Identification of a Novel Epitope in the Thyroid-stimulating Hormone Receptor Ectodomain Acting as Intramolecular Signaling Interface*§

Gunnar Kleinau‡, Holger Jäschke‡, Susanne Neumann§, Jens Lättig‡, Ralf Paschke‡, and Gerd Krause‡

From the ‡Forschungsinstitut für Molekulare Pharmakologie, D-13125 Berlin, Germany, the §II Department of Medicine, Universitätsklinikum Leipzig, D-04103 Leipzig, Germany, and §NIDDK, National Institutes of Health, CEB, Bethesda, Maryland 20892

Glycoprotein hormone receptors (GPHRs) differ from the other seven transmembrane receptors mainly through a complex activation mechanism that requires the binding of a large hormone toward a large N-terminal ectodomain. The intramolecular mechanism of the signal transduction to the serpine domain upon hormone binding at the ectodomain is not understood. To identify determinants at the GPHR ectodomain that may be involved in signal transduction, we first searched for homologous structural features. Based on high sequence similarity to the determined structures of the Nogo-receptor ectodomain and the intermolecular complex of the Interleukin-8 ligand (IL8) and the N-terminal peptide of the IL8 receptor (IL8RA), the hypothesis was developed that portions of the intramolecular components, Cysteine-box-2 and Cysteine-box-3, of the GPHR ectodomain interact and localize at the interface between ectodomain and serpine domain. Indeed, point mutations within the D403EFN406 motif at Cysteine-box-3 of the thyrotropin receptor resulted in increased basal cAMP levels, suggesting that this motif may be important for transduction of the signal from the ectodomain to the transmembrane domain. New indications are provided about the tight spatial cooperation and relative location of the new epitope and other determinants at the thyrotropin receptor ectodomain, such as the leucine-rich repeat motif Ser281 and the cysteine boxes. According to the high sequence conservation, the results are of general relevance for the signal transduction mechanism of other glycoprotein hormone receptors such as choriogonadotrophic/luteinizing hormone receptor and follicle-stimulating hormone receptor.

The glycoprotein hormone receptors (GPHRs)1 CG/LHR, FSHR, TSHR, and the leucine-rich repeat-containing glycoprotein receptors constitute a subfamily of family A of G-protein-coupled receptors (1). Understanding the specific molecular mechanisms, starting with binding of the heterodimeric hormone to the large ectodomain, and knowledge about the changes in interactions between the ectodomain and transmembrane domain are prerequisites for understanding the specific activation mechanism of GPHRs. Furthermore, it is instrumental in generating new ideas for pharmacological intervention. An x-ray structure of the large ectodomain is not yet available. Based on sequence similarities, the large N-terminal portion of the TSHR seems to consist of five structural components: (i) an N-terminal C-b1 (hTSHR: 1–54), (ii) a 230-amino acid-spanning LRR motif (hTSHR: 55–297) with nine repeats back-to-back followed by (iii) the central C-b2 (hTSHR: 280–316), (iv) the TSHR-specific insertion region (hTSHR: 317–366), and (v) the C-b3 (hTSHR: 370–410) located close to TMH1 (2). For GPHRs it has been demonstrated that the major binding region for the large hormones hCG/hLH, hTSH, and hFSH is located in the extracellular leucine-rich repeat region (3). The crystal structures of 14 proteins containing LRR motifs show that LRRs fold into a curved shape with a parallel β-sheet on the concave face and with various secondary structures, including α-helix, 3–10 helix, and pII helix, on the convex face (4). Exposed side chains on the concave face are supposed to contact the ligands and provide selective interaction patterns for hormone receptor binding (5). The structures of hCG (PDB accession number 1QFW (6)) and hFSH (PDB accession number 1FL7 (7)) have been determined. Data of peptide studies and mutagenesis provide established knowledge about direct interaction of the hormone β-subunits and the LRRs (8, 9), whereas the hormone α-subunit is supposed to be oriented toward the serpine domain and to be involved in ligand-induced signaling (10, 11). However, the LRR sequence is only one portion in the middle of the complete N-terminal sequence, and there are additional structural components between the LRRs and the TMH1 of the serpine domain whose three-dimensional structure is unknown. Moreover, it is not completely understood how the intramolecular signal transduction toward the serpine domain of the GPHR takes place.

The central C-b2 is located back-to-back following the LRR region. Mutants at Ser281, directly preceding the first cysteine...

hLH, human lutropin; hFSH, human follicle-stimulating hormone receptor; hTSH, human thyroid-stimulating hormone receptor; C-b1, C-b2, C-b3, Cysteine-box-1, -2, -3; LRR, leucine-rich repeat; TMH, transmembrane helix; SCA, specific constitutive activity; IL, interleukin; PDB, Protein Data Bank; wt, wild type; FACS, fluorescence-activated cell sorter; IP, inositol phosphate; PBS, phosphate-buffered saline.
in C-b2, are known to act as a switch for constitutive activity (12). The structural interrelation between the constitutive active mutant S281N and the necessary presence of the complete LRR motif has been shown by deletion studies for the hCG/LHR (13). N-terminal truncations of the TSHR ectodomain (14) and mutational studies at corresponding positions of the leucine-rich repeat-containing glycoprotein receptor 2, the CG/LHR, and the FSHR demonstrated the importance of the Ser281 region for the maintenance of a conformational switch in all four glycoprotein hormone receptors (15, 16). Recent studies suggest that amino acids Pro276 and Ser277 in CG/LHR (TSHR: Pro280, Ser281) are constituents of a loop-like epitope that can act as an activation switch (15). This is confirmed by substitution of a proline residue, Pro276, (TSHR: Pro280) adjacent to Ser277 (TSHR: Ser281) of the CG/LHR receptor with a glycine that exerts less structural restraint and leads to constitutive receptor activation and higher agonist affinity. Mutations of the TSHR cysteines Cys283 and Cys284 to serine led to a 2-fold increase of basal TSHR activity (17).

Disulfide bonds are involved in the quaternary structure of the TSHR (18). Although there are currently no direct data to indicate which of the single cysteines form pairs, a number of indirect lines of evidence permit global assignments of disulfide bridges (19, 20). Mutations of Cys283, Cys284 (C-b2) and Cys398 Cys408 (C-b3) have dramatic effects on the TSHR structure and TSH binding. The hypothesis that Cys301 (C-b2) and Cys390 (C-b3) are paired is supported by recent evidence that intramolecular cleavage of the TSHR releases a “C peptide” located between these two residues (21). Further evidence is provided by the fact that mutation of either Cys301 or Cys390 produces the identical effect of reduced hTSH binding affinity (22). Moreover, the CG/LHR receptor of New World monkey Callithrix jacchus (23) is missing exon 10 encoding the particular cysteine corresponding to Cys301 of TSHR. Additionally, this receptor is simultaneously also missing the cysteine corresponding to Cys390 TSHR. Taken together, the two neighboring cysteines Cys283 and Cys284 of C-b2 are very likely paired either to Cys284 and Cys408 of C-b3 or in reversed order to Cys408 and Cys283 whereas the Cys301 (C-b2) is very likely paired with Cys390 (C-b3). For the large extracellular N-terminal tail of GPHRs, only molecular models for the LRR have been suggested (24, 25), based on the first available LRR crystal structure of the ribonuclease inhibitor (26).

To provide support for the molecular understanding of the signal transduction process upon hormone binding at the glycoprotein hormone receptor ectodomain, we decided to determine how the LRR region cooperates with other fragments of the ectodomain, like C-b2 and C-b3. To identify determinants in the ectodomain that may be involved in the intramolecular signal transduction and to obtain indications for their spatial cooperation, we searched for an optimal structural LRR template and for additional homologous structural motifs in the ectodomain. Sequence similarities between the central C-b2 and C-b3 with the IL8/CXCL8 chemokine and IL8RA/CXCR1 in a structure complex (PDB accession number 1ILQ (27)) of an IL8-IL8RA fragment were used as constraint. For the TSHR ectodomain model, the following disulfide bridges were used as constraints: Cys24/Cys51, Cys24/Cys105, and Cys108/Cys115. Conjugate gradient minimizations using the hFSH model as template. For the hTSH model, disulfide bridges between cysteines were incorporated based on the template: α-subunit, Cys31/Cys58, Cys89/Cys94, Cys39/Cys106, Cys26/Cys105, Cys49/Cys155, Cys39/Cys125, Cys57/Cys303, Cys71/Cys105, and Cys108/Cys155. Conjugate gradient minimizations were performed until it converged at a termination gradient of 0.05 kcal/mol Å. The AMBER 7.0 force field was used. The geometric quality of the models was controlled by the PROCHECK (31) software.

Modeling of Hormone-Receptor Ectodomain Interactions—Components of homologous models were assembled manually or by comparative modeling using functional data and complementary side chain properties with the biopolymer module or automatically using the docking module FlexX of the Sybyl program package. The electrostatic potentials (calculated by a probe radius of 2.0 Å) were created at the Connolly surface of all three GPHR LRRs and corresponding homologs by the MolCAD module of the Sybyl program package. The hormones hTSH, hCG, and hFSH were initially placed close to the corresponding GPHR LRRs according to their complementary electrostatic potentials. The hormones were docked during a constrained molecular dynamics simulation. Putative interaction pairs TSHR Lys209–Asp111TSH, CG/LHR Glu206–Arg115CG, FHSR Lys179–Asp111FSH, Asp110–Arg115FSH were as constraints according to experimental data (5, 32), allowing complementary side chain interaction (repulsion and attraction) for the remaining portions. The models were scored according to their consistency to available experimental binding data. All assembling calculations were performed in a vacuum.

The assembled ectodomain-ligand models of the three GPHRs were soaked with water in a periodic boundary box. Initially the ectodomain atoms were kept fixed to relax the water during minimization. Later on, the entire system was studied. Minimizations were performed as described above. Molecular dynamics simulations were performed at 300 K for 1 ns. For both, the AMBER 7.0 force field was used; the geometric quality of the model was controlled by the PROCHECK software (31). Site-directed Mutagenesis—Mutations were introduced into the human TSHR via a QuikChange site-directed mutagenesis kit (Stratagene). TSHR-pSVL (33) was used as template. PCR products containing the mutations were digested with BspTI and EcoR91I and used to replace the analogous BspTI/EcoR91I fragment.
in the wild type TSHR-pSVL vector. Sequences of mutated TSH receptor vectors were verified by dideoxy sequencing with Big Dye Terminator Cycle Sequencing chemistry (ABI Advanced Biotechnologies, Inc., Columbia, MD). Searcing reactions were analyzed on a genetic analyzer ABI 310 (ABI Advanced Biotechnologies).

The mutated receptors were cloned in the expression vector pSVL and transiently expressed in COS-7 cells. Characterization of the constructs was performed by determination of cAMP and phospholipase C-inositol phosphate accumulation, TSH binding, and cell surface expression. The wt receptor and empty pSVL vector were used as controls. Levels for cell surface expression were determined by flow cytometry.

**Cell Culture and Transfection—** COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO2 incubator. Cells were seeded of DNA/well for 105 cells/well) with 0.5 g of DNA/well. COS-7 cells were transfected using FuGENETM reagent (Roche Applied Science).

**FACS Analysis—** 48 h after transfection, nonpermeabilized cells were detached from the dishes using 1 mM EDTA and 1 mM EGTA in PBS and transferred in Falcon 2054 tubes. Before incubation with the primary antibody, cells were washed once with PBS containing bovine serum albumin and 0.1% NaCN3. For permeabilized cell assay, in the first step cells were fixed with 1% paraformaldehyde for 10 min on ice following an incubation with PBS containing 0.1% bovine serum albumin, 0.1% NaCN3, and 0.2% saponin for 30 min. Saponin was supplemented in all subsequent buffers. Subsequently, cells were incubated for 1 h with a mouse anti-human TSHR antibody (10 μg/ml, 2C11; Serotec, Oxford, UK). Cells were washed twice and incubated for 1 h in the dark with a fluorescein-conjugated Fab(′), rabbit antirabbit IgG (Serotec). Before FACS analysis (FACscan; BD Biosciences), cells were washed twice and fixed with 1% paraformaldehyde. Receptor expression was determined by fluorescence intensity; the percentage of signal positive cells corresponded to transfection efficiency.

**Radioligand Binding Assay—** Competitive binding studies were performed as previously described (34). Data were analyzed assuming a one-site binding model using the fitting module of SigmaPlot 2.0 for Windows (35).

**cAMP Accumulation Assay—** Measurement of cyclic AMP (cAMP) accumulation was performed 48 h after transfection as previously described (34).

**Stimulation of IP Formation—** Transfected COS-7 cells were incubated with 2 μCi/ml myo[H]inositol (Amersham Biosciences) for 8 h. Thereafter, cells were preincubated with serum-free Dulbecco’s modified Eagle’s medium containing 10 mM LiCl2 for 30 min. Stimulation with bTSH for 1 h was performed with the same medium supplemented with 100 milliunits/ml bTSH. Evaluation of basal and TSH-induced increases in intracellular IP levels was performed by anion exchange chromatography as described (36). IP values are expressed as the percentage of radioactivity incorporated from [H]inositol phosphates (IP1–3) over the sum of radioactivity incorporated in IPs and phosphatidylinositols. 

**Specific Constitutive Activity (SCA)—** The assays for determination of Bmax values and basal cAMP accumulation were performed as follows: 7-COS-7 cells were transiently transfected in 24-well plates (0.5 × 105 cells/well) with various concentrations of wt and mutant DNA (50, 100, 200, 300, 400, and 500 ng/well). For radioligand binding assays, cells were incubated in the presence of 180,000–200,000 cpm of125I bovine thyrotropin (bTSH) supplemented with 5 milliunits/ml nonlabeled bTSH. Specific binding was determined by subtraction of radioactivity incorporated in IPs and nonlabeled bTSH. The effect of expression level on basal cAMP accumulation was investigated by using the fitting module of Graph Pad Prism 3.0 (Graph Pad Software, San Diego, CA) to determine the optimal structural template for the LRR domain of the GPHR. The LRR motif from the Nogo-receptor ectodomain (PDB entry 1O2N) (29) was identified from 14 different LRR structures (4) as the best matching template to the LRR features of all three human GPHRs (Table I, hNogo-receptor: hCG/LHR, hFSHR, hTSHR).

**RESULTS**

**Hormone-bound LRR Complex—** Studies were carried out to examine the optimal structural template for the LRR domain of the GPHR. The LRR motif from the Nogo-receptor ectodomain (PDB entry 1O2N) (29) was identified from 14 different LRR structures (4) as the best matching template to the LRR features of all three human GPHRs (Table I, hNogo-receptor: hCG/LHR, hFSHR, hTSHR).

Not only did the nine typical repeats match best to the sequence of the three receptors but also the cysteine box of the Nogo-receptor structure, which is N-terminal directly attached to the LRR, showed a best fit to C-b1 of the GPHR sequences. The putative disulfide bridges between the cysteines Cys324, Cys331, Cys337, and Cys453 in the homologous TSHR model (Fig. 1, a and b) stabilize an anti-parallel and parallel β-strand as integral parts of the LRR, where the latter participates as an additional parallel strand (LRR0) to the convex binding face.

According to the LRR pattern rule, an additional β-strand (named b-strand X) is adopted at the C-terminal side for all three GPHRs. Based on this match, nine complete LRRs (LRR-IIX) plus one additional β-strand (LRR0) at the N terminus plus one at the C-terminal side (LRRXX) form 9 + 2 β-strands lining the inner convex surface of the hormone-binding region (Fig. 1a). The TSHR LRR sequence not only shows fewer sequence similarities (PAM250 matrix) to the previous RI template (22%) and other LRR templates compared with the new Nogo-receptor template (34%) but the new LRR model for the GPHRs also offers, as the only one, a much larger radius for the inner convex arch of the hormone-binding region. This resulting radius is a shallow deflection like a “scythe blade” shape rather than a “horseshoe” shape, as demonstrated by the superimposition of TSHR models using both templates (Fig. 1b). The LRR structure of the Nogo-receptor ectodomain contains inside the LRR a “Phe spine” (green aromatic rings) for the stabilization of the fold, instead of the missing helices at the concave outer face (Fig. 1a and Table I). The importance of phenylalanines from such a Phe spine for the overall fold of LRRs, as well as for the GPHRs, is demonstrated by a homozygous mutation at the CG/LHR, where a F194V mutant causes male pseudohermaphroditism by lost trafficking to the membrane (38).

Examination of complementary shapes and properties of electrostatic potentials for LRRs in our docking studies (see supplementary material) and functional data from mutations of all three GPHR subtypes, TSHR, CG/LHR, and FSHR and their hormones (Table II), resulted finally in a refined complex model for LRR-hormone with parallel orientation to each other (Fig. 1c). This is only possible by the widened radius of the scythe blade-shaped LRR. The β-subunit of the hormone is thereby oriented with a large face that also includes amino acids of the “determinant loop” (β-TSH Asn109,Asp114 (25, 32, 39)) toward the β-strands of the LRR (Fig. 1c, TSHR). Residues for hTSH, hCG/hLH, and hFSH and their counterparts at the receptors that mainly take part in complementary intermolecular hormone-LRR recognition in our models are given in Table II. The models are consistent with previous mutation studies of the hormone (32, 40) and the LRRs of the GPHR (5, 41–43).
TABLE I
LRR repeat-wise sequence alignments of N-terminal Cysteine-box-1, LRR motifs of GPHRs from the hNogo-receptor ectodomain

The sequence from the hNogo-receptor ectodomain (PDB entry 1OZN) best matched the LRR of the GPHR according to sequence similarity, sequence length/repeat, and number of repeats out of 14 LRR structures. An additional N-terminal repeat (named LRR0) including Cys11 provided by the Cysteine-box-1 (background grey) is a new structural feature. An additional ß-strand occurs at the C-terminal end (LRRX). The back-to-back following sequence of Cysteine-box-2 (background grey) and the template sequences for malonyl coenzyme and chemokine sequences (Eotaxin2, IL8) homologies are given. Sequence names of crystal structures are underlined. Beside the number of each repeat (repeats 0–X) the patterns of LRR sequence similarity positions are given by x1xLxLxLxLxL. Highly conserved residues like leucine are marked by upper case letters. Conserved phenylalanine residues forming a Phe spine are marked by bold F. Lower case “n” and “a” sequences contain only less conserved N and A. Additional residues in the loop region occurring in the template and GPHR sequences are marked as x. The ß-strands are indicated by ß. The potential binding site comprises residue positions x1–x5.

| N-terminal Cysteine-box-1 | res No |
|---------------------------|-------|
| hNoggR                    | 26    |
| hTSHR                     | 23    |
| hFSHR                     | 17    |
| hCGLHR                    | 19    |

| LRR repeat No. | Sequence similarities | hNoggR | hTSHR | hFSHR | hCGLHR | LRR0  | LRR1  | LRR2  | LRR3  | LRR4  | LRRV  | LRRVI | LRRVII | LRRVIII | LRRIX | LRRX  |
|----------------|-----------------------|--------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|--------|--------|-------|-------|
|                | x1 x2 L x3 L x4 x5   |        |       |       |        | V T T | Q R I | T I L | E Q L | H T L | Q Y L | T H L | N O P | M T L | Q M Y |
|                |                       | 30     | 60    | 84    | 133    | 157   | 154   | 147   | 129   | 123   | 181   | 190   | 205   | 203   | 198   | 268   |
|                |                       | V     | Q     | T     | K F L | Q L Y | F I L | L L D | Q L V | L T L | L Y L | L T L | R D L | D A V | N O P | R K L |
|                |                       | N     | S     | E     | F     | Y     | L     | I     | L     | L     | F     | K     | T     | V     | L     | L     |
|                |                       | T     | I     | T     | G     | L     | L     | D     | I     | D     | L     | F     | H     | S     | E     | L     |
|                |                       | S     | R     | I     | Y     | V     | I     | S     | T     | G     | I     | Y     | L     | M     | L     | A     |
|                |                       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |

| Structure templates of Cysteine-box-2 |
|--------------------------------------|
| Malonyl coenzyme                     | 198  |
| IL8                                  | 9    |

\[ V \text{PSHC} \]  
\[ \text{CIKTYSKFPF} \]
This orientation is consistent with several functional data of the hormones previously reported as important features for signaling to the ecto- and serpentine domains, like locations of glycosylation sites (44) and an accessible C-terminus of the α-subunit (10, 11). Moreover, residues that are highly conserved among hormones and among GPHRs interact in our model. Identical complementary charged residue pairs fitting between the additional repeat LRR0 in hTSHR and hormone are observed for hTSHR-hTSH and hFSHR-hFSH interaction. Furthermore, charged residues of the hormone’s determinant loop spatially fit to the LRR position with complementary charges (Table II). Finally, the electrostatic potentials that led to this parallel orientation between hormone and LRR also provide an explanation for the promiscuity of hormone binding caused by some point mutations in LRRs (5, 43) (see also Supplemental Figs. 5–7).
Complementary pairs of charged residues

Complementary pairs of charged residues for hTSH, hCG/LH, and hFSH and their receptors (hTSHR, hCG/CRH, and hFSHR, mainly take part in hormone-LRR interaction, according to our new parallel docking models. Positions that have been demonstrated experimentally to participate in hormone binding are indicated by appropriate references. Residues with negative charge are shown in black background and white text.

Identical complementary charged residue pairs between hormone and receptor at position x5 at LRR0 are observed for hTSH-TSHR and hFSH-FSHR interaction (indicated by #). Residues in a row are not only involved in pair-wise intermolecular interactions but also in multiple residue inter- and intramolecular interaction. Residues of the determinant loop (&) of the hormones are fit to position x5 of LRR VII (hTSH-hTSHR) and to spatial adjacent position x6 of LRRV and VII (hCG-hLHC/LHR) or to position x5 of LRRVI (hFSH-hFSHR) (see also Supplemental Figs. 5–7).

Table II: Relative Location of Signaling Epitopes in TSHR Ectodomain

| Receptor | Hormone |
|----------|---------|
| TSHR LRR| position | residue | reference | hTSH | hCG/LHR | reference |
| LRR0 | x5 | D43 | | R54 | | |
| LRRII | x3 | E107 | | K104 | | |
| LRRV | x6 | K209 | 5 | & D111 | 32 |
| LRRX | x3 | D276 | | R60 | | |
| CCG/LHR | LRR | position | residue | reference | hCG/LH | |
| LRRII | x2 | E102 | 42 | R109 | | |
| LRRV | x3 | E154 | 40 | R60 | | |
| LRRV | x5 | D157 | 5 | & R114 | 40 |
| LRRX | x3 | D228 | 41 | R63 | | |
| FSHR | LRR | position | residue | reference | hFSH | |
| LRR0 | x5 | E34 | | R63 | | 44 |
| LRRIII | x5 | K104 | 5 | & D106 | | |
| LRRV | x3 | D150 | | K72 | | |
| LRRV | x5 | K179 | 5 | & D106 | | |
| LRRX | x3 | R246 | | E27 | | |

Cysteine-box-2 and Cysteine-box-3 Interact and Are Located Close to the Transmembrane Domain—Next we asked whether additional structural templates exist in the N-terminal ectodomain of the TSHR. Systematic searches with fragmented portions of the ectodomain in the Protein Data Bank for sequence similarities identified a new homologous structural template for C-b2 and C-b3 of the TSHR ectodomain, based on the structure of the complex between chemokine IL8/CXCL8 and chemokine receptor fragment IL8RA/CXCR1 (PDB entry 1ILQ) solved by NMR solution methods (27). Chemokine ligand structure, like MIP-2 (PDB entry 1MIP), Rantes (PDB entry 1RT0), and Eotaxin-3 (PDB entry 1G2S) show a common extended-like conformation for sequences directly following the two characteristic cysteines. These are involved in chemokine-ligand-receptor interaction. A sequence similarity to the central C-b2 of the TSHR ectodomain was identified exactly for this region with the consensus sequence pattern C283CAFKNQKKI292 (Fig. 2a).

Moreover, also for the bound peptide, representing a portion of the IL8RA N-terminal tail, a high sequence similarity to the TSHR receptor sequence of C-b3 was identified with the consensus sequence pattern P400XSDEFNPC408 (Fig. 2, b and c). This structural template applied to the TSHR ectodomain model by substituting amino acid side chains from C-b2 (C283CAFKNQKKI292) and C-b3 (P400XSDEFNPC408) resulted in homologous model residues with complementary properties matching as interacting side chains like the aromatic interaction of Phe286 in C-b2 and Phe405 in C-b3 (Fig. 2e).

Subsequently using this template, Cys408 of C-b3 is ideally placed close to Cys283 (or Cys284) of C-b2 to form a disulfide bridge (Fig. 2e). Our findings support the hypothesis of disulfide bridges between cysteines Cys283/Cys284 of C-b2 either to Cys398/Cys408 or to the reverse order, Cys408/Cys398 of C-b3 (21).

The TSHR sequence S278YPSC283 directly following the last β-strand of LRRX and preceding Cys283 of C-b2 (including Ser281) matched best with sequences of structural fragments containing a turn/loop conformation, such as SVPSHC from the malonyl coenzyme (PDB entry 1MLA) and VLPSHC from a stem cell factor (PDB entry 1EXZ) (Fig. 2, a and b, and Table I). These findings confirm previous suggestions that Ser281 is an integral part of a turn or loop conformation (15). Adapted to our model, such a turn links the LRR and C-b2 and places the C-terminal portion of the LRR domain directly above the linked C-b2 and C-b3. The function of this turn as a potential spatial pivot or hinge between LRR and C-b2/C-b3 is consistent with the data for mutational activation at Ser281 (12). The assembled model provides support for a very compact structural arrangement of the C-b1, LRR, Ser281 turn/loop, C-b2, and C-b3 proximal to the extracellular end of TMH1 (Fig. 2d). Subsequently the residues P400EFNPC408 of the C-b3 are located particularly at prominent interface positions of the ectodomain (Fig. 2e), closest to the transmembrane domain. The hydrophilic residues Asp403, Glu404, and Asn406 were therefore hypothesized to very likely participate in the intramolecular signal transduction from the ectodomain toward the serpentine domain.

New Switch for Constitutive Activity Induced by Mutations Identified in the Ectodomain—To provide support for this hypothesis, we experimentally addressed the three amino acids, Asp403, Glu404, and Asn406 in the ectodomain by site-directed mutagenesis. To investigate the functional properties of these residues in the intramolecular signal transduction, the positions of the acidic residues Asp403 and Glu404 were replaced by a non-polar and a basic amino acid, alanine and lysine, respectively (Table III). For Asn406, a substitution to alanine was characterized.

The cell surface expression of the transfected TSHR constructs compared with the wt TSHR were as follows: wt TSHR set at 100%; D403A, 27.2 ± 0.9%; D403K, 12.3 ± 0.8%; E404A, 95.4 ± 4.6%; E404K, 35.3 ± 1.0%; N406A, 66.4 ± 3.6% (Fig. 3a and Table III). After permeabilization of the cells, receptors with a strong impaired cell surface expression (D403A, D403K, and E404K) showed an increased FACS signal up to 60% compared with the wt TSHR, suggesting an increased accumulation within the cells (Fig. 3b). To confirm our assumption of an intracellular receptor accumulation, we performed confocal laser scanning microscopy in HEK 293 cells. In brief, for mutants D403A, D403K, and E404K a strong intracellular receptor accumulation after permeabilization was observed. In contrast, E404A showed a cell surface expression comparable with the wt TSHR before and after permeabilization. This is in accordance with the results obtained by FACS analysis. However, for mutant N406A with a cell surface expression of 66% (wt TSHR set at 100%) the difference between non-permeabilized and permeabilized cells was not consistent. This is most likely because the reduced cell surface expression for N406A was only very slight compared with the other mutants.

Three new mutations (D403A, E404K, and N406A) in the ectodomain of the TSHR, causing constitutive cAMP activity,
were identified. The mutant with the highest constitutive activity is N406A with a 5- to 6-fold over basal cAMP production (Fig. 3D). Moreover, after stimulation with 100 milliunits/ml of hTSH, a maximal cAMP response of 90% of the wt receptor could be detected (Fig. 3E). Substitution of the TSHR-specific position Glu^{404} by lysine resulted in a 4- to 5-fold increase of basal cAMP production. In contrast, the replacement of Glu^{404} by alanine had no significant effect on cAMP production. Mutant D403A showed a constitutive activation of the cAMP-signaling cascade (2-fold over wt TSHR basal). The hTSH-induced cAMP response was half of the wild type. A strongly reduced cAMP production was detected by the D403K mutant.

To investigate the effects of these mutations on the basal receptor signal transduction more precisely, we calculated the specific constitutive activity. The calculation was based on a linear increase of basal cAMP accumulation of higher densities of receptors at the cell surface. An increase of basal activity was strongly reduced cAMP production was detected by the D403K mutant.

To investigate the effects of these mutations on the basal receptor signal transduction more precisely, we calculated the specific constitutive activity. The calculation was based on a linear increase of basal cAMP accumulation of higher densities of receptors at the cell surface. An increase of basal activity was determined as follows: wt TSHR (slope = 0.0024 ± 0.0002) <N406A (slope = 0.031 ± 0.0003) <D403A (slope = 0.032 ± 0.004) <E404K (slope = 0.042 ± 0.004) (Fig. 3F). Stimulated with higher concentrations of hTSH, the activated TSHR also
coupled to the phospholipase C-inositol phosphate pathway (IP1–3). No constitutive activation of the IP pathway was observed for any of the mutants (Fig. 3F). After stimulation with 100 milliunits/ml bTSH, the mutations E404K and N406A showed decreased IP1–3 accumulation (Fig. 3G), which correlates with the cell surface expression. The mutant E404A had similar characteristics as the wt receptor. For the mutations D403A and D403K, no IP1–3 response could be detected. These findings were in accordance with the impaired cell surface expression of these two mutants.

In summary, we have identified a complete epitope with three extracellular constitutively activating mutants, D403A, E404K, and N406A, in the N-terminal portion of the TSHR. Aspartate 403 and asparagine 406 are highly conserved in the GPHR family, and the D403A and N406A mutations lead to similar levels of constitutive activity. In contrast, the E404A mutation does not affect basal cAMP accumulation, whereas the E404K mutation leads to an increased basal cAMP accumulation compared with the wt TSHR.

**DISCUSSION**

Using data from comparative modeling, sequence analysis, and reported site-directed mutagenesis studies, we have provided in this report strong indications explaining the spatial cooperation and relative location of molecular components in the GPHR ectodomain involved in receptor activation. Moreover, we have presented molecular models for the N-terminal TSHR components like the C-b1, the LRR motif, the C-b2 including Ser281 and a C-terminal epitope of C-b3 based on homologous structural features. By extensive consideration of known functional data, these structural features were assembled to a tightly packed molecular model. This led to the identification of new key residues participating in intramolecular signal transduction at the interface toward the serpentine domain. By a combined modeling/mutagenesis approach, we demonstrated the functional importance of the N-terminal epitope D403EFNPC408 of C-b3 component of the TSHR ectodomain for the intramolecular signaling processes in the TSHR. The next following structural component, C-b2, is attached to the C-b1 via the Ser281 turn/loop and C-b3 is connected to C-b2 via the Ser281 turn/loop active receptor. This may support the idea of a mechanism in which a certain spatial adjustment between the LRR motif and the following C-b2 (and remaining components of the ectodomain) via the Ser281 turn/loop is responsible for the TSHR activation. This is consistent with our model, in which a LRR-bound hormone may also influence the spatial adjustment between LRR and C-b2/C-b3 (Fig. 2d).

**Epitope D403EFNPC408 of Cysteine-box-3 Is Localized at the Interface between the Ectodomain and Serpentine Domain**

Based on new structural features for the ectodomain, identified by sequence homology with a complex of the IL8 ligand and the IL8RA peptide, we formed the hypothesis that the structural components C-b2 and C-b3 interact and are located close to TMH1. The structural consequence of this model is a potential disulfide bridge between Cys283 and Cys408 or Cys284 and Cys408. Our findings are in agreement with suggestions of disulfide bridges between C-b2 and C-b3 based on biochemical data (48, 49). Moreover, because the C-terminal end of the LRR is connected to C-b2 via the Ser281 turn/loop and C-b3 is connected with the transmembrane domain TMH1, we have postulated a tightly packed structure including the C-terminal end of the LRRs and the C-b2 and C-b3 of the ectodomain. Subsequently, the epitope D403EFNPC408 of C-b3 of the ectodomain was structurally localized at the interface between ectodomain and serpentine domain; it was therefore proposed as an important mediator for the intramolecular signal transduction from the ectodomain to serpentine domain (Fig. 2e). In the assembled models the two linked C-b2/C-b3 motifs are arranged across the serpentine domain. Their relative location may ac-

**TABLE III**

| Transfected construct | Cell expression FACS | 125I-bTSH binding Bmax | cAMP accumulation | IP accumulation |
|-----------------------|----------------------|-------------------------|------------------|----------------|
|                       | % of TSHR wt         | % of TSHR wt            | Basal            | Stimulated     |
|                       |                      |                        |                  |               |
| TSHR wt               | 100                  | 100                     | 1                | 1             |
| D403A                 | 27.22 ± 0.90         | 35.5 ± 2.1              | 1.9 ± 0.1        | 6.8 ± 0.7     |
| D403K                 | 12.29 ± 0.75         | 12.2 ± 0.9              | 0.9 ± 0.1        | 3.2 ± 0.4     |
| E404A                 | 95.40 ± 4.60         | 89.0 ± 4.6              | 0.6 ± 0.2        | 16.7 ± 0.6    |
| E404K                 | 35.32 ± 0.96         | 49.7 ± 3.5              | 4.8 ± 1.0        | 13.4 ± 0.7    |
| N406A                 | 66.40 ± 3.60         | 70.7 ± 7.1              | 5.2 ± 1.3        | 12.8 ± 0.6    |
count for an interaction with the embedded conformation of ECL2, which fits to data of an ECL2 interaction with the ectodomain reported for CG/LHR (16). Although the linked C-b2/C-b3 motifs are constrained by the fact that they are connected to TMH1, some altered orientations across the extracellular loops should be considered until non-ambiguous contact points are known (Supplemental Fig. 8, arrows). Therefore, interactions of C-b2/C-b3 with the other ECLs, as well as

FIG. 3. Characteristics of mutants at the TSHR epitope Asp⁴⁰³-Asn⁴⁰⁶. A, cell surface expression by FACS analysis of wt and mutated TSHRs in COS-7 cells. B, comparison of cell surface expression of wt TSHR and mutated TSHR in non-permeabilized (np) and permeabilized (p) cells by FACS analysis and confocal laser scanning microscopy (HEK 293 cells). FACS data are presented as mean ± S.E. of three independent experiments, each performed in duplicate. After permeabilization of the cells, receptors with impaired cell surface expression (D403A, D403K, E404K) showed an increased FACS signal up to 60% compared with the wt TSHR. In confocal laser scanning microscopy experiments, TSHR mutants D403A, D403K, and E404K show strong intracellular receptor accumulation after permeabilization. This is in accordance with the results obtained by FACS analysis. C, Bmax values were determined by homologous competitive binding experiments. D, the TSHR has been described to be basal active in cAMP signaling. Therefore, elevated basal cAMP levels of cells expressing the wt TSHR in comparison with the pSVL vector alone can be observed after transient transfection of COS-7 cells. Because of the basal activity of the wt TSHR, cAMP levels are expressed as -fold over wt TSHR basal level. E and G, maximum increases in cAMP and IP1–3 levels were determined after stimulation with 100 milliunits of bTSH/ml. F, IP1–3 basal activity. H, effect of receptor density at the cell surface on basal cAMP accumulation. COS-7 cells were transiently transfected with increasing amounts of plasmids encoding wt TSHR or mutant TSHRs. Values were expressed as a function of receptor density at the cell surface, and lines were calculated by linear regression analysis. All data are presented as mean ± S.E. of three independent experiments, each performed in duplicate. The values for slopes are as follows: TSHR (0.0024 ± 0.0002) < N406A (0.031 ± 0.003) < D403A (0.032 ± 0.004) < E404K (0.042 ± 0.004).
simultaneous access of the hormone to C-b2/C-b3 and to one of the ECLs, are feasible and correspond to previous results (50). This would also fit to reported data of ligand (relaxin-3) binding to both the N-terminal ectodomain and ECL2 of leucine-rich repeat-containing glycoprotein receptor 7 (51).

New Intramolecular Switch for Constitutive Activity in the TSHR Ectodomain—Our major finding is a new switch for constitutive activity identified in the TSHR ectodomain. Three amino acids of an epitope in the ectodomain (Asp403, Glu404, Asn406) within the C-b3 component in proximity to TMH1 can act as switches for constitutive activity of the TSHR induced by point mutations. The residues Asp403 and Asn406 are highly conserved in GPHR and show similar constitutive activity for alanine mutants. The TSHR-specific glutamate at position 404 shows constitutive activity for lysine mutation, but not for an alanine substitution. Subsequently, the side chains of consecutive acidic residues Asp403 and Glu404 are differently orientated in space and/or are differently tightly packed. Mutation of Asp403 with a smaller neutral amino acid (alanine) causes a constitutive active mutant, very likely by loss of side chain interaction. In contrast, a lysine mutant at position Asp403 with a more bulky and basic side chain strongly disturbs the receptor expression, probably by a bulky/electrostatic repulsion, which obviously only has an effect on the overall fold but no effect on basal activity. Therefore it is likely that Asp403 and Asn406 constrain the partial inactive receptor state by a side chain interaction via H-bonds toward a tightly packed environment, such as extracellular loops or TMHs. On the contrary, at the TSHR-specific residue Glu404, the mutant with a smaller neutral side chain, E404A, behaves as the wt TSHR. However, the mutant with the bulkier basic residue, E404K, behaves as constitutive active mutant, obviously by electrostatic/bulk repulsion effects. Therefore, Glu404 does not seem to be involved in constraining the partial inactive state by its side chain interaction.

Ser281 Turn/Loop and the New Epitope Asp403-Asn406 in Cysteine-box-3 Are in Spatial Proximity—The results of our study show that the Ser281 turn/loop and the epitope Asp403-Asn406 are assembled in close spatial proximity at the interface between ectodomain and serpentine domain (Fig. 2d). Compared with Ser281, this epitope to a certain extent reacts differently to mutations and obviously has additional capabilities to induce a constitutive activity. The latter can be seen in previous observations (14) where stepwise N-terminal truncations of the TSHR ectodomain caused a 5-fold specific constitutive activation of the TSHR by deleting the Ser281 region. An additional increase to 6-fold specific constitutive activation was observed by those fragments in which Asp403 and/or Asn406 were also deleted. In conclusion, the stepwise truncation of structural components of the ectodomain led to a graduated constitutive activation (partial activation) of the TSHR cAMP pathway. Single mutations at position Ser277 (Ser281 in TSHR) cause constitutive activation. Their strength depends on size and property of the mutated side chain (47). In contrast to Ser281, which seems to be involved in constraining a loop/turn conformation (15), Asp403 and Asn406 seem to interact with other partners.

Asp403 and Asn406 Are Members of a Constraining Network at the Interface between Ectodomain and Serpentine Domain—From our study we cannot exclude an interaction of Asp403 and Asn406 with other N-terminal portions. However, based on our and other data available from reported mutagenesis studies, we suggest that they very likely form a common tightly interacting structural interface together with the extracellular loops and/or TMH residues. Already minor conformational changes (by mutants or by hormone binding) lead to receptor activation.
We suggest a scenario where this tight structural interface of the ectodomain (C terminus LRR, Ser281-turn/loop, C-b2, C-b3) and serpentine domain/extracellular loops constrains the native basal receptor state. Presumably it mediates the signaling upon hormone binding by release/formation of hydrogen bonds between ectodomain and serpentine domain. These predictions follow from the concept that receptor activation may involve disruption of a constraining hydrogen bond network between the TMHs of the serpentine domain that stabilizes the partial inactive basal state while promoting the formation of a new set of hydrogen bonds that stabilizes the active state. We suggest that this constraining hydrogen bond network in the TSHR has an increased density toward the compact ectodomain. Asp403 and Asn406 seem to be members of this constraining network at the interface between ectodomain and serpentine domain.

The known constitutive active mutant switch position at Ser281 is 100% conserved to a serine in the hCG/LHR (Ser277) and hFSHR (Ser273). According to the 100% conservation of the newly identified residues Asp403 and Asn406 in GPHRs, we strongly assume that these residues are of general relevance for the signal transduction mechanism of other glycoprotein receptors such as lutropin and follitropin receptors.

Acknowledgments—We thank Eileen Bösenberg for excellent technical assistance. We thank BRAHMS Diagnostica (Berlin, Germany) for providing 125I-bTSH.

REFERENCES

1. Sakudlinski, M. W., Fremont, V., Remin, C., and Weintraub, B. D. (2002) Physiol. Rev. 82, 473–502
2. Themmen, A. P. N., and Huhtaniemi, I. T. (2000) Endocr. Rev. 21, 551–583
3. Braun, T., Schofield, P. R., and Sprengel, R. (1991) EMBO J. 10, 1885–1890
4. Enkhbayar, P., Kamiya, M., Osaki, M., Matsumoto, T., and Matsushima, N. (2000) Proteins Struct. Funct. Bioinf. 54, 394–403
5. Smits, G., Campillo, M., Govaerts, C., Janssens, V., Richter, C., Vassart, G., Pardo, L., and Costagliola, S. (2003) EMBO J. 22, 2692–2703S
6. Nishi, S., Nakabayashi, K., Hseue, A. J., and Maruo, T. (2003) Mol. Cell. Endocrinol. 205, 139–144
7. Ho, S. C., Van Sande, J., Lefort, A., Vassart, G., and Costagliola, S. (2002) J. Biol. Chem. 277, 3958–3964
8. Rapoport, B., Chazenbakh, G. D., Jaume, J. C., and McLachlan, S. M. (1998) Endocr. Rev. 19, 673–716
9. Bozon, V., Couture, L., Pajot-Auge, E., Richard, F., Remy, J. J., and Salesse, R. (2002) Protein Expression Purif. 25, 114–123
10. Zhang, R., Buckco, E., and Dufau, M. L. (1996) J. Biol. Chem. 271, 5755–5760
11. Tanaka, K., Chazenbakh, G. D., McLachlan, S. M., and Rapoport, B. (1998) J. Biol. Chem. 273, 1959–1963
12. Nagayama, Y., and Rapoport, B. (1992) Endocrinology 131, 548–552
13. Gromoll, J., Wistuba, J., Darden, T. A., Merz, K. M., Stanton, R. V., Cheng, A. L., Vincent, J. J., Crowle, M., Tsui, V., Gojvilk, H., Ruder, R. J., Duan, Y., Pitera, J., Massova, J., Seibel, G. L., Singh, U. C., Weiner, P. K., and Kollman, P. A. (2002) AMBER 7, University of California, San Francisco
14. Vlaeminck-Guillem, V., Ho, S. C., Rodien, P., Vassart, G., and Costagliola, S. (1997) Mol. Endocrinol. 11, 1425–1436
15. Zeng, H., Ji, I., and Ji, T. H. (2005) FEBS Lett. 575, 547–551
16. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Son, W. A., and el Tayar, N. (1995) J. Biol. Chem. 270, 20620–20631
17. Kobe, B., and Deisenhofer, J. (1993) Nature 366, 751–756
18. Skelton, N. J., Quan, C., Reilly, D., and Lowman, H. (1999) Structure Fold Des. 7, 157–168
19. He, X., Banaz, J. F., Mc Dermott, G., Park, J. B., Wang, K., Tessier-Lavigne, M., He, Z., and Garcia, K. C. (2003) Neuron 38, 177–185
20. Case, D. A., Pearlman, D. A., Caldwell, J. W., Cheatham, T. E., III, Wang, J., Ross, W. S., Simmerling, C. L., Darden, T. A., Merz, K. M., Stanton, R. V., Cheng, A. L., Vincent, J. J., Crowle, M., Tsui, V., Gohilk, H., Ruder, R. J., Duan, Y., Pitera, J., Massova, J., Seibel, G. L., Singh, U. C., Weiner, P. K., and Kollman, P. A. (2002) AMBER 7, University of California, San Francisco
21. Acknowledgements—We thank Eileen Bösenberg for excellent technical assistance. We thank BRAHMS Diagnostica (Berlin, Germany) for providing 125I-bTSH.