An N-Linked Glycosylation Motif from the Noncleaving Luteinizing Hormone Receptor Substituted for the Homologous Region (Gly367 to Glu369) of the Thyrotropin Receptor Prevents Cleavage at Its Second, Downstream Site

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The thyrotropin receptor (TSHR) exists in two forms (single polypeptide and two subunits), whereas the lutropin/chorionic gonadotropin receptor (LH/CGR) is a single chain. Recent data suggest that the TSHR cleaves at two sites. We mutagenized selected chimeric TSH-LH/CGR to localize the cleavage sites in the TSHR. All 23 receptors mutated in the estimated vicinity of the upstream site cleaved into two subunits as determined by 125I-TSH cross-linking to intact cells. In contrast, in a series of mutations homologous to the noncleaving LH/CGR, the downstream TSHR cleavage site localized to three amino acids (GQE367–369). Remarkably, group substitution of these residues, but not substitution of individual residues, abolished cleavage. Moreover, the mutation that prevented cleavage (GQE367–369NET) transposed a motif (NET181–183) that is glycosylated in the LH/CGR. TSHR cleavage or noncleavage after substitution of GQE367–369 with other triplets (AAA, NQE, and NQT) was consistent with a role for N-linked glycosylation at this site.

In summary, our data (i) support the concept that the TSHR cleaves at two sites, (ii) relate TSHR residues GQE367–369 to cleavage at the second, downstream site, and (iii) suggest that cleavage or noncleavage at site two is related to N-linked glycosylation. These findings provide new insight into the evolutionary divergence of two closely related receptors.

The thyrotropin receptor (TSHR) and lutropin/chorionic gonadotropin (LH/CGR) receptor are closely related members of the G protein-coupled receptor family with glycoprotein hormone ligands. Both the TSHR and LH/CGR receptor have large, heavily glycosylated ectodomains with leucine-rich repeats and are encoded by numerous exons (1–3). Despite their common evolutionary background, these receptors have several remarkable differences aside from the specificity of their ligands. For example, disease-causing autoantibodies to the TSHR are common, whereas autoantibodies to the LH/CGR receptor are extremely rare. Thus, engagement of the TSHR by autoantibodies is the direct cause of thyrotoxicosis in Graves’ disease, an organ-specific autoimmune disorder affecting only humans. Another striking difference exists at the structural level. As detected by TSH cross-linking to the surface of intact cells, the functional TSHR exists in two forms; a single chain receptor and a receptor with two subunits (4, 5). In contrast, the LH/CGR receptor exists only in a single chain form (reviewed in Segaloff and Ascoli (6)). Variable proportions of single chain and two-subunit forms of the TSHR are also observed on immunoprecipitation or immunoblotting of mammalian cell extracts (7–11). The two-subunit TSHR involves a ligand-binding, glycosylated A subunit and a membrane-associated B subunit linked by disulfide bonds (12). Because the TSHR is encoded by a single mRNA species (13–16), the A and B subunits must be formed by intramolecular cleavage, a process believed to involve a matrix metalloprotease (17).

Recent evidence raised the surprising possibility that cleavage of the TSHR into A and B subunits does not occur at a single site as previously suspected, but at two sites (18), a feature unique to currently known members of the family of G protein-coupled receptors. In the present study, we sought to identify more precisely the location of each putative cleavage site using a strategy involving mutagenesis of selected chimeric TSH-LH/CGR receptors.

MATERIALS AND METHODS

TSH Receptor Expression—To increase the level of receptor expression, we deleted the 5′- and 3′-untranslated regions of chimeric receptor TSH-LHR-5 (19) by transposing its A/III-SpeI fragment (domains D and E) (see Fig. 1) with the corresponding fragment in TSHR-5′3′TR-NEOECE (20). Mutations in domain D of chimeric receptor TSH-LHR-5 were generated by PCR using overlapping primers and Pfu DNA polymerase (Stratagene, San Diego, CA). The nucleotide sequences of the PCR fragments were confirmed by the dideoxyribonucleotide termination method (21). Plasmids were stably transfected with Lipofectin (Life Technologies, Inc.) into Chinese hamster ovary (CHO) cells cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum and standard antibiotics. Selection was with 400 μg/ml G418 (Life Technologies, Inc.). Surviving clones (>100 per 100-mm diameter culture dish) were pooled and propagated for further study.

For our initial series of mutations in domain E of chimeric receptor TSH-LHR-4, we used the E1, E2, and E3 mutations (see Fig. 3A) previously constructed in the wild-type TSHR (22). These mutations were transposed into the cDNA for TSH-LHR-4 (Ecorv-XhoI fragment). Subsequent finer mutations in the E domain of TSH-LHR-4 were made by PCR using overlapping primers, with replacement of the A/III-SpeI fragment. The nucleotide sequences of all PCR products were

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1 The abbreviations used are: TSHR, thyrotropin receptor; TSH, thyrotropin, LH, lutropin; CG, chorionic gonadotropin; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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confirmed and stably transfected CHO cell lines were generated as described for the domain D mutations.

**Covalent Cross-linking of Radiolabeled TSH**—Highly purified bovine TSH (5 μg, 30 units/mg of protein) was radiolabeled with 125I to a specific activity of ~80 μCi/μg of protein using the Bolton-Hunter reagent (4400 Ci/mmol; DuPont NEN) according to the protocol of the manufacturer. Confluent 100-mm diameter dishes of TSHR-expressing cells were incubated for 2 h at 37°C with 5 μCi of 125I-TSH in 5 ml of modified Hanks' buffer (without NaCl), supplemented with 280 mM sucrose and 0.25% bovine serum albumin (binding buffer). Unbound 125I-TSH was removed by rinsing the cells three times with ice-cold binding buffer. Disuccinimidyl suberate (1 mM; Sigma) in 10 mM sodium phosphate buffer, pH 7.4, containing the protease inhibitors phenylmethylsulfonyl fluoride (100 μM), leupeptin (1 μg/ml), aprotinin (1 μg/ml), and pepstatin A (2 μg/ml) (all from Sigma) was then added for 20 min at room temperature. The cross-linking reaction was terminated by the addition of 20 mM ammonium acetate (final concentration).

After cross-linking, the cells were rinsed twice with phosphate-buffered saline and scraped into 10 mM Tris, pH 7.5, containing the same protease inhibitors. Cells were homogenized using a Polytron homogenizer and centrifuged for 5 min at 4°C (500 × g). The supernatant was centrifuged (15 min, 10,000 × g, 4°C), and the pellet was resuspended in 50 μl of 10 mM Tris, pH 7.5. After the addition of Laemmli buffer containing 0.7 μl β-mercaptoethanol (30 min at 42°C), the samples were subjected to 7.5 or 10% SDS-PAGE and autoradiography using Kodak XAR-5 x-ray film (Eastman Kodak Co.).

**RESULTS AND DISCUSSION**

The basis for the present study was our previous observation that cleavage into A and B subunits did not occur in a chimeric receptor (TSH-LHR-6) (Fig. 1) in which the carboxyl-terminal portion of the TSHR ectodomain (amino acid residues 261–418; arbitrary domains “D” and “E”) was replaced with the corresponding region of the LH/CG receptor (19, 23). On the other hand, cleavage still occurred when either domain D or domain E (residues 261–362 and 363–418; chimeric receptors TSH-LHR-4 and TSH-LHR-5, respectively) were substituted on an arbitrary domain (18), suggesting that cleavage sites 1 and 2 were in TSHR domains D and E, respectively.

Because elimination by mutagenesis of only one cleavage site would not be sufficient to abrogate receptor cleavage, we mutated regions in the vicinity of putative site 1 on a background of TSH-LHR-5. Conversely, we mutated regions in the vicinity of putative site 2 on a background of TSH-LHR-4. By this means, we were able to determine the structural significance of each mutation on receptor cleavage into subunits, as determined by 125I-TSH cross-linking to monolayers of intact, stably transfected cells. As a general principle for mutagenesis, we replaced TSHR residues with the homologous residues of the noncleaving LH/CG receptor. Residues common to both receptors were unaltered. In the case of a 50-amino acid region that is unique to the TSHR and lacking in the LH/CG receptor, substitutions could not be performed, so we introduced alanine residues in most instances. It is noteworthy that this 50-amino acid segment (estimated to be between residues 317 and 366) is very hydrophilic and can be deleted from the wild-type TSHR without loss of ligand binding and function (24) and without preventing cleavage into A and B subunits (5).

The deglycosylated TSHR A subunit is ~35 kDa in size (18, 25), which would place cleavage site 1 at approximately amino acid residue 335 (numbering includes a 21-residue signal peptide). On this basis, we initially targeted residues 330–338 for alanine scanning mutagenesis (Fig. 2A). None of these individual substitutions prevented cleavage of chimeric receptor TSH-LHR-5, as determined by release of the TSH-linked A subunit on disulfide bond reduction (Fig. 2B). Note that, consistent with previous TSH cross-linking studies to intact cells (4, 5, 18, 26), only a portion of the TSHR on the cell surface cleave into two subunits, with TSH also binding to single polypeptide chain TSHR. Because of the possibility that a single amino acid residue may be insufficient to disrupt a cleavage site, we next...
performed multiple substitutions over a wider area between residues 324 and 352, also without abolishing cleavage. Finally, we mutagenized TSH-LHR-5 even further afield (residues 317–323), well upstream of the calculated cleavage site and up to the carboxyl terminus of the D domain (residues 353–362), also without effect (Fig. 2).

Mutagenesis of TSH-LHR-4 in the vicinity predicted to harbor putative cleavage site 2 (Fig. 3A) identified residues 367–375 (E1 subdomain) as being related to TSHR subunit formation (Fig. 3B). Subdivision of the E1 region into two segments next identified residues 367–371 (E1a segment) to be associated with ectodomain cleavage. The subsequent substitution of individual alanines at residues 367, 368, 369, and 371 (residue 370 being conserved) did not prevent cleavage, suggesting that multiple amino acids contributed to this event. Therefore, to refine further the cleavage-related residues, we created two overlapping mutants within the E1a segment, E1a1 (GQE367–369NET) and E1a2 (ELK369–371T-Y) (Fig. 3A). Abrogation of cleavage in the former identified residues 367–369 to be involved in cleavage at putative site 2 (Fig. 3B).

The inability of single amino acid substitutions to prevent cleavage at putative site 2, as well the involvement of multiple amino acids in this cleavage site, was instructive in view of our inability to localize putative cleavage site 1 despite the generation of 21 new receptors mutagenized in the anticipated region. Given the vast number of additional permutations that might be required to characterize site 1, further mutagenesis was daunting. Moreover, a number of possibilities existed. (i) Cleavage site 1 could be upstream of amino acid residue 317. However, in this case the deglycosylated A subunit would be 33 kDa or less, smaller than observed by multiple investigators (11, 18, 25). (ii) The specificity of cleavage at site 1 was “relaxed.” (iii) Our hypothesis for two cleavage sites was wrong, despite the strong supporting evidence that we had obtained previously (18).

To exclude the possibility of only a single cleavage site, we introduced the E1a1 mutation (GQE367–369NET), known to prevent cleavage of TSH-LHR-4, into the wild-type TSHR. Thus, if this segment was the sole site related to TSHR cleavage, its introduction into the wild-type TSHR should prevent cleavage. Conversely, if cleavage still occurred in the wild-type TSHR harboring the GQE367–369NET mutation, there must be two cleavage sites in the TSHR ectodomain. Cross-linking of 125I-TSH to this new construct clearly indicated cleavage (Fig. 4), proving the existence of cleavage site 1.

Remarkably, the mutation (GQE367–369NET) that abrogates cleavage at site 2 introduces a consensus sequence for an N-linked glycosylation site (N-X-(S/T)). A carbohydrate side chain in this vicinity could, therefore, prevent ectodomain cleavage, for example by steric hindrance of a cleavage enzyme. As men-

Fig. 3. A, amino acid substitutions introduced in the region of putative cleavage site 2 in the TSHR. Mutations were made in the E domain of chimeric receptor TSH-LHR-4 (Fig. 1). The mutations shown in bold prevent cleavage (completely, or nearly completely in the case of GQE367–369NQT), as determined by TSH cross-linking. In the E1, E2, and E3 mutations, “−” represents no substitution because the amino acid residues are the same in both receptors. In E2, “z” represents the absence of an amino acid which is not present in the LH/CG receptor. B, radiolabeled TSH cross-linking to intact CHO cells stably expressing the receptors described in A. Cross-linked products were reduced to dissociate the subunits in cleaved receptors. The upper two panels depict autoradiograms of 7.5% gels, the lower two panels show material electrophoresed in 10% gels.
tional mutations at the GQE367–369 triplet to explore further receptor on the cell surface. We, therefore, introduced addition in TSH-LHR-4 was caused by the introduction of an cell homogenates. These observations reinforced the value of other bands (presumably synthetic or degradation products) in subunit because of a weak signal and the presence of many B subunit that we obtained did not definitively identify the B linked glycan were noninformative. Thus, two antibodies to the GQE367–369NET substitution in the TSHR transposes NET291–293 from the LH/CG receptor, a motif (AAA) that would not be a potential glycosylation site (Fig. 3A). In contrast to the non cleaving receptor, TSHR-LH/CG NET substitution did cleave (Fig. 3B).

Attempts to determine directly by immunoblotting or immunoprecipitation whether or not the GQE367–369NET substitution in TSH-LHR-4 was caused by the introduction of a N-linked glycan were noninformative. Thus, two antibodies to the B subunit that we obtained did not definitively identify the B subunit because of a weak signal and the presence of many other bands (presumably synthetic or degradation products) in cell homogenates. These observations reinforced the value of the TSH cross-linking to intact cells which sees only mature receptor (23, 30). One possibility is that TSHR cleavage, including the release of a small polypeptide between cleavage sites 1 and 2, may be related to the very common occurrence of disease-causing autoantibodies, a phenomenon rarely, if ever, en countered with other members of the G protein-coupled receptor family.

In summary, data obtained from 33 new TSHR variants stably expressed in CHO cells (i) strongly support the concept of two cleavage sites in the TSHR; (ii) identify three amino acid residues in the TSHR that, when substituted with the homologous residues of the LH/CG receptor, prevent cleavage at the second, downstream cleavage site (site 2); and (iii) reveal that cleavage or noncleavage at this second site appears related to N-linked glycosylation. To our knowledge, the presence or absence of glycosylation represents a novel mechanism by which two closely related receptors have evolved into having a difference in subunit structure. Greater understanding of its structural features will also contribute to a better understanding of why the TSHR is a pivotal autoantigen in human autoimmune disease.

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FIG. 4. The presence of the GQE367–369NET mutation does not prevent cleavage of the wild-type TSHR. Cross-linked [125]TSH-TSHR products were reduced and subjected to PAGE (10%) and autoradiography.

FIG. 5. Radiolabeled TSH cross-linking to intact CHO cells expressing receptors mutated at TSHR residues GQE367–369 to explore the potential role of N-linked glycosylation in preventing cleavage at site 2. Mutations are either in the wild-type TSHR or chimeric receptor TSHR-LH/4 in which cleavage site 1 is eliminated. NQT would and NQE would not represent an N-linked glycosylation motif. A, one experiment in which inhibition of cleavage by GQE367–369NQT in TSHR-LH/4 is incomplete. B, one of two experiments in which the GQE367–369NQT in TSHR-LH/4 appears to totally inhibit receptor cleavage. Cross-linked [125]TSH-TSHR products were reduced and subjected to PAGE (7.5%) and autoradiography.
TSH Receptor Intramolecular Cleavage Sites

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