Contacts between Extracellular Loop Two and Transmembrane Helix Six Determine Basal Activity of the Thyroid-stimulating Hormone Receptor*§

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A number of alanine mutations in extracellular loop two (ECL2) of the thyroid-stimulating hormone receptor (TSHR) were found to increase or decrease basal activity when compared with the wild type receptor. K565A was identified as a mutant with decreased basal activity, and strongly impaired hormone induced signaling activity. To gain insights into how ECL2 mutants affect basal activity, we focused on constitutively activating pathogenic mutant I568V in ECL2, which exhibits elevated basal activity. Because our molecular model suggests that Ile-568 is embedded in an environment of hydrophobic residues provided by transmembrane helix bundle, we tested mutants in this region to identify potential interaction partner(s) for Ile-568. Indeed, the double mutant I568V/I640L (ECL2/TMH6) suppresses the increased basal activity exhibited by I568V alone. We suggest a spatial and functional relationship between ECL2 and TMH6 in which side chain interaction between Ile-568 and Ile-640 constrains the receptor in a conformation with low basal activity. Although the single mutant I640L exhibits basal activity lower than wild type, its differently branched and bulkier side chain complements the reduced side chain bulk in I568V, restoring wild type basal activity to the double mutant. This scenario is confirmed by the reciprocal double mutant I640V/I568L. The combination of basally increased activity of I640V and basally decreased activity of mutant I568L also restores basal activity of wild type TSHR. These and other mutant phenotypes reported here support a spatial and functional relationship (ECL2/TMH6) suppresses the increased basal activity exhibited by I568V alone. We suggest a tight hydrophobic interaction for Ile-568 in the partially active wild type basal conformation. A structural model of the serpentine domain of TSHR based on the rhodopsin structure (21) orients the ECL2 between the TMHs, where Ile-568 is located at the tip of ECL2 and is embedded in an environment of hydrophobic residues provided by TMH1, -2, -6, and -7. To identify hydrophobic residues at the TMHs that may interact with Ile-568 to constrain the basal conformation, we systematically tested amino acids at TMH1, -2, -6, and -7 that are located within a feasible interaction distance with Ile-568 suggested by the putative receptor structure. Indeed, the double mutant I568V/I640L suppressed constitutive cAMP signaling of I568V to wild type level, suggesting a structural and functional interplay between ECL2 and TMH6. Our findings provide new insights into structure-function relationships in hormone receptor; TSH, thyroid-stimulating hormone; bTSH, bovine TSH; TSHR, TSH receptor; 7TM, seven transmembrane receptor; TMH, transmembrane helix; ECD, extracellular domain; ECL, extracellular loop; ICL, intracellular loop; CAM, constitutively activating mutant; IP, inositol phosphate; WT, wild type; FACS, fluorescence-activated cell sorter.

The glycoprotein hormone receptors (GPHRs)³ LHCGR, FSHR, and TSHR constitute a subfamily of the 7TMRS. Members of the GPHRs are characterized by several common structural and functional features (1–3). Apart from the serpentine domain consisting of seven TMHs, three ECLs, three ICLs, and the intracellular C-terminal tail (see Fig. 1), the unique characteristic of GPHRs is a large N-terminal ECD, which is responsible for binding of the heterodimeric hormones (4). The GPHRs activate mainly Gαs and Gαq (2, 5, 6).

TSHR is activated by TSH, CAMs (7, 8), mutants causing promiscuous hormone activity (9), antibodies (10), tryptic action (11, 12), small ligands (13), and deletions of epitopes in the ECD (14) or the serpentine domain (15, 16). Here, for the first time, we provide a systematic evaluation of important functional properties of amino acids within the ECL2 of TSHR to gain insights into how ECL2 affects the signaling activity.

Most pathogenic CAMs reported in the ECLs of TSHR are hydrophobic amino acids (ECL1, I486M, I486F (17); ECL2, I568T, I568V (17, 18); ECL3, N650Y, V656F (19, 20)). Disruption of hydrophobic interactions caused by mutations at these positions may be responsible for structural rearrangements resulting in constitutive receptor activation. Because I568T, I568V in ECL2 are pathogenic CAMs (17, 18), we assumed a tight hydrophobic interaction for Ile-568 in the partially active wild type basal conformation. A structural model of the serpentine domain of TSHR based on the rhodopsin structure (21) orients the ECL2 between the TMHs, where Ile-568 is located at the tip of ECL2 and is embedded in an environment of hydrophobic residues provided by TMH1, -2, -6, and -7. To identify hydrophobic residues at the TMHs that may interact with Ile-568 to constrain the basal conformation, we systematically tested amino acids at TMH1, -2, -6, and -7 that are located within a feasible interaction distance with Ile-568 suggested by the putative receptor structure. Indeed, the double mutant I568V/I640L suppressed constitutive cAMP signaling of I568V to wild type level, suggesting a structural and functional interplay between ECL2 and TMH6. Our findings provide new insights into structure-function relationships in hormone receptor; TSH, thyroid-stimulating hormone; bTSH, bovine TSH; TSHR, TSH receptor; 7TM, seven transmembrane receptor; TMH, transmembrane helix; ECD, extracellular domain; ECL, extracellular loop; ICL, intracellular loop; CAM, constitutively activating mutant; IP, inositol phosphate; WT, wild type; FACS, fluorescence-activated cell sorter.
GPHRs and highlight the importance of interactions between ECL2 and TMH6 for basal activity of TSHR.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—The TSHR mutants were constructed by PCR mutagenesis using the human TSHR plasmid TSHR-pSVL as template as described previously (22) PCR fragments were digested with BspTI and Eco91I (MBI Fermentas, Vilnius, Lithuania). The obtained fragments were used to replace the corresponding fragments in the WT TSHR-pSVL constructs. Mutated TSHR sequences were verified by dideoxy sequencing with dRhodamine terminator cycle sequencing chemistry (ABI Advanced Biotechnologies, Inc., Columbia, MD).

Cell Culture and Transient Expression of Mutant TSHRs—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Paisley, UK) at 37 °C in a humidified 5% CO2 incubator. Cells were transiently transfected in 12-well plates (1 × 105 cells/well) or 48-well plates (0.25 × 105 cells/well) with 1 μg of respective 0.25 μg of DNA/well using the Genelamper® transfection reagent (Stratagene, Amsterdam, The Netherlands).

FACS Analyses—The TSH receptor cell surface expression level was quantified on a FACS flow cytometer. Transfected cells were detached from the dishes with 1 mM EDTA and 1 mM EGTA in phosphate-buffered saline and transferred into Falcon 2054 tubes. Cells were washed once with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN3 and then incubated at 4 °C for 1 h with a 1:200 dilution of a mouse anti-human TSHR antibody (2C11, 10 mg/liter, Serotec Ltd., Oxford, UK) at 37 °C in a humidified 5% CO2 incubator. Cells were transiently transfected in 24-well plates (0.5 × 105 cells/well) or 48-well plates (0.25 × 105 cells/well) with 1 μg of respective 0.25 μg of DNA/well using the Genelamper® transfection reagent (Stratagene, Amsterdam, The Netherlands).

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cAMP Accumulation Assay—For cAMP assays, cells were grown and transfected in 48-well plates. Forty-eight hours after transfection, cells were preincubated with serum-free Dulbecco’s modified Eagle’s medium containing 1 mM 3-isobutyl-1-methyloxanthine (Sigma) for 20 min at 37 °C in a humidified 5% CO2 incubator. Subsequently, cells were stimulated with 100 milliunits/ml bTSH (Sigma) for 1 h. Reactions were terminated by aspiration of the medium. The cells were washed once with ice-cold phosphate-buffered saline and then lysed by the addition of 0.1 n HCl. Supernatants were collected and dried. cAMP content of the cell extracts was determined using the cAMP AlphaScreen™ assay (PerkinElmer Life Sciences, Zaventem, Belgium) according to the manufacturer’s instructions.

Stimulation of Inositol Phosphate Formation—Forty hours after transfection, cells were incubated with 2 μCi/ml myo[3H]inositol (18.6 Ci/mmol, Amersham Biosciences, Braunschweig, Germany) for 8 h. Thereafter cells were preincubated with serum-free Dulbecco’s modified Eagle’s medium without antibiotics containing 10 mM LiCl for 30 min. Stimulation by bTSH was performed in the same medium containing 100 milliunits/ml bTSH (Sigma) for 1 h. Intracellular inositol phosphate (IP) levels were determined by anion exchange chromatography as described (23). IP values are expressed as the percentage of radioactivity incorporated from [3H]inositol-phosphates (IP1–3) over the sum of radioactivity incorporated in IPs and phosphatidylinositols.

Linear Regression Analysis of Constitutive Activity as a Function of TSHR Expression (Slopes)—The constitutive activity is expressed as basal cAMP formation as a function of receptor expression determined by 125I-bTSH binding. COS-7 cells were transiently transfected in 24-well plates (0.5 × 105 cells/well) with increasing concentrations of WT or mutant TSHR DNA (50, 100, 150, 200, 250, and 300 ng/well). For radioligand binding assays, cells were incubated in the presence of 160,000–180,000 cpm/ml of 125I-bTSH (BRAHMS Diagnostica, Germany) supplemented with 5 milliunits/ml nonlabeled bTSH (Sigma). For cAMP assays, 48 h after transfection, cells were incubated with serum-free Dulbecco’s modified Eagle’s medium containing 1 mM 3-isobutyl-1-methyloxanthine (Sigma) for 1 h. Cells were washed once with phosphate-buffered saline and then lysed using 0.1 n HCl. Supernatants were collected and dried. The cAMP levels were determined using the cAMP AlphaScreen Assay (PerkinElmer Life Sciences) according to the manufacturer’s instructions. Basal cAMP formation as a function of receptor expression was analyzed according to Ballesteros et al. (24) using the linear regression module of GraphPad Prism 2.01 for Windows.

Molecular Modeling—The methods of molecular modeling procedures for TSHR serpentine domain have been previously described (25). The sheet-like fold of ECL2 and its general localization between the transmembrane helices were kept as in rhodopsin based on rhodopsin structure-consistent results for different accessibility of two CC chemokine receptor 5 (CCR5) antibodies, each specific for the two different β-strand epitopes of ECL2 of CCR5 (26–28).

RESULTS

Alanine and Phenylalanine Mutations within ECL2

All ECL2 residues (Fig. 1) were substituted by alanine (Table 1), and hydrophobic amino acids were also substituted with phenylalanine (supplemental Table 1). Wild type and mutated TSHRs were assessed after transient expression in COS-7 cells for cell surface expression, basal and TSH-stimulated cAMP, and IP accumulation. Cells transfected with a construct encoding the empty pSVL vector were used as controls.

We did not consider amino acids Cys-569 and Pro-571 of ECL2 in this mutagenesis approach since previous studies at both amino acids for the TSHR and the LHCG (29, 30) demonstrated that they are essential for correct folding of the receptors. For the FSHR, the threonine mutant of Pro-519 (TSHR: Pro-571) is known as an inactivating pathogenic mutant caused by intracellular trapping of the receptor (31). Amino acid Cys-569 is disulfide-linked to the conserved Cys-494 of TMH3 (Ballesteros and Weinstein number (32) C3.25).
Interaction between ECL2 and TMH6 and Basal Activity of TSHR

Cell Surface Expression—FACS analyses revealed that the mutants show a cell surface expression in the range of 50–110% of WT TSHR with exception of mutants Y563A and I568A (Y563A 8.4%, I568A 18.7% of WT) (Table 1). We assume that the inactivity of the Y563A mutant is strongly related to its low number of receptors at the cell surface. Therefore, this mutant was not considered in further functional description.

CAMP Accumulation—Alanine and phenylalanine mutants of Ile-568 are characterized by increased basal Go<sub>a</sub>-mediated CAMP signaling when compared with WT (Table 1, supplemental Table 1). In contrast, mutants S561A, S562A, K565A, S567A, and M572A displayed decreased basal CAMP accumulation. For K565A, no basal cAMP signaling was observed (Table 1). TSH-mediated signaling is comparable with wild type or slightly decreased for all mutants (not under 50% when compared with maximum of WT) except mutant K565A with strongly impaired signaling activity.

IP Accumulation—Basal inositol phosphate levels of all mutants were comparable with that of the WT TSHR (Table 1). Ligand-stimulated IP accumulation is markedly reduced by alanine mutants of Ser-561, Ser-562, Leu-565, Leu-570, and Met-572 (Table 1) and by V566F (supplemental Table 1).

TABLE 1

Functional characterization of alanine mutants at ECL2

| Transfected construct | Cell surface expression (FACS) | CAMP accumulation (relative to WT basal) | IP accumulation (relative to WT basal) |
|-----------------------|-------------------------------|-----------------------------------------|----------------------------------------|
|                       |                               | Basal | Stimulated | Basal | Stimulated |
| WT                    |                               | 1     | 15.1 ± 0.1 | 1.8 ± 0.4 | 42.3 ± 4.5 |
| S561A                 |                               | 0.5 ± 0.01 | 10.9 ± 0.4 | 1.8 ± 0.3 | 5.8 ± 0.7 |
| S562A                 |                               | 0.5 ± 0.05 | 12.4 ± 0.6 | 1.9 ± 0.4 | 6.7 ± 1.2 |
| Y563A                 |                               | 0.6 ± 0.3 | 2.2 ± 0.5 | 1.9 ± 0.4 | 1.7 ± 0.5 |
| K565A                 |                               | 0.4 ± 0.1 | 2.1 ± 0.3 | 1.7 ± 0.2 | 2.5 ± 0.5 |
| S567A                 |                               | 1.4 ± 0.1 | 13.3 ± 0.6 | 1.9 ± 0.4 | 3.2 ± 0.4 |
| S566A                 |                               | 0.4 ± 0.1 | 11.6 ± 3.4 | 2.7 ± 0.4 | 27.6 ± 2.5 |
| I568A                 |                               | 2.2 ± 0.3 | 8.4 ± 0.8 | 1.7 ± 0.4 | 1.7 ± 0.3 |
| L570A                 |                               | 1.5 ± 0.1 | 9.4 ± 1.5 | 1.7 ± 0.4 | 7.1 ± 1.3 |
| M572A                 |                               | 0.2 ± 0.1 | 8.6 ± 0.7 | 1.8 ± 0.5 | 5.3 ± 0.6 |
| D573A                 |                               | 1.3 ± 0.1 | 11.1 ± 0.7 | 1.7 ± 0.4 | 28.7 ± 2.6 |
| T574A                 |                               | 1.7 ± 0.1 | 13.2 ± 1.3 | 1.9 ± 0.7 | 43.2 ± 2.2 |
| E575A                 |                               | 1.3 ± 0.3 | 10.3 ± 1.7 | 2.4 ± 0.5 | 37.2 ± 3.2 |
| pSVL                  |                               | 0.4 ± 0.1 | 0.6 ± 0.01 | 2.0 ± 0.6 | 2.0 ± 0.6 |

COS-7 cells were transfected with the WT TSH receptor or described mutant TSH receptors. Functional assays were carried out as described under "Experimental Procedures." Because of the basal activity of the WT TSHR, CAMP levels are expressed as relative to WT TSHR basal (set at 1). Increase in cAMP and IP levels was determined after stimulation with 100 milliunits/ml bovine TSH. The TSH receptor cell surface expression was quantified on a FACS flow cytometer. Data are given as mean ± S.D. of two independent experiments, each carried out in duplicate. The pSVL vector was used as a control.

FIGURE 1. Alignment of amino acids of the ECL2 and a scheme of GPHRs. a, gray background, mutated amino acids of the ECL2 at TSHR; underlined, different amino acids between the human TSHR, LHCG, and FSHR. The sequence of bovine rhodopsin (bOPSD) indicates the structural alignment to the rhodopsin structure. b, general scheme of GPHRs. The serpentine domain of GPHRs consists of three ECLs 1–3, the TMHs 1–7, and three ICLs 1–3. The common structural characteristics of the large N-terminal ECD (Met-1–Asp-410) of all GPHRs can be subdivided into (amino acids and numbers are human (h) TSHR-specific). Shown are: the cysteine-box 1 (C-b1) (Cys-24–Cys-41); the leucine-rich repeat (LRR) (Pro-280–Cys-301); the central cysteine-box 2 (C-b2) (Pro-280–Cys-301); the cysteine-box 2/3 linker (C-b2/3) (Asn-302–Ile-389); and the cysteine-box 3 (C-b3) (Cys-390–Cys-408).
Interaction between ECL2 and TMH6 and Basal Activity of TSHR

Potential Interaction Partners for Constitutively Activating Pathogenic Mutant I568V

We demonstrate that in addition to the known pathogenic CAMs I568T, I568V (17, 18), mutants I568A, I568F (Table 1, supplemental Table 1) are CAMs. Therefore, we hypothesized a tight hydrophobic interaction for Ile-568 with its counterpart(s) to constrain the basal WT conformation since slight side chain alterations at Ile-568 lead to a release of these constraints. Moreover, we assumed that similar modifications at the counterpart side chains (shorter, longer, or bulkier when compared with WT TSHR) might also result in constitutive activation of the TSHR. The molecular model of the TSHR suggests that ECL2 is plugged nearly horizontally into the transmembrane domain on the extracellular side. Isoleucine 568 is located at the tip of ECL2 and is directly flanked by the conserved Cys-569, which is disulfide-bridged to Cys-494 at TMH3 and embedded between hydrophobic residues of TMH1, -2, -6, and -7. The side chain of Ile-568 points downwards in a hydrophobic cleft between the TMH1, -2, -6, and -7, and toward potential interaction partners Leu-417 (TMH1), Ile-470 (TMH2), Ile-640 (TMH6), and Val-664 (TMH7) (Fig. 2). Thus we tested these four potential hydrophobic interaction partners for Ile-568 constructing valine or alanine mutants (Table 2). Indeed, the I640V mutant in TMH6 with reduced side chain length was a CAM (basal activity 240%, WT TSHR set at 100%). Interestingly, further modifications of Ile-640 to methionine (elongated but flexible side chain), leucine (different branching), and phenylalanine (angled side chain) are not CAMs (Table 3). The I640L single mutant showed a decreased cAMP basal activity (basal activity ~40%, WT TSHR set at 100%, Table 3). Now, we opted to test whether this phenotype affects mutant I568V. Combining the CAM I568V in ECL2 and I640L in TMH6 in the double mutant I568V/I640L resulted in restoration of the WT level of basal cAMP signaling (Table 3). The cAMP accumulation of the double mutant I568V/I640L after hormone stimulation was comparable with I568V (Table 3), whereas the decreased level of the IP accumulation was comparable with the single mutant I640L (Table 3).

![Schematic representation of the localization of Ile-568 at ECL2 and potential interaction partners in the transmembrane region.](Image)

TABLE 2
Functional characterization of single mutants at TMH1, 2, 6 and 7

| Location | Transfected construct | Cell surface expression (FACS) | CAMP accumulation (relative to WT basal) | IP accumulation |
|----------|-----------------------|-------------------------------|------------------------------------------|----------------|
|          |                       | % of WT | Basal | Stimulated | Basal | Stimulated |
| TMH1     | L417V                 | 98.1 ± 7.4 | 0.9 ± 0.1 | 10.1 ± 2.1 | 2.1 ± 0.0 | 1.1 ± 0.0 |
| TMH2     | I470V                 | 98.8 ± 7.5 | 2.4 ± 0.3 | 10.1 ± 2.1 | 1.6 ± 0.2 | 0.6 ± 0.1 |
| TMH6     | I640V                 | 95.9 ± 8.6 | 1.1 ± 0.3 | 6.4 ± 1.1 | 1.6 ± 0.2 | 0.6 ± 0.1 |
| TMH7     | V664A                 | 75.5 ± 8.5 | 0.6 ± 0.1 | 0.6 ± 0.1 | 2.0 ± 0.0 | 2.1 ± 0.0 |

TABLE 3
Functional characterization of single and double-mutants at ECL2 and TMH6

| Location | Transfected construct | Cell surface expression (FACS) | CAMP accumulation (relative to WT basal) | IP accumulation |
|----------|-----------------------|-------------------------------|------------------------------------------|----------------|
|          |                       | % of WT | Basal | Stimulated | Basal | Stimulated |
| TMH6     | I640F                 | 74.2 ± 7.6 | 0.9 ± 0.1 | 8.7 ± 0.2 | 0.9 ± 0.1 | 15.7 ± 0.8 |
| TMH6     | I640M                 | 59.6 ± 2.6 | 1.2 ± 0.2 | 9.4 ± 1.6 | 1.2 ± 0.2 | 15.7 ± 0.8 |
| TMH6     | I640L                 | 92.5 ± 4.7 | 0.4 ± 0.1 | 7.8 ± 1.1 | 0.4 ± 0.1 | 15.7 ± 0.8 |
| TMH6     | I640V                 | 92.1 ± 2.0 | 2.9 ± 0.6 | 10.7 ± 1.5 | 2.9 ± 0.6 | 15.7 ± 0.8 |
| ECL2     | I568V                 | 90.8 ± 2.1 | 2.9 ± 0.5 | 12.3 ± 0.6 | 2.9 ± 0.5 | 15.7 ± 0.8 |
| ECL2     | I568L                 | 101.0 ± 1.2 | 0.4 ± 0.04 | 10.7 ± 1.6 | 0.4 ± 0.04 | 15.7 ± 0.8 |
| ECL2/TMH6 | I568V/I640L            | 76.1 ± 4.8 | 1.1 ± 0.0 | 11.2 ± 1.4 | 1.1 ± 0.0 | 15.7 ± 0.8 |
| ECL2/TMH6 | I568L/I640V            | 98.5 ± 5.0 | 1.0 ± 0.1 | 9.8 ± 1.6 | 1.0 ± 0.1 | 15.7 ± 0.8 |
|          |                       | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 1.6 ± 0.2 |

Increase in cAMP and IP levels was determined after stimulation with 100 milliunits/ml bovine TSH. Data are given as mean ± S.D. of two independent experiments, each carried out in duplicate. The pSVL vector was used as a control.
Next, we tested the single mutants I568L, I640V and the double mutant I640V/I568L. Whereas I568V and all other known mutants at position 568 are CAMs (I568T, I568A, I568F (17) (Table 1, supplemental Table 1)), mutant I568L is basally inactive (Table 3). This phenotype corresponds exactly to the phenotype of I640L. The combination of CAM I640V and mutant I568L with decreased basal cAMP activity in reciprocal double mutant I640V/I568L restored the basal cAMP activity of wild type. Linear regression analysis of TSHR mutants I568V and I640V confirmed increased basal cAMP activity (supplemental Fig. 1). The expression level for mutants I568L, I640V, and I568L/I640V is close to 100%, and the signaling capacity induced by TSH is about 70% of wild type.

**DISCUSSION**

Numerous data for different 7TMRs have shown the importance of ECL2 for receptor signaling (33–36). The ECL2 of the LHCGR has been reported to be involved in hormone binding (30, 37) and to be essential in signal transmission processes (38). For the first time, the present study aimed to characterize systematically the amino acids within the ECL2 of TSHR by site-directed mutagenesis to provide deeper insights into the functional and structural interrelations during the processes of TSHR activation and intramolecular signal transduction.

Lysine 565 in ECL2 Is a Key Player in the Intramolecular Signaling Processes of the TSHR—The alanine scan of ECL2 revealed K565A to be the most impaired mutant with respect to basal and hormone-induced activity (Table 1). We suggest two scenarios that might explain the low activity of K565A. (i) The cascade of constituents involved in the signal transmission process starting from the ligand-occupied ECD to intracellular effectors is interrupted by mutation of Lys-565 via breaking of hydrogen bonds or of electrostatic salt bridge interactions. (ii) Lys-565 is important for the formation of the active receptor state conformation by binding to a new interaction partner after hormone-induced signal initiation. The mutant is unable to stabilize the active receptor structurally and/or functionally.

Mutant K565A at ECL2 is basally inactive and shows an impaired TSH-induced response. The recently published mutants K660D (TMH7), E409K, and D410K (extreme C terminus of the ECD) (39) express a similar phenotype. The suggested inactivation mechanisms for mutant K565A at ECL2 can also be applied to these mutants, and a functional interplay in the WT receptor during the hormone-induced activation process between these two positively and two negatively charged amino acids at the extracellular side of TSHR is conceivable.

**Mutants with Both Decreased Basal Goq- and Decreased Hormone-induced Goq Activity**—We identified mutants in ECL2 that are characterized by impaired basal Goq-mediated signaling activity (Table 1). In addition to our findings for ECL2, several other mutants with decreased basal activity but with cell surface expression comparable with the WT receptor have been characterized at ICL2, at the junction between TMH5/6 and ICL3 and in the interior transmembrane domain (40–43). It is noteworthy that most of the mutants exhibiting decreased basal cAMP accumulation also exhibit decreased ligand-mediated Goq activity (Table 1). However, hormone-stimulated cAMP accumulation is not affected in the same range as IP

![Figure 3. Molecular model based scheme of WT and mutant interactions between ECL2 (position 568) and TMH6 (position 640).](image-url)

**FIGURE 3.** Molecular model based scheme of WT and mutant interactions between ECL2 (position 568) and TMH6 (position 640). a, in the WT receptor, the hydrophobic side chain interaction between Ile-640 (TMH6) and Ile-568 (ECL2) is essential to adjust TMH6 toward ECL2 into the basal active conformation. b, reduction of side chains by mutants I568V at ECL2 or by I640V as the counterpart in TMH6 leads to a release of the intertwined interaction and to a constitutive receptor activation by movement of TMH6. c, the slight side chain alteration from isoleucine to leucine at position 640 by an additional branching at the tip of the side chain (β-β-atom instead of a β-β-atom) causes a different intertwined interaction toward Ile-568 at ECL2. The additional γ-methyl group constrains TMH6 in an opposite direction when compared with the state of release and activation and thus is moved to a more inactive position preventing the adjustment of the basal active conformation. d, the reciprocal single mutant I568L in ECL2 shows exactly the same phenotype as I640L e, although in the double mutant I640L/I568V the side chain of 568 is reduced, the additional methyl group of leucine enables an intertwined interaction between the two branched side chains, which is reflected by its WT behavior. The same phenotype could be observed for the reciprocal double mutant I568L/I640V, also indicating an intertwined interacting mechanism.
Interaction between ECL2 and TMH6 and Basal Activity of TSHR

The mutant I640V showed constitutive activation. Ile-640 is located at TMH6 close to Pro-639 of TSHR, which corresponds to the highly conserved proline P6.50 (Ballesteros and Weinstein number (32)) in GPCR family one and is responsible for causing a kink at TMH6. The side chain Ile-640 points toward ECL2. Based on our data, we suggest that in the WT receptor, a hydrophobic side chain interaction between Ile-640 (TMH6) and Ile-568 (ECL2) is essential to adjust TMH6 toward ECL2 in the basal active conformation (Fig. 3a). Reduction of side chain length at the interface between TMH6 and ECL2 by I640V at TMH6 or by I568V as the counterpart at ECL2 leads to a release of this interaction, resulting in constitutive receptor activation, very likely by movement of TMH6 (Fig. 3b). Our observation of a decreased basal activity with mutant I640L can be explained by our model as well. The slight side chain alteration from isoleucine to valine at position 640 by an additional branching at the tip of the side chain (a γ-C-atom instead of a β-C-atom) causes a different interlocked interaction with Ile-568 in ECL2. The additional γ-methyl group constrains TMH6 in an opposite direction when compared with the release and activation state, and thus it is moved to a more inactive position, preventing the adjustment of the basal active conformation (Fig. 3c).

Although in the double mutant I640L/I568V the side chain of 568 is reduced, the additional methyl group of leucine enables an interaction between the two branched side chains, which is reflected by its WT behavior (Fig. 3e).

To validate our suggested scenario, we designed the reciprocal single mutants I568L, I640V, and the corresponding double mutant I640V/I568L. We hypothesized that in the case of intertwining side chain contact between Ile-640 and Ile-568, an interlocking takes place by introducing the differently branched side chain of leucine (branch already on the tip of side chain, Fig. 3) by mutant I640L on TMH6 (Fig. 3c). In that case, a similar effect should also occur at the counterpart at ECL2 by mutant I568L, resulting in a decreased basal activity (Fig. 3d).

Setting to threonine and valine are known as pathogenic (17, 18). In our model, Ile-568 is pointing downwards, directly into a cleft between the transmembrane helices. To identify possible hydrophobic residues in the TMHs that interact with Ile-568 as counterparts, we systematically introduced side chain alterations at potential interaction sites of Ile-568 with TMH1 (Leu-417), TMH2 (Ile-470), TMH6 (Ile-640), or TMH7 (Val-664) (Fig. 2 and Table 3).

The mutant I640V showed constitutive activation. Ile-640 is located at TMH6 close to Pro-639 of TSHR, which corresponds to the highly conserved proline P6.50 (Ballesteros and Weinstein number (32)) in GPCR family one and is responsible for causing a kink at TMH6. The side chain Ile-640 points toward ECL2. Based on our data, we suggest that in the WT receptor, a hydrophobic side chain interaction between Ile-640 (TMH6) and Ile-568 (ECL2) is essential to adjust TMH6 toward ECL2 in the basal active conformation (Fig. 3a). Reduction of side chain length at the interface between TMH6 and ECL2 by I640V at TMH6 or by I568V as the counterpart at ECL2 leads to a release of this interaction, resulting in constitutive receptor activation, very likely by movement of TMH6 (Fig. 3b). Our observation of a decreased basal activity with mutant I640L can be explained by our model as well. The slight side chain alteration from isoleucine to valine at position 640 by an additional branching at the tip of the side chain (a γ-C-atom instead of a β-C-atom) causes a different interlocked interaction with Ile-568 in ECL2. The additional γ-methyl group constrains TMH6 in an opposite direction when compared with the release and activation state, and thus it is moved to a more inactive position, preventing the adjustment of the basal active conformation (Fig. 3c).

Although in the double mutant I640L/I568V the side chain of 568 is reduced, the additional methyl group of leucine enables an interaction between the two branched side chains, which is reflected by its WT behavior (Fig. 3e).

To validate our suggested scenario, we designed the reciprocal single mutants I568L, I640V, and the corresponding double mutant I640V/I568L. We hypothesized that in the case of intertwining side chain contact between Ile-640 and Ile-568, an interlocking takes place by introducing the differently branched side chain of leucine (branch already on the tip of side chain, Fig. 3) by mutant I640L on TMH6 (Fig. 3c). In that case, a similar effect should also occur at the counterpart at ECL2 by mutant I568L, resulting in a decreased basal activity (Fig. 3d). Subsequently, the reciprocal double mutant I568L/I640V (CAM I640V) should restore the wild type basal cAMP activity (Fig. 3e). Indeed, whereas I568V and all other known mutants at position 568 are CAMs (I568T,I568A,I568F (17) (Table 1, supplemental Table 1)), the reciprocal mutant I568L at ECL2 is basally inactive and thus identical to mutant I640L at TMH6. Moreover, the combination of CAM I640V with the decreased basal active mutant I568L in the double mutant I640V/I568L confirmed our prediction by restoring the basal cAMP activity of wild type as shown for double mutant I640L/I568V (Table 3). This reverse approach is consistent with our model and supports the suggested scenario presented in Fig. 3, c and e. Moreover, the finding is also supported by the WT sequence of homologous leucine-rich-repeat containing G-protein coupled receptors (LGR4, -5, -and 6) that contain our tested reciprocal pair of "WT rescue" amino acids (leucine at ECL2 and valine at TMH6) at the corresponding positions 568 and 640 of TSHR (for alignment, see supplemental Fig. 3).

TMH6 May Glide along ECL2 According to Different Receptor Activity States—An established common mechanism for family A 7TMRs is an activation-associated see-saw movement of TMH6 around the conserved proline P6.50 (TSHR Pro-639) as a pivot. Our mutants are reflecting a supple interface where TMH6 is gliding along ECL2 in a raster of three different receptor states. a, in the activated state, the rather constrained basal state is released either by mutants with reduced side chains or by a hormone-induced ECL2 shift. Since we identified residue Lys-565 at ECL2 as involved in forming a hormone-induced activated conformation, a slight backbone displacement is also feasible at ECL2 (small arrows). Such an induced slight lever-like shift around the essential proline 571 of ECL2 would be sufficient to release the stabilized interaction between TMH6 and ECL2 and allows the see-saw movement of TMH6 for complete activation. b, the hydrophobic side chain interaction of Ile-640 (TMH6) and Ile-568 (ECL2) is constraining the basal (partially active) conformation of wild type TSHR. The TMH6 in this partial activity state is slightly tilted to the vertical axis. c, the decreased basal activity is provoked by a differently branched bulkier side chain (I640L) at TMH6 by further interlocking with Ile-568, which likely causes a slight movement of TMH6 in the opposite direction to the activation conformation and diminishes G-protein interaction (Fig. 3, c and d).
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...the formation of a hormone-induced activated conformation, at least a side chain movement, but also a slight backbone displacement, is likely at that particular ECL2 site (Fig. 4a). Moreover, since ECL2 is plugged into the transmembrane domain and is intercepted at three anchor points (N-terminal TMH4, C-terminal TMH5, and disulfide bridge at Cys-569) consisting of a fold-essential proline (Pro-571), a slight lever-like shift during hormone-induced activation is conceivable at least for a portion of ECL2. Such an induced slight shift of ECL2 would be sufficient to release the suggested interaction between TMH6 (Ile-640) and ECL2 (Ile-568) in the basal conformation, thus allowing a further gliding of TMH6 along ECL2 and the subsequent see-saw movement of TMH6 for complete activation (Fig. 4a).

Our mutants reflect a dynamic interface where TMH6 glides along ECL2 according to three different receptor states. The basal activity is constrained by side chain interaction (Fig. 4b). The decreased basal activity is provoked by a differently branched bulkier side chain of Ile640L in TMH6 (Fig. 4c). The side chain interlocking of mutant Ile640L with Ile-568, which likely causes a slight movement of TMH6 in the opposite direction when compared with the activation, diminishes G-protein interaction. This side chain interlock cannot be established by the Ile40F and Ile40M mutants because their differences in side chain branching and flexibility when compared with the leucine mutant. The aromatic ring system is angled at the β-carbon of the phenyl side chain at position 640 and cannot interlock with Ile568V. Methionine is characterized by an unbranched side chain and more flexibility in side chain orientation. In the activated state, the constraint is released by mutants with reduced side chains at either of the counterparts or by hormone-induced ECL2 shift, allowing movement of TMH6, which opens the intracellular access for G-proteins (Fig. 4d).

Taken together, we identified amino acids at ECL2 and TMH6 of TSHR, which are sensitive for signaling, and we suggest an activation mechanism at the interface between TMH6 and ECL2, in which TMH6 glides along ECL2 according to the different receptor activation states. Our findings that ECL2 and TMH6 interact and their dynamic interface for signaling are due to the high sequence conservation (49) are very likely con-...
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