	Competion by peptide for ^{125}I-TSH binding to native TSHR (most potent)	142
286–305	Competion for ^{125}I-TSH binding	142
292–311	Neutralization of TBI in immunized rabbits; Affinity enrichment with peptide of antibodies that inhibit TSH binding and function	320
322–341	Competion for ^{125}I-TSH binding by murine anti-TSHR antisera	388
341–358	Competion for ^{125}I-TSH binding by serum of chickens immunized with peptide	172
344–364	Direct ^{125}I-TSH binding to peptide	386
367–386	Neutralization of TBI in immunized rabbits; Affinity enrichment with peptide of antibodies that inhibit TSH binding and function	320
367–415	Competion for ^{125}I-TSH binding by murine anti-TSHR antisera	388
372–397	Competion for ^{125}I-TSH binding by serum of chickens immunized with peptide	172
397–415	Neutralization of TBI in immunized rabbits	320
649–661	Competion for ^{125}I-TSH binding by serum of chickens immunized with peptide	172

detection of the TSHR in cell homogenates is insufficient to confirm cell surface expression of mutated TSHR.

2. Homologous substitutions of TSHR residues with the corresponding residues of a closely related molecule (*e.g.*, the LH/CGR) are most likely to lead to retention of the native conformation of the molecule.

3. There is general agreement that for a mutation or deletion to be informative, the conformational integrity of the altered TSHR must be demonstrated. When an alteration in the TSHR leads to *reduced* TSH binding, it is necessary to demonstrate unaltered reactivity with another ligand. Under these circumstances, two approaches have been used: (i) Parallel testing of the biological response to TSH or TSHR autoantibodies (54, 163, 166). In our view it is not always easy to interpret the data obtained, especially when polyclonal serum IgG is used in a very sensitive and capricious bioassay and when the data are presented in relative rather than in absolute values. (ii) Parallel testing of hCG *binding* to TSHR with LH/CGR substitutions (134, 162). In this approach a reduction in TSH binding affinity with parallel increase (or no change) in hCG binding affinity provides the strongest evidence that mutations involve ligand-binding residues. The weakness of this approach is that it cannot determine contact residues common to TSH and LH/CG binding. These two ligands have a common A subunit.

3. Interpretation of data. Taking the above principles into account, a few simple, but important, conclusions can be distilled from the confusing mass of data (Fig. 5).

1. From chimeric TSH-LH/CGR data, important TSH contact points are present in the midregion (residues 171–260; domain C) and carboxyl-terminal segments (residues 261–418; domains D and E) of the TSHR ectodomain (418 residues including signal peptide) (33, 134).

2. Homologous substitutions in the TSHR with the corresponding regions of the LH/CGR convincingly localize some TSH contact residues in the ectodomain midregion to amino acids between 201–211 and between 222–230 (162). These findings, within the LRR portion of the TSHR, are supported by the molecular modeling of the region (17).

3. Nonhomologous substitutions or deletions, with TSHR conformation (confirmed by retention of autoantibody responsiveness), strongly suggest that residues between 295–302 and 387–395, in particular Tyr 385 (54), contain TSH contact residues in the C-terminal third of the TSHR ectodomain. These findings are supported by homologous (LH/CGR) substitutions within this region (60).

In our view, a number of other conclusions require confirmation and must presently be regarded as speculative, in particular:

1. There is the concept that the TSH-binding site involves a low-affinity "agonist face" at the N terminus and an "antagonist face" of high affinity at the C terminus of the TSHR ectodomain (57, 164, 165). The basis for this conclusion is that mutations at the N terminus (predominantly between residues 30–45) are associated with a lower TSH binding affinity with unchanged TSH-induced signal transduction. We have a number of reservations about this interpretation of the data. Thus: (i) Thermodynamically, high-affinity TSH binding is likely to reflect the sum of all ligand-receptor contact residues, and not just a few residues at the TSHR C terminus. Indeed, mutations at the N terminus that decrease TSH binding affinity (164, 165) speak against the C terminus being "a", or "the", high affinity binding site in and of itself. If the latter were correct, N-terminal mutations would not affect high-affinity TSH binding. The observation that substitution or deletion of one or a few amino acids abolishes high-affinity TSH binding does not establish these residues to be, in themselves, "a" high-affinity binding site. These amino acids can be an important component contributing to high-affinity TSH binding, or their alteration could have steric or allosteric effects on TSH binding.

2. It is difficult to understand how N-terminal mutations can lead to a lower TSH binding affinity without affecting the sensitivity (EC_{50}) for TSH stimulation of cAMP generation (165). Further, some receptors appear to be "immaculate," with TSH stimulating cAMP generation (albeit weakly) despite undetectable TSH binding. We have never observed such a phenomenon in ~60 systematically performed TSHR substitutions, all studied in stably, rather than transiently, transfected cells (33, 60, 69, 134, 162, 167).

3. The concept that Cys301 and Cys390 contribute to the TSH-binding site requires confirmation. Thus, it is possible

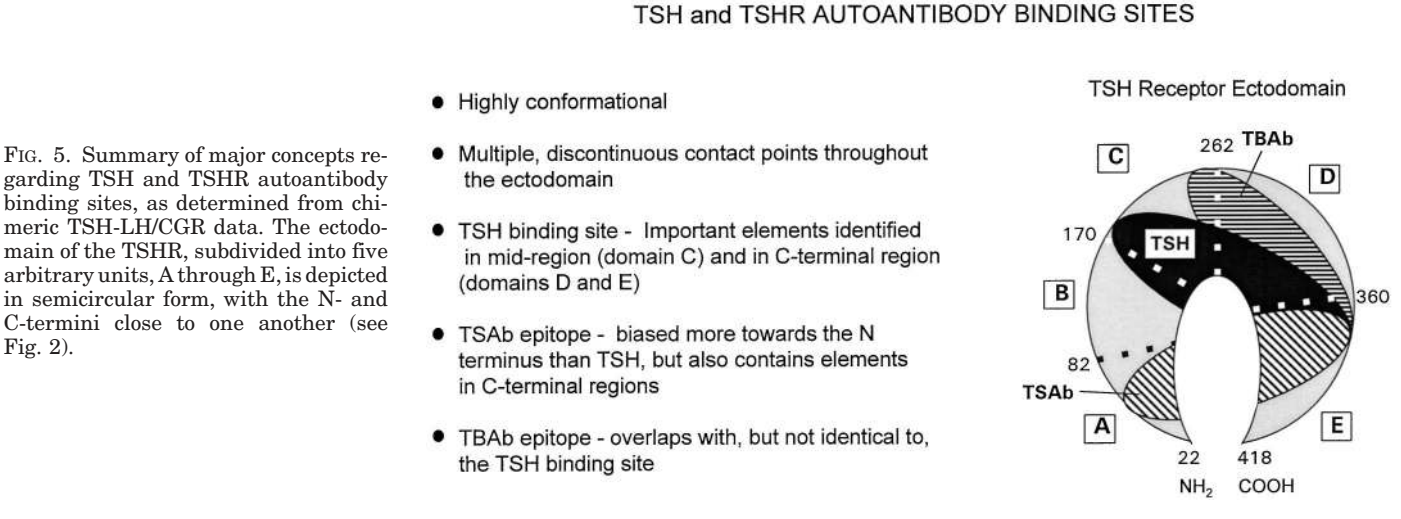

FIG. 5. Summary of major concepts regarding TSH and TSHR autoantibody binding sites, as determined from chimeric TSH-LH/CGR data. The ectodomain of the TSHR, subdivided into five arbitrary units, A through E, is depicted in semicircular form, with the N- and C-termini close to one another (see Fig. 2).

that these two cysteines are paired and contribute to linkage of the two TSHR subunits after deletion of a C peptide (*Section II.C*), suggesting that they are important for stabilization of the molecule rather than being TSH contact residues.

4. Data obtained using TSHR synthetic peptides are frequently cited to support TSHR mutagenesis findings (reviewed in Ref. 57). As noted above, peptides encompassing more than 75% of the linear TSHR ectodomain sequence have been implicated in TSH binding and function. Given the scope of the present peptide literature, it is not difficult to find a citation for a particular region of interest.

E. Autoantibody epitopes

The subject of TSHR autoantibody epitopes is even more complex than the TSH-binding site because it has long been recognized that there are multiple forms of TSHR autoantibodies: those capable of activating the TSHR (168, 169) and nonstimulating antibodies that block TSH binding and action (170, 171). "Neutral" TSHR autoantibodies that neither activate the receptor nor block TSH action may also possibly occur in autoimmune thyroid disease. As for the TSH-binding site, two main approaches have been used to define the binding site(s) for TSHR autoantibodies, synthetic peptides, and TSHR mutagenesis.

1. Synthetic peptides. Peptides have been used in four general manners (Tables 2 and 3): (i) direct binding of patients' IgG to TSHR peptides by ELISA or binding in solution; (ii) inhibition, or "neutralization" by peptides of TSHR autoantibody binding or activity; (iii) affinity enrichment and characterization of TSHR autoantibodies using immobilized peptides; and (iv) study of the consequences of immunizing animals with TSHR peptides (40, 54, 172, 173).

In our view, there are a number of difficulties in interpreting the data obtained, for the following reasons.

1. TSHR autoantibodies in serum (typically ng/ml) are a very small proportion of the total IgG concentration (8–16 mg/ml). Consequently, sera are used at high concentrations and background binding values are high (149), reducing confidence in the specificity of the detection system.

2. Autoantibody neutralization studies are potentially more significant, but the very high concentration of peptide necessary for neutralization (typically 10^{-5} to 10^{-4} M) also reduces confidence in interpretation of the data.

3. Affinity enrichment should, theoretically, provide definitive data, but we have observed nonspecific enrichment of TSHR autoantibodies using bacteria and liver membranes (*Section IV.B.4*).

4. Although an epitope typically involves ~20 amino acids, using synthetic peptides, more than 180 and 330 amino acids have been implicated for stimulating (Table 2) and blocking (Table 3) autoantibodies, respectively. Indeed, every one in a panel of 29 TSHR peptides is recognized by patients' sera to a significantly greater extent than by sera from normal individuals (149). Some sera in a series of patients with newly diagnosed hypothyroidism recognized up to 16 different 20-mer peptides (158).

5. Screening of a hexapeptide mimotope library found the sequences EEFDDA, ETFDDA, EHFDDA to be most effective in neutralizing autoantibody binding (112). These nonspecific peptides are very rich in charged amino acids, E (Glu), D (Asp), and H (His), and suggest that TSHR autoantibodies are particularly influenced by such amino acids.

2. Mutagenesis of the TSHR. The strategy used to identify TSHR autoantibody epitopes is the same as that described above for the TSHR-binding site. Again, the mass of infor-

TABLE 2. Synthetic peptides implicated in TSAb interaction with the TSH receptor

TSHR residues	Description of study	Ref.
29–57	Patient IgG binding to peptide by ELISA	389
31–50	TSI neutralization by peptide; affinity enrichment with peptide of FRTL5-binding antibody from patients' sera	390
32–56	Patient IgG binding to peptide by ELISA	242, 391
91–119	TSI neutralization by peptide; affinity enrichment with peptide of FRTL5-binding antibody from patients' sera	390
103–111	TBI neutralization by peptide; Peptide immunoprecipitation by patient IgG	392
172–202	Patient IgG binding to peptide by ELISA	389
181–200	Patient IgG binding to peptide by ELISA; Affinity enrichment of TSI with immobilized peptide	149
287–304	TSI neutralization by peptide; affinity enrichment with peptide of FRTL5-binding antibody from patients' sera	390
331–350	Patient IgG binding to peptide by ELISA	393
333–343	TSI neutralization; Direct binding by patients' sera	394
338–353	Patient IgG binding to peptide by ELISA; TSI neutralization	395
352–366	Patient IgG binding to peptide by ELISA	40, 54
354–367	TSI neutralization by peptide; affinity enrichment with peptide of FRTL5-binding antibody from patients' sera (also recognizes 287–304!)	390
354–367	Patient IgG binding to peptide by ELISA; TSI neutralization	395
357–372	Patient IgG binding to peptide by ELISA	99
359–371	Patient IgG binding to peptide by ELISA	389
362–376	Patient IgG binding to peptide by ELISA	99
373–388	Patient IgG binding to peptide by ELISA	99
376–394	Patient IgG binding to peptide by ELISA	149
377–397	Patient IgG binding to peptide by ELISA	99
398–417	Patient IgG binding to peptide by ELISA	389

TABLE 3. Synthetic peptides implicated in TBAb interaction with the TSH receptor

TSHR residues	Description of study	Ref.
61–81	Hypothyroid patients' IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	158
151–170	Hypothyroid patients' IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	158
181–200	Patient IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	149, 158
301–320	Hypothyroid patients' IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	158
361–380	Hypothyroid patients' IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	158
376–394	Hypothyroid patients' IgG binding to peptide by ELISA; affinity enrichment of autoantibodies with peptide	158

mation in the literature is very confusing and intimidating. To simplify the subject and to convey the essence of these data, we only describe what we perceive to be appropriate conclusions based on strong evidence. We also subdivide the data according to antibody type. At present, we feel most confident in the following conclusions regarding stimulatory and blocking antibodies; most are derived from studies on TSH-LH/CGR chimeras (Fig. 5).

a. Stimulatory autoantibodies. (i) The epitope(s) for stimulatory autoantibodies overlaps with, but is not precisely the same as, the TSH binding site (138, 139). (ii) The N-terminal third of the TSHR ectodomain (amino acids 22–160 after signal peptide deletion; domains A and B) is necessary for TSHR autoantibody stimulation (138) (Fig. 5). Similar data were obtained with the homologous substitution of residues 22–165 (139). (iii) Although it is a common perception (reviewed in Ref. 159) that stimulatory autoantibodies bind only to the N terminus of the TSHR ectodomain (as opposed to C-terminal binding by blocking antibodies), stimulatory autoantibodies bind to discontinuous segments in both the N terminus and the C terminus (138). (iv) A number of N-terminal amino acid residues appear to be involved in the activity of (at least some) stimulatory autoantibodies, namely, Ser 25-Glu 30 (167) and Thr 40 (165).

b. TSH-blocking autoantibodies. (i) The epitope(s) for blocking autoantibodies on the TSHR ectodomain overlaps with, but is not precisely the same as, the TSH-binding site and the stimulatory autoantibody epitope (138, 139). Overlap between these three ligands is greatest in the C-terminal region of the TSHR ectodomain (138). (ii) Consistent with the close overlap between the TSH-binding site and the blocking autoantibody epitope in the C terminus, residues that appear to be involved in TSH binding, in particular residues 295–302, 385, and 387–395 (54), are also important in blocking autoantibody activity (165). (iii) The great emphasis placed on blocking autoantibody binding to the C terminus of the TSHR ectodomain (reviewed in Ref. 57) obscures the finding that these autoantibodies also bind to the N terminus or midregion of the TSHR ectodomain (residues 1–261) (138).

c. General comments. (i) TSH binding inhibition (TBI) by an autoantibody does not indicate whether the antibody is a thyroid stimulator or a TSH blocker. Obviously, TSH blockers will have TBI activity, but so may stimulating antibodies. (ii) For the same reasons cited for their use in defining the TSH-binding site, we have less confidence in data obtained with TSHR synthetic peptides than with the TSHR holoreceptor, TSHR ectodomain, or large fragments of the TSHR ectodomain. Given the large number of peptides with which patients' sera are reported to interact, it is inevitable that some of these peptides will contain residues within the autoantibody epitopes(s). However, it is difficult to identify important peptides from the mass of data. (iii) One peptide that has generated great interest is the so-called "immunogenic peptide" corresponding to TSHR residues 352–366 (40) or 352–367 (54). This peptide (YYVFFEEQEDEIIGF) is highly immunogenic when injected into rabbits and is reported to be recognized by the majority of TSHR autoantibodies (40, 41). The immunogenic peptide is now known to be within the C peptide, presumed to be released following intramolecular cleavage at two sites (see above). Therefore, if this sequence is indeed recognized by TSHR autoantibodies, it must be on the single-chain, not two-subunit, form of the receptor on the cell surface. Our experience in detecting specific Graves' IgG binding to the TSHR, whether by flow cytometry (103), ELISA, immunoblot, or immunoprecipitation (our unpublished data) is less than favorable. To our knowledge, peptide 352–366 has not been used to absorb out TSHR autoantibody activity, a critical experiment. (iv) The N-terminal and midregion of the TSHR ectodomain (TSHR-261; residues 22–261) can neutralize TSHR autoantibody inhibition of TSH binding to the TSH holoreceptor (74). As mentioned above, TSH binding-inhibitory activity does not discriminate between autoantibody-stimulatory and -blocking activities. Regardless of the type of autoantibody in these sera (stimulatory autoantibodies are far more common than the blocking variety), the data would appear to conflict with chimeric receptor data indicating that stimulatory and blocking autoantibodies bind to both the N-terminal and C-terminal regions of the TSHR (138). However, the affinity of the interaction between TSHR-261 and autoantibodies is not known, and this antigen may include a sufficient part of the epitope(s) to neutralize autoantibody binding to the holoreceptor. (v) There have been modifications in the position of proponents of the viewpoint that stimulatory autoantibodies interact only with the N-terminal region of the TSHR ectodomain and not to segments throughout the ectodomain. Thus, before antithyroid drug therapy, 32% of Graves' sera have recently been reported to contain thyroid-stimulatory autoantibodies (TSAbs) that interact with segments in the C-terminal half of the ectodomain (118), a figure that rises to 78% after antithyroid drug therapy (174) (see below).

V. TSHR Autoantibody Assays

A. Historical background

In 1956, a pivotal year in thyroid autoimmunity, Graves' disease was found to be caused by a serum stimulator with a duration of action longer than that of TSH (175). The identification of LATS as an IgG molecule in 1964 (176, 177)

ushered in the novel concept that humoral autoimmunity could lead to glandular hyperfunction. Shortly thereafter, the TSHR was discovered (178), and in the early 1970s became the focus of intensive study (179–183). It was, therefore, a logical progression that the two phenomena were linked. Thus, in 1970 LATS IgG was found to mimic TSH in activating thyrocyte adenylate cyclase (184, 185). Subsequently, Graves' IgG was observed to compete for TSH binding to the thyroid membranes, confirming its interaction with the TSHR (138, 139, 186).

Not only were thyroid-stimulatory TSHR Igs (TSAb, or TSI) found in Graves' disease, but a few sera were noted to contain IgG that inhibited TSH activation of thyroid adenylate cyclase (170) and to be associated with hypothyroidism (171, 187). Such molecules are now commonly referred to as TSH blocking autoantibodies (TBAbs). Autoantibodies reported to stimulate thyroid growth independent of the adenylate cyclase pathway caused a flurry of interest in the early 1980s (188, 189). Based on the available evidence, most investigators (including ourselves) remain unconvinced that "growth" antibodies are a separate species from conventional thyroid-stimulating autoantibodies, which, of course, also induce thyroid growth. Nevertheless, despite strong contrary arguments (190, 191), the debate as to their existence continues at low intensity (192).

It would be anticipated that the availability in the 1990s of cell lines stably expressing the human TSHR would consolidate the classification of TSHR autoantibodies present in the sera of patients with autoimmune thyroid disease. Unfortunately, this has not been the case. After first summarizing the development and present state of the field, we provide our perspective in an attempt to simplify a subject that has become confusing even to those working in the area.

B. In vivo and in vitro bioassays

1. Thyroid stimulating Ig (TSI).

a. Bioassays using animals in vivo, thyroid slices, and thyroid membranes. Historically, TSHR autoantibodies were first detected in a bioassay involving the ability of injected patient's serum to release radioiodine from guinea pig thyroids *in vivo* (175, 193). Adaptation of this procedure to mice established the "McKenzie bioassay" for TSH (194) and for LATS (195), leading to a surge in clinical studies on the pathogenesis of Graves' disease. However, this *in vivo* bioassay was cumbersome and had a relatively low sensitivity. Sensitivity for LATS, now referred to as thyroid stimulating autoantibodies (TSAb or TSI), in the McKenzie assay was improved by an ingenious modification based on the observation in 1964 that LATS activity could be adsorbed by human thyroid tissue extracts (177). This "LATS protector" assay (196) compensated for the use of heterologous (human) IgG in the mouse bioassay. In the early 1970s, sera with LATS-protector activity were shown to stimulate human thyroid function *in vitro* (197) and, in heroic experiments inconceivable today, *in vivo*. In the latter experiments, the authors used themselves in lieu of the mouse bioassay by prelabeling their own thyroids with radioiodine and infusing themselves with up to 500 ml of Graves' patient serum (198)!

At the same time, attempts were underway to establish *in vitro* bioassays for TSI, including quantification of secretory 'colloid' droplet formation and cAMP production in human thyroid tissue slices (199), or adenylate cyclase activation in thyroid plasma membranes (170). However, these human thyroid slice assays had suboptimal precision and were impractical whereas the plasma membrane adenylate cyclase assays lacked sensitivity.

b. Thyroid cells in monolayer culture. In 1976, the excellent precision and sensitivity of the cAMP response to TSH in thyroid cell monolayer cultures was noted to be an advantage relative to the above mentioned *in vivo* and *in vitro* bioassays (200). Indeed, a bioassay for TSH (201) and TSI (202, 203) was soon developed with these cells, made practical by the use of cryopreserved human thyroid cells (203). A modification of this technique, incubation of cells in sodium chloride-free medium, further improved the sensitivity of the TSI assay (204). Remarkably, despite the fact that nearly 50% of Graves' sera fail to stimulate the mouse thyroid (205), a variant (FRTL5) of the FRTL rat thyroid cell line (206) proved to be valuable and practical in a bioassay for TSI (207). For some sera, however, discrepancies are observed between assays employing human thyrocytes and FRTL5 cells (208).

c. Cell lines expressing the recombinant TSHR. The stable expression of the human TSHR in CHO cells (discussed above) has combined the advantage of detecting TSI activity with a homologous receptor and the ready availability of FRTL5 cells. Indeed, TSHR-expressing CHO cells, easier to propagate and more sensitive than FRTL5 cells, are now the standard bioassay for measuring TSI activity in patients' sera (120, 209).

2. TSH-blocking autoantibodies. At present, blocking autoantibodies can only be detected by bioassay. This assay quantitates inhibition by autoantibodies of the increase in cAMP production (210) or the iodide uptake in response to a standard concentration of TSH (211). Typically, monolayers of FRTL5 cells (212) or CHO cells stably transfected with the human TSHR (138, 213) are used in the blocking autoantibody assay.

C. Indirect TBI assays

1. Present TBI assays. It has been nearly 25 yr since TSHR autoantibodies were found to compete for radiolabeled TSH binding to the TSHR (138, 139, 186). This observation has been developed into the TBI assay, whose simplicity, precision, and cost effectiveness has made it the most widely used TSHR autoantibody assay in present use. The ability to use a crude detergent extract of porcine thyroid membranes in solution and the development of a highly potent, affinity-purified ligand (214, 215) have led to the present popularity of the TBI assay. However there are a number of limitations to this assay. In particular, the TBI assay (i) does not correlate very well with the TSI bioassay, the gold-standard for assessing thyroid-stimulatory activity (115, 117, 120, 216, 217); (ii) does not discriminate between thyroid-stimulating and TSH-blocking autoantibodies; (iii) remains an indirect assay for TSHR autoantibodies, unlike direct assays for TPO autoantibodies presently in widespread use; (iv) uses TSHR and TSH from heterologous species (porcine TSHR and bo-

vine TSH) that may influence the detection of autoantibodies to the human TSHR (115, 120, 217); and (v) would benefit from greater sensitivity.

2. Future TBI assays. Recent developments offer the prospect for some improvement in the current TBI assay.

a. Use of the recombinant human TSHR. Despite the lapse of 8 yr since the expression of a functional, recombinant human TSHR, most TBI assays still use solubilized porcine thyroid TSHR. The main reason for this delay, in our view, has been the inordinate attention paid to the technically easier expression systems, namely TSHR antigen produced in bacteria, insect cells, and cell-free translation systems, or as synthetic peptides (see above). None of these approaches has provided effective antigen. Further, although the recombinant TSHR expressed in mammalian cells does provide excellent antigen, its use in TBI assays employing intact CHO cells in monolayer culture (121) is cumbersome, and insufficient antigen can be extracted from CHO cell membranes to make practical an assay with solubilized TSHR (116, 122). One promising approach has been the use of fermentors to propagate very large numbers of mouse myeloma cells expressing the human TSHR (116).

Very recently, these problems have been overcome. The overexpression of the TSH holoreceptor in CHO cells (32) combined with their direct extraction with detergent from the cell monolayers can now provide large quantities of highly effective antigen, and it is no longer necessary to use the porcine TSHR in the TBI assay (217). TBI values for serum from 30 individuals with suspected Graves' disease correlated closely when tested with solubilized human and porcine TSHR. Further studies are required to determine whether there is an advantage to use human, rather than porcine, TSHR in the TBI assay. The ultimate TBI assay is likely to involve use of the human TSHR with human (h) rather than bovine (b) TSH as a ligand. At present, however, hTSH is a less effective ligand than bTSH, even when tested with the human TSHR (218)! By Medical Research Council standards, the specific activities of bTSH and hTSH are ~30 and ~5 U/mg protein, respectively. Another such example in nature is chicken insulin, which is more effective than human insulin on the human insulin receptor. 'Superanalogs' of human TSH have recently been developed (219) and may provide the ligand needed for an homologous, totally human TBI assay.

D. Direct assays for TSHR autoantibodies

There is currently no assay by which TSHR autoantibodies can be assayed by direct binding to antigen, such as ELISA or newer automated 'platform' techniques. It is interesting to consider the background to this situation. Over many years, efforts to detect the direct binding of autoantibodies to the TSHR have been fraught with difficulty. For example, immunoblotting or immunoprecipitation of thyroid tissue or cell extracts with patients' sera (220, 221), has not revealed antigen consistent with the presently known structure of the TSHR. Even with recombinant TSHR preparations, it has been difficult to obtain a strong, specific signal with many sera that are clearly positive in a TBI assay (77, 78, 92, 222). This difficulty in directly detecting autoantibodies contrasts with excellent TSHR recognition by antibodies from immunized animals, in particular mAbs (27, 55, 126, 127). In our view, three major factors have contributed to these difficulties, two of which have now been overcome.

1. Low TSHR content of thyroid tissue. Thyroid tissue contains relatively few TSHRs, and this material has not been purified for use in a direct autoantibody assay. The ability to express and purify recombinant TSHR (see above) has obviated the problem.

2. Use of recombinant TSHR forms that are not optimally recognized by TSHR autoantibodies (see above). The high level expression of the TSH holoreceptor and TSHR ectodomain variants in mammalian cells now provides antigen that is well recognized by human autoantibodies (32, 74).

3. Very low levels of TSHR autoantibodies in patients' sera. Even when the amount of excellent TSHR antigen is not a limiting factor, development of a direct assay for TSHR autoantibodies will not be a straightforward task. Thus, studies to detect directly TSHR autoantibodies by flow cytometry with CHO cells overexpressing the TSHR (103) have confirmed the concept (223), still not widely appreciated, that TSHR autoantibodies are present at much lower concentrations in patients' sera than are autoantibodies to TPO. More recent studies have determined that only nanogram amounts of an TSHR ectodomain variant were required to neutralize TBI activity in patients' sera (74). By this approach, only rare, highly potent Graves' sera contain TSHR autoantibody levels as high as 1–5 μg/ml. These data are consistent with the report that only two of many potent sera tested contained TSHR autoantibodies that could be detected by immunofluorescence with thyroid cells in monolayer culture (102). In contrast, TPO autoantibody levels can reach 1 mg/ml (224).

Another limitation of a direct TSHR autoantibody assay, like the indirect TBI assay, is that the function of the autoantibodies cannot be determined. In addition, direct assays using a large protein cannot distinguish among autoantibodies with different epitopes. Therefore, discrimination (*e.g.*, between stimulating and blocking TSHR autoantibodies) will require the expression of a variety of TSHR variants. Selected chimeric TSH-LH/CGRs (138, 139, 164) are ideal candidates for this purpose. Recent reports on the use of chimeric receptors to determine the clinical outcome of therapy in Graves' patients are provocative and potentially important (118, 174) and await confirmation.

In addition to indicating the difficulties that will be faced in establishing direct TSHR autoantibody assays, the notion of a very low TSHR autoantibody concentration has important implications for future studies to understand the pathogenesis of Graves' disease. Thus, as hypothesized previously (223), their low levels suggest that TSHR autoantibodies arise at an early stage of the autoimmune process. Support for this concept is provided by restricted κ- or λ-light chain usage (104–106) and restriction to the IgG1 subclass (225) of TSHR autoantibodies in many patients.

E. Confusion on the terminology and types of TSHR autoantibodies

TSHR autoantibodies can be classified according to their function. In addition, since the molecular cloning of the TSHR, it has become possible to discern epitopic differences even within a population of autoantibodies with similar function. The expanding description of TSHR autoantibodies with subtle differences is causing considerable and, in our view, unnecessary confusion. For the reasons summarized below, we do not believe that present evidence requires changes in the primary classification of functional TSHR autoantibodies.

1. Classification of TSHR autoantibodies by function. For many years, it has been clear that there are two primary types of functional TSHR autoantibodies (see above): (i) autoantibodies that activate the TSHR and cause hyperthyroidism (TSI or TSAb); and (ii) more rarely, autoantibodies that block TSH binding and action, thereby causing atrophic hypothyroidism (TBAbs).

Such diverse functions are likely to be mediated by autoantibodies with nonidentical epitopes, a concept confirmed in studies using chimeric TSH-LH/CGRs (138, 139). Nevertheless, there is considerable overlap between both types of autoantibodies and the TSH-binding site, particularly in the carboxyl-terminal region of the TSHR ectodomain (Fig. 5).

This simple classification is complicated by reports of autoantibodies with other functional differences.

a. Thyroid growth autoantibodies. As mentioned above, the general view (with which we agree) does not support that thyroid growth autoantibodies are distinct from thyroid-stimulatory antibodies (TSAbs or TSI). Nevertheless, advocacy for such antibodies continues (159). Moreover, growth autoantibodies have recently been reported to be detected more effectively with FRTL5 rat thyroid cells than with cells expressing the recombinant human TSHR (118). In evaluating this evidence, it should be appreciated that cAMP (induced by TSH) increases growth in FRTL5 thyroid cells, but not in nonthyroidal cells.

b. Thyroid blocking autoantibodies that do not interact with the TSHR. Such antibodies are reported to inhibit a variety of thyroid functions without inhibiting TSH binding (Refs. 210, 211, 226, 227; discussed in Ref. 228). One reservation in accepting these antibodies as a separate category is that very potent, nonspecific blockers of thyroid function exist in human serum (201) and may still be present in IgG samples prepared by ammonium sulfate or polyethylene glycol precipitation. Moreover, their interaction with an unknown, thyroid-specific antigen must be postulated; otherwise, such antibodies would inhibit adenylate cyclase activity, or postadenylate cyclase activity, in every tissue in the body.

c. TSH binding-inhibitory Igs (TBIIs). Recently, TBII have been classified as a group separate from thyroid-stimulating and TSH-blocking autoantibodies (159, 213, 229, 230). A corollary of this concept is that thyroid-stimulating autoantibodies do not interact at all with the TSH-binding site and do not affect TSH binding. We are confused by, and do not agree with, this classification. Thus, it is well established that not all autoantibodies that inhibit TSH binding to the TSHR are stimulatory. Indeed, TSH-blocking autoantibodies have this function. However, it is difficult to understand how a nonstimulating antibody can inhibit TSH binding and not reduce the response to TSH stimulation, the definition of a "blocking" antibody. We suggest that the lack of thyroid stimulation by some antibodies with TBI activity that arise in a recently developed mouse model of hyperthyroidism (231) can be explained by the observations made in previous decades that some autoantibodies to the human TSHR (LATS) preferentially interact with the human, and not the mouse, TSHR (see above). It is also difficult for us to accept on present evidence that the TBI assay is not measuring at least some stimulating autoantibodies.

d. Thyroid-blocking autoantibodies that can be converted into thyroid stimulators by the binding of divalent anti-IgG molecules (76, 213). This very interesting variant of TSHR-blocking autoantibodies, described thus far only in Japanese patients, requires further investigation and may shed light on the role of dimerization in TSHR activation. Regardless of the reason for their difference from other TSHR-blocking autoantibodies (perhaps a subtle difference in epitope), these molecules are blockers of TSH action *in vivo* and, therefore, fall within this functional classification.

e. Neutral TSHR autoantibodies, which neither activate the TSHR nor block ligand action, are also a logical possibility and there is, indeed, evidence for their existence (232).

2. Classification of TSHR autoantibodies by epitope. It is not surprising, perhaps, that autoantibodies with slightly different epitopes can still have similar functions. To simplify this expanding subject, we suggest that such antibodies remain classified primarily according to their function and secondarily according to other characteristics, such as epitopes. To date, thyroid-stimulating autoantibodies with different epitopes have been described:

1. Stimulatory autoantibodies whose binding and function are influenced by the homologous substitution at amino acid residues 25–30 with the corresponding residues of the LH/CGR (167). TSHR autoantibodies in 57% (33/58) of Graves' patients were observed to be sensitive to this mutation (167, 233). There was no association between this epitopic difference and patient age, sex, the presence or absence of hyperthyroidism, or ophthalmopathy (233).

2. Stimulatory autoantibodies have also been divided into "homogeneous" stimulators that recognize only the N terminus of the TSHR ectodomain and "heterogeneous" stimulators whose epitope encompasses both the − and C termini of the TSHR ectodomain (118). This epitopic difference was noted with a TSH-LH/CGR chimera (homologous substitution of TSHR residues 90–165). Of 66 patients studied, 32% were unaffected by this substitution (group A) (118). With antithyroid drug therapy, 78% of patients (29/37) became "type A" (174). This group of patients was reported to be more responsive to such therapy (174). These interesting findings require confirmation.

VI. Monoclonal Autoantibodies to the TSHR Ectodomain

A. Introduction

There is no aspect of research into the TSHR that has generated more controversy, for reasons described below. Since the early 1980s, a variety of approaches have been used to obtain two broad categories of TSHR mAbs, murine and human:

1. mAbs generated by immunizing mice. The uses of such antibodies are (i) to identify and characterize the TSHR in tissues, cells, and membranes by techniques such as immunoblotting, immunoprecipitation, and immunohistochemistry. In general, mAbs provide more specific signals (with a lower background) than do polyclonal antisera, a major advantage to studying a protein that is present at very low concentrations in tissue; (ii) to affinity purify TSHR from thyroidal and nonthyroidal tissues and cells; and (iii) to analyze TSHR receptor variants by means of mAbs with known epitopes.

2. Human mAbs. Such mAbs are representative of the repertoire of TSHR autoantibodies in the sera of patients with autoimmune thyroid disease, primarily Graves' disease. Consequently, the value of human TSHR mAb is immense. In addition to the uses described above for mouse mAb, monoclonal human TSHR autoantibodies will be essential (i) to map precisely the TSHR epitopes recognized by functionally different populations of serum autoantibodies, as has been done for TPO autoantibodies (reviewed in Refs. 234 and 235). The epitopes of mouse mAb may not be precisely the same as those of naturally occurring autoantibodies; (ii) to determine the three-dimensional structure of TSHR autoantibodies, already accomplished for one TPO autoantibody (236). In addition, if it proves to be feasible, crystallization of autoantibody-TSHR complexes (an exceptionally difficult undertaking) would provide the definitive structure of the epitopes recognized by different forms of TSHR autoantibodies; (iii) to study the influence of autoantibodies on antigen presentation to TSHR-specific T cells (237) that control TSHR autoantibody production. Progress is presently being made in this field for TPO autoantibodies (238); (iv) to identify and characterize the heavy and light chain genes coding for TSHR autoantibodies. This information, already available for a wide repertoire of TPO autoantibodies (reviewed in Refs. 234 and 235), will enable investigation of a possible association between Ig germline gene usage and TSHR epitope specificity (particularly important in the case of stimulatory and blocking antibodies) and may also contribute to understanding the genetic background and inheritance of Graves' disease.

B. Mouse TSHR mAbs

Many different approaches have been taken to generate mouse mAbs to the TSHR. In initial reports, mice were immunized with thyroid membrane extracts (189, 239) or with TSH in an antiidiotypic approach (240–242). Despite a high level of competence in these earlier very difficult studies, it is unclear, in our view, whether or not they generated effective or specific antibodies. For example, very high mAb concentrations were required to demonstrate interaction with antigen (189, 239, 240, 242), and the specificity of some mAbs was tested by competition with $\sim 10^{-6}$ M TSH (189, 240), a concentration that fully competes for TSH binding to plastic. Other mAbs were IgM whose high avidity can lead to nonspecific interactions (240, 242). None of these antibodies convincingly recognized the TSHR on immunoblotting. Finally, the use of these antibodies has faded from the literature.

In contrast to immunization with thyroidal TSHR preparations, immunization with different forms of the recombinant TSHR has been more successful. Unequivocally effective mAbs have been generated and evaluated by numerous criteria including immunoprecipitation, immunoblotting, immunohistochemistry, and ELISA (27, 28, 55, 126, 127, 243). However, with two exceptions (55, 243), none of these mAbs were shown to interact with the native, conformationally intact TSHR. Overall, excellent reagents are now available, particularly for detecting the denatured TSHR.

C. Human monoclonal TSHR autoantibodies

1. Immortalization of human B cells by fusion with a myeloma cell partner. Early reports on the generation of B cell clones secreting TSHR-specific mAbs involved fusing peripheral blood mononuclear cells (PBMC) from Graves' patients with either mouse (244) or human (245, 246) myeloma cell lines. Human-human fusions to immortalize B cells are notoriously unstable, and there have been no further reports on the clones with weak or ambiguous specificity that were obtained. In 1982, fusion of human B cells with a mouse myeloma partner, also a very difficult undertaking, was reported to be successful in obtaining clones representative of TSAbs and TBAbs (244), a feat recently repeated by the same laboratory (230). Independent confirmation is awaited.

2. Immortalization of human B cells by infection with Epstein-Barr virus (EBV). The ability to readily immortalize B cells with EBV was a very attractive approach and, in the mid-1980s, numerous laboratories attempted to obtain human TSHR mAbs from patients' PBMC by this method. Most attempts (including our own) failed and were not reported. Nevertheless, over the next decade, there were reports on the successful production of TSHR mAbs, or putative TSHR mAbs, with either TSAbs, TBAbs, or mixed activities (247–253). Some of these studies achieved cloning of the human mAb secreting human B cells (248–250, 252, 253). A major handicap with EBV-transformed B cells is that they secrete predominantly IgM, a very large and sticky molecule that can easily lead to false positive data. Many of the human mAbs produced were IgM, but some were IgG (250, 251). A recent technical advance, removal of IgM-secreting B cells, has greatly facilitated the cloning of EBV-immortalized B cells of IgG class (252, 253).

3. Summary of data derived from studies on the cloning of human TSHR autoantibodies. A number of important observations or conclusions have been made from the above studies: (i) Human mAbs obtained can have TSAb activity, TBAb activity, or both activities; (ii) Stimulating mAb autoantibodies

(TSAbs) can be obtained that do not inhibit TSH binding (230, 253). Conversely, some blocking autoantibodies (TBAbs) do not compete well for TSH binding (250); (iii) Interestingly, TSAb-secreting B cells can be isolated from patients with myxedema and TBAb-secreting B cells can be isolated from hyperthyroid patients (252); (iv) There is a high frequency of B cells capable of producing TSHR antibodies in the peripheral blood of patients with Graves' disease (251); (v) The heavy and light chain genes for a number of mAbs have been sequenced and characterized (249), the first such report for TSHR autoantibodies.

D. Interpretation of the data on human TSHR-specific mAbs

Because of the importance of human TSHR mAbs, it is essential that there be no ambiguity in the specificity of the autoantibodies that are being reported with increasing frequency in the literature. For example, published nucleotide sequences of TSHR autoantibodies can seriously complicate the field if these antibodies are not, in fact, specific for the TSHR. Therefore, while not negating the validity of previous findings, we suggested a number of criteria for confirmation of the cloning of disease-associated, human TSHR autoantibodies (254):

1. Monoclonal TSHR autoantibodies should be of IgG class, like their functional serum counterparts. The high avidity of IgM molecules can also be associated with nonspecific, low-affinity interactions.

2. Monoclonal human TSHR autoantibodies should be functional at nanogram per ml concentrations, the concentration of human TSHR autoantibodies in serum (74). Many studies on human mAbs to the TSHR have only demonstrated biological activity at far higher concentrations.

3. Monoclonal human TSHR autoantibody activity should be removed from serum or tissue culture medium by adsorption with specific antigen but not with control antigen. A variation of this theme would be adsorption with an anti-κ but not with an anti-λ antiserum, or *vice versa*. Such studies are lacking in previous reports.

4. Functional monoclonal human TSHR autoantibodies should be active after complete purification. cAMP stimulation assays can be influenced by non-Ig factors in serum (201, 255). Moreover, it was reported (256) and is common experience that high concentrations of normal IgG can inhibit TSH binding.

5. The heavy- and light-chain genes of the TSHR autoantibody should be expressed, and the purified, recombinant proteins should be functional at nanogram/ml concentrations. "Rescue" of Ig genes by PCR of hybridomas, immortalized B cell lines, or plasma cells *in vivo* are now established procedures. We appreciate that this criterion is very stringent. However, it is especially important if the preceding criteria have not been met.

E. Why is the subject of TSHR mAbs controversial?

There are two aspects to this issue:

1. Uncertainty about the specificity of the antibodies. Aside from the excellent mouse mAbs to the TSHR that have been generated in recent years, we have reservations about the specificity of some of the initial mouse mAbs reported. In addition, we remain uncertain of the specificity of the human TSHR mAbs generated, none of which presently meet all of the criteria that we have suggested. In our view, this issue should be regarded as part of the normal scientific process rather than being controversial. Readers should appreciate the great technical difficulties involved in these studies and, even when skillfully done, some studies may prove disappointing upon subsequent reevaluation. For example: (i) The specificity of some human TSHR autoantibodies secreted in culture (251) may now be doubted because the TSHR antigen used for screening was shown later not to be recognized by TSHR autoantibodies in patients' sera (100). (ii) The high frequency of TSHR autoantibody B cells in peripheral blood (230, 251) should be viewed with caution because of the subsequent determination of the very low concentration of TSHR autoantibodies in patients' sera (74). (iii) The specificity of some human mAbs tested by immunoblotting (246) is now less certain in the light of new information on TSHR structure.

2. The use of earlier, ambiguous studies to claim priority for important concepts. In our view, it is this practice that has led to acrimony in the field of TSHR mAbs. In particular, two papers (189, 244) are heavily cited, even at the present time, to have predicted that blocking and stimulating autoantibodies have different epitopes and recognize different components of the TSHR. Aside from the fact that this concept existed before these publications, the specificity of these mAbs is open to question. Thus, functional effects were observed at extremely high mAb concentrations and interaction with the TSHR was tested by competition with 10^{-6} M TSH (189, 244). Moreover, immunoblots with these antibodies are inconsistent with the presently known structure of the TSHR (161). To our knowledge, the only independent confirmation of mAb function was in a cytochemical assay (257, 258), a difficult and variable assay no longer in use. Indeed, use of these mAbs faded from the literature in subsequent years. Rather than discovering different epitopes for TSAbs and TBAbs, the novel concept that was proposed with these mAbs was that stimulatory autoantibodies interact with a ganglioside component of the TSHR (244), a concept that awaits confirmation.

F. Source of B cells used to obtain human TSHR monoclonal autoantibodies

All previous attempts to generate human mAbs to the TSHR have used PBMC from patients with autoimmune thyroid disease, particularly Graves' disease. However, in autoimmune thyroid disease, it is the thyroid gland that is enriched with thyroid autoantibody-secreting B lymphocytes (reviewed in Ref. 58). In particular, thyroidal B cells (unlike PBMC) are activated, spontaneously secrete TSHR autoantibodies (259), and are likely to be most appropriate for cell fusion. Incidentally, the present debate in the literature as to whether (260–262) or not (263, 264) TSHR autoantibodies are secreted by the Graves' thyroid revolves around the detection of an autoantibody arterio-venous

(A-V) gradient across the thyroid bed. It should be appreciated that detection of an A-V gradient for IgG, a molecule with a very long half-life (up to 3 weeks) and a very large pool size, is a near impossibility.

VII. T Cell Epitopes

A. Background

Although antibodies to the TSHR ectodomain are the direct cause of hyperthyroidism in Graves' disease, the initiation and/or the amplification of this process must involve T cells (265). Understanding the mechanism whereby T cells regulate TSHR autoantibody production is, therefore, of vital importance for future progress toward therapeutic immune intervention in this disease. Unlike antibodies that interact predominantly with conformational epitopes on intact antigen, T cells recognize short, linear peptides bound to autologous major histocompatibility complex (MHC) molecules on antigen-presenting cells. Furthermore, large protein antigens require proteolysis or "processing" to produce peptides suitable for binding within the MHC groove (~20 amino acids for MHC class II molecules). Consequently, as for any other antigen or autoantigen, recognition of the TSHR by T cells *in vivo* requires T cells with receptors specific for TSHR peptides as well as autologous, MHC-class II positive cells capable of processing and presenting antigen. T cell responses to antigen *in vitro* are assessed by their proliferation (measured as [^{3}H]thymidine incorporation) or production of cytokines (such as the autocrine T cell growth factor interleukin, IL-2). The ultimate goal is to characterize TSHR-specific T cells in terms of (i) the linear amino acid epitope recognized by the T cells; (ii) the MHC molecule(s) involved in antigen presentation to the T cells; (iii) the genes encoding the T cell receptor α- and β-chains; (iv) the effector function of the T cells, namely cytokine(s) produced and/or "help" provided for autoantibody synthesis. This component is, obviously, most important in the induction of disease.

B. Approaches to studying T cell responses

In common with other studies in humans, investigation of TSHR-specific T cell responses in Graves' patients poses several problems. However, there are also a few advantages.

1. Source of lymphoid tissue.

a. PBMC. These cells are the most readily available for study, but have the disadvantage that they are likely to contain very small numbers of TSHR-specific T cells. It should be appreciated that T cell studies in rodents are performed after immunization using lymphocytes from the draining lymph node or spleen.

b. Thyroid tissue. A potential advantage in studying human autoimmune thyroid disease is the occasional availability of thyroid tissue that (relative to PBMC) contains a lymphocyte population enriched in activated T cells, including thyroid-specific T cells (for example Refs. 266–270).

2. Requirement for antigen-specific T cell clones. The detailed characterization of TSHR-specific T lymphocytes can only be performed on antigen-specific clones, a procedure requiring a continuous supply of cells bearing autologous MHC molecules. In man, the most convenient source of such antigen-presenting cells are B cells immortalized with EBV. Such immortalized B cells can present peptide-MHC complexes, but their capacity to capture and internalize intact exogenous antigen for processing is limited. Two different approaches have recently been used to address these problems, namely the use of (i) TSHR synthetic peptides and (ii) systems that present to T cells the "naturally" processed peptides derived from whole antigen.

C. TSHR synthetic peptides

The requirement for processing whole antigen can be bypassed by using synthetic, overlapping peptides based on the predicted amino acid sequence of the TSHR. T cell responses to TSHR synthetic peptides have been studied in (i) nonselected lymphocytes from PBMC (271–278) and (ii) T cell lines and clones generated from PBMC by repeated cycles of stimulation with bacterial TSHR ectodomain and/or peptides (276, 279, 280), or by expansion of *in vivo* activated lymphocytes from the thyroid (281).

Many of the T cell proliferative responses [Stimulation Indices (SI)] are small, and in some cases there is no difference between patients and controls in the response to specific peptides (282). Nevertheless, analysis of results from different investigators reveals some commonality in the TSHR peptides recognized by patients', but not by control, T cells (271, 274, 277, 283) (Table 4). Particularly striking are responses to peptides in the regions of amino acid residues 202–222 and 244–266. Also of interest are the observations concerning comparable recognition of TSHR peptides in Graves' patients and family members (278), as well as in Graves' patients before and after different types of therapy (277).

Aside from the above mentioned regions of the TSHR, it is somewhat disappointing that TSHR peptides recognized by Graves' patients' T cells encompass the entire range of the ectodomain and, in some studies, the transmembrane region (279). These data suggest extensive T cell epitope diversity and bode poorly for devising therapeutic strategies involving TSHR peptides of the type proposed for other autoimmune diseases (284). Moreover, some T cell epitopes identified on the basis of a low-level response to synthetic peptides *in vitro* may not correspond to peptides processed *in vivo* from whole antigen (see below). Thus, the most important TSHR-specific T cell epitopes in human autoimmunity may still need to be defined and may ultimately prove to be more restricted than suggested by presently available data.

D. Endogenous antigen processing

The importance of "naturally" processed peptides was recently highlighted by the comparison of T cell clones generated using the full-length acetylcholine receptor α-subunit (a component of the major autoantigen involved in myasthenia gravis) *vs.* clones generated using synthetic peptides (285). Clearly, a more pathophysiological approach is to

TABLE 4. Synthetic TSHR peptides that stimulate proliferation in Graves' (but not control) PBMC, T cell lines, or T cell clones

Lymphocytes	TSHR ectodomain amino acid residues				Transmembrane	Ref.
	20–100	101–200	201–300	300–314		
PBMC	62–81		202–221 230–249 244–263 272–291			271
PBMC	32–50 44–64			328–348 344–364		272
PBMC				316–330		273
PBMC		152–157	207–222			274
PBMC		113 . . .[a]	. . . 237 293 . . .	. . . 386		275
T cell line	31 . . .	. . . 169		338 . . .	. . . 420 441–661	279
PBMC, T cell lines and clones		158–176	237–252 248–263	343–362 357–376		276–278, 280
PBMC	52–71	142–161	202–221 247–266			283

[a] *Dotted lines* between the first and last amino acid residues indicate the range spanned by pools of peptides.

study T cell epitopes presented by cells that have themselves processed the antigen.

Processing and presentation of T cell epitopes *in vivo* involve "professional" antigen-presenting cells. Dendritic cells, usually resident in lymphoid organs, play an important role in the initiation of T cell responses (reviewed in Ref. 286). Because limited numbers of dendritic cells can be isolated or cultured from peripheral blood, investigation of their role in thyroid autoantigen presentation has only been performed for thyroglobulin (Tg) (287). However, it is possible that dendritic cells may be involved in the *in vitro* proliferative responses of PBMC to TSHR ectodomain (produced in bacteria) and in the generation of T cell lines and clones (276, 277).

It is not generally appreciated that, at least in secondary T cell responses, antibodies may play a critical role in antigen presentation to T cells. Thus, macrophages internalize (and subsequently process) antigens by phagocytosis and antigen/antibody complexes via their Fc receptors. More importantly, membrane-bound antibodies on B lymphocytes provide a powerful system for specific antigen "capture" (reviewed in Ref. 288) (Fig. 6). Indeed, a recombinant TPO-specific autoantibody has been shown to capture and present TPO (238). Not only can antibodies capture antigen for processing and presentation, but they are able to modulate antigen processing and thereby enhance or suppress presentation of different T cell determinants (237, 289–291). The potentially important role of TSHR autoantibodies in antigen presentation to T cells has yet to be explored.

Finally, "nonprofessional" cells may also be involved in antigen presentation. In the case of autoimmune thyroid disease, thyrocytes "aberrantly" express MHC class II molecules (292) and have been shown to function as antigen-presenting cells (266, 293). These observations provided the impetus for stably transfecting immortalized B cells with the cDNA for TPO or the TSHR (281, 294–296). TPO-expressing immortalized B cells have been used to derive nonclonal T cell lines (295) and to test previously isolated T cell clones specific for TPO (294). Furthermore, such B cell lines have been used to identify TSHR-specific T cells among a panel of clones isolated from activated intrathyroidal T cells by expansion with IL-2 and anti-CD3 (281).

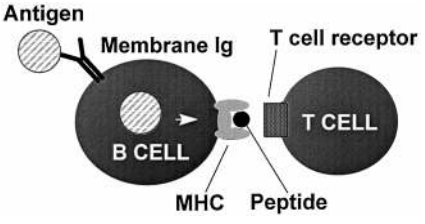

FIG. 6. Membrane-bound antibodies on B lymphocytes provide a powerful system for specific antigen capture and presentation to T cells.

E. TSHR-specific T cell receptor genes and cytokine production

Limited information is available on the T cell receptor variable (V) genes used by TSHR-specific T cells. Thus far, five different T cell receptor Vα genes are reported to be used by a PBMC-derived T cell line responding to peptides from the N-terminal and C-terminal portions of the TSHR ectodomain, as well from the transmembrane region (279).

Humoral responses (such as TSHR autoantibody production) and cell-mediated responses (like T cell cytotoxicity) are controlled by T cells that secrete different cytokines (reviewed in Ref. 297). Briefly, Th1 cells, which regulate cell-mediated responses, secrete interferon (IFN)-γ and little IL-4 (Fig. 7). In contrast, Th2 cells, which provide "help" for antibody production, secrete IL-4 and little IFN-γ. The detection of mRNA for IL-4 in numerous Graves' glands (298–300) provides indirect evidence for a Th2-type response in this disease and is in accordance with the role of autoantibodies in causing hyperthyroidism. Direct evidence for polarization away from Th1 responses comes from the analysis of T cell clones. Thus, T cell clones responsive to the TSHR ectodomain, like many other human clones, appeared to be "Th0-like" in that they produced IL-4 as well as IFN-γ (280). Furthermore, thyroid-derived TSHR-specific T cells, identified using TSHR-expressing autologous B cells, were predominantly of Th2-type (281). It should be pointed out that "deviation" toward a Th2 response in Graves' disease is counter to the prevailing concept in human autoimmunity in

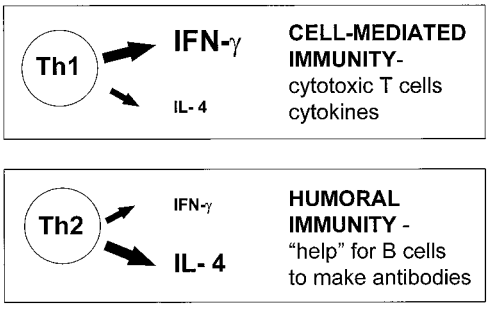

FIG. 7. Cytokine profiles in cell-mediated and humoral immunity.

general, in which Th1 responses are believed to dominate (301).

F. Summary

T cells specific for the TSHR are less well characterized than those specific for TPO. Apart from the difficulties associated with studying human rather than murine responses, the very low serum levels of TSHR autoantibodies (74, 102, 103) suggest that the frequency of TSHR-specific T cells is similarly limited. This information, together with the very wide spectrum of TSHR synthetic peptides reported to be recognized by T cells in Graves' disease, reduces confidence that the T cell epitopes have truly been identified. In addition, for Graves' disease, the most meaningful, but the most difficult, "readout" of T cell function will be the ability of TSHR-activated T cells to induce specific autoantibody synthesis by autologous B cells.

VIII. Molecular Mimicry

An important and long-standing issue is whether or not autoantibodies to the TSHR arise in response to a foreign protein, bacterial or viral, with a structure similar to that of the human TSHR (reviewed in Ref. 302). Such molecular mimicry could relate to a B cell (autoantibody) or T cell epitope. Most interest has involved the former. Antigenic cross-reactivity between the TSHR and a variety of infectious agents has been reported, including the HIV-1 nef protein (303). The most intriguing observations have involved the gram-negative bacterium *Yersinia enterocolitica*.

Antibodies to *Y. enterocolitica* in patients with autoimmune thyroid disease were first noted in 1974 in Scandinavia (304). Because of the high incidence of *Y. enterocolitica* infections in this region, evidence for an association between *Y. enterocolitica* antibodies and thyroid disease was sought, and found, in countries with a low incidence of infections (305, 306). Interest in this phenomenon accelerated with the observation that sera with *Yersinia* antibodies interacted with thyroid epithelium (307) and with the demonstration of a saturable TSH-binding site on *Yersinia* organisms (308) that could be competed for by sera with TSHR autoantibodies (309, 310). Further, mice immunized with *Y. enterocolitica* produced antibodies that interacted with the TSHR (311). At face value, these data appear to provide an overwhelming case for molecular mimicry between the TSHR and *Y. enterocolitica*.

Evidence is emerging on the identity of the *Y. enterocolitica* protein(s) cross-reacting with TSHR autoantibodies. Maximal TSH binding was observed with permeabilized *Yersinia*, suggesting that the reactive proteins were not expressed on the surface of the bacterium (308). Subsequently, Graves' serum reactivity was found to be greatest with *Yersinia* outer membrane virulence proteins encoded by the pYV plasmid (312). However, both of these observations were contradicted in a study suggesting that TSHR cross-reactive proteins were expressed on the surface of *Yersinia* and were encoded by chromosomal genes, not by plasmids (313).

Purification of the *Yersinia* envelope protein cross-reactive with the TSHR revealed it to be low molecular mass (5.5 kDa) bacterial lipoprotein (LP) (314), the most abundant protein in the bacterium cell wall, encoded by a gene highly conserved among gram-negative organisms. Determination of the partial (~50%) amino acid sequence of *Yersinia* LP confirmed its identity with LP from other gram-negative bacteria. Remarkably, however, antibody cross-reactivity with the TSHR was found only for *Yersinia* LP and not for LP from other bacteria, leading to the suggestion that apparent LP-TSHR cross-reactivity was related to the fatty acid component of LP (314). Further characterization of this protein is awaited with interest.

Taken together, the data on the relationship between the TSHR and *Y. enterocolitica* are intriguing and potentially important. However, in our view, a number of potential difficulties with these studies exist. For example:

1. The apparent TSH-binding site on *Yersinia* is of low affinity, comparable to that observed on plastic dishes (157). In addition, TSH binding is not restricted to *Yersinia*, but is observed with a wide variety of organisms (310).

2. Antibodies induced by immunization with *Yersinia* were tested with a nonglycosylated TSHR preparation (311) shown subsequently not to interact with TSHR autoantibodies (100).

3. Cross-reactivity between purified *Yersinia* LP and the TSHR has not been studied with autoantibodies in Graves' sera, a difficult proposition. Moreover, there is no amino acid homology between *Yersinia* LP and the TSHR.

4. Other studies have found no unique pattern of serological reactivity against *Yersinia* membrane proteins in patients with autoimmune thyroid disease, raising the possibility that cross-reactivity with *Yersinia* in Graves' disease may occur at the T cell epitope level rather than via autoantibodies (315). However, the phenomenon of cross-reactivity between *Y. enterocolitica* and the TSHR is based on observations on the humoral immune response. In addition, the ability of a peptide (in conjunction with MHC molecules) to induce a T cell response may depend more on its conformation than on its amino acid sequence (316). Cross-reacting T cell mimotopes between an infectious organism and the TSHR may, therefore, be very difficult to discern simply from their primary amino acid sequences.

5. Molecular mimicry between *Y. enterocolitica* and the TSHR does not explain the frequent detection of autoantibodies to TPO and, to a lesser extent, Tg in Graves' disease. T cell epitope spreading to cryptic determinants on an antigen (317) is a common explanation for the occurrence of these other thyroid autoantibodies. However, if correct, why

does spreading remain restricted to TPO and Tg, and not to other thyroidal, or nonthyroidal, antigens?

These reservations aside, however, molecular mimicry between an infectious organism and the TSHR remains an attractive hypothesis that requires further investigation.

IX. Animal Models of Graves' Disease

Animals that spontaneously develop diseases similar to those occurring in humans have provided a major impetus to the study of human autoimmunity. For example, animal models exist for lupus erythematosus, diabetes mellitus type I, ankylosing spondylitis, and thyroiditis. Until very recently, the spontaneous development of TSHR autoantibodies that can cause hyperthyroidism has been limited to humans and has seriously hampered investigation of the pathogenesis of Graves' disease. To appreciate this handicap, it is only necessary to consider the field of diabetes type I with all the literature related to the NOD mouse deleted. With the absence of a spontaneous animal model for Graves' disease, the past three decades have seen many attempts to develop induced models for this disease. Among these approaches, and not covered in this review, were attempts to generate antiidiotypic antibodies to TSH.

A. Immunization with soluble TSHR antigen and adjuvant

The traditional immunological approach to develop an animal model of an autoimmune disease is to immunize the animal with soluble antigen in adjuvant. The classic example was the first induction of thyroiditis (in rabbits) using human thyroglobulin and complete Freund's adjuvant (318). After the cloning of the TSHR, numerous attempts have been made to develop an animal model of Graves' hyperthyroidism. Rabbits and mice immunized with human TSHR peptides, or protein expressed in bacteria and/or in insect cells, develop serum antibodies that react with receptor preparations in ELISA (for example Refs. 40, 96, 126, and 319). Moreover, serum antibodies and murine mAbs from these animals have provided invaluable reagents for receptor characterization, including immunohistochemistry, Western blotting, and immunoprecipitation. In terms of generating functional antibodies, TBI and TSAb activity (86, 97, 126, 320–322), as well as small increases in T_3 and/or T_4 levels (322–324), could be demonstrated in the sera of some mice immunized with TSHR and adjuvant. However, a serious pitfall associated with these findings was revealed when it was shown that false positive results could be obtained in assays using serum rather than purified IgG (325).

Despite the use of different receptor preparations, different mouse strains (including NOD mice that are genetically predisposed to develop endocrine autoimmunity), and different adjuvants, this approach has not succeeded in mimicking Graves' hyperthyroidism (Table 5). One consequence of immunization with the TSHR ectodomain is lymphocytic infiltration of the thyroid, which is also observed after immunization with Tg (reviewed in Ref. 326) and TPO (327). Based on the possibility that the human TSHR ectodomain may be unsuitable for the experimental induction of Graves' disease in animals, the murine TSHR has been cloned and expressed in insect cells (108, 328). Future experiments will determine the success of this strategy. At present, it is clear that traditional immunization with human TSHR ectodomain in adjuvant fails to mimic the particular type of autoimmune response that occurs *in vivo*.

B. Genetic immunization

An alternative to immunization with a protein and adjuvant is to inject "naked DNA," such as a plasmid containing the cDNA for the respective protein (329). Upon injection, usually intraperitoneal, the DNA enters cells that subsequently express on their surface the protein coded for by the DNA. Preliminary reports indicate that injection of TSHR cDNA in mice induced TBI and TSAb activity that were unequivocally present in the IgG fraction of serum (243).

C. Severe combined immunodeficiency (SCID) mouse model of Graves' disease

Another approach to developing an animal model of Graves' disease involves the use of mice with SCID. Such mice accept xenografts of human tissue because they lack mature T and B cells (reviewed in Ref. 330). Human IgG class autoantibodies to Tg and TPO develop in SCID mice engrafted with suspensions of Graves' or Hashimoto blood lymphocytes, thyroid lymphocytes, or even intact thyroid tissue (331–335). More importantly, some mice xenografted with Graves' thyroid tissue develop TSAb activity and tran-

TABLE 5. Immunization with soluble human TSHR antigen and adjuvant in attempts to establish an animal model of Graves' hyperthyroidism

Antigen	H2 type	TBI (% inhibition)	TSAb	Serum T_3/T_4	Ref.	Thyroid histology
TSHR ectodomain (insect cell)	d	35 ± 16%	ND	Slight increase 7.3 + 0.8	323	No infiltrate
	s	33 ± 13% (*vs.* ~8%)	ND	2.4 + 0.5 (T_4; μg/dl)	322	No infiltrate
GEJ-TSHR cell line (detergent solublized)	s d, k, q, b	~20%	ND	Transient, variable changes	396	Infiltrates (strain and sex dependent)
TSHR ectodomain (bacterial)	d	~50%	Absent	Low T_4	255, 319, 325	Thyroiditis
	g	Absent	Absent	Low T_4		Thyroiditis
	k, b	Absent	Absent	T_4 unchanged		No change
TSHR ectodomain (insect cell)	s	~40%	ND	T_4 unchanged	321	No infiltrate
	d	~20%	ND	T_4 unchanged		No infiltrate

ND, Not determined.

TABLE 6. SCID mice (CB-17) engrafted with Graves' thyroid tissue with and without T cell lines or lymphocytes from the peripheral blood (PBMC) or bone marrow

Antigen	TBI (% inhibition)	TSAb (% increase)	Serum T_3/T_4	Thyroid histology	Ref.
+ PBMC	ND	121 ± 42%	121 ± 35	Small grafts	337
+ control lines	ND	113 ± 58%	136 ± 26	Small grafts	
+ TSHR-lines	ND	232 ± 18%	131 ± 33 (T_3; ng/dl)	Grafts enlarged	
+ N − PBMC	ND	77%, 98%	36, 45		338
+ GD − PBMC	ND	315 ± 90%	63.1 ± 4.1	Fibrosis	
+ GD − Bone Marrow cells	ND	1080 ± 239%	82.5 ± 20.3 (T_4; nmol/liter)	Hypertrophy	

ND, Not determined; GD, Graves' disease.

sient hyperthyroxinemia (335). However, the production of thyroid autoantibodies, including TSAb, is lost about 8–10 weeks after engraftment. This loss of function is likely to be related to the rapid decline in T cell function shortly after lymphocyte transfer (reviewed in Ref. 330). Another pitfall with SCID mouse models of autoimmunity is the variability between individual mice (331, 336).

In attempts to induce a more intense response, two new approaches have been used (Table 6).

1. SCID mice engrafted with thyroid tissue together with PBMC or T cell clones. Mice that received TSHR-specific T cell lines (but not the control T cell lines) developed low but detectable levels of TSAb. However, although the thyroid grafts increased in size, serum T_3 levels were unchanged (337). A major difficulty of this potentially interesting approach is the need to develop T cell clones from PBMC some time before surgery or, alternatively, to use MHC-matched T cell clones and thyroid tissue.

2. Simultaneous xenotransplantation of Graves' thyroid tissue and autologous bone marrow cells. Such experiments have induced higher, but still variable, levels of thyroid autoantibodies including TSAb. Moreover, T_4 levels were higher compared with mice receiving PBMC at least for the 8-week period of study (338).

Despite these drawbacks, not to mention the difficulty and expense of maintaining the mice in a sterile environment, SCID mice have provided the opportunity to study a number of important parameters in autoimmune thyroid disease, including lymphocyte homing (339) and the effects of regulatory cell populations (340). In addition, SCID mice have been used to characterize T cells in functional " thyroid organoids" produced by resuspending thyroid cell suspensions in a solubilized basement membrane preparation (341). However, the necessity for using MHC-matched T cell clones and thyroid grafts (337), as well as the difficulty (and possibly ethics) of obtaining autologous bone marrow cells (338), limits the general application of either of these approaches.

D. Immunization with live syngeneic fibroblasts expressing the TSHR and class II molecules

Very recently, an animal model has been developed that clearly mimics Graves' hyperthyroidism (Fig. 8). This major step forward was achieved by the ingenious approach of immunizing mice with fibroblasts stably transfected with the cDNA for the human TSHR and syngeneic MHC class II

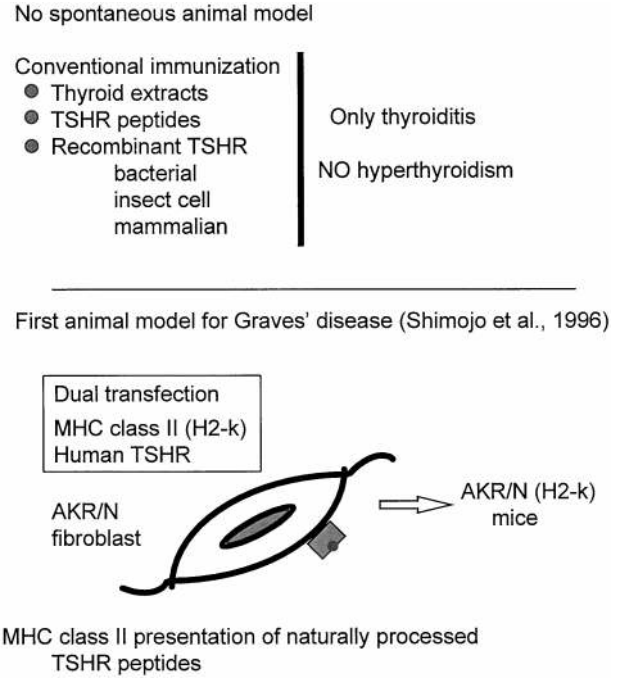

FIG. 8. Animal model for Graves' disease.

TSHR (Table 7) (231). The basis for this approach was the initial report in 1983 (292) of "inappropriate" class II expression on thyroid cells of Graves' or Hashimoto patients, but not from control individuals.

Several important features of this model need to be emphasized. Mice immunized with fibroblasts transfected with the TSHR cDNA alone, like those receiving vector-transfected cells, did not develop elevated T_4 and T_3 levels or TSHR autoantibodies. In contrast, the majority of mice immunized with fibroblasts expressing both class II and the TSHR had readily detectable TBI activity in their sera. Moreover, ~25% of the mice were clearly thyrotoxic with elevated T_4 and T_3 levels, detectable TSAb activity, and thyroid hypertrophy (231). For the first time, therefore, an animal model has been established in which a proportion of individuals have the immunological and endocrinological hallmarks of Graves' hyperthyroidism. One reservation about the "Chiba" mouse model for Graves' disease is that, unlike in Graves' disease in humans, there is no lymphocytic infiltration.

TABLE 7. Induction of Graves' hyperthyroidism in AKR/N mice by immunization with live fibroblasts expressing syngeneic MHC class II and human TSHR

Fibroblasts	TBI (% inhibition)	TSAb (% of basal)	T_4 (μg/dl)	Thyroid histology
MHC II−, TSHR−	7.3 ± 5.4%	~100%	2.5 ± 0.5	No change
MHC II+, TSHR−	4.9 ± 2.4%		2.0 ± 0.4	No change
MHC II+, TSHR+				
18/24 mice	59.3 ± 27.2%	~90%	2.1 ± 0.7	No change
6/24 mice	70.1 ± 12.3%	~350%	12.9 ± 3.4	Hypertrophy

X. Ectodomain Mutations and Polymorphisms

In contrast to mutations in the membrane-spanning segments of the TSHR (reviewed in Refs. 10 and 11), there is relatively little information on naturally occurring, functional mutations or polymorphisms in the ectodomain of the receptor.

A. Spontaneous ectodomain mutations leading to enhanced TSHR function

The clinical presentation of hyperthyroidism, especially in neonates or in young individuals, without evidence for autoimmunity raises the possibility of a mutation in the TSHR. A "gain of function" mutation in one allele of the TSHR is sufficient to cause hyperthyroidism. Thus far, in the TSHR ectodomain, Ser-281 has been identified as a critical amino acid residue whose mutation to Thr, Asn (61), or Ile (62) leads to constitutive activation of the receptor and hyperthyroidism. These data are consistent with the concept that in the absence of ligand the ectodomain serves to constrain TSHR activity (reviewed in Ref. 10). The Pro-52-Thr polymorphism (see below) has been suggested to be associated with an enhanced response to TSH stimulation (342). To date, however, no instances of clinical or subclinical hyperthyroidism have been reported in individuals with this mutation.

B. Spontaneous ectodomain mutations leading to decreased TSHR function

In contrast to gain-of-function mutations in a single allele that readily come to clinical attention, homozygosity or compound heterozygosity (different mutations in each allele) is necessary for clinical presentation of TSHR mutations leading to reduced function. It is possible, therefore, that "loss-of-function" mutations in a single allele are more common than presently recognized. Resistance to TSH stimulation with compensated hypothyroidism has been reported in compound heterozygotes with mutations at Cys-41 (343), Arg-109 (344), Pro-162 (345), Ile-167 (345), Cys-390, and Asp-410 (343), as well as in a neonate with a homozygous mutation at Pro-162 (343). Congenital hypoplasia with overt hypothyroidism has also been observed in a compound heterozygote with a mutation of Cys-390 in one allele together with a deletion mutation at residues 406–412, leading to a shift in reading frame and premature termination of translation (346).

When the functional effects of these spontaneously occurring mutations are compared with those of mutations introduced experimentally into the TSHR in previous studies, an interesting feature is revealed. In both circumstances, mutation of either Cys-41 (69, 165) or Cys-390 (163) abolished or markedly diminished TSH binding and signal transduction. The observation, that a TSHR with a spontaneous mutation at Asp-410 binds TSH with normal high affinity but has reduced signal transduction via adenylate cyclase (343), is consistent with data obtained using chimeric TSH-LH/CGRs in which substitution of the C terminus of the ectodomain reduced signal transduction without affecting TSH binding (33).

C. Nonfunctional polymorphisms in the TSHR ectodomain

The frequency of nonfunctional TSHR ectodomain polymorphisms in the population is unknown. Most interest has involved a Pro-52-His mutation in the TSHR ectodomain because it was suggested to be associated with a predisposition toward Graves' disease (347–349). However, further analysis revealed it to be a polymorphism unrelated to disease that is present in ~8–12% of the population (350, 351). It is highly unlikely that germline polymorphisms can be related to autoimmune disease because protein harboring such amino acids will be recognized as self during development of the immune system.

D. TSHR ectodomain splice variants

On Northern blot analysis, in addition to the full-length (4 kb) TSHR mRNA, smaller transcripts are also readily detected (3, 4). Of these, the dominant 1.3-kb transcript has been cloned and its nucleotide sequence determined (130, 131). This sequence codes for a 253-amino acid polypeptide comprising TSHR exons 1–8 spliced to an unidentified tract (presumably part of an intron) that contributes the C-terminal 22 amino acids. Lacking the membrane-spanning segments of the TSHR, it was speculated that TSHR ectodomain "variant 253" could be secreted by thyrocytes and could play a role in the autoimmune response to the TSHR in Graves' disease (130, 131). Detection of the 1.3-kb, but not the 4-kb TSHR transcript, in orbital tissue also raised the possibility that this TSHR variant played a role in the pathogenesis of Graves' ophthalmopathy (GO) (352).

The hypothesis that the human TSHR ectodomain 253-residue variant was of pathogenetic importance was weakened by the fact that the isolated TSHR ectodomain is not secreted, at least by transfected nonthyroidal cells (84, 110). The recent observation that progressive C-terminal truncation of the TSHR restores TSHR ectodomain secretion revitalizes this theory. However, in our view, given the low number of TSHRs on thyroid cells, the absence of a high turnover rate (at least in cultured cells) and the expected rapid renal clearance of a protein of its size, it is likely that

levels of TSHR variant 253 in plasma will be extremely low and probably nondetectable by present means. Nevertheless, its potential pathogenetic importance remains, especially in regard to local antigen presentation to thyroid-infiltrating T cells.

Deletion of 25 amino acid residues, corresponding approximately to exon 2, has also been observed in a dog TSHR (15). The importance of this TSHR ectodomain variant is unknown.

XI. Extrathyroidal Manifestations of Graves' Disease

The TSHR is the most logical of the thyroid-specific proteins to be involved in the pathogenesis of GO. This is because autoantibodies to the TSHR and eye involvement are observed almost exclusively in Graves' disease. The past 40 yr have witnessed waves of enthusiasm for the concept of TSHR expression in orbital tissue. Before recognition of the autoimmune basis for Graves' disease, this condition was believed to be caused by a pituitary factor (353). Indeed, in the early 1970s, a factor that produced exophthalmos in experimental animals was found to copurify with TSH (354). This activity was also inherent to a proteolytic fragment of TSH (354). Moreover, TSH was reported to bind to (355), and activate (356), the TSHR on plasma membranes prepared from guinea pig orbital tissues. TSH and TSH fragment binding to the TSHR in this tissue was enhanced by IgG from Graves' patients (355, 357) (a phenomenon difficult to understand in the present state of knowledge). Also at an early stage, LATS (now known to be TSAb) was postulated to be related to ophthalmopathy (193).

The subsequent two decades was occupied by a debate as to which orbital tissue cell type (muscle, fibroblast, or adipocyte) was the target of the immune system in GO, with extraocular muscle and orbital fibroblasts receiving the lion's share of investigation (reviewed in Ref. 358). However, the concept of a role for the TSHR in GO languished to some extent because clinical studies failed to find an association between TSHR autoantibodies and the development of GO (reviewed in Ref. 358).

In recent years, the molecular cloning of the TSHR, together with the availability of the PCR, has led to a resurgence of interest in the TSHR in GO. Initially, TSHR mRNA (359–361) or the 1.3-kb TSHR transcript variant (352, 362) was identified in retroorbital muscle and connective tissue. In some studies, evidence was also presented for the expression of TSHR protein in this tissue (360, 363–365). Moreover, TSHR autoantibody production was observed after xenografting of Graves' retroorbital tissue into SCID mice, suggesting the presence in this tissue of TSHR-specific B cells (366).

Nevertheless, the field remained controversial because of variable results from different laboratories (reviewed in Ref. 367), the detection of TSHR in a variety of nonthyroidal tissues, including adipocytes, skin fibroblasts, and lymphocytes (reviewed in Refs. 368 and 369), and because it became increasingly recognized that PCR is too sensitive a procedure on which to base a conclusion of TSHR expression in a particular tissue. Thus "illegitimate transcription" of only a few mRNA molecules for genes not expressed to any significant extent in a tissue can be detected by PCR. For all these reasons, it became apparent that the direct detection of TSHR protein, and not detection of mRNA by RT-PCR, was required to prove TSHR expression in retroorbital tissue. Unfortunately, detection of TSHR in nonthyroidal tissue is difficult and subject to artifact (reviewed in Ref. 13).

Adipose tissue has been the most neglected component of the retroorbital cell troika, probably because it is difficult (literally) to come to grips with this very friable tissue. Adipose tissue is also less prominent than extraocular muscles as a discrete structure on computed tomography or magnetic resonance imaging scan. Most important, the huge fat content of adipocytes makes study of their membranes and proteins very challenging. Despite these obstacles and relative lack of attention, there was much preexisting evidence for TSHR expression on adipocytes, which consequently could be involved in the pathogenesis of GO. Thus (i) the volume of adipose tissue is increased in many severely affected orbits (370, 371); (ii) there are convincing data that adult guinea pig (372–374) and human (375) fat cells express TSHR protein; (iii) TSHR mRNA can be detected in rat epididymal fat (376), guinea pig retroperitoneal fat (377), and in orbital fat from a patient with Graves' disease (378) by Northern blotting without the need for PCR. Moreover, this mRNA, when reverse-transcribed, has been cloned and expressed from guinea pig (377) and rodent (376) fat cell cDNA libraries; and (iv) neonatal human adipocytes are sensitive to the metabolic effects of TSH (379) and TSHR autoantibodies (380). Of importance, this sensitivity greatly diminishes by 4–10 weeks after delivery (379).

In summary, physiologically significant TSHR protein expression by adipocytes has been demonstrated unequivocally in guinea pigs and in neonatal human cells. Inappropriate expression in adult, retroorbital adipocytes cannot be excluded. Support for this concept is the important demonstration that hormone-induced differentiation of 3T3-L1 mouse preadipocytes (381) and primary cultures of rat preadipocytes (382) is associated with expression of a functional TSHR.

Very recently, the availability of effective mouse mAbs to the TSHR appears to have resolved this issue by the direct observation of TSHR in orbital fibroblasts with the characteristics of preadipocytes (383, 384). This function is lost after repeated passage of the cells (384). In another report on the immunodetection of the TSHR in orbital fibroblasts, it is unclear whether or not these cells are, in fact, preadipocytes (385). Confirmation of these findings is awaited and will be very important for future investigations into the pathogenesis of GO.

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Note Added in Proof

Very recent unpublished data from our laboratory suggest that, instead of two distinct cleavage sites in the TSHR, there may be degra-

dation back from cleavage site 1 through the putative C peptide to the region of site 2. These observations are consistent with evidence (as yet unpublished) presented by M. Misrahi *et al.*, INSERM, Le Kremlin-Bicetre France at the 1998 Endocrine Society meeting.

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