A Conserved Asn in Transmembrane Helix 7 Is an On/Off Switch in the Activation of the Thyrotropin Receptor*

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The thyrotropin (TSH) receptor is an interesting model to study G protein-coupled receptor activation as many point mutations can significantly increase its basal activity. Here, we identified a molecular interaction between Asp633 in transmembrane helix 6 (TM6) and Asn672 in TM7 of the TSHr that is crucial to maintain the inactive state through conformational constraint of the Asn. We show that these residues are perfectly conserved in the glycohormone receptor family, except in one case, where they are exchanged, suggesting a direct interaction. Molecular modeling of the TSHr, based on the high resolution structure of rhodopsin, strongly favors this hypothesis. Our approach combining site-directed mutagenesis with molecular modeling shows that mutations disrupting this interaction, like the D633A mutation in TM6, lead to high constitutive activation.

The strongly activating N674D (TM7) mutation, which in our modeling breaks the TM6-TM7 link, is reverted to wild type-like behavior by an additional D633N mutation (TM6), which would restore this link. Moreover, we show that the Asn of TM7 (conserved in most G protein-coupled receptors) is mandatory for ligand-induced cAMP accumulation, suggesting an active role of this residue in activation. In the TSHr, the conformation of this Asn residue of TM7 would be constrained, in the inactive state, by its Asp partner in TM6.

The TSH, LH/CGr and FSH receptors constitute a subfamily of G protein-coupled receptors (GPCR) characterized by large amino-terminal ectodomains responsible for high affinity binding of their natural agonists, the glycoprotein hormones (1, 2). These ectodomains are made essentially of leucine-rich repeats, a protein fold frequently found to be involved in protein-protein interactions (3–5). The serpentine portion of these receptors, responsible for signal transduction, comprises seven transmembrane helices, showing significant similarity with the transmembrane portions of rhodopsin-like GPCRs; they are therefore grouped with them into family 1 of GPCRs.

The glycohormone receptors thus present a clear dichotomy between the agonist-binding and signal-transduction domains, with the mechanism of interaction between the two domains, responsible for receptor function, remaining largely unknown. Sequences displaying strong similarities with the glycohormone receptors have been identified in Anthopleura elegansissima, Drosophila, and Caenorhabditis elegans (6–9). Related receptors have also been discovered in mammals (10, 11). But for all of these cases, the nature of the agonists remains to be identified.

Several characteristics make the TSH receptor an interesting system to study the mechanisms of receptor activation: (i) the wild type receptor has been shown to display significant basal activity (12–14); (ii) mutations involving more than 20 different residues have been shown to increase its constitutive activity, causing autonomous thyroid adenomas or non-autoimmune hereditary hyperthyroidism (15); (iii) loss of function mutations have also been described, abolishing basal activity (16) or affecting agonist-induced response (17, 18).

Interestingly, naturally occurring activating mutations are more rarely found in the LH/CGr (19), and almost never found the FShr (20). Indeed, no spontaneous activating mutation has been identified in the FSH receptor except for a single case with an unusual phenotype (21). These data reflect a difference in the structural constraints keeping the various glycoprotein hormone receptors in the inactive state. The wild type TSH receptor displaying constitutive activity and being easily activated by mutations would be the less constrained, followed by the LH receptor, devoid of constitutive activity but susceptible to activation by mutations. The FSH receptor would be the most constrained, as it is silent in the absence of stimulation by agonist and difficult to activate by mutations.

The high resolution crystal structure of bovine rhodopsin determined recently (22) provides for the first time a detailed atomic description of a GPCR molecule in an inactive conformation, and represents a solid basis for modeling the structures of other rhodopsin-like GPCRs. Such models can then be used to help rationalize the many observations made on the relations between conserved sequence feature and functional properties. Conservation of functionally important sequence motifs within this receptor family has been interpreted as meaning that the basic characteristics of the rhodopsin fold, as
well as the molecular mechanisms leading to receptor activation are similar in the different receptor subtypes. Among such conserved sequence motifs are prolines in transmembrane helices (TM) 6 and 7 and charged residues in TM2 and TM3 (the Asp and Arg of the canonical “DRY” motif). However, considering the extreme diversity of the natural agonists of the different receptors it has also been accepted that different receptor subtypes may have evolved quite specific structural and functional features, probably reflected in the specific sequence signatures of each subtype.

Multiple alignments of the glycohormone receptor sequences reveal one such signature: a conserved Asp residue in TM6 at position 6.44 (corresponding to residue 633 in the TSHr sequence; see “ Experimental Procedures”). Most other GPCRs harbor a Phe or a Tyr at this position. Natural mutations of sequence; see “Experimental Procedures”). Most other GPCRs

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(29). Ionizable groups in the helices were modeled as uncharged except for Asp2.50, Glu3.37, Glu3.49, Arg3.50, Asp6.30, and Asp6.44.

This residue was identified by two numbers: the first (1 through 7) corresponds to the helix in which it is located; the second indicates its position relative to the most conserved residue in that helix, arbitrarily assigned to 50. For instance, N7.49 is the asparagine in transmembrane helix 7 (TM7), 50 residues from the highly conserved proline (6.44). Residues D2.50 correspond to Asp4.46 in the human TSHr sequence; S3.36 to Ser405, S3.39 to Ser408, T6.43 to Thr522, D6.44 to Asp633, N7.45 to Asp670, and N7.49 to Asn674.

Molecular Modeling and Molecular Dynamics Simulation of the Transmembrane Bundle—The atomic model of the transmembrane domain of the TSHr was built by comparative modeling techniques, using as template the atomic coordinates of the transmembrane domain of bovine rhodopsin (22). The sequences of the 2 proteins in this region were aligned so as to equivalence the positions of the following conserved residues: Asn65,Asn1.50(22) (the superscripts represent the residue numbering in rhodopsin structure PDB code 1F88, and human TSHr sequence, respectively, and 1.50 is the numbering in the stand-

standardized nomenclature), Asp83-Asp5.00(44), Arg3.50(29), Trp3.49, Trp5.046, Pro1.50(29), Arg2.50(29), Pro2.50(29), Pro3.50(29), and Pro5.50(29). The conformations of these side chains were kept as in the rhodopsin crystal structure. Those of the non-conserved amino acids were built using a rotamer library specific for α-helices. (29). Ionizable groups in the helices were modeled as uncharged except for Asp2.50, Glu3.37, Glu3.49, Arg3.50, Asp6.30, and Asp6.44.

To believe residual strain was arising from suboptimal positioning of the side chains, the resulting model was first subjected to energy minimization (1000 steps), and then to a simulated annealing proce-

shakes bond constraints on all bonds, &s integration time

To start the simulation protein portion was kept fixed while the

order to mimic the hydrophobic environment of the membrane, these

sequences were carried out in the presence of explicit methane mole-

ules, and using periodic boundary conditions. The periodic box was

~ 73 Å × 63 Å × 52 Å in size, and contained between 4219 and 4241 methane molecules in addition to the transmembrane domain. Similar conditions have been recently used to mimic the membrane environment in molecular dynamics simulations of the potassium channel (30).

To start the simulation protein portion was kept fixed while the

molecules were energy minimized (500 steps), then heated to

300 K for 15 ps and equilibrated for another 35 ps. Following this, the same procedure was repeated on the entire protein-solvent system but

with an equilibration run of 250 ps; a 250 ps production trajectory was then generated at constant volume using the Particle Mesh Ewald method for computing electrostatic interactions (31). For analysis pur-

poses, structures were collected every 2 ps. The simulations were per-

formed with the Sander module of AMBER 5 (32), the all-atom force

field (33), SHAKE bond constraints on all bonds, a 2 fs integration time step, and constant temperature of 300 K coupled to a heat bath.

Site-directed Mutagenesis of the TSH Receptor—Plasmids encoding the various TSHr mutants were constructed by site-directed mutagenesis using two subsequent PCR amplifications rounds. This procedure requires two partially overlapping complementary primers containing the mutation and two external primers. Two distinct PCRs are performed on TSHr template by using in one tube the direct mutagenic primer and the external reverse primer and in the other tube the reverse mutagenic primer and the external direct primer. One µl of each PCR product was mixed and used as template in a subsequent PCR amplification with the two external primers. The resulting amplified fragment contains the desired mutation and can be cloned into a pSVL expression vector with BstEIII and BamHI into the pSVL expression vector (Amersham Pharmacia Biotech, Freiburg, Germany) containing the wild type TSHr (34) using standard procedures. All PCR-generated receptor frag-

ments were verified by sequencing before transfection.

Transfection and Assays—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%),

EXPERIMENTAL PROCEDURES

Numbering Scheme of GPCRs—The standardized numbering system of Ballesteros and Weinstein (28) was used throughout to identify residues in the transmembrane segments of different receptors. Each
TM6

|       | 6.30 | 6.44 | 5.50 | 6.60 | 7.33 | 7.45 | 7.49 | 7.53 |
|-------|------|------|------|------|------|------|------|------|
| TSHr (human) | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| TSHr (mouse)  | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| LIFr (human)  | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| LIFr (mouse)  | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| LIFr (chicken) | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| FSHr (human)  | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| FSHr (rat)    | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| FSHr (chicken) | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| fnr (newt)    | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| fnr (salmon)  | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| fnr (bass)    | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| fnr (sea anemone) | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| fnr (C. elegans) | VTKKRMAVSLI | FTDPCDASFLPSYASAI | KPLTVV | SKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |
| rhodopsin (bovine) | KSKLIVLF | YPDSSICNTSLV | ATFFKYA | TAKAPF | CR |

**RESULTS**

**Sequence Alignment of the Glycohormone Receptor Subfamily**

Multiple sequence alignment of the sixth and seventh transmembrane helices of 44 glycophorine and closely related receptors was performed. Fig. 1 shows the alignment of representative sequences, together with that of bovine rhodopsin. Inspection of this alignment reveals two key sequence motifs characteristic of the rhodopsin-like family: the P6.50 in TM6 and the NPXXY motif in TM7. A noteworthy difference is the presence of Asp at position 6.44 instead of the more common Phe or Tyr, which represent more than 50% of the rhodopsin-like receptor found in the GPCRDB (38). D6.44 is completely conserved throughout the glycohorine family of GPCR, from *C. elegans* to *human*, with only a single exception, the Drosophila DLG1 sequence (accession number U47005(6)), which contains an Asn residue. Interestingly, this change is correlated with the replacement of the highly conserved Asn at position 7.49 by Asp. Swapped mutations of this kind suggest interaction between the corresponding residues in the three-dimensional structures (26, 27). We could indeed verify that in the rhodopsin structure, the C<sub>6</sub> of Phe<sub>6.44</sub> and Asn<sub>7.49</sub> (the conserved N of the NPXXY motif) were facing each other at a distance of 11 Å, sufficient to allow direct interaction between the side chains. Given that a series of amino acid substitutions at D6.44 found in thyroid adenomas result in a significant increase in the constitutive activity of the TSHr, we then made the hypothesis that interactions between the D6.44 and N7.49 side chains are important in maintaining the receptor in its inactive conformation.

**Molecular Dynamics Simulation of the Transmembrane Region of the TSHr**

In order to investigate the proposed interaction between D6.44 and N7.49 in the context of the entire helix bundle, the
molecular model of the transmembrane domain of the TSHr was built, based on the high-resolution structure of bovine rhodopsin. This model was then subjected to unrestrained molecular dynamics simulations in the presence of explicit methane molecules mimicking the apolar membrane environment (see "Experimental Procedures"). Having ascertained that the helical segments conserved their secondary structure and that the bundle remained well packed and maintained the rhodopsin fold throughout the trajectory, we analyzed the polar interactions made between TM6 and TM7. In particular, we computed the average hydrogen-acceptor distances in conformations along the trajectory, and the fraction of the conformations in which the bond was formed.

All throughout the simulation D6.44 and N7.49 were seen to form a hydrogen bond between the D6.44 O and N7.49 N atoms, with an average O-H distance of 1.9 Å (see Fig. 2 for a representative structure). The TM6-TM7 interaction involved in addition a more complex hydrogen bond network. N7.45, located one helix turn prior to N7.49, formed 2 H-bonds. One, with D6.44 (N6.44 O-H-O6.44; average distance 2.0 Å) and another with N7.49 (N7.45 O-H-O6.44; average distance of 1.9 Å). The hydrogen bond between D6.44 and N7.45, thus provides a second polar interaction between TM6 and TM7. In addition, the D6.44 O was seen to form an H-bond with O-H group of the conserved S3.39, in TM3 (average distance of 1.8 Å), establishing a polar interaction between TM3 and TM6 as well.

Experimental Probing of the 6.44–7.49 Interaction

The existence and functional role of this predicted TM6-TM7 interaction was probed by mutating the residues involved and testing the functional consequences after transfection of the mutant constructs in COS-7 cells. The following mutants were engineered: (i) the D6.44N/N7.49D double mutant, made in order to mimic the situation of the Drosophila receptor in the context of the human TSHr; (ii) D6.44N and N7.49D single mutants, introduced in order to explore the effects of the individual mutations; (iii) D6.44A and N7.49A side chain deletion mutants engineered to test the effects of eliminating altogether...
the interactions between these side chains and any other residue. Following observations made from our molecular dynamics simulation of the TSHr and other studies on the LH/CG receptor (24) we designed two additional mutants, the single N7.45D substitution and the double substitution D6.44N/N7.45D. This was done in order to explore the possibility of a direct interaction between D6.44 and N7.45.

Cell Surface Expression of the Mutants

Cell surface expression of the mutated receptors was measured by FACS analysis using the BA8 monoclonal antibody which recognizes a conformational epitope in the NH2-terminal domain of the receptor. The data are representative of at least four different experiments. Values represent mean cell fluorescence, normalized to the value obtained for wt TSHr (error bars: S.E.).

Fig. 3. Level of expression of the receptors. Cell surface expression of wt TSHr and the different mutants measured by FACS using the BA8 monoclonal antibody which recognizes a conformational epitope in the NH2-terminal domain of the receptor. The data are representative of at least four different experiments. Values represent mean cell fluorescence, normalized to the value obtained for wt TSHr (error bars: S.E.).

The constitutive activity of the wt and mutant receptors was measured by FACS analysis using the BA8 monoclonal antibody which recognizes a conformational epitope in the NH2-terminal domain of the TSHr (1), we do not expect a modification of its affinity following mutations in the transmembrane region. Also, similar results were obtained with another monoclonal antibody (3G4), recognizing a different epitope in the ectodomain (not shown). Hence, it is assumed that observed fluorescence changes will be in direct relation with the number of receptors present at the cell surface. As shown in Fig. 3, mutants D6.44N, D6.44N/N7.49D, and N7.49A are expressed at levels comparable (above 50%) to that of the wild type TSHr. In contrast, mutants N7.49D, D6.44A, and the D6.44N/N7.45D double mutant display a reduced expression, between 15% and 30% of the wild type receptor expression. The level of expression of N7.45D is too low (specific fluorescence of about 3% of wt TSHr) to allow reliable normalization of functional results, it will therefore not be considered further.

Basal and TSH-stimulated cAMP Accumulation in COS-7 Cells Transfected with the Mutants

The constitutive activity of the wt and mutant receptors was characterized by measuring intracellular cAMP accumulation in transiently transfected COS-7 cells. As constitutive activity is linearly dependent on the number of receptors at the cell surface (39), we have normalized the basal activity of each construct using cell surface expression data yielded by FACS analyses (see "Experimental Procedures"). This allows to compute constitutive activity on a per receptor basis, and to compare it to that of the wild type receptor.

Mutants D6.44N, D6.44N/N7.49D, and N7.49A display basal activity similar to that of the wild type (Fig. 4A). Strikingly, the single mutants N7.49D and D6.44A show a dramatic increase in constitutive activity, reaching more than 15 times that of wild type TSHr, when normalized to the level of expression (Fig. 4B). Although the N7.49D mutant, bearing aspartate residues at both positions 6.44 (as in wild type) and 7.49 (introduced by mutation), is among the strongest constitutive mutant of the TSHr ever identified (37), it is remarkable that addition of the D6.44N mutation on the N7.49D background reverses completely the phenotype back to a wild type-like behavior. The D6.44N/N7.45D can also be considered as a constitutive mutant as its basal activity is more than five times that of wt TSHr (Fig. 4B).

We then tested the ability of TSH to stimulate the various constructs. Fig. 5 illustrates typical concentration-response curves and the corresponding EC50 are summarized in Table I. All mutants except N7.49A are stimulated by TSH in a way similar to the wild type. While starting from a higher basal level, the N7.49D mutant is activated efficiently by TSH, reaching comparable Emax (not shown). All EC50 values were in the range 0.4–1 milliunits/ml of TSH (Fig. 5, A and B, and Table I). Although the N7.49A mutant displays wt-like constitutive activity, TSH is unable to elicit signal transduction, as only very moderate stimulation if any can be observed at 100 milliunits/ml, a saturating concentration for wt TSHr (Fig. 5C).

Hormone Binding

The binding properties of the different receptors were tested by homologous competition using 125I-bovine TSH as tracer. As shown in Fig. 6, all constructs tested were able to bind TSH efficiently.

Mutants D6.44N and D6.44N/N7.49D bind TSH with similar affinity as the wild type receptor, as measured by the IC50 test (IC50 of about 3 milliunits/ml, see Table I and Fig. 6A). Noticeably, the various constitutive mutants display a significantly lower IC50 (below 1 milliunits/ml, see Table I and Fig. 6B). Increase in apparent affinity with no significant modification of hormone potency has been observed in most constitutive mutants of the TSH receptor (40). Interestingly, mutant N7.49A binds TSH with high affinity, even slightly higher than the wt receptor (Fig. 6A and Table I), although this mutant is completely unable to be activated by the hormone.

It should be noted that the residues identified here as key actors in the activation mechanism of the TSHr have also been studied by several groups on the LH receptor (24, 41). Overall these studies are in agreement with the results presented here. Note, however, that these studies did not consider double mutants in different TMs, which in our view are crucial to the identification of TM6-TM7 interactions.

Molecular Dynamics Simulations of the Transmembrane Region of Mutant Receptors

In an effort to interpret, in structural terms, the results of the site-directed mutagenesis experiments, the molecular dy-
Data were normalized between basal and maximum values (experiment out of at least three, performed independently. Results were cAMP increase in the different cell lines. The curves represent a typical experiment out of at least three, performed independently. Results were analyzed by nonlinear regression using the GraphPad Prism software. Data were normalized between basal and maximum values (E_max) in panel A and B. A, receptors with (close to) normal basal activity. B, receptors with strongly increased basal activity, together with the wt receptor. C, wt TSHr and N7.49A mutant. Absolute cAMP values are shown in this panel, demonstrating the inability of TSH to induce a significant response in the mutant.

The TM7 Switch in Activation of the TSH Receptor

FIG. 5. TSH stimulation of the different receptors. TSH-induced cAMP increase in the different cell lines. The curves represent a typical experiment out of at least three, performed independently. Results were analyzed by nonlinear regression using the GraphPad Prism software. Data were normalized between basal and maximum values (E_max) in panel A and B. A, receptors with (close to) normal basal activity. B, receptors with strongly increased basal activity, together with the wt receptor. C, wt TSHr and N7.49A mutant. Absolute cAMP values are shown in this panel, demonstrating the inability of TSH to induce a significant response in the mutant.

The D6.44N/N7.49D Double Mutant Receptor—The representative structure for the trajectory of this mutant receptor TM region features a hydrogen bond network opposite to that seen in the wild type simulation (see Fig. 2). The negatively charged D7.49 interacts with the neutral N6.44, through a N_6.44-H-O_7.49 hydrogen bond (average distance, 1.8 Å). In addition, its O_53 and O_52 atoms take turns in forming an H-bond with the N_53 group of N7.45 (average minimum distance of 1.9 Å). Furthermore the O_53 of N6.44 forms hydrogen bonds to both the N_52 group of N7.45 (2.2 Å), and the O_51 of S3.39 (1.9 Å), mimicking the hydrogen bond network seen in the wt simulations.

The N7.49D Mutant Receptor—The molecular dynamics simulation of the constitutive N7.49D mutant receptor shows a dramatic change in the hydrogen bond network linking TM6 and TM7 (see Fig. 2). Due to the repulsion between the 6.44 and 7.49 side chains, both an aspartate in this mutant, the D6.44 side chain has moved away from TM6 and toward TM3, forming two new persistent H-bonds. One with S3.36 (O_3.36-H-O_6.44, average distance 1.7 Å), and one with S3.39 (O_3.39-H-O_6.44, average distance 2.1 Å). On the other hand, D7.49 forms H-bonds with the N_52 group of N7.45 (average distance 1.8 Å) and with the O_53 of T6.43 (average distance 2.2 Å), via its O_51 and O_52 atoms, respectively.

The D6.44N/N7.45D Double Mutant Receptor—In this mutant receptor polar interactions between helices TM6 and TM7 are maintained throughout the trajectory, but their pattern differs from that observed in the wt. They are formed between residues N6.44 and T6.43 on the one hand, and D7.45 and N7.49 on the other. An H-bond forms persistently between the N6.44 N_52H and the D7.45 O_51 (average distance, 1.9 Å). N6.44 also H-bonds to N7.49 in a sizable fraction of the conformations, but not as persistently as in the wt simulations. In addition, the D7.45 O_53 hydrogen bonds persistently to the T6.43 O_52 (average distance, 1.7 Å). To form this pattern of interactions, TM6 and TM7 have undergone a small local rearrangement, which now enables H-bond formation between the N7.49 N_52H and D2.50 O_51 (average distance 2.5 Å). The latter residue is the highly conserved Asp in TM2, found in most rhodopsin-like GPCRs (see “Discussion”).

The D6.44A Mutant Receptor—In the absence of a polar side chain at 6.44, the interactions of TM6 with N7.45 and N7.49 in TM7 can no longer be made. As a consequence, the latter polar side chains rearrange their respective conformations, so as to find new H-bonding partners. N7.49 rotates toward TM2, forming an H-bond between its N_52H group and the D2.50 O_52 (average distance 2.0 Å) (Fig. 2). This interaction too is facilitated by a local rearrangement of TM7 which brings 7.49 closer to 2.50 than in the wt simulation (data not shown).

The N7.49A Mutant Receptor—Unlike for the D6.44A mutant, in this mutant, polar interactions between TM6 and TM7 are maintained through H-bonds of D6.44 to N7.45 (N_6.44-H-O_7.49, average distance, 1.8 Å) and to T6.43 (O_6.43-H-O_51, average distance, 1.9 Å) (see Fig. 2).

In summary, the nonconstitutive mutant receptors (D6.44N, D6.44N/N7.49D, and N7.49A) maintain the TM6-TM7 interaction through a complex hydrogen bond network involving the side chains at positions 3.39, 6.43, 6.44, 7.45, and 7.49. Moreover, as in the wt simulation, the side chain of residue 7.49 is always maintained close to TM6 (except in the N7.49A mutant), essentially through direct partnership with residue 6.44.
used in the study of other receptors (42–44). Interestingly, they supported the idea that the same N7.49 residue would interact with D2.50, one of the most conserved residues in rhodopsin-like GPCRs. The apparent incompatibility between these two mutually exclusive interactions find an explanation in the study of additional mutants (see below).

Our molecular dynamics study of the helix bundle of the TSHr based on the crystal structure of rhodopsin strongly supports the D6.44-N7.49 interaction. In simulations of the wild type TSHr structure, the D6.44-N7.49 interaction was observed in all conformations of the trajectory, indicating that this putative partnership fits well the rhodopsin template, under our simulation conditions. In agreement with experimental observations, simulation of the D6.44NN7.49D double mutant shows that the interaction between these amino acids is preserved when the two side chains are exchanged, as found in the Drosophila receptor. It should be noted that our modeling study predicts that a 6.44-7.49 interaction would be preserved in the D6.44N single mutant, and, indeed, when tested experimentally, this mutant showed a wild type-like behavior. This observation is of particular significance, considering that most if not all other mutations at this position induce constitutive activity in the TSH or LH receptors (35, 41). The structural rationale explaining why only Asn is tolerated at position 6.44 in the glycohormone receptors gives support to the local structure proposed here and suggests an explanation as to how the D6.44NN6.49D double substitution may have occurred stepwise in evolution in the Drosophila receptor.

Our modeling points to additional partners to account for the phenotype of some of the constitutive mutants identified here. The strongest constitutive activity is associated with a complete lack of the 6.44-7.49 interaction (like the N7.49D and D6.44A mutants). Weakening of this bond as observed in the D6.44NN7.49D double mutant causes increase in constitutive activity but to a smaller extent, which indicates that this mutant is not completely released from its ground state constraints. In our N7.49D simulation, D6.44 interacts with serine residues S3.36<sup>505</sup> and S3.39<sup>508</sup>, in TM3. Interestingly, mutations of S3.36 into Arg or Asn were found to constitutively activate the receptor (40, 45). It is likely that the 3.36-6.44 interaction observed in the N7.49D mutant would be strongly favored in a S3.36<sup>505</sup>R mutant. Anionic S3.36-D6.44 interaction would be more stable than the D6.44-N7.49 (wt situation), therefore redirecting the side chain of D6.44 toward TM3 and releasing N7.49.

Sequestration of N7.49 Is Implicated in the Inactive Conformation of the TSHr—Overall the modeling and experimental data converge toward the idea that, in the TSHr, a direct D6.44-N7.49 interaction is required to maintain the inactive state of the receptor, and that breaking this link results in high constitutive activity. How does this view agree with current notions of activation of other rhodopsin-like GPCRs?

Despite the fact that residue D6.44 is not conserved throughout the family (in most cases it is a Phe), mutation of 6.44 into Ala leads also to constitutive activation in other rhodopsin-like receptors (46, 47). Moreover, in the opsins, 6.44 has been shown to be linked to the activation process, being involved in color sensitivity (48, 49). The mechanism by which Asp (in the glycohormone receptors) or Phe (in most of the others) might exert similar functions cannot be determined from the present study. A provocative explanation could be that sequestration of the side chain of N7.49 by H-bonding to D6.44 in the inactive TSHr would be replaced by the existence of a steric shield in the other GPCRs.

An Active Role of N7.49 in Signal Transduction—Mutation of N7.49 into Ala casts another light on the role of the TM6-TM7...
motif in the activation of the TSHr. Although the N7.49A mutant binds TSH with wild type-like affinity, it is totally refractory to activation by the hormone. This indicates that disrupting the 6.44-7.49 interaction (which is de facto absent here) is not sufficient to trigger activation, but that N7.49 is fulfilling a specific role during activation, which cannot be achieved by an Ala side chain. Work on various rhodopsin-like GPCRs has suggested that, upon activation, N7.49 would interact with the highly conserved Asp of TM2 (D2.50) and also with the perfectly conserved Asn of TM1 (N1.50) (42, 43). As already mentioned, using a similar experimental strategy as the one described here, it has been shown in these studies that inactivation by a D2.50N mutation could be rescued by an additional N7.49D mutation. Moreover, it has been proposed that D2.50 undergoes a change in H-bonding upon light activation of rhodopsin (50).

A Model of the Early Steps of Activation—We can summarize the different points discussed above in a simple sequence of intramolecular events that would be necessary to trigger the conformational changes leading to activation.

(i) In the inactive state of the receptor, N7.49 is locked away from TM2. In the glycophorin receptor family, this conformational constraint is achieved by a direct interaction with D6.44.

(ii) A necessary step in activation would be the release of this interaction. This could happen either upon ligand binding, or due to mutation of a key residue. For instance, the D6.44A mutation abrogates completely the interaction. Noticeably, this mutation leads to almost maximal activation (2/3 of activity after full stimulation by the hormone).

(iii) N7.49 would then reorient toward the 1.50-2.50 motif, where it could modify the polar equilibrium and subsequently cause a reorganization of the inter-helical H-bond network. This rearrangement would in turn induce the conformational changes of the TM bundle known to be associated with activation (i.e. motions of TM3 and TM6 (51, 52)). In the N7.49A mutant, the side chain of N7.49 would not be able to establish the interaction(s) normally fostered by the Asn, and no transition to the active state is observed. Whether this scenario applies to the physiological activation of the receptor by its natural agonist remains to be demonstrated.

The 2.50-7.49 interaction is well supported by numerous studies on various receptors. However, a survey of the GPCR data base yields about 100 sequences of rhodopsin-like receptors with an Asp residue at both the 2.50 and 7.49 positions. In this case a direct 2.50-7.49 interaction is impossible. It has been shown that an Asn to Asp substitution at position 7.49 leads to modification of the signaling characteristics in GPCRs: changing the N7.49PXXY motif for D7.49PXXY leads to changes in the coupling specificity of the GnRH receptor (53). This indicates that the active conformation achieved by receptors bearing an Asp in 7.49 may be somewhat different from that of those bearing an Asn, in agreement with the current concept that GPCRs may achieve multiple active states with the potential to activate different G proteins (52).

When integrated with the current literature, the present results point to the 1.50-2.50-7.49 motif as an important actor in the activation of rhodopsin-like GPCRs. Additional players are likely to be involved as well, which remain to be identified. Among these are positively charged residue(s), participation of structural water molecules and specific protonations. In this context, it was suggested that D2.50 would interact with the conserved R3.50 of the DRY motif at the base of TM3 (54, 55). However, since the Cα of these two residues are separated by more than 2Å in the crystal structure of rhodopsin, we do not currently favor such a possibility. Positively charged residues are often found at the cytoplasmic end of TM6 and could possibly interact with the 1.50-2.50-7.49 motif after a rigid body motion of TM6 of the kind which is believed to occur upon activation (56–58). It is noteworthy that a water molecule is present in close vicinity of D2.50 in one of the two monomers of the rhodopsin crystal structure (22). Also, it is known that protonation events do take place upon activation of GPCRs. One of the protonation site is most probably the highly conserved D3.49 of the DRY motif (59). Recent studies on the β2-adrenergic receptor indicate that protonation of an additional negatively charged residue is most probably implicated in activation (60). D2.50 is a good candidate for the “missing” protonated residue in adrenergic receptors and has been proposed to be protonated in rhodopsin (50, 61). Protonation of D2.50 could be a necessary step in the activation process, as it has been shown for several receptors that a D2.50N mutation severely impairs the signaling properties, while a D2.50E mutation can be tolerated (44). Protonation of D2.50 would account for the observation that the N1.50-D2.50-N7.49 motif tolerates N7.49D substitutions in many systems, including the TSH receptor. As we were not able to include the various putative additional players in the molecular dynamics simulations, we could not observe a 2.50-7.49 interaction in our model of the N7.49D mutant. In all fairness, the absence of these players in our simulations and for that matter, also that of the entire extracellular domain and of an explicit lipid environment, are limitations that must be kept in mind also when considering other conclusions deduced from the simulations.

The Active Conformation of the Wild Type Receptor May Be Different from that of D6.44 Mutants—Of potential interest is the observation that the N7.49A mutant, while it cannot be activated by the hormone, still displays constitutive activity similar to the wild type TSHr. This implies that the interactions achieved by the side chain of N7.49 would be dispensable for the basal activity of the wild type receptor, whereas they are required for that of the ligand induced activity. The existence of multiple active conformations of GPCRs has been well documented in the case of TSHr mutants (16, 62) as well as in many other GPCRs (see Ref. 52 for review). The present observation suggests that “noisy” receptors like the unliganded wild type TSHr might achieve an active conformation via a route involving residues distinct from those implicated in full agonist-induced activation.

In conclusion, we have identified a conserved motif central to the activation of the TSHr receptor. Our molecular modeling provides a structural framework for understanding how the D6.44-N7.49 link, by sequestering the side chain of N7.49, would keep it in an “inactive” conformation. Upon activation, this side chain would be released, and free to adopt its “active” conformation, which involves interactions with the N1.50-D2.50 motif. Further rearrangements of the intramolecular network, which remain to be defined, lead to exposure of surfaces allowing interaction between the receptor and its cognate G protein(s). We suggest that in other GPCRs, a similar mechanism might operate, but involving a different type of partner for N7.49 in the inactive state. Now that rhodopsin provides a reliable template for modeling the inactive conformation of GPCRs, it is our hope that further molecular dynamics simulations coupled to site-directed mutagenesis experiments will help in elucidating their active conformation(s).

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