Thyrotropin Receptor Cleavage at Site 1 Involves Two Discontinuous Segments at Each End of the Unique 50-Amino Acid Insertion*

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Among the glycoprotein hormone receptors, only the thyrotropin receptor (TSHR) cleaves (at two sites) into disulfide-linked A and B subunits. A 50-amino acid insertion unique to the TSHR ectodomain (residues 317–366) plays no role in ligand binding or signal transduction, but its deletion abrogates cleavage at Site 1, closely upstream of the insertion. We sought to define the region within the 50-amino acid tract involved in TSHR cleavage at Site 1. Mutation of small segments within this region previously failed to prevent cleavage at Site 1. We, therefore, divided the 50-amino acid insertion into quartiles and deleted each one individually (TSHR residues 317–327, 328–338, 339–350, and 351–362). As determined by covalent cross-linking of 125I-TSH to intact cells expressing the mutant receptors, none of these deletions prevented TSHR cleavage at Site 1. Neither did larger deletions of quartiles 1 + 2, 2 + 3, and 3 + 4. However, qualitative differences in the extent of receptor cleavage suggested that quartiles 1 and 4 were playing a greater role in cleavage at Site 1 than were the middle two quartiles. In support of this hypothesis, deletion of these two discontinuous segments almost completely eliminated TSHR cleavage at Site 1.

In conclusion, intramolecular cleavage at Site 1 requires the presence of the N-terminal and C-terminal quartiles of the 50-amino acid insertion unique to the TSHR. Taken together with previous observations, our data suggest that this tract may provide a discontinuous binding site for a protease that clips the TSHR at Site 1.

Three unusual features distinguish the thyrotropin (TSH) receptor from the other glycoprotein hormone receptors. First, stimulating autoantibodies mimic the action of its ligand TSH and cause Graves’ disease, one of the most common autoimmune diseases affecting humans (reviewed in Ref. 1). Second, a variable proportion of TSH receptors (TSHR) on the cell surface cleave into two subunits (A and B) that remain linked by disulfide bonds (2–4). Intramolecular cleavage of the TSHR occurs at two separate sites with the loss of a putative polypeptide fragment (C peptide) (5). Finally, relative to the other glycoprotein hormone receptors, the TSHR contains an insertion of 50 amino acids in the vicinity of residues 317–366 (low homology makes the exact boundaries difficult to define). This insertion, bordered by cysteine residues and with characteristics of an hydrophilic external loop, can be deleted without affecting ligand and autoantibody binding and receptor activation (6).

Whether all three of these features are inter-related is an important unanswered question that may yield clues regarding the pathogenesis of Graves’ disease. Characterization of the mechanism and sites of TSHR cleavage is, therefore, of pathophysiologic interest. There is evidence that TSHR cleavage (at an undetermined site) involves a matrix metalloprotease (7). The putative TSHR C peptide has not been isolated or characterized, possibly because it is degraded. Recently, a direct association between TSHR cleavage and the 50-amino acid insertion has been found. Thus, cleavage at Site 1 is dependent upon and appears to occur closely upstream of, this 50-residue tract (8). However, there is no specific motif for cleavage at Site 1 in that every amino acid in this region can be replaced without abrogating cleavage. Cleavage at Site 2, which also lacks a specific motif, is not dependent on the 50-amino acid insertion but can be prevented by replacing TSHR amino acid residues 367–369 with the N-linked glycosylation motif at the corresponding location in the noncleaving lutropin/choriogonadotropin receptor (9).

In the present study, we sought to define the region within the 50-amino acid insertion involved in TSHR cleavage at Site 1. Surprisingly, we found that almost the entire tract needs to be present for cleavage to occur. However, cleavage is most dependent on the N-terminal and C-terminal quartiles of the 50-amino acid segment. Because there is no amino acid specificity at the cleavage site itself, our data suggest that the 50-amino acid insertion in the TSHR may function as a discontinuous binding site for a protease that clips the TSHR at Site 1.

MATERIALS AND METHODS

TSH Receptor Mutations—All the following deletion mutations were introduced into a TSHR unable to cleave at Site 2 (GQE367–369NET) (9); amino acid residues 317–327, 328–338, 339–350, 351–362, 317–338, 339–362, 328–350, and 317–328 + 351–362. DNA fragments containing these deletions were generated by polymerase chain reaction using overlapping primers and Pfu DNA polymerase (Stratagene, San Diego, CA) or, when necessary, AmpliTaq Gold (Perkin-Elmer). Templates were plasmids containing the cDNA for the wild-type TSHR modified by the introduction of three restriction sites (10) (upstream DNA fragment) and the same receptor with the mutation GQE367–369NET (9) (downstream DNA fragment). The joined DNA fragments were restricted with AflII and SpeI and substituted for the corresponding fragment in the wild-type TSHR cDNA with the 5’- and 3’-untranslated ends deleted in the expression vector pECE-Neo (11). The nucleotide sequences of the polymerase chain reaction-generated fragments, and adjacent restriction sites were confirmed by the dideoxynucleotide termination method (12). The construction of TSHR with deletion of residues 317–366 and the GQE367–369NET substitution (TSHR Δ317–366-NET) has been described previously (6, 8).

Plasmids were stably transfected into Chinese hamster ovary (CHO)
cells with Superfect (Qiagen, Santa Clarita CA). Selection was with 400 
mg/ml G418 (Life Technologies, Inc.). Surviving clones (>100/100-mm 
diameter culture dish) were pooled and propagated for further study. 
Cells were cultured in Ham's F-12 medium supplemented with 10% 
fetal calf serum (fetal calf serum), penicillin (100 units/ml), gentamicin 
(50 g/ml) and amphotericin B (2.5 g/ml). 

Covalent Cross-linking of Radiolabeled TSH—Confluent 100-mm dia-
meter dishes of TSHR-expressing cells were incubated for 2.5 h at 37 
°C with ~5 μCi of 125I-TSH followed by cross-linking with disuccinimidyl 
suberate (1 mol, Sigma) and processing as described previously in detail 
(5). After the addition of Laemmli sample buffer (13) containing 0.7 M 
β-mercaptoethanol (30 min at 50 °C), the samples were electrophoresed 
on 10% SDS-polyacrylamide gels (Bio-Rad). Prestained molecular weight 
markers (Bio-Rad) were included in parallel lanes. We precalibrated these 
markers against more accurate unstained markers to obtain the molecu-
lar weights indicated in the text. Radiolabeled proteins were visualized by 
autoradiography on Biomax MS x-ray film (Eastman Kodak Co.). 

RESULTS

Deletion of the entire TSHR 50-amino acid insertion (residues 317–366) (8), but not mutagenesis of 3–5 amino acid residues 317–327, 328–338, 339–350, and 351–362). Four residues (363–366) most distal to cleavage Site 1 were left intact to 
preserve a restriction site necessary for plasmid constructs. 

The 50-amino acid region, and the horizontal lines are the deletions. 
Note that the four amino acids (363–366) remain to preserve a restriction site for 
plasmid constructs. Deletions were performed on a background of a 
TSHR mutant (GQE367–369NET) in which cleavage at downstream Site 
2 is eliminated (9) (see also Fig. 5). Abrogation of cleavage at Site 1 can 
then be determined, because elimination of both cleavage sites creates 
a largely noncleaving TSHR. Panel B, radiolabeled TSH cross-linking to 
cell-surface TSHR. CHO cells expressing the wild-type TSHR express 
both cleaved (two subunit) and uncleaved, single polypeptide chain 
forms of the receptor on the cell surface (Ref. 3; reviewed in Ref. 1). 
Similarly, for the above deletion mutants, cleaved and single chain 
holoreceptors were detected by autoradiography after covalent cross-
linking of 125I-TSH to monolayers of intact CHO cells ("Materials and 
Methods"). TSH cross-links primarily to the A subunit of the cleaved 
receptor, which is separated from the B subunit under denaturing and 
reducing conditions used for polyacrylamide gel electrophoresis (7.5%) 
of the ligand-receptor complexes.

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Deletion of individual quartiles in the TSHR 50-amino acid insertion does not abrogate cleavage at Site 1. Panel A, schematic representation of the amino acid residues deleted (317–327, 328–338, 339–350, and 351–362). The clear segments indicate the 50-amino acid region, and the horizontal lines are the deletions. Note that the four amino acids (363–366) remain to preserve a restriction site for plasmid constructs. Deletions were performed on a background of a TSHR mutant (GQE367–369NET) in which cleavage at downstream Site 2 is eliminated (9) (see also Fig. 5). Abrogation of cleavage at Site 1 can then be determined, because elimination of both cleavage sites creates a largely noncleaving TSHR. Panel B, radiolabeled TSH cross-linking to cell-surface TSHR. CHO cells expressing the wild-type TSHR express both cleaved (two subunit) and uncleaved, single polypeptide chain forms of the receptor on the cell surface (Ref. 3; reviewed in Ref. 1). Similarly, for the above deletion mutants, cleaved and single chain holoreceptors were detected by autoradiography after covalent cross-linking of 125I-TSH to monolayers of intact CHO cells ("Materials and Methods"). TSH cross-links primarily to the A subunit of the cleaved receptor, which is separated from the B subunit under denaturing and reducing conditions used for polyacrylamide gel electrophoresis (7.5%) of the ligand-receptor complexes.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Larger deletions with the 50-amino acid insertion also do not abrogate TSHR intramolecular cleavage. Panel A, schematic representation of the amino acid residues deleted. Panel B, radiolabeled TSH was cross-linked to intact cells stably expressing TSHR with deletions of quartiles 1 and 2 (amino acid residues 317–338), quartiles 2 and 3 (residues 328–350), and quartiles 3 and 4 (residues 339–362). Again, all deletions were made on a background of a receptor with the mutation (GQE367–369NET), which eliminates cleavage at Site 2. For comparison, TSH cross-linking is shown to a TSHR with the entire 50 amino acids deleted on a background of GQE367–369NET (TSHR Δ317–366 NET) (8). Note that in the experiment shown, a small proportion of the TSHR Δ317–366 NET molecules have undergone cleavage and are more clearly evident on longer exposure and when a larger amount of material relative to the other mutants is applied to the gel. However, the extent of cleavage is much less than with the smaller deletions.
polypeptide chain (uncleaved) and two subunit (cleaved) receptors (Fig. 1B).

Based on the above results, we turned to larger, rather than smaller, deletions in the TSHR 50-amino acid insertion. For this purpose we constructed three TSHR with deletions of quartiles 1 and 2 (amino acid residues 317–338), quartiles 2 and 3 (residues 328–350), and quartiles 3 and 4 (residues 339–362) (Fig. 2A). Again, all deletions were made on a background of a receptor with the mutation (GQE367–369NET) that eliminates cleavage at Site 2. As with the smaller deletions, radiolabeled TSH cross-linking to the surface of intact cells indicated that none of these much larger deletions prevented TSHR cleavage at Site 1 (Fig. 2B).

At this juncture, on re-evaluating data from repeated experiments showing an inability to prevent cleavage at Site 1, we noted that there appeared to be quantitative differences in the degree of TSHR cleavage occurring with the different deletions (see for example Figs. 1 and 2). Densitometric analysis confirmed this impression (Fig. 3). Thus, deletion of the middle two quartiles (residues 328–350) had no effect on the ratio of uncleaved to cleaved receptors relative to the TSHR without deletions. In contrast, deletion of either quartiles 1 and 2 (residues 317–338) or quartiles 3 and 4 (residues 339–362) significantly reduced the extent of TSHR cleavage into two subunits (p < 0.025 and p < 0.05, respectively).

These data suggested that quartiles 1 and 4 were playing a greater role in cleavage at Site 1 than were the middle two quartiles. To test this hypothesis, we generated a new TSHR with deletions of discontinuous quartiles 1 and 4 (residues 317–327 and 351–362) (Fig. 4A). In confirmation of this hypothesis, the effect of these two discontinuous deletions was to almost completely eliminate TSHR cleavage at Site 1 (Fig. 4B). As observed previously with deletion of the entire 50-amino acid segment (8), prevention of cleavage at Site 1 by outer quartile deletion is not absolute, with a trace of cleaved receptor still being evident.

**DISCUSSION**

Only the TSHR, and not the other closely related glycoprotein hormone receptors, undergoes proteolytic cleavage at the cell surface. However, mutagenesis studies have been unable to define a specific amino acid motif at either of the two cleavage sites in the TSHR ectodomain (8, 9). TSHR cleavage must, therefore, be caused by a proteolytic enzyme with relaxed specificity at its catalytic site yet which interacts preferentially with the TSHR and not with the other glycoprotein hormone receptors. This likelihood is supported by evidence for the involvement of a matrix metalloproteinase in TSHR cleavage at the cell surface (7, 14). Matrix metalloproteinases have broad amino acid sequence specificities (for example, Refs. 15–17).

The concept that the specificity of TSHR cleavage relates to the binding of a protease to this receptor rather than to a protease with a specific catalytic site is supported by the observation that transposition of an N-linked glycosylation site from the noncleaving LH/CG receptor to the corresponding region of the TSHR abrogates cleavage at Site 2 (9). Thus, a glycan moiety could provide steric hindrance to the binding of a protease. Similarly, it is possible that the 50-amino acid insertion could contain a protease binding site because deletion of this tract eliminates cleavage at Site 1 (8). It is intriguing, however, that each of these two modifications to the TSHR appears to
eliminate cleavage at only one of the two sites. These data suggest that either two distinct proteases are responsible for TSHR cleavage, or alternatively, cleavage involves two separate binding sites for the same protease.

Another possible role for the 50-amino acid insertion in TSHR cleavage is that this segment has intrinsic protease activity and clips the receptor at an adjacent site. The present data do not support this hypothesis. Thus, every quartile of the 50-amino acid insertion can be deleted without abrogating TSHR cleavage. Deletion of a catalytic element is unlikely to leave TSHR cleavage at Site 1 unaffected or only partially affected.

We suggest a model (Fig. 5) that integrates the present data with the following previously known features of the TSHR ectodomain. (i) The insertion (residues 317–366) is a very hydrophilic structure that can be deleted without affecting the conformational integrity of the TSHR (6) or loss of of the ligand binding A subunit (6, 18), which is linked by disulfide bridges to the membrane-spanning B subunit; (ii) although the precise residues at cleavage Sites 1 and 2 are unknown, these sites appear to lie close to each end of the 50-amino acid insertion (8, 9, 18); and (iii) for the A and B subunits to remain linked after cleavage, the cysteine residues involved in linkage must lie outside the two cleavage sites. Indeed, the cysteines most likely to link the A and B subunits are closely upstream of cleavage Site 1 (Cys-283, Cys-284, Cys-301 on the A subunit) and downstream of cleavage Site 2 (Cys-390, Cys-398, Cys-408 on the B subunit) (reviewed in Ref. 1).

Taken together, these data suggest that the 50-amino acid insertion is an external loop on the surface of the TSHR, with its first and fourth quartiles being drawn together by disulfide bonding. Consequent to intramolecular cleaving, all or most of this insertion is removed like a polyp clipped at its base. The present observations raise the possibility that the first and fourth quartiles (the base of the polyp) constitute a binding site for a protease that clips the receptor at Site 1. Further cleavage at Site 2, whether by the same or a different protease, releases the polyp from the receptor in the form of a C peptide, which either remains intact or is degraded.

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