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The Thyrotropin Receptor Hinge Region Is Not Simply a Scaffold for the Leucine-Rich Domain but Contributes to Ligand Binding and Signal Transduction

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The glycoprotein hormone receptor hinge region connects the leucine-rich and transmembrane domains. The prevalent concept is that the hinge does not play a significant role in ligand binding and signal transduction. Portions of the hinge are redundant and can be deleted by mutagenesis or are absent in certain species. A minimal hinge will be more amenable to future investigation of its structure and function. We, therefore, combined and progressively extended previous deletions (Δ) in the TSH receptor (TSHR) hinge region (residues 277–418). TSHRΔ287–366, Δ287–371, Δ287–376, and Δ287–384 progressively lost their response to TSH stimulation of cAMP generation in intact cells, consistent with a progressive loss of TSH binding. The longest deletion (TSHRΔ287–384), reducing the hinge region from 141 to 43 amino acids, totally lost both functions. Surprisingly, however, with deletions extending from residues 371–384, constitutive (ligand-independent) activity increased severalfold, reversing the suppressive (inverse agonist) effect of the TSHR extracellular domain. TSHR-activating point mutations I486F and I568T in the first and second extracellular loops (especially the former) had reduced activity on a background of TSHRΔ287–371. In summary, our data support the concept that the TSHR hinge contributes significantly to ligand binding affinity and signal transduction. Residues within the hinge, particularly between positions 371–384, appear involved in ectodomain inverse agonist activity. In addition, the hinge is necessary for functionality of activating mutations in the first and second extracellular loops. Rather than being an inert linker between the leucine-rich and transmembrane domains, the TSHR hinge is a signaling-specificity domain. (*Molecular Endocrinology* 22: 1171–1182, 2008)

THE TSH RECEPTOR (TSHR), a member of the G protein-coupled receptor superfamily, is the primary regulator of thyrocyte function and growth and is also the target of receptor-activating autoantibodies in Graves' disease (reviewed in Refs. 1 and 2). The hormone and autoantibody binding sites lie in the large extracellular domain (residues 22–418 after deletion of a 21-amino-acid signal peptide) linked by a hinge region to the serpentine transmembrane domain (TMD) (see Fig. 1). The amino-terminal two thirds of the TSHR ectodomain contains multiple leucine-rich repeats flanked by cysteine clusters, a feature common to the glycoprotein hormone receptor subfamily. The TSHR hinge region (residues 277–418) is particularly large, approximately 50 amino acid residues longer than in the other glycoprotein hormone receptors. Much is known about the three-dimensional structure of the TSHR. The TMD has been modeled on the crystal structure of rhodopsin (3, 4). The complete ectodomains of the glycoprotein hormone receptor ectodomains have not been crystallized because of difficulty in generating properly folded, soluble recombinant material (5, 6). However, the leucine-rich domain (LRD) component of the TSHR ectodomain (equivalent to the A-subunit generated by intramolecular cleavage) is amenable to large-scale production in native form (7), and a module comprising residues 22–260 has recently been crystallized (8) with a three-dimensional structure closely resembling the previously crystallized FSH receptor (FSHR) LRD (6).

The most enigmatic region of the glycoprotein hormone receptors is the hinge region, the structure of which is difficult to model because of low homology with other proteins in the database. It should be emphasized that the hinge region is absent in the crystal structure of the FSH-FSHR LRD complex (6) as well as in the complex of an activating TSHR autoantibody bound to the TSHR LRD (8). Nevertheless, based on this information, a prevalent concept of glycoprotein hormone activation is that ligand does not bind to the

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Abbreviations: CAM, Constitutively active mutation; ECL, extracellular loop; FSHR, FSH receptor; LHR, LH receptor; LRD, leucine-rich domain; mAb, monoclonal antibody; SCA, specific constitutive activity; SSD, signaling and specificity domain; TMD, transmembrane domain; TSHR, TSH receptor.

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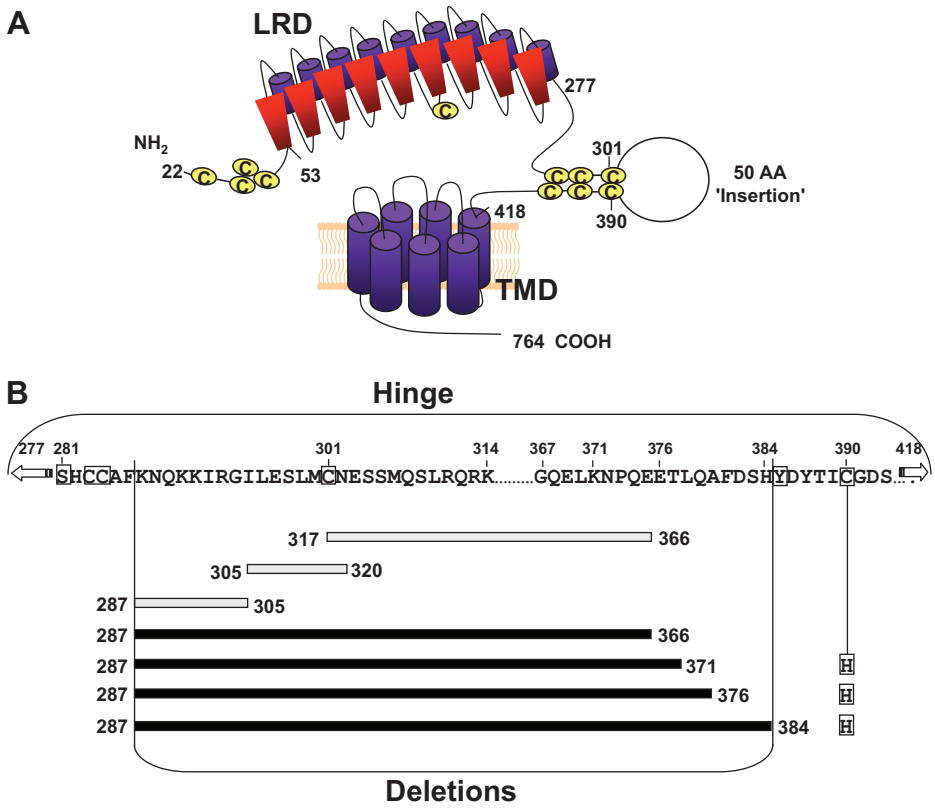

Fig. 1. TSHR Hinge Deletion Mutations

A, Schematic representation of the TSHR and deletion mutations in the hinge region (not to scale). The LRD is flanked by cysteine clusters. The hinge region is depicted between the LRD and the serpentine TMD. Amino acid numbering includes the 21-residue signal peptide. The exact junction between the LRD and hinge region is uncertain. The crystal structure of TSHR 22–260 appears to contain the entire LRD (8), whereas the homologous FSHR LRD terminates at the equivalent of TSHR residue 277 (6). Using the latter boundary, within the 141-amino-acid hinge region, the residues between C301 and C390 are believed to form a loop (2, 40). Relative to the other glycoprotein hormone receptors, this loop contains an insertion of 50 additional amino acids (AA). Posttranslational intramolecular cleavage of the TSHR deletes a poorly defined portion of this loop (C peptide region) leaving an N-terminal A-subunit linked by disulfide bonds to a largely transmembrane B-subunit (13, 14). B, Important amino acid residues are depicted in the TSHR hinge. Cysteine residues are boxed. Also boxed are S281 and Y385; mutations of the former lead to high ligand-independent, constitutive activity (37, 38), and mutations of Y385, which is sulfated, reduces TSH binding and function (19, 20). TSHR deletion mutations reported previously are shown as *gray bars*; D317–366 (12) and D305–320 (17). Data on TSHRΔ287–305 are unpublished (Tanaka, K., C.-R. Chen, S. McLachlan, and B. Rapoport). Deletion mutations made in the present study are shown in *black*. An additional set of mutations superimposed on the indicated deletion mutations involved C390H (*boxed*).

hinge region, which is simply a scaffold for the LRD (reviewed in Ref. 9). Ligand bound to the LRD impacts the extracellular loops (ECLs) of the TMD. Moyle *et al.* (9) describe numerous inconsistencies between a scaffold model and previously reported functional data and suggest an alternative concept more consistent with the latter data. In this alternative model, the hinge region contains part of the ligand binding site and (rather than the ligand itself) is involved in receptor activation (10). Clearly, a crystal structure is incontrovertible. However, a suggested resolution to this paradox is that the isolated FSHR LRD lacking the hinge contains a cryptic ligand binding site that is sterically hindered by the other components of the extracellular domain (11).

One approach to studying the poorly understood, yet vitally important, glycoprotein hormone receptor hinge region is to reduce its complexity by generating a functional receptor with a minimal hinge. Significant portions of the hinge region are known to be redundant. The TSHR hinge (~141 amino acid residues) can be reduced in size without loss of function by deletion of a 50-amino-acid insertion that is absent in the other glycoprotein hormone receptors (12). Indeed, this insertion, as well as poorly defined adjacent regions in the hinge, are removed during posttranslational processing of the mature TSHR into disulfide-linked A- and B-subunits (13, 14). In most species studied, the gonadotropin receptor hinge regions are only about 90 amino acid residues in length. However, the marmoset

LH receptor (LHR) lacking exon 10 (15) and the salmon FSHR (16) are even smaller. Based on these naturally occurring small hinge regions, Moyle *et al.* (10) generated a functional analog of the rat FSHR with a hinge of only 63 amino acid residues.

In the present study, considering the foregoing information as well as other experimental data, we progressively reduced the TSHR hinge region from 141 to 43 amino acid residues. The data obtained with this minimal TSHR hinge provide strong evidence to support the Moyle concept that the hinge region is a signaling and specificity domain (SSD). In particular, the TSHR hinge contributes significantly to ligand binding affinity and also influences signaling in terms of the extent of TSHR constitutive (ligand-independent) activity.

RESULTS

TSHR Hinge Deletion Mutations

Based on homology to the FSHR LRD three-dimensional structure (6), the N terminus of the TSHR hinge region is at residue 277. Entry of the TSHR ectodomain into the plasma membrane at approximately residue 418, implying a hinge region of 141 amino acids (Fig. 1A). Previously, TSHR residues 317–366 have been deleted without loss of TSH binding or function (12). TSH binding to the mature receptor on the cell surface is also retained after deletion by mutagenesis of TSHR amino acids 305–320 (17) and 287–305 (Tanaka, K., C.-R. Chen, S. McLachlan, and B. Rapoport, unpublished data). Trypsin treatment of intact cells also removes the latter two segments in the TSHR without alteration in TSH binding and function (18).

In the present study, we explored the effect of the combined deletion of all of these segments (residues 287–366; total of 80 amino acids) on TSHR binding and function (Fig. 1B).

Preliminary flow cytometric data revealed that TSHRΔ287–366 is expressed on the surface of transiently transfected COS-7 cells. We did not consider deletion in the TSHR hinge further upstream of residue 287 because of the critical cysteine doublet C283,C284 (Fig. 1B). However, further deletions downstream of residue 366 were potentially feasible up to residue 384, retaining residue Y385 known to be critical for TSH binding (19, 20). Consequently, we generated progressive downstream deletion mutations in the TSHR hinge region, namely Δ287–371, Δ287–376, and Δ287–384 (Fig. 1B).

TSH Stimulation of TSHR Hinge Deletion Mutations

After transient transfection of COS-7 cells in monolayer culture, TSH stimulation of the wild-type TSHR increased intracellular cAMP levels with a half-maximal effect at approximately 0.1 mU/ml (Fig. 2). Despite

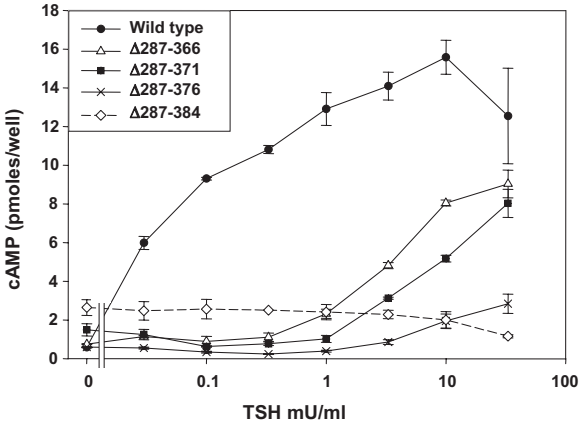

Fig. 2. TSH Stimulation of TSHR Hinge Deletion Mutations

COS-7 cells transiently transfected with plasmids expressing the wild-type TSHR or TSHR with hinge region deletion mutations were stimulated for 1.5 h with the indicated concentrations of TSH (see *Materials and Methods*). Data represent the mean + SD of intracellular cAMP levels in duplicate wells of cells. All cultures and cAMP determinations were performed in the same assay. The data shown are representative of two experiments with similar results.

deletion of 80- and 85-amino acid residues in the hinge regions of TSHRΔ287–366 and TSHRΔ287–371, respectively, these mutant receptors still responded vigorously to TSH stimulation but with a sensitivity two to three orders of magnitude lower than the wild-type receptor. TSHRΔ287–376 with a slightly more extensive hinge region deletion (90 of 141 amino acid residues) was barely responsive, even at the highest TSH concentration, whereas the TSHRΔ287–384 with the largest deletion of 98 residues was totally unresponsive. Remarkably, however, TSHRΔ287–384 and, to a lesser degree, TSHRΔ287–371 appeared to have increased ligand-independent constitutive activity compared with the wild-type TSHR.

Because C301 lies within the foregoing deletions, its pair, C390, remains an orphan with potential deleterious consequences to TSHR structure and function. We, therefore, wished to restore balance by eliminating C390 with a C390H mutation. This mutation was chosen because it does not alter ligand binding or receptor activation when combined with deletion of the homologous upstream cysteine residue in the rat LHR (10). TSHR C390S mutations, on the other hand, markedly reduced TSH binding and receptor function (21, 22), including constitutive activity (22). TSH stimulation of TSHRΔ287–371,C390H; TSHRΔ287–376,C390H; and TSHRΔ287–384,C390H resulted in cAMP response profiles (Fig. 3) similar to those in TSHR deletion mutants without this cysteine substitution (Fig. 2). Again, increased constitutive activity relative to the wild-type TSHR was evident in TSHRΔ287–384 and TSHRΔ287–371 even in the presence of C390H substitutions.

Ligand-Independent, Constitutive Activity of TSHR with Hinge Deletion Mutations

The surprising observation of apparently elevated constitutive activity in some of the TSHR hinge region deletion mutants required confirmation by determining their specific constitutive activity (SCA) relative to their level of cell surface expression (23). As before (Figs. 2 and 3), uncorrected basal intracellular cAMP levels were increased in some deletion mutants, particularly in TSHRΔ287–384, with or without the C390H substitution (Fig. 4A). Flow cytometric analysis using a monoclonal antibody (mAb) (CS-17) with an epitope upstream of TSHR residue 287 (24) revealed variable but detectable levels of receptor expression in all TSHR mutants, including TSHRΔ287–376 and TSHRΔ287–384 (Fig. 4B) with minimal or absent TSH responses (Figs. 2 and 3). Adjusting cAMP values for the level of TSHR expression revealed a six-fold increase in TSHRΔ287–384 and a 2- to 3-fold increase in TSHRΔ287–371 SCA values (Fig. 4C). Remarkably, TSHRΔ287–376, with a hinge region deletion of intermediate length did not have an increased SCA value relative to the wild-type TSHR. The C390H mutation did not alter the SCA in any TSHR mutant. CS-17 used for flow cytometry was previously recognized to reduce TSH-independent TSHR cAMP generation, even with TSHR point mutations that greatly enhance constitutive activity (24). This inverse agonist activity of CS-17 was also evident with TSHRΔ287–384 in that it suppressed cAMP generation by this deletion mutant

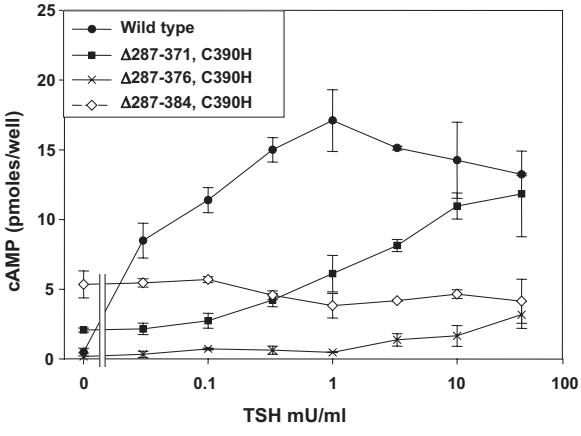

Fig. 3. TSH Stimulation of TSHR Hinge Deletion Mutations with C390H Substitutions

COS-7 cells transiently transfected with plasmids expressing the wild-type TSHR or TSHR with C390H substitutions superimposed on hinge region deletion mutations were stimulated for 1.5 h with the indicated concentrations of TSH (see *Materials and Methods*). The rationale for the C390H mutations is described in *Results*. Data represent the mean ± SD of intracellular cAMP levels in duplicate wells of cells. All cultures and cAMP determinations were performed in the same assay. The data shown are representative of two experiments with similar results.

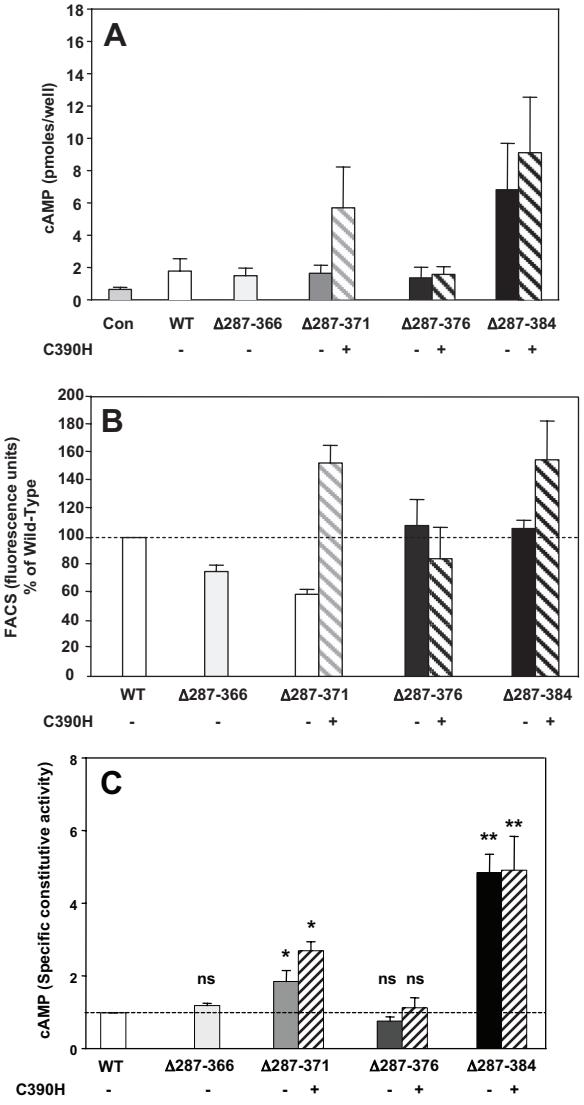

Fig. 4. Selected TSHR Hinge Deletion Mutations Increase SCA

A, Uncorrected cAMP levels. Intracellular cAMP levels were determined in transiently transfected COS-7 cells expressing the wild-type TSHR (WT) and receptors with the indicated deletions, with or without a C390H substitution. Con represents cells transfected with empty vector. *Bars* represent the mean ± SE of cAMP values from three separate experiments (two, two, and four wells in each experiment). B, Flow cytometric quantitation of TSHR expression using aliquots of the same cells shown in A. Detection was with murine mAb CS-17 whose epitope lies upstream of TSHR residues 287. *Bars* represent the mean ± SE of values from three separate transfections. C, SCA calculated from the data shown in A and B (see *Materials and Methods*). SCA of the wild-type (WT) TSHR is normalized to 1, and values for the other receptors are expressed relative to the wild type. *Bars* represent the mean ± SE of values from three experiments (two, two, and four wells in each experiment). Statistical analyses were performed using the Mann-Whitney rank sum test. Significance *vs*. WT: *, $P = 0.002$; **, $P = 0.001$; ns, not significant.

(Fig. 5). Similar suppression was observed with TSHRΔ287–371 (data not shown).

TSH Binding to TSHR Hinge Deletion Mutants

The foregoing data raised the question as to whether the cAMP response to TSH stimulation as well as the level of TSHR constitutive activity was related to TSH binding to the TSHR deletion mutants. Because the C390H mutations did not influence the basal or TSH-stimulated cAMP values in the TSHR deletion mutants, we did not study TSH binding to receptors with these mutations. Consistent with numerous previous reports, specific binding of radiolabeled TSH to the wild-type TSHR expressed on monolayers of transiently transfected COS-7 cells revealed a high-affinity TSH binding site with a dissociation constant K_d of 0.4 ± 0.9 mU/ml TSH (mean ± SE; n = 5), with a representative experiment shown in Fig. 6. Specific TSH binding to all TSHR deletion mutants was too low for accurate analysis, although clearly evident but with reduced affinity for TSHRΔ287–366 and TSHRΔ287–371.

Functional Effect of Hinge Deletion on TSHR-Activating Point Mutations

Point mutations in different regions of the TSHR can increase constitutive activity in terms of increased cAMP generation and can cause clinical disease. Because TSHRΔ287–384 with the largest deletion in the hinge region had increased constitutive activity (Fig. 4C), we examined the functional effect of this deletion

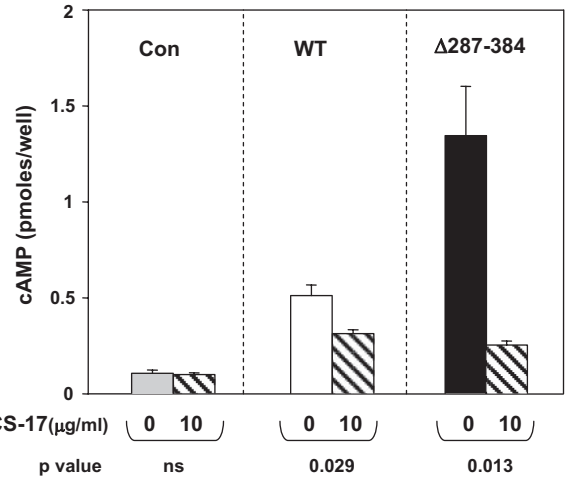

Fig. 5. The Inverse Agonist CS-17 Suppresses the Elevated Constitutive Activity of TSHRΔ287–384

COS-7 cells transiently transfected with empty vector (Con), the wild-type TSHR (WT), and TSHRΔ287–384 were incubated for 1 h in medium with or without mAb CS-17 (10 μg/ml). Each *bar* represents the mean + SE of intracellular cAMP values from triplicate wells of cells. *P* values shown were determined by Student's *t* test. Similar data were obtained in a second experiment.

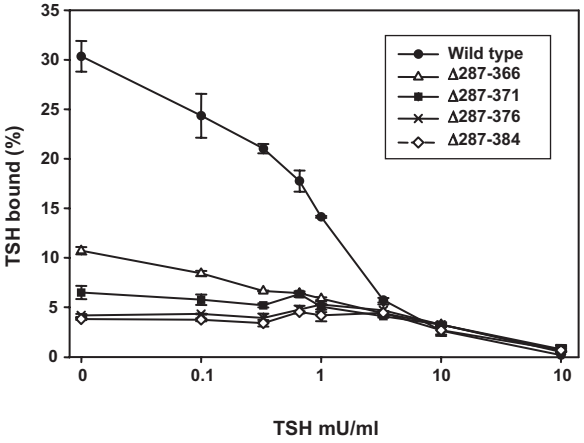

Fig. 6. TSH Binding to TSHR Hinge Deletion Mutations

COS-7 cells were transiently transfected with plasmids expressing the wild-type TSHR or TSHR with the indicated hinge region deletion mutations. Radiolabeled TSH binding was determined in duplicate wells of cells in monolayer in the absence and presence of the indicated concentrations of unlabeled bovine TSH. Specific TSH binding is shown after subtraction of binding in the presence of 100 mU/ml TSH (<2% of total cpm added). Each point represents the mean ± SD of duplicate wells of cells. The data shown are representative of five similar experiments.

on a number of naturally occurring TSHR mutants with increased constitutive activity. We tested mutations in the hinge region (S281I) and ECLs 1, 2, and 3 (I486F, I568T, and V656F, respectively) as well as an intracellular mutation, A623I, in the third intracellular loop. As anticipated, basal cAMP levels were increased relative to the wild-type TSHR in TSHRΔ287–384 and to an even greater extent in all of the point mutations studied (Fig. 7A), including when corrected for the level of cell surface expression as determined by flow cytometry (Fig. 7B) to determine their SCA (Fig. 7C). Superimposition of these point mutations on TSHRΔ287–384 generated an unexpected finding (Figs. 7, A–C). The SCA of the I486F mutation (ECL1) was markedly reduced. In contrast, the hinge region deletion had no significant effect on the SCA of the other activating mutations, S281I, I568T, V656F and A623I. TSHRΔ287–371 also had increased constitutive activity, although to a lesser extent than TSHRΔ287–384 with the longer deletion within the hinge region. The combined effect of the activating point mutations together with TSHRΔ287–371 resembled that with TSHRΔ287–384, but with some differences. Correcting basal cAMP activities (Fig. 8A) for their relative flow cytometric levels of expression (Fig. 8B), the SCA for I486F was almost completely eliminated by the TSHRΔ287–371 deletion (Fig. 8C). Moreover, the SCA of S281I and I568T (ECL2) were now significantly reduced. As with TSHRΔ287–384, the SCAs of V656F (ECL3) and A623I (intramembrane helix 6) were not significantly altered (Fig. 8C).

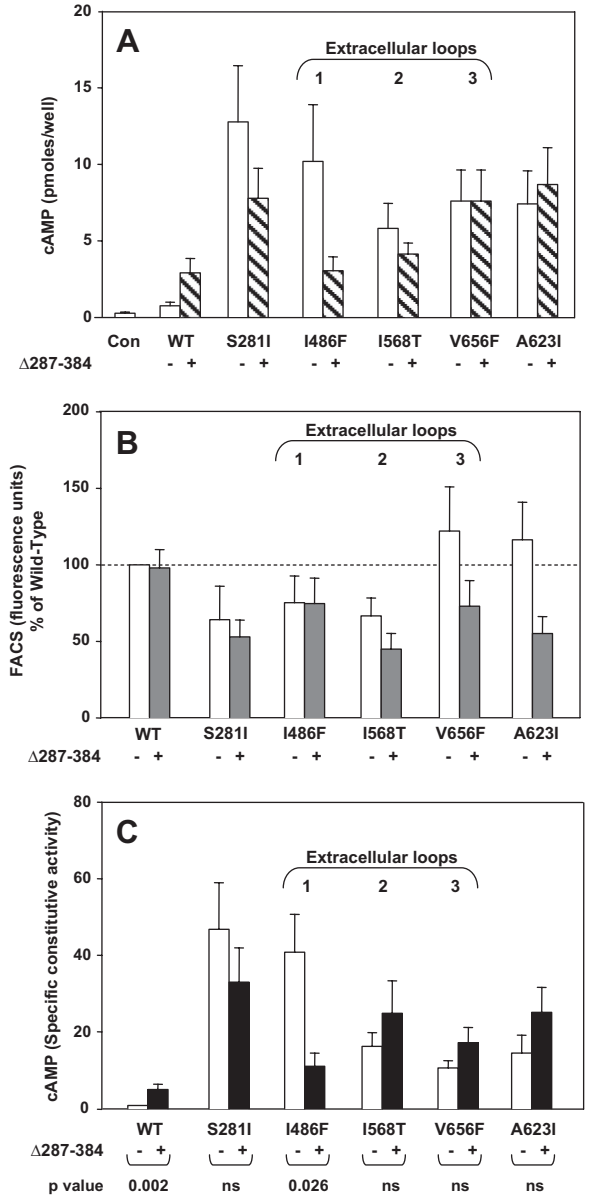

Fig. 7. Functional Effect of Deletion of TSHR Hinge Region Residues 287–384 on TSHR-Activating Point Mutations

A, Uncorrected cAMP levels. Intracellular cAMP levels were determined in transiently transfected COS-7 cells expressing the wild-type TSHR (WT) and receptors with the indicated point mutations, with or without deletion of hinge regions residues 287–384 (TSHRΔ287–384). Con represents cells transfected with empty vector. *Bars* represent the mean + SE of cAMP values from six experiments, each involving triplicate wells of cells. B, Flow cytometric quantitation of TSHR expression using aliquots of the same cells shown in A. Detection was with murine mAb CS-17 whose epitope lies upstream of TSHR residue 287. *Bars* represent the mean + SE of values from three separate transfections. C, SCA calculated from the data shown in A and B (see *Materials and Methods*). SCA of the wild-type (WT) TSHR is normalized to 1, and values for the other receptors are expressed relative to the wild type. *Bars* represent the mean + SE of values from six experiments, each involving triplicate wells of cells. Statistical analyses were performed using the Mann-Whitney rank sum test.

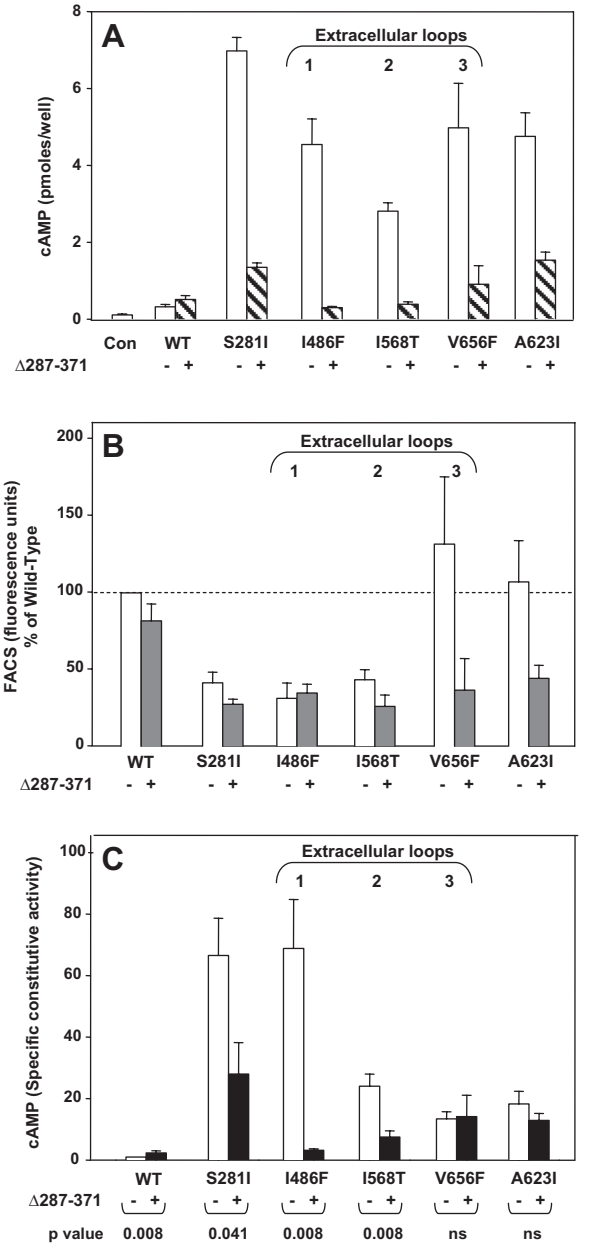

Fig. 8. Functional Effect of Deletion of TSHR Hinge Region Residues 287–371 on TSHR-Activating Point Mutations

A, Uncorrected cAMP levels. Intracellular cAMP levels were determined in transiently transfected COS-7 cells expressing the wild-type TSHR (WT) and receptors with the indicated point mutations, with or without deletion of hinge regions residues 287–371 (TSHRΔ287–371). Con represents cells transfected with empty vector. *Bars* represent the mean ± SE of cAMP values from four or five experiments, each involving triplicate wells of cells. B, Flow cytometric quantitation of TSHR expression using aliquots of the same cells shown in A, as described for Fig. 7B. *Bars* represent the mean + SE of values from three separate transfections. C, SCA calculated from the data shown in A and B (see *Materials and Methods*). SCA of the wild-type (WT) TSHR is normalized to 1, and values for the other receptors are expressed relative to the wild type. *Bars* represent the mean + SE of values from four or five experiments, each involving triplicate wells of cells. Statistical analyses were performed using the Mann-Whitney rank sum test.

As reported previously (25), TSH stimulation of the wild-type TSHR with the I486F mutation did not greatly increase the already elevated cAMP level (Fig. 9). However, the combination of the Δ287–371 deletion in the TSHR hinge region together with I486F had reciprocal inhibitory effects. Both the high I486F constitutive activity and the TSHRΔ287–371 cAMP response to TSH stimulation were nearly abrogated (Fig. 9) despite receptor expression on the cell surface (see Fig. 8B). These data indicate a dominant effect of the I486F mutation on TSH activation of the TSHR. Not surprisingly because TSHRΔ287–384 with the longer hinge region deletion does not respond to TSH stimulation (Fig. 2), this receptor remained unresponsive to TSH when combined with I486F (Fig. 9).

DISCUSSION

The ectodomains of glycoprotein hormone receptors have ligand binding LRDs of similar size and structure. In contrast, the hinge region components of the ectodomains in this GPCR subfamily vary greatly in size, and their three-dimensional structure is poorly understood. The TSHR has the largest hinge region, approximately 141 amino acids in length *vs.* approximately 90 residues in the gonadotropin receptors. Remarkably, in some species (marmoset and salmon), the LHR and FSHR hinge regions are even smaller. On this basis, Moyle *et al.* (10) generated a functional analog of the rat FSHR with a hinge of only 63 amino acid residues. Because of apparent redundancy in the glycoprotein hormone receptor hinge region, generating receptors with smaller hinges that still traffic normally to the cell surface may provide insight into the controversial functional role of this region. Previous studies have revealed that the human TSHR tolerates a number of deletions within the hinge region, namely residues 287–305 (Tanaka, K., C.-R. Chen, S. McLachlan, and B. Rapoport, unpublished data), 305–320 (17), and 317–366 (12).

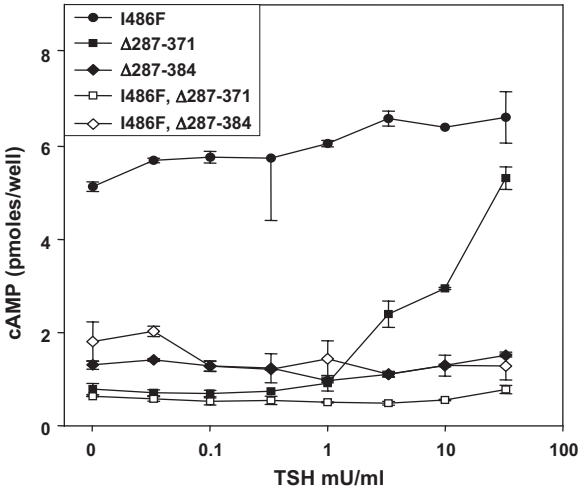

Fig. 9. TSH Stimulation of TSHR Hinge Deletion Mutations Combined with the I486F Mutation

COS-7 cells transiently transfected with plasmids expressing the indicated TSHR were stimulated for 1.5 h with the indicated concentrations of TSH. Data represent the mean ± SD of intracellular cAMP levels in duplicate wells of cells (see *Materials and Methods*).

In the present study, we tested the limit to which TSHR hinge deletions are compatible with cell surface expression and function. Having found that a receptor with the sum of the above mentioned deletions (TSHRΔ287–366) retained responsivity to TSH stimulation of cAMP generation in intact cells, we progressively extended the TSHR hinge region deletions further downstream to residue 384 (Fig. 1). Residues known to be critical for TSHR expression or function (C283, C284, and Y385) (19, 20, 26) precluded further upstream or downstream extension of the deletions. Nevertheless, despite deleting as much as 70% of the hinge region (only 43 of 141 residues remaining), TSHR expression and trafficking to the cell surface was similar to that of the wild-type TSHR. Mutating C390 to balance deletion of C301 had no consistent effect on TSHR expression on the cell surface, supporting the concept that these two cysteines form a disulfide bond that, unlike others in the extracellular domain, are not critical for receptor folding and function (reviewed in 2).

Investigation of TSHR with extensive hinge regions deletions led to a number of novel observations with challenging implications. First, despite cell surface expression similar to that of the wild-type TSHR, progressive deletions within the TSHR hinge (residues 287–366 through 287–384) progressively reduced sensitivity to TSH-stimulated cAMP generation with a corresponding reduction in TSH binding. These findings require reconciliation with the prevailing concept (based on the crystal structure of an FSH-FSHR LRD complex) that the glycoprotein hormone binding sites on their cognate receptors lie entirely within the LRDs of the latter (6). One possibility is that mutations within the TSHR hinge region lead to allosteric changes within the LRD that distort the TSH binding site. Another possibility is that not all glycoprotein hormone binding sites (FSH but not TSH) are contained entirely within the receptor LRD. Evidence supporting the latter alternative is that a chimeric receptor (TSH-LHR-9) containing the TSHR hinge region combined with the LHR LRD binds TSH with high affinity, similar to that with the wild-type TSHR (27). Even stronger evidence is that the purified TSHR LRD (amino acid residues 1–289) does not bind TSH with an affinity sufficient for detection (7). This receptor component (equivalent to the A-subunit) generated in eukaryotic cells is conformationally intact as evident by its recognition by thyroid-stimulating autoantibodies (28, 29). Crystallization of TSHR 1–260 with a monoclonal thyroid-stimulating autoantibody with TSHR residues 1–260 confirms this

interaction (8); however, whether TSHR 1–260 also binds TSH is unknown.

It should be emphasized that lack of TSH binding to the isolated TSHR LRD does not imply noninteraction between these two molecules. Extensive and detailed mutagenesis studies identify individual amino acid contact points on the TSHR (30, 31). However, the number of residues identified is considerably smaller than the FSH footprint on the FSHR (25 amino acid residues) (6). Therefore, ligand contact points in the TSHR hinge region cannot be excluded. Even if the TSHR LRD contains the major portion of the TSH footprint, reduction in the latter by deletion of only a few residues within the hinge region could greatly reduce TSH binding affinity. Another intriguing and provocative suggestion is that, at least for the FSHR, the isolated LRD exposes a cryptic ligand binding site that is obscured when the hinge is also present (11). That is, the physiological ligand binding site is different from that determined with the free LRD.

The second novel finding in the present study is the unanticipated increase in ligand-independent constitutive activity with extensive deletions within the TSHR hinge region. The role of the TSHR ectodomain in high TSHR constitutive activity has received considerable attention. Earlier studies tested, and refuted, the hypothesis that intramolecular cleavage of the TSHR hinge region with deletion of residues 317–366 exposes the N terminus of the B-subunit as a tethered ligand with close homology to that on the thrombin receptor (32). Indeed, the TSHR ectodomain was subsequently found to have the opposite effect, namely functioning as an inverse agonist that suppresses constitutive activity (23, 33). Evidence suggested that the N-terminal two thirds of the ectodomain (primarily the LRD, equivalent to the A-subunit) was the inverse agonist. Thus, progressively longer deletions beginning at the N terminus of the TSHR led to a jump in constitutive activity when the deletion extended downstream to residue 286 (23). Further downstream deletions to residue 415, the entire ectodomain, did not lead to a further increase in TSHR constitutive activity. With this background, in the present study, we did not anticipate that TSHR hinge region deletions would modify constitutive activity. In all the deletions that we performed, TSHR residues 1–286 remain intact. It is of interest that CS-17, a mAb with inverse agonist activity for the TSHR (24), retains this property with the minimal hinge TSHR (Δ287–384) with high constitutive activity. A portion of the CS-17 epitope has been localized to lie within TSHR amino acid residues 260–289 (24), at the N terminus of the hinge region (8). This observation supports the concept that the TSHR hinge region plays a role in ectodomain inverse agonist activity.

What is the mechanism by which deletion of opposite regions of the TSHR ectodomain, N-terminal LRD and C-terminal hinge, both release suppression of constitutive activity? As for any mutational study, allosteric effects cannot be excluded. Deletion of the LRD may alter the conformation of the hinge region and vice versa. However, based on past and present data, we propose that the hinge region, rather than the TSHR N terminus (residues 1–286), is the immediate inverse agonist that impacts the serpentine region of the receptor, thereby restraining constitutive activity. As shown in the present study, deletion of a critical component in the hinge region removes this silencing effect with a consequent increase in constitutive activity. We suggest that deletion of the TSHR N terminus secondarily alters the conformation of the hinge region or its position relative to the serpentine region with a similar alteration in receptor constitutive activity. Support for this notion is that by virtue of its primary sequence, the hinge region is likely to be closely related to the serpentine TMD, as suggested by a numbers of models or schematic concepts that include all components of the glycoprotein hormone receptors (6, 9, 10, 34). Analysis of functional data on ligand activation of the glycoprotein hormone receptors also suggests that the hinge region has a direct role in receptor activation and does not serve as an uninvolved scaffold for the LRD (reviewed in Ref. 9).

The present study also provides evidence for particular components in the TSHR hinge and serpentine regions that are involved in ectodomain suppression of constitutive activity. Increased constitutive activity (release of suppression) occurred only when the hinge region deletion extended to residue 371 and, especially, to residue 384. No enhancement in constitutive activity occurred when much of the TSHR hinge region (residues 287–366) was deleted. We, therefore, suggest that the TSHR tethered inverse agonist that directly suppresses constitutive activity lies in the vicinity of residues 371–384. In terms of which components in the TSHR serpentine region are impacted by the hinge region, a number of spontaneously occurring single amino acid point mutations in the ECLs of the TSHR increase constitutive activity and can cause clinical disease (25, 35, 36). These constitutively activating mutations (CAMs) are nonfunctional when introduced into a TSHR whose entire ectodomain has been deleted, suggesting direct contact between the ectodomain and the ECLs is necessary for increased receptor activity (23). The present data confirm this phenomenon and indicate that the hinge region component of the ectodomain is necessary for CAM function (Fig. 10). However, this requirement appears to be limited to the CAM at the N terminus of the hinge region (S281I) as well as to the CAMs in the first and second ECLs (I486F and I568T, respectively). Of these CAMs, I486F is most dependent on the TSHR hinge region. Moreover, abrogation of CAM function is most readily seen with the smaller hinge deletion (TSHRΔ287–371) than with the more extended deletion (TSHRΔ287–384). Indeed, with the latter, S281I and I568T activities are little affected. A possible explanation for the apparent paradox that the larger hinge region deletion has a lesser effect on CAM activity is that two separate mechanisms are operative

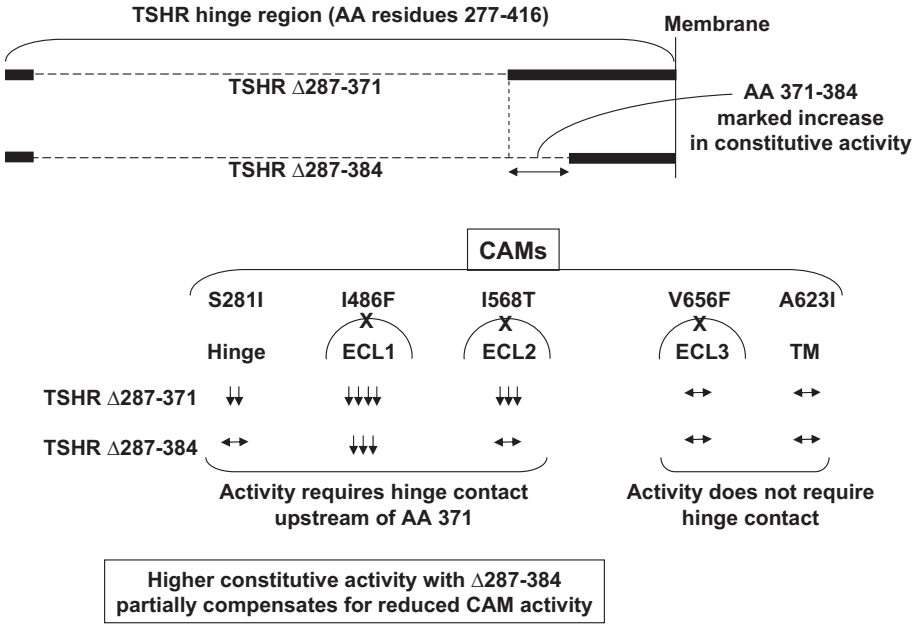

Fig. 10. Schematic Depiction of Data Obtained by Combining Hinge Region Deletions with CAMs
Hypotheses that may explain these data are included in the text.

(Fig. 10). First is increased constitutive activity produced by deletion of residues in the vicinity of 371–384, near the C terminus of the hinge region. The second effect involves contact between S281I, I486F, and I568T with the hinge region, particularly upstream of TSHR residue 371. Our observations may reflect the algebraic sum of these two effects, namely that the higher constitutive activity with the longer deletion (TSHRΔ287–384) partially compensates for reduced CAM activity. Moreover, point mutations at TSHR hinge residue 281 are especially potent activators of the TSHR, attaining levels equivalent to maximal TSH stimulation (37, 38). It is possible that the remaining portion of the TSHR hinge (residues 385–418) or the TSHR N terminus (in a primary or secondary manner) are necessary for functionality of the activating mutations in ECL3. Indeed, mutations at residues 403, 404, and 406 have been found to increase TSHR constitutive activity (39, 40).

In summary, the present study provides novel mechanistic information regarding TSHR structure and function, as follows. 1) The data establish limits for a minimal TSHR hinge region (greatly reduced from 141 to 43 amino acid residues). This information will be useful for future studies by other investigators. 2) High-affinity TSH binding requires contact with the TSHR hinge region. These findings challenge the prevalent concept based on crystal structure and mutagenesis data that ligand does not bind to the hinge region of the glycoprotein hormone receptors. 3) TSHR tethered inverse agonist activity involves the hinge region. These data contrast with previous evidence that the LRD, not the hinge, is the major inverse agonist. 4) An important component of the TSHR hinge region contributing to inverse agonist activity appears to be in the vicinity of residues 371–384. 5) These same residues (371–384) are also important for TSH binding to the TSHR. 6) The TSHR hinge region is necessary for some ECL mutations leading to increased constitutive activity. Previous data did not distinguish between the contributions of the hinge region and the LRD. 7) Only the constitutive activating mutations in ECL1 and ECL2, but not ECL3, depend on the TSHR hinge region. Previous studies involving deletion of the entire ectodomain implicated all three ECLs. 8) Of the ECL CAMs, I486F in ECL1 is the most dependent on hinge contact.

Taken together, our study, supported by recent mutagenesis data (41), provides strong evidence that the TSHR hinge is not simply a linker with "little or no role in ligand binding and signaling other than to prevent the LRD from dissociating from the TSHR" (9), supporting the suggestion that a preferable term for hinge is a SSD (10).

MATERIALS AND METHODS

Construction and Expression of TSHR Mutants

The human TSHR cDNA (42) with three introduced restriction sites (27) and the H601 polymorphism converted to Y601 was introduced into the vector pcDNA5/FRT (Invitrogen, Carlsbad, CA). For this purpose, the cDNA was excised with *Eco*RI (blunted with Klenow DNA polymerase) and *Xba*I. The vector was cut with *Nhe*I (blunted) and *Xba*I. The *Xba*I site in the vector cloning site was made available by removal of a second, downstream *Xba*I site by mutation without codon alteration. Deletions in the TSHR of amino acid residues 287–366

(TSHRΔ287–366), 287–371 (TSHRΔ287–371), 287–376 (TSHRΔ287–376), and 287–384 (TSHRΔ287–384) were performed by overlap PCR using Pfu Ultra (Stratagene, La Jolla, CA). The cDNA was restricted with *Afl*II (codon 260) and *Xba*I (after 3′-end stop codon) and substituted for the same fragment in pcDNA5/FRT-TSHR. Mutations of C390H were introduced into TSHRΔ287–371, TSHRΔ287–376, and TSHRΔ287–384 using the QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA). Gain-of-function TSHR mutants S281I (37) I486F and I568T (25), A623I (35), and V656F (36) were introduced into the wild-type TSHR as well as into TSHRΔ287–384 and TSHRΔ287–371 using the same mutagenesis kit. All mutations were confirmed by nucleotide sequencing.

Although the pcDNA5/FRT is designed for stable transfections, we performed transient transfections because of difficulty in generating CHO cell lines stably expressing the TSHR. Plasmids were transiently expressed in COS-7 cells using FuGENE HD (Roche, Indianapolis, IN). Cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamycin (50 μg/ml), and fungizone (2.5 μg/ml) and were tested approximately 48 h after transfection.

Cultured Cell cAMP Assays

COS-7 cells expressing the wild-type TSHR and TSHR mutants were transferred into 96-well plates approximately 24 h after transfection and 24 h before assay. Cells from the same transfection were also plated in 6-cm culture dishes to monitor the transfection efficiency by flow cytometry (see below). For bioassay, the culture medium described above was replaced with DMEM supplemented with 1 mM isobutyl methylxanthine, 10 mM HEPES, and, where indicated in the text, bovine TSH (Sigma-Aldrich, St. Louis, MO). Mock transfected COS-7 cells were included as controls. After 60 min at 37 C, the medium was aspirated and intracellular cAMP was extracted with 0.2 ml 95% ethanol. The extracts were evaporated to dryness and resuspended in 0.1 ml Dulbecco's PBS (pH 7.5), and samples (12 μl) were assayed using the LANCE cAMP kit according to the protocol of the manufacturer (Perkin-Elmer, Shelton, CT). SCA (23) was calculated as (cAMP in TSHR transfected cells − cAMP in control vector transfected cells)/(flow cytometric geometric mean fluorescence in TSHR transfected cells − fluorescence in control vector transfected cells). SCA values were adjusted in proportion to that of the wild-type TSHR, which was normalized to a value of 1. Statistical analyses of SCA values between TSHR were performed using the Mann-Whitney rank sum test for nonnormal values (SigmaPlot9; Systat, San Jose, CA).

Flow Cytometry

Transiently transfected COS-7 cells were harvested from six-well plates using 1 mM EDTA, 1 mM EGTA in PBS. After washing twice with PBS containing 10 mM HEPES (pH 7.4), 2% fetal bovine serum, and 0.05% NaN_3, the cells were incubated for 30 min at room temperature in 100 μl of the same buffer containing 1 μg of either normal mouse IgG or mAb CS-17 (epitope upstream of TSHR residue 289) (24). After rinsing, the cells were incubated for 45 min with 100 μl fluorescein isothiocyanate-conjugated goat antimouse IgG (1:100) (Caltag, Burlingame, CA), washed, and analyzed using a Beckman FACScan flow cytofluorimeter. Cells stained with propidium iodide (1 μg/ml final concentration) were excluded from analysis.

TSH Binding to Transfected Cells

COS-7 cells transiently transfected with plasmids expressing the wild-type TSHR or TSHR mutants were grown to confluence in 24-well plates. Medium was aspirated and replaced with 250 μl binding buffer (Hanks' buffer with 250 mM sucrose substituting for NaCl to maintain isotonicity and 0.25% BSA) containing about 12,000 cpm [^{125}I]TSH (BRAHMS, Berlin Germany). After incubation for 1–2 h at room temperature, cells were rapidly rinsed three times with binding buffer (4 C) and solubilized with 0.5 ml 1 N NaOH, and radioactivity was then measured in a γ-counter. Nonspecific binding was determined using COS-7 cells transfected in parallel with the vector alone.

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REFERENCES

1. Vassart G, Dumont JE 1992 The thyrotropin receptor and the regulation of thyrocyte function and growth. Endocr Rev 13:596–611
2. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM 1998 The thyrotropin receptor: interaction with thyrotropin and autoantibodies. Endocr Rev 19: 673–716
3. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M 2000 Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289:739–745
4. Govaerts C, Lefort A, Costagliola S, Wodak SJ, Ballesteros JA, Van Sande J, Pardo L, Vassart G 2001 A conserved Asn in transmembrane helix 7 is an on/off switch in the activation of the thyrotropin receptor. J Biol Chem 276:22991–22999
5. Rapoport B, McLachlan SM, Kakinuma A, Chazenbalk GD 1996 Critical relationship between autoantibody recognition and TSH receptor maturation as reflected in the acquisition of mature carbohydrate. J Clin Endocrinol Metab 81:2525–2533
6. Fan QR, Hendrickson WA 2005 Structure of human follicle-stimulating hormone in complex with its receptor. Nature 433:269–277
7. Chazenbalk GD, Jaume JC, McLachlan SM, Rapoport B 1997 Engineering the human thyrotropin receptor ectodomain from a non-secreted form to a secreted, highly immunoreactive glycoprotein that neutralizes autoantibodies in Graves' patients' sera. J Biol Chem 272:18959–18965
8. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, Bolton J, Reeve M, Nakatake N, Evans M, Richards T, Powell M, Miguel RN, Blundell TL,

Furmaniak J, Smith BR 2007 Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. Thyroid 17:395–410
9. Moyle WR, Lin W, Myers RV, Cao D, Kerrigan JE, Bernard MP 2005 Models of glycoprotein hormone receptor interaction. Endocrine 26:189–205
10. Moyle WR, Xing Y, Lin W, Cao D, Myers RV, Kerrigan JE, Bernard MP 2004 Model of glycoprotein hormone receptor ligand binding and signaling. J Biol Chem 279:44442–44459
11. Lin W, Bernard MP, Cao D, Myers RV, Kerrigan JE, Moyle WR 2007 Follitropin receptors contain cryptic ligand binding sites. Mol Cell Endocrinol 260–262: 83–92
12. Wadsworth HL, Chazenbalk GD, Nagayama Y, Russo D, Rapoport B 1990 An insertion in the human thyrotropin receptor critical for high affinity hormone binding. Science 249:1423–1425
13. Chazenbalk GD, Tanaka K, Nagayama Y, Kakinuma A, Jaume JC, McLachlan SM, Rapoport B 1997 Evidence that the thyrotropin receptor ectodomain contains not one, but two, cleavage sites. Endocrinology 138: 2893–2899
14. de Bernard S, Misrahi M, Huet JC, Beau I, Desroches A, Loosfelt H, Pichon C, Pernollet JC, Milgrom E 1999 Sequential cleavage and excision of a segment of the thyrotropin receptor ectodomain. J Biol Chem 274: 101–107
15. Zhang FP, Rannikko AS, Manna PR, Fraser HM, Huhtaniemi IT 1997 Cloning and functional expression of the luteinizing hormone receptor complementary deoxyribonucleic acid from the marmoset monkey testis: absence of sequences encoding exon 10 in other species. Endocrinology 138:2481–2490
16. Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y 1999 The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem Biophys Res Commun 265:366–371
17. Tanaka K, Chazenbalk GD, McLachlan SM, Rapoport B 2000 Evidence that cleavage of the thyrotropin receptor involves a "molecular ruler" mechanism: deletion of amino acid residues 305–320 causes a spatial shift in cleavage site 1 independent of amino acid motif. Endocrinology 141:3573–3577
18. Chen CR, Chazenbalk GD, McLachlan SM, Rapoport B 2003 Evidence that the C terminus of the A subunit suppresses thyrotropin receptor constitutive activity. Endocrinology 144:3821–3827
19. Kosugi S, Ban T, Akamizu T, Kohn LD 1991 Site-directed mutagenesis of a portion of the extracellular domain of the rat thyrotropin receptor important in autoimmune thyroid disease and nonhomologous with gonadotropin receptors. Relationship of functional and immunogenic domains. J Biol Chem 266: 19413–19418
20. Costagliola S, Panneels V, Bonomi M, Koch J, Many MC, Smits G, Vassart G 2002 Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors. EMBO J 21:504–513
21. Kosugi S, Ban T, Akamizu T, Kohn LD 1991 Further characterization of a high affinity thyrotropin binding site on the rat thyrotropin receptor which is an epitope for blocking antibodies from idiopathic myxedema patients but not thyroid stimulating antibodies from Graves' patients. Biochem Biophys Res Commun 180: 1118–1124
22. Ho SC, Goh SS, Su Q, Khoo DH 2005 Cysteine 390 mutation of the TSH receptor modulates its ectodomain as an inverse agonist on the serpentine domain with decrease in basal constitutive activity. Mol Cell Endocrinol 245:158–168
23. Vlaeminck-Guillem V, Ho SC, Rodien P, Vassart G, Costagliola S 2002 Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. Mol Endocrinol 16:736–746
24. Chen CR, McLachlan SM, Rapoport B 2007 Suppression of thyrotropin receptor constitutive activity by a monoclonal antibody with inverse agonist activity. Endocrinology 148:2375–2382
25. Parma J, Van Sande J, Swillens S, Tonacchera M, Dumont J, Vassart G 1995 Somatic mutations causing constitutive activity of the thyrotropin receptor are the major cause of hyperfunctioning thyroid adenomas: identification of additional mutations activating both the cyclic adenosine 3′,5′-monophosphate and inositol phosphate-Ca^{2+} cascades. Mol Endocrinol 9:725–733
26. Kosugi S, Ban T, Akamizu T, Kohn LD 1992 Role of cysteine residues in the extracellular domain and exoplasmic loops of the transmembrane domain of the TSH receptor: effect of mutation to serine on TSH receptor activity and response to thyroid stimulating autoantibodies. Biochem Biophys Res Commun 189: 1754–1762
27. Nagayama Y, Wadsworth HL, Chazenbalk GD, Russo D, Seto P, Rapoport B 1991 Thyrotropin-luteinizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for TSH receptor function. Proc Natl Acad Sci USA 88:902–905
28. Chazenbalk GD, Wang Y, Guo J, Hutchison JS, Segal D, Jaume JC, McLachlan SM, Rapoport B 1999 A mouse monoclonal antibody to a thyrotropin receptor ectodomain variant provides insight into the exquisite antigenic conformational requirement, epitopes and in vivo concentration of human autoantibodies. J Clin Endocrinol Metab 84:702–710
29. Chazenbalk G, McLachlan S, Pichurin P, Rapoport B 2001 A "prion-like" shift between two conformational forms of a recombinant thyrotropin receptor A subunit module: purification and stabilization using chemical chaperones of the form reactive with Graves' autoantibodies. J Clin Endocrinol Metab 86:1287–1293
30. Smits G, Campillo M, Govaerts C, Janssens V, Richter C, Vassart G, Pardo L, Costagliola S 2003 Glycoprotein hormone receptors: determinants in leucine-rich repeats responsible for ligand specificity. EMBO J 22: 2692–2703
31. Sanders J, Bolton J, Sanders P, Jeffreys J, Nakatake N, Richards T, Evans M, Kiddie A, Summerhayes S, Roberts E, Miguel RN, Furmaniak J, Smith BR 2006 Effects of TSH receptor mutations on binding and biological activity of monoclonal antibodies and TSH. Thyroid 16:1195–1206
32. Chazenbalk GD, Tanaka K, McLachlan SM, Rapoport B 1999 On the functional importance of thyrotropin receptor intramolecular cleavage. Endocrinology 140: 4516–4520
33. Zhang M, Phuong K, Tong T, Fremont V, Chen J, Narayan P, Puett D, Weintraub BD, Szkudlinski MW 2000 The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. Endocrinology 141: 3514–3517
34. Nunez MR, Sanders J, Jeffreys J, Depraetere H, Evans M, Richards T, Blundell TL, Rees SB, Furmaniak J 2004 Analysis of the thyrotropin receptor-thyrotropin interaction by comparative modeling. Thyroid 14: 991–1011
35. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G 1993 Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. Nature 365:649–651

36. Fuhrer D, Holzapfel HP, Wonerow P, Scherbaum WA, Paschke R 1997 Somatic mutations in the thyrotropin receptor gene and not in the Gsα protein gene in 31 toxic thyroid nodules. J Clin Endocrinol Metab 82: 3885–3891
37. Kopp P, Muirhead S, Jourdain N, Gu WX, Jameson JL, Rodd C 1997 Congenital hyperthyroidism caused by a solitary toxic adenoma harboring a novel somatic mutation (serine281→isoleucine) in the extracellular domain of the thyrotropin receptor. J Clin Invest 100:1634–1639
38. Duprez L, Parma J, Costagliola S, Hermans J, Van Sande J, Dumont J, Vassart G 1997 Constitutive activation of the TSH receptor by spontaneous mutations affecting the N-terminal extracellular domain. FEBS Lett 409:469–474
39. Kleinau G, Jaschke H, Neumann S, Lattig J, Paschke R, Krause G 2004 Identification of a novel epitope in the thyroid-stimulating hormone receptor ectodomain acting as intramolecular signaling interface. J Biol Chem 279:51590–51600
40. Mueller S, Kleinau G, Jaeschke H, Neumann S, Krause G, Paschke R 2006 Significance of ectodomain cysteine boxes 2 and 3 for the activation mechanism of the thyroid-stimulating hormone receptor. J Biol Chem 281:31638–31646
41. Mueller S, Kleinau G, Jaeschke H, Krause G, Paschke R 2007 The hinge region of the TSHR—importance for TSH binding and receptor activation. Horm Res 68(Suppl 3):9 (Abstract)
42. Nagayama Y, Kaufman KD, Seto P, Rapoport B 1989 Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. Biochem Biophys Res Commun 165:1184–1190

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