From Molecular Details of the Interplay between Transmembrane Helices of the Thyrotropin Receptor to General Aspects of Signal Transduction in Family A
G-protein-coupled Receptors (GPCRs)*

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Transmembrane helices (TMHs) 5 and 6 are known to be important for signal transduction by G-protein-coupled receptors (GPCRs). Our aim was to characterize the interface between TMH5 and TMH6 of the thyrotropin receptor (TSHR) to gain molecular insights into aspects of signal transduction and regulation. A proline at TMH5 position 5.50 is highly conserved in family A GPCRs and causes a twist in the helix structure. Mutation of the TSHR-specific alanine (Ala-5935.50) at this position to proline resulted in a 20-fold reduction of cell surface expression. This indicates that TMH5 in the TSHR might have a conformation different from most other family A GPCRs by forming a regular α-helix. Furthermore, linking our own and previous data from directed mutagenesis with structural information led to suggestions of distinct pairs of interacting residues between TMH5 and TMH6 that are responsible for stabilizing either the basal or the active state. Our insights suggest that the inactive state conformation is constrained by a core set of polar interactions among TMHs 2, 3, 6, and 7 and in contrast that the active state conformation is stabilized mainly by non-polar interactions between TMHs 5 and 6. Our findings might be relevant for all family A GPCRs as supported by a statistical analysis of residue properties between the TMHs of a vast number of GPCR sequences.

G-protein-coupled receptors (GPCRs) are mediators between extracellular stimuli and intracellular signaling cascades. Knowledge concerning the mechanisms of receptor and G-protein activation has grown exponentially in the past few decades (1–6). GPCRs are anchored in the membrane, and different parts of GPCRs are responsible for specific intra- as well as intermolecular functions during a sequential signal transduction process. Despite individual and selective properties of particular receptors in each of these steps, general aspects are common for family A GPCRs. A “global toggle-switching” mechanism is proposed based on a vast amount of experimental and structural data (7) whereby a vertical see-saw movement of transmembrane helix (TMH) 6 occurs around a pivot. Consequently, spatial movement of particular TMHs relative to each other characterizes receptor activation; this occurs to the greatest extent between TMHs 5, 6, and 7, respectively (8, 9). The conformations and structural rearrangements are supported by amino acids acting as “microswitches” (10). Some of these residues are well known for family A GPCRs such as the highly conserved NPXXY (TMH7) and DRY (TMH3) motifs. Different GPCR conformations are causally related to different signaling activity states (3, 10, 11). Furthermore, differences between activated conformations depend on particular interacting ligands, and they are probably involved in determination of G-protein subtype preferences (12–17). The previously published GPCR structures of inactive receptor conformations (for reviews, see Refs. 15, 18, and 19) compared with the recently published active conformation of opsin and β2-adrenergic receptor (20, 21) confirmed a distinct spatial movement of specific transmembrane helices relative to each other, in particular of TMHs 5 and 6, during receptor activation.

However, underlying details and general questions of GPCR signal transduction still remain open. For instance, which residues constrain the inactive state, and which are responsible for movement of the helices? Structural and functional specificities caused by different residues at conserved positions of GPCR subtypes are also of interest for further investigation (22, 23). Therefore, our aim was to characterize the interface of amino acids between TMH5 and TMH6 of the thyrotropin receptor (TSHR) in detail to gain deeper insight into properties that are responsible for the inactive (or basal) and the active conformations. This we studied by site-directed mutations, structural homology modeling, and by statistical analysis of a vast number of GPCR sequences.

As in other GPCRs, the TSHR topology is characterized by a serpentine domain comprising seven TMHs connected by three intracellular loops and three extracellular loops (24). In
addition, a few amino acids that are highly conserved in other family A GPCRs such as the proline at position 5.50 in TMH5 or the essential tryptophan at position 6.48 in TMH6 are different in the TSHR. They are replaced in the TSHR by an alanine (Ala-593) at position 5.50 and by a methionine at position 6.48 (Met-637). Furthermore, by analyzing available functional data for TSHR residues in TMH5 and TMH6 in a glycoprotein hormone receptor (GPHR) information resource (25), it becomes apparent that several amino acids that form the interface between the activation-important TMHs 5 and 6 have not yet been investigated. Therefore, we analyzed those amino acids and performed site-directed mutagenesis followed by functional characterization of the mutant receptors. In combination with previous findings, our data provide (i) hints for details of interacting partners at transmembrane helices 5 and 6, (ii) insight into general aspects and specific details of signaling mechanisms during TSHR activation, and (iii) structural and functional consequences of variations at conserved positions in family A GPCRs. Finally, this study focused on the causal relationship among amino acid sequence, the three-dimensional structural properties, and the particular functions proceeding at the interfaces along the transmembrane helices of both the TSHR and other family A GPCRs.

EXPERIMENTAL PROCEDURES

Molecular Homology Models of Different TSHR Conformations—Crystal structures in the inactive conformation serving for GPCR homology modeling have been published for several family A GPCRs (26–34). These structures were silenced by keeping the receptors in a rigid conformation (thermostabilized) by one of the following methods: (a) inverse agonists were used as ligands, (b) receptors were modified by silencing mutations, or (c) they were fused with lysozyme (for reviews, see Refs. 18, 19, 35, and 36). In contrast, the new x-ray structure of opsin lacks the inverse agonist ligand retinal and has structural features predicted for an active receptor conformation (20). For structural comparisons between an inactive TSHR and an activated TSHR conformation, we used homology models for both an inactive TSHR model based on rhodopsin (Protein Data Bank code 2I35) and an activated model using opsin (Protein Data Bank code 3CAP) as a template.

For TMH5, two different conformational models were generated. Model 1 has the proline bulge at position 5.50 from the template structures, and model 2 has a regular \( \alpha \)-helical conformation around position 5.50 due to the lack of a proline compared with opsin/rhodopsin (Fig. 1). Gaps of missing residues in the loops of the template structure were closed by the “Loop Search” tool in Sybyl 8.1 (Tripos Inc., St. Louis, MO). Side chains and loops of homology models were subjected to conjugate gradient minimizations (until converging at a termination gradient of 0.05 kcal/mol \( \times \) Å) and molecular dynamics simulation (4 ns) by fixing the backbone of the TMHs. Finally, the models were minimized without constraints using the AMBER 7.0 force field. To facilitate comparison with other GPCRs, we used both the amino acid numbering scheme of the entire TSHR with its signal peptide and the Ballesteros-Weinstein nomenclature for family A GPCRs (37).

![FIGURE 1. Transmembrane helix 5 exerts different conformations dependent on sequence specificities at position 5.50. Two superimposed TSHR homology models (TMH3, TMH4, TMH5, and TMH6 are displayed) displaying possible different conformations of the second half of TMH5 toward the extracellular side. TSHR model 1 (cyan TMH5) is based on the rhodopsin template. In most of the family A GPCRs, a proline (Pro593 5.50; cyan stick) is located at TMH5 position 5.50. As a consequence, a proline-supported bulge caused by a missing H-bond to the backbone can be observed; it causes a twist of the preceding part of TMH5 (cyan) in comparison with a regular \( \alpha \)-helix. TSHR model 2 (orange TMH5) shows a regular \( \alpha \)-helix for TMH5 (without a bulge) because in the TSHR an alanine (Ala593 5.50) can be found at this position instead of a proline. This lack of twisted conformation affects the constituents of the interfaces between TMH3 and TMH5, TMH4, or TMH6, respectively. As a result, the spatial orientations of side chains (sticks) are remarkably different in the models. Side chain Leu-587 5.44 (circled in red) is pointing toward the membrane in model 1, but in model 2, it points to TMH6. L587V mutation data of constitutive activation (Ref. 46 and Fig. 5) are consistent with the \( \alpha \)-helical conformation in the second model.](image-url)
Signal Transduction and Regulation at TSHR

Cell Culture and Transfection—HEK 293 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Biochrom) at 37 °C in a humidified 5% CO2 incubator. For determination of intracellular CAMP accumulation, cells were cultured for 48 h and stimulated for 1 h at 37 °C with stimulation buffer (DMEM supplemented with 10 mM HEPES, 0.5% BSA, 0.25 mM 3-isobutyryl-1-methylxanthine) alone or stimulation buffer containing increasing concentrations of bovine TSH (1 μIU/ml to 0.1 IU/ml; Sigma Aldrich). Stimulation medium was aspirated, and reactions were stopped by lysis of the cells with 0.1% trifluoroacetic acid, 0.005% Triton X-100 for 30 min at 4 °C. Supernatants were collected, heated at 95 °C for 10 min, dried in a rotation vacuum concentrator (Alpha-RCV, Martin Christ Gefriertrocknungsanlagen GmbH), and finally stored at −20 °C until use. After reconstitution and acetylation of the samples (40), the cAMP content was determined using cAMP-125I-tyrosyl methyl ester (10,000 cpm; IBL International) and polyclonal rabbit anti-cAMP antibody (final dilution, 1:160,000). Samples were incubated overnight at 4 °C. The antibody-bound fraction was precipitated using 50 μl of Saccel (IBL International). The radioactivity of the precipitate was determined in a γ-counter.

Determination of [3H]Inositol Phosphate—After transfection, cells were cultivated for 24 h. The DMEM was supplemented with 74 kBq/ml myo-[2-3H]inositol (Amersham Biosciences), and cells were incubated for an additional 24 h. Cells were washed with stimulation buffer (culture medium with 10 mM HEPES, 0.5% BSA, and 10 mM LiCl) and then stimulated for 60 min at 37 °C with bovine TSH in stimulation buffer. Stimulation medium was removed, and cells were lysed with 0.1 M NaOH. By subsequent addition of 0.2 M formic acid and dilution buffer (5 mM sodium tetraborate, 0.5 mM EDTA), the appropriate conditions (pH and ion strength) were adjusted. The lysates were spun, and supernatants were subjected to
anion exchange chromatography on Sep-Pak Accell™ Plus QMA cartridges (Waters). [3H]Inositol 1,4,5-triphosphate was eluted with 0.1 M formic acid and 0.4 M ammonium formate. Radioactivity was determined in a liquid scintillation counter. For normalization, the individual counting results were related to the total amount of myo-[2-3H]inositol incorporated into the HEK 293 cells.

Confocal Laser Scanning Microscopy—After transfection, cells were incubated overnight, washed once with PBS, and transferred immediately into a self-made chamber (details are available on request). For the co-localization of the TSH receptor with the plasma membrane marker trypan blue, live cells were covered with 1 ml of PBS, and trypan blue was added to a final concentration of 0.05%. After 1 min of incubation, GFP and trypan blue signals were visualized at room temperature using a Zeiss LSM510 invert confocal laser scanning microscope (objective lens, 100×/1.3 oil; optical section, <0.9 μm; multitrack mode; GFP: λ excitation, 488 nm, argon laser; band pass filter, 500–530 nm; trypan blue: λ excitation, 543 nm, helium–neon laser; long pass filter, 560 nm). The overlay of both signals was computed using the Zeiss LSM510 software (release 3.2; Carl Zeiss AG, Jena, Germany). Images were imported into Photoshop software (release 6.0; Adobe Systems Inc., San Jose, CA), and contrast was adjusted to approximate the original image.

Immunoprecipitation, EndoH and PNGaseF Treatment, SDS-PAGE, and Immunoblotting—After transfection, cells were cultivated for 24 h, washed twice with PBS-CM (PBS, 1 mM MgCl2, 0.1 mM CaCl2, pH 7.4) and lysed for 1 h with 1 ml of lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, pH 8.0) supplemented with 0.5 mM PMSF, 0.5 mM benzamidine, 1.4 μg/ml aprotinin, and 3.2 μg/ml trypsin inhibitor). Insoluble debris were removed by centrifugation (30 min at 12,000 × g). The supernatant was supplemented with a 1:200 dilution of monoclonal mouse anti-TSHR antibody (4C1 (1 mg/ml), Serotec) coupled to protein A-Sepharose CL-4B beads (3.5 mg/1 ml of PBS), and the samples were incubated overnight. Receptors were precipitated (2 min at 700 × g) and washed once with 1 ml of washing buffer 1 (50 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 0.5% (v/v) Triton X-100, pH 8.0) and twice with 1 ml of washing buffer 2 (50 mM Tris–HCl, 1 mM EDTA, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, pH 7.4). Precipitated receptors were treated with EndoH or PNGaseF according to the supplier’s recommendations or left untreated. Samples were supplemented with RotiLoad sample buffer (Carl Roth GmbH and Co. KG), incubated for 5 min at 95 °C, and analyzed by SDS-PAGE (8% SDS) and immunoblotting. Immunoblots were carried out as described (41) using a 1:200 dilution of a monoclonal mouse anti-human TSHR antibody directed against an extracellular domain (2C11 (1 mg/ml), Serotec) and a 1:10,000 dilution of DyLight680-conjugated goat anti-mouse IgG (1 mg/ml; Pierce) as secondary antibody. The quantification was done using an infrared imager (Odyssey 2.1, LI-COR Biosciences).

Determination of Specific Constitutive Activity (SCA)—Because of the linear relationship between basal cAMP accumulation and receptor cell surface expression (41), basal cAMP was normalized to cell surface expression for each of the constructs. To this end, specific basal (constitutive) activity was defined as described recently by Mueller et al. (42). The values were normalized to the basal activity of the WT TSHR, which was set to 1.

RESULTS

Side Chain Variations of Alanine S93 to Proline, Valine, and Glycine Cause Fold Defects or Constitutive Activation of Signaling—In most of the family A GPCRs, a proline is found at position 5.50 in TMH5 (for a review, see Ref. 7). Like all other GPHRs, the TSHR possess an alanine in TMH5 instead of a proline. Therefore, we exchanged Ala-5935.50 to proline but also replaced this alanine by larger and smaller side chains to reveal insights into the functionality of this position.

Cell Surface Expression—Cell surface expression of the mutants A593P, A593V, and A593G (Table 1) was decreased in comparison with the wild type; in particular, mutant A593P reached only 6% of the wild type level. The fact that cell surface expression of mutant A593P was strongly reduced could be explained by a folding defect of the receptor caused by the mutation. In this case, the mutant receptor should be recognized and retained by the quality control system of the early secretory pathway and finally subjected to proteolysis by the endoplasmic reticulum-associated degradation pathway. To address the question of whether the mutant is retained intracellularly, the wild type and mutant receptors were C-terminally tagged with GFP. HEK 293 cells were transiently transfected with the constructs, and co-localization of the GFP fluorescence signals of the receptor with those of the plasma membrane marker trypan blue was analyzed by confocal laser scanning microscopy (Fig. 2A).

In the case of the wild type TSHR–GFP, both plasma membrane and intracellular signals were detectable, demonstrating that the receptor reaches the cell surface. The intracellular sig-

### Table 1

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<th>Cell surface expression, FACS percentage of TSHR WT</th>
<th>cAMP accumulation</th>
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Functional characterization of TSHR mutations at Ala-593 in TMH5 (position 5.50)

HEK 293 cells were transiently transfected with the WT TSHR or mutated TSHRs. The TSHR cell surface expression levels were quantified by flow cytometry measurements and expressed as the percentage of the wild type control. Data represent mean values of two independent experiments each carried out in duplicates ± S.E. Accumulation of cAMP following TSH stimulation of intact cells was measured by a cAMP RIA. Data represent mean values of two independent experiments, each carried out in duplicates ± S.E. EC50 values are shown as the geometric mean (95% confidence limits). The assignment of the SCA was analyzed as described by Mueller et al. (42).
FIGURE 2. Receptor mutant A593P is unable to leave early secretory pathway in transiently transfected HEK 293 cells due to misfold. A, subcellular localization of the constructs TSHR-GFP and TSHR-A593P-GFP by confocal laser scanning microscopy. The GFP fluorescence signals of the receptors (green; left panel) were recorded and computer-overlaid with trypan blue plasma membrane signals (red; center panel). Co-localization is indicated by yellow color (right panel). The horizontal (x) scans show representative cells. Scale bar, 10 μm. Similar data were obtained in three independent experiments.

B, quantification of the untagged TSHR and mutant A593P at the cell surface by flow cytometry measurements. Plasma membrane receptors of 1 × 10⁶ cells were quantified using a monoclonal mouse anti-TSHR antibody and detected by SDS-PAGE/immunoblotting using a monoclonal mouse anti-TSHR antibody and DyLight680-conjugated goat anti-mouse IgG. Columns represent mean values of three independent experiments (± S.E.). C, glycosylation state analysis of the untagged TSHR and mutant A593P TSHR. Receptors were immunoprecipitated from cell lysates using a monoconal mouse anti-TSHR antibody and detected by SDS-PAGE/immunoblotting using a monoclonal mouse anti-TSHR antibody and DyLight680-conjugated goat anti-mouse IgG. The immunoblot is representative of three independent experiments. D, verification of the identity of the protein bands for the untagged TSHR. The receptors were precipitated and detected as described above. Samples were treated with EndoH or PNGaseF prior to SDS-PAGE or left untreated (−). High mannose-glycosylated receptors are EndoH- and PNGaseF-sensitive (§); complex glycosylated receptors are EndoH-resistant and PNGaseF-sensitive (§). Non-glycosylated receptors are indicated (§). The immunoblot is representative of three independent experiments.

nals may represent transport intermediates en route to the cell surface and/or a misfolded receptor population present even in the case of the wild type receptor. In contrast, only intracellular signals were detectable in the case of mutant TSHR-A593P-GFP. In agreement with these results, only the wild type untagged TSHR could be detected at the cell surface in flow cytometry measurements using an antibody directed against the TSHR N-terminal tail (Fig. 2B). If the mutant receptor is indeed retained in the early secretory pathway, only immature glycosylated forms of the receptors should be present. To address this question, the untagged receptors were precipitated from transiently transfected HEK 293 cells and detected by SDS-PAGE/immunoblotting (Fig. 2C). For the wild type receptor, two immunoreactive protein bands were detected. The upper band represents mature complex glycosylated receptors, and the lower band represents immature high mannose-glycosylated receptors (identity of the protein bands was verified by analysis of the glycosylation state using EndoH and PNGaseF treatments; see Fig. 2D). In the case of mutant A593P, only high mannose forms were found, demonstrating that the receptor is unable to leave the early secretory pathway. These data indicate that the A593P mutation indeed causes a folding defect that is recognized by the quality control system of the early secretory pathway.

CAMP Accumulation Determined by RIA Measurements—We determined basal and TSH-induced cAMP accumulation to characterize signaling properties of the mutants. The maximum cAMP accumulation of the mutants A593V and A593G after stimulation with bovine TSH was comparable with that of wild type (Table 1).

For the mutants A593V, A593G, and A593P, we also measured concentration-response curves (10 points; Fig. 3). The mutant EC₅₀ values were comparable with wild type. For the mutant A593P, decreased basal and maximum values were obtained; these might be caused by the lower cell surface expression levels (Table 1).

Mutation A593G induced constitutive TSHR activation, which was confirmed by SCA determination (Table 1). Mutant A593P also showed a 3-fold increase in SCA with a cell surface expression of around 7% compared with wild type.

Regular α-Helical Conformation of TMH5 in TSHR—Taking these functional data into consideration, we conclude that TSHR alanine 593 likely causes a different helix conformation in TMH5 compared with other family A GPCRs that have the conserved proline at this position.

Moreover, an initial TSHR model (model 1) based on the available GPCR crystal structures resulted in a twisted helix 5 caused by a proline-induced bulge, which is not consistent with particular TSHR mutation data. In model 1, the side chain of Leu-587 points toward the membrane (Fig. 1), but even the slight side-chain alteration L587V led to constitutive activation. However, a regular α-helical conformation of TMH5 in a modified TSHR model (model 2) showed more consistency with the mutation data. In this second model, the spatially shifted side-chain orientation of Leu-587 points toward TMH6 and interacts with Leu-645, mutation of which also led to con-
Determination of IP accumulation was done by inositol incorporation studies, and results for cAMP accumulation were measured by RIA. Data are given as means ± S.E. of two independent experiments, each carried out in duplicate. EC50 values are shown as the geometric mean (95% confidence limits). ND, not determined.

**FIGURE 4. Amino acids constituting potential interface between helices 5 and 6 of TSHR.** The homology model of the TSHR serpentine domain (white backbone) with amino acid side chains (sticks) potentially constituting the interface between TMH5 and TMH6 (both helices are colored brown) shows the spatial localization and orientation of side chains. Residues that were not investigated previously but studied here by side-chain browning the interface between TMH5 and TMH6 (both helices are colored brown) are depicted in green, and their labels are bold and enlarged. ICL, intracellular loop; ECL, extracellular loop; Nt, N-terminal tail; Ctr, C-terminal tail.

**TABLE 2**

Functional characterization of TSHR mutations in TMH5 and TMH6

HEK 293 cells were transiently transfected with the wild type or mutant TSHRs. The cell surface expression levels of the receptors were quantified by flow cytometry measurements and expressed as the percentage of the wild type control. Accumulation of cAMP following TSH stimulation was determined by cAMP RIA. Determination of IP accumulation was done by [3H]inositol incorporation studies, and results for cAMP accumulation were measured by RIA. Data are given as mean ± S.E. of two independent experiments, each carried out in duplicate. EC50 values are shown as the geometric mean (95% confidence limits). ND, not determined.

![Signal Transduction and Regulation at TSHR](image)

- **cAMP accumulation**
  - Basal n-fold wt
  - EC50 n-fold wt
  - TSH-stimulated percentage of TSHR
  - SCA
- **IP accumulation**
  - Basal n-fold WT
  - TSH-stimulated percentage of TSHR

**Transfected construct**

- **Ballesteros-Weinstein numbering**
- **Cell surface expression, percentage of TSHR WT**

**WT TSHR**

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<th>Transfected construct</th>
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<th>cAMP accumulation</th>
<th>IP accumulation</th>
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<td>F594I</td>
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* Amino acids with decreased basal signaling activity by mutation.
* Mutations with an increased or slightly increased EC50 compared with wild type.
whereas mutants F594I and F634I showed slightly increased EC50 values. Four mutants (F594I in TMH5 and F634I, A638V, and I648V in TMH6) showed decreased basal signaling activity of TSHR (Table 2), whereas A644V was characterized by a slightly increased constitutive activity of 1.7-fold over wild type.

IP Determination—Single side-chain substitutions F634I, A638V, F642I, and A644V in TMH6 and F594I in TMH5 were found to decrease the capability of the TSHR for basal cAMP accumulation and to partially decrease the capability of the TSHR for TSH-stimulated cAMP accumulation. Therefore, for these mutations, we also measured the Gq-mediated IP accumulation, which is known to be of physiological relevance to thyroid growth (44). All of these mutations led to a strong decrease in the maximum IP accumulation induced by bovine TSH in a range between 8 and 41% compared with wild type.

Sequence Conservation and Spatial Distribution of Polar and Non-polar Residues Analyzed in Family A GPCR Sequences—According to the model of the active TSHR conformation, the following direct side-chain interactions occur: Leu-5805.37–Ile-6486.59, Ile-5835.40–Ile-6486.59, Leu-5875.44–Leu-6456.56, Phe-5945.51–Phe-6346.45, (and Met-6376.48), Cys-5985.55–Ile-6306.41, and Tyr-6015.58–Met-6266.37 (the first residue of each pair is located in TMH5, and the second is located in TMH6). The dynamic process of helical movement in the transition between both conformations (inactive/active) can be studied in detail using structural morphings to visualize the helical shifts and side-chain reorientations that potentially occur during TSHR activation.

However, the majority of mutations at these positions led to (partial) receptor inactivation (Table 2 and Figs. 5 and 6), supporting their significance to the active conformation. It can be concluded that interactions between helices 5 and 6 are essential for stabilization of the active state, and it is of note that these wild type residues are exclusively hydrophobic. Therefore, we analyzed the spatial distribution of the conserved polar and non-polar residues in family A GPCRs. The frequencies were calculated from a set of 574 family A GPCRs with a sequence similarity of 70% or less in the transmembrane helix regions. A set of polar residues in TMHs 1, 2, 3, and 7 is conserved throughout family A and most likely helps to stabilize the inactive state (Fig. 7). Non-polar residues are conserved in TMHs 3, 5, and 6 and form a non-polar interface.

DISCUSSION

One of the important questions of signal transduction by GPCRs relates to the details of the structural-functional interplay between TMH5 and TMH6. Here we ask the question of which of the residues that are forming the interface of TMH5 and TMH6 are responsible for stabilization of the basal state and which residues are responsible for movement of the helices to support the active conformation. Inspection of the research resource for glycoprotein receptors (43) revealed for the TSHR signal transduction and regulation at TSHR.
that 13 of the potential 24 interface residues have previously been characterized by mutations. Receptor mutations are known to generate active and inactive receptor conformations. Thus, we hypothesized that mutational analysis of the remaining amino acids would then complete the data for the interface between TMH5 and TMH6 and would provide insights into the intramolecular mechanisms underlying stabilization and activation. Here we combined the structural data of inactive and active GPCR conformations and the statistical occurrence of amino acid properties at helix interfaces in a large set of GPCR sequences with functional data from the TSHR to pinpoint determinants that regulate different receptor conformations.

Interplaying Transmembrane Helices 5 and 6 Regulate Receptor Conformations and Activity States—In agreement with others (45), we assume that constitutively activating mutations (CAMs) lead to a disturbance of the GPCR ground state by supporting the active conformation. In contrast, inactivating mutations that do not impair receptor cell surface expression, binding properties, or direct interactions with the G-protein might prevent stabilization of the activated state, which is normally conducted by the wild type amino acid. In other words, inactivating mutations are indicators of wild type amino acids acting as stabilizers or conductors of activated conformation(s). We mapped the functional data from this study together with previously published mutagenesis studies onto the TSHR homology models (Fig. 5) to reveal deeper insights into the role and interplay between TMH5 and TMH6 (see Fig. 5B for references of mutants at particular positions). We found that particular interacting pairs of residues also show concurrent functional data by mutations. Our conclusions from the combined functional-structural insights for pairwise residue interactions between TMH5 and TMH6 follow.

The side-chain interaction between leucine 5875.44 (TMH5) and leucine 6456.56 (TMH6) close to the extracellular site is supported by constitutive TSHR activation induced by mutations at both positions (46). This interaction was disrupted by side-chain alterations of either Leu-5875.44 or Leu-6456.56 and led finally to an activated receptor conformation. Mutation of the two alanines, Ala-6386.49 and Ala-6446.55, in TMH6 to valine, an enlargement of the side chain, resulted in partial inactivation of TSHR whereby A644V mediated a slight increase in basal cAMP accumulation. These modifications cause steric repulsion to surrounding amino acids and likely cause steric hindrance of the spatial rearrangement of TMH6. In contrast, side-chain substitutions of the two phenylalanines 5945.51 and non-polar (hydrophobic) residues (shown in red and green, respectively) are mapped onto the three-dimensional structure of inactive bovine rhodopsin (Protein Data Bank code 1U19). The frequencies were calculated from a set of 574 family A GPCRs with a sequence similarity of 70% or less in the transmembrane helix regions. A, a set of polar residues in TMHs 1, 2, 3, and 7 is conserved throughout family A, and these residues most likely help to stabilize the inactive state. Non-polar residues are conserved in TMHs 3, 5, and 6 and form a non-polar interface that is probably important for stabilizing the active state. B, the conservation of these central polar core residues and the non-polar interfaces throughout family A suggests a fundamental role for them in activity regulation.

**FIGURE 7. Spatial distribution of conservation degree of polar and non-polar residues in 574 family A GPCRs.** The frequency of occurrence for polar and non-polar (hydrophobic) residues (shown in red and green, respectively) are mapped onto the three-dimensional structure of inactive bovine rhodopsin (Protein Data Bank code 1U19). The frequencies were calculated from a set of 574 family A GPCRs with a sequence similarity of 70% or less in the transmembrane helix regions. A, a set of polar residues in TMHs 1, 2, 3, and 7 is conserved throughout family A, and these residues most likely help to stabilize the inactive state. Non-polar residues are conserved in TMHs 3, 5, and 6 and form a non-polar interface that is probably important for stabilizing the active state. B, the conservation of these central polar core residues and the non-polar interfaces throughout family A suggests a fundamental role for them in activity regulation.
(TMH5) and 634.6-45 (TMH6) led to a partially decreased TSHR signaling capability (Table 2), suggesting the involvement of these opposing positions (Fig. 5) in the maintenance of an active TSHR conformation by aromatic-hydrophobic residue interactions. Both residues might be essential for a "microswitch" mechanism together with TMH6 residues (see above). Furthermore, the residues Tyr-601.5-58 (TMH5) and Met-626.6-37 (TMH6) are located spatially close to each other two turns along toward the intracellular side. For both residues, CAMs and inactivating mutations are reported, indicating that these residues are likely to be involved in stabilizing the activated as well as basal conformations (dual functionality; Fig. 5). It is particularly interesting that these residues change their relative spatial orientation during the activation-related helical movement at the intracellular end. In the active conformation, the amino acid Tyr-601.5-58 points toward TMH3 and forms an interaction network with Arg-5193.50 (TMH3) and Tyr-6787.73 (TMH7) (Fig. 6). For Tyr-605.6-62 (TMH5) and Ile-622.6-33 (TMH6), inactivating mutations have been reported (see Fig. 5B references). With respect to their location close to the intracellular side, they are potential points for direct G-protein interaction (13). Close to the intracellular end of TMH6, CAMs are known at Asp-6196.30, a residue thought to participate in a helix-capping mechanism (47) or to establish a salt bridge with Arg-5193.50 (TMH3) (for a review, see Ref. 7) in the inactive conformation. The potential interaction partner in the inactive state, Arg-5193.50 (TMH3), forms a hydrogen bond with Tyr-601.5-58 (TMH5) in the active conformation (Fig. 6C). It can be summarized that the arginine at position 3.50 (TMH3) is a key player for stabilization of both the inactive and active conformations and also directly interacts with the α-subunit of G-proteins (21). With respect to family A GPCRs without a positively charged amino acid residue at this position, alternative mechanisms must be assumed (for reviews, see Refs. 48 and 49).

For one functional observation, a detailed explanation cannot be fully provided by a monomeric TSHR model. The side chain of Phe-6426.53 points toward the membrane, and mutation to isoleucine led to partial TSHR inactivation. Only a potential dimer arrangement of the TSHR where the contact interface would be defined at or close to TMH6 could provide a direct (intermolecular) interaction partner. TSHR dimer conformations have been shown to be obligate (50), and it has been demonstrated that intermolecular contacts in the transmembrane region are the main determinants of such TSHR and GPHR dimer arrangements (51-53). Known phenomena published for the TSHR support the hypothesis of TMH6 contacts between TSHR monomers like the occurrence of pathogenic CAMs at TMH6 residues, which also point toward the membrane (F631V (54) or I635V (55)). For such mutations, mechanisms of activation might be determined by the modification of the potential monomer-monomer interface related to activity states of the TSHR (56). Our mutation at Phe-6426.53 supports this hypothesis.

Position 5.50 and TMH5 Are Significant for Receptor Signal Transduction—The TMH5 of TSHR lacks a proline that is conserved in most other family A GPCRs. The proline mutation of Ala-5935.50 in TMH5 led to a misfolded TSHR. Our initial TSHR model based on a proline-containing structural template showed inconsistencies with other TMH5 mutation data as well. Therefore, for TMH5 of the TSHR, we suggest an alternative model, model 2, which has a regular α-helix for TMH5 in contrast to the available crystal structures of family A GPCRs. Interestingly, the mutants A593G and A593P showed an increased basal activity, which indicates the importance of orientation and adjustment of TMH5 in relation to other helices for the activation mechanism. Furthermore, at TSHR position Ala-593.5-50, the pathogenic CAM A593N (57) and the pathogenic inactivating mutation A593V (58) have been reported. Based on our functional characterization of the A593G and A593V mutations, we can extract two important results related to these pathogenic findings. (i) The pathogenic inactivating mutant A593V is characterized by a decreased expression and decreased basal activity (not TSH-induced stimulation), which might therefore be a main reason for the observed mild congenital primary hypothyroidism. (ii) This impeded basal activity is likely caused by altered interaction with the counterpart amino acid Val-5093.40 in TMH3 (59). From our models, we assume that contact between the side chains Ala-5935.50 and Val-5093.40 is involved with regulation of the basally active conformation. Introduction of a valine at position 5.50 in TMH5 led to a more retained interaction between the two valine side chains by interlocking and as a consequence of restriction of the TMH5-TMH3 interplay at this particular site. Thus, the capacity for release or an adequate flexibility of this interacting residue pair, 5.50-3.40, might be important for receptor activity regulation in the TSHR. This is supported by our constitutive active mutations A593G and A593P and the pathogenic CAMs A593N (58) and V509A (60). Moreover, this principle finding corresponds with recent insights from the crystal structure of an activated β2-adrenergic receptor conformation where position 5.50 in TMH5 and the region around position 3.40 were found to be directly related to receptor signaling regulation (60). This insight from the comparison of recent GPCR structures is in accordance with results of studies on the histamine H1 receptor (61).

Polar Side-chain Interactions between TMHs 2, 3, 6, and 7 Constrain Inactive Conformation, whereas Hydrophobic Interactions between TMHs 5 and 6 Constrain Active Conformation—From the above described and discussed observations, a main message can be extracted: only two mutations in TMH5 are reported to cause slight constitutive TSHR activation. In contrast, several mutations are reported for TMH5 that lead to receptor signaling inactivation. Based on these facts as well as the higher number of direct contacts in the activated compared with the inactive TSHR model, we here suggest a dominantly "stabilizing" function of TMH5 for TMH6 in the active state. How is this hypothesis in accordance with and supported by previous findings for the TSHR?

In the TSHR, a methionine is located at position 6.48 in TMH6 instead of a tryptophan, which is found in around 80% of the rhodopsin-like GPCRs (22). This tryptophan moves slightly during activation and stabilizes the active GPCR conformation by forming new interactions with TMH5 (for a review, see Ref. 62). It is assumed that in the active state the aromatic tryptophan interacts with position 5.47 in TMH5. The significance of
this tryptophan in ligand-induced signal transduction was shown for rhodopsin (63) and other GPCRs like the adenosine receptor (64) and 5-HT2 receptor (65). Furthermore, this tryptophan is also known to be involved in the maintenance of the basal state, which has been investigated for the thyrotropin-releasing hormone receptor subtypes 1 and 2 (66). Methionine 6.48 in the TSHR (Met-6376.48) might undergo comparable movement toward TMH5 during activation as assumed for the tryptophan in other GPCRs and interact in the active state with aromatic residues in helices 5 and 6 (Phe-5945.51 and Phe-6346.44). Interestingly, introduction of an aromatic and bulky tryptophan to TSHR at position 6.48 instead of the methionine cannot be tolerated spatially and leads to repulsion by surrounding amino acids and hence causes constitutive receptor activation (46).

It is known that conserved hydrophilic polar amino acids in the TSHR transmembrane region like Asp-6336.44 (TMH6), Asn-6747.49 (TMH7), Asp-4603.50 (TMH2), and Arg-5193.50 (TMH3) are mandatory for the signal transduction process (67–70). Arginine 519 at position 3.50 is a key player in many family A GPCRs. Aspartate 4603.50, Asn-6747.49, and Asp-6336.44 interact (68, 70) by hydrophilic interactions and stabilize the inactive conformation (Fig. 6C). For these amino acids, several CAMs have been identified by mutagenesis studies or by naturally occurring mutations.

From Details of Microswitches in TSHR to General Intramolecular GPCR Activation Mechanism—Taking this detailed information from functional studies and structural insights into account, we suggest the following scenario for activation of the TSHR in the transmembrane region. Polar contacts between TMHs 2, 3, 6, and 7 preserve the inactive receptor state. Water molecules might participate in this interaction network as suggested for rhodopsin (71), β2-adrenergic receptor (72), and the MC4R (73). Signal induction (by ligands or mutation) must release such constraints for movement of TMH6 to take place (Figs. 5 and 6). Induced by this release, the intracellular end of TMH6 moves outward as observed in the active opsin crystal structure. Whereas TMH6 is likely to be involved in stabilization of both the inactive and the active state, TMH5 functions as an “active state stabilizer” in interplay with TMH6 (Fig. 6C). In contrast to the outward movement of TMH6, the TMH5 undergoes a slight inward movement (Fig. 6A). This bidirectional rearrangement is locked by specific and mostly non-polar residue interactions with the TMH5-TMH6 interface (Fig. 6C), which might also prevent a movement of TMH6 back into the inactive conformation. In consequence, the TSHR can be subdivided into two main regions: 1) The inactive state is constrained by a polar core set between TMHs 2, 3, and 7 (Fig. 6C, pink half), 2) stabilization of the active state is supported by hydrophobic interfaces between TMH5 and TMH6 (Fig. 6C, green half). Particular amino acids contribute to this process of helical rearrangement, which might work sequentially as revealed by insights from studies on rhodopsin (74).

To assess whether our data can be generally applied to family A GPCRs, we statistically analyzed the occurrence of polar and non-polar residues in all TMHs in a large set of family A GPCRs. The amino acid sequences of the transmembrane helices of 574 family A GPCRs were taken, and the frequency of polar and non-polar amino acids at each position of the transmembrane helices was calculated (Fig. 7). Indeed, a set of polar residues in TMHs 1, 2, 3, and 7 are conserved throughout family A and most likely help to stabilize the inactive state of the family A GPCRs. Non-polar residues are conserved in TMHs 3, 5, and 6 and form a non-polar interface that seems to be important for stabilizing the active state of family A GPCRs. Conservation of these central polar core residues and the non-polar interfaces throughout family A suggests their fundamental role in regulating family A GPCR activity.

Taken together, we have described mechanisms of TSHR activity regulation at the molecular level. Our results together with previous data enabled us to assign particular residues of the TMH5-TMH6 interface as interacting pairs with functional relevance. Interacting residues showing constitutive activation by mutation indicate determinants for stabilization of the basal (non-active) conformation. Residue pairs showing inactivating mutations indicate their involvement in forming the active conformation. We suggest that the inactive TSHR conformation (and that of other family A GPCRs) is constrained by interacting polar residues between TMHs 2, 3, and 6, whereas interacting hydrophobic residues between TMH5 and TMH6 constrain the active state. We also suggest that the TMH5 in TSHR does not have a proline-induced helical kink and bulge and has a different conformation compared with other GPCRs. A detailed understanding of intramolecular mechanisms for GPCR activity regulation is of vital importance because it should also reveal molecular knowledge for better understanding dysfunctions of GPCRs.

REFERENCES