RESEARCH ARTICLE

The intramolecular agonist is obligate for activation of glycoprotein hormone receptors

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Abstract

In contrast to most rhodopsin-like G protein-coupled receptors, the glycoprotein hormone receptors (GPHR) have a large extracellular N-terminus for hormone binding. The hormones do not directly activate the transmembrane domain but mediate their action via an, thus, far only partially known Tethered Agonistic Ligand (TALI). The existence of such an intramolecular agonist was initially indicated by site-directed mutation studies and activating peptides derived from the extracellular hinge region. It is still unknown precisely how TALI is involved in intramolecular signal transmission. We combined systematic mutagenesis studies at the luteinizing hormone receptor and the thyroid-stimulating hormone receptor (TSHR), stimulation with a drug-like agonist (E2) of the TSHR, and structural homology modeling to unravel the functional and structural properties defining the TALI region. Here, we report that TALI (a) is predisposed to constitutively activate GPHR, (b) can by itself rearrange GPHR into a fully active conformation, (c) stabilizes active GPHR conformation, and (d) is not involved in activation of the TSHR by E2. In the active state conformation, TALI forms specific interactions between the N-terminus and the transmembrane domain. We show that stabilization of an active state is dependent on TALI, including activation by hormones and constitutively activating mutations.

KEYWORDS
G protein-coupled receptors, glycoprotein hormone receptors, hormone receptors, tethered agonist, thyroid-stimulating hormone

Abbreviations: 7TMD, seven-transmembrane helices domain; CAM, constitutively active mutant; EL, extracellular loop; GPCR, G protein-coupled receptor; GPH(R), glycoprotein hormone (receptor); LRR(D), leucine-rich repeat (domain); TALI, tethered agonistic ligand.
1 | INTRODUCTION

Glycoprotein hormone (GPH) receptors (GPHR) are central in hypothalamus-pituitary-gland axes transmitting the signals mediated by the thyroid-stimulating hormone (thyrotropin [TSH]), luteinizing hormone (lutropin [LH]), choriodonadotropin (CG), and the follicle-stimulating hormone (foliliotropin [FSH]) via their respective receptors (GPCR) because of sequence similarities in highly conserved parts of the seven-transmembrane helices domain (7TMD).\(^1\) In contrast to most other rhodopsin-like GPCR, GPHR present with a large N-terminus which contains 11 leucine-rich repeats (LRR) organized in a specific LRR domain (LRRD) and a so-called “hinge region” connecting the LRRD with transmembrane helix 1 (TM1) (reviewed in Kleinau et al\(^3\)). Upon GPH binding to the N-terminus, the 7TMD changes into its active conformation and mediates G-protein activation.\(^4,5\) The N-terminus structure and the GPH-binding mode are known from solved crystal structures of the FSHR N-terminus/FSH and TSHR LRRD/autoantibody complexes.\(^6-8\) Unfortunately, there is still no structural information available that illustrates how the N-terminus is arranged in relation to the 7TMD and how both interplay during intramolecular signal transmission.

Several previous studies indicated that GPH do not directly interact with and activate the 7TMD.\(^9\) Additional complexity is added by the fact that autoantibodies against the N-terminus and single point mutations or deletions within the N-terminus can activate GPHR.\(^6,10-14\) Therefore, it is assumed that parts of the N-terminus itself may act as the 7TMD ligand.\(^9,15-17\) Because some artificially generated deletions and truncations of the N-terminus can increase TSHR activity, it was proposed that the N-terminus functions as an intramolecular inverse agonist inhibiting 7TMD signaling until ligand binding at the N-terminus.\(^15,17-19\) An alternative hypothesis was that the N-terminus contains a so-called “intramolecular agonistic unit”\(^3,16,20\) which is exposed following hormone binding and eventually activates the 7TMD. We have shown that deletion of the entire N-terminus did not activate the LHR, which rejects the hypothesis of an inhibitory function of the N-terminus and supports the alternative hypothesis of an “intramolecular agonistic unit.”\(^9\) Recently, substantial progress was made by peptide studies showing that a peptide (termed “p10”), derived from the very conserved C-terminal sequence of the hinge region, can activate both the full-length GPHR and the 7TMD without the N-terminus.\(^21\) In addition, modification of p10 by a naturally occurring inactivating Glu-to-Lys substitution (E354K in LHR\(^22\)) blocked GPH- and autoantibody-induced TSHR activation.\(^21\) Thus, this study extended and refined previous studies with the rat LHR in which precisely this transition between the TM1 and the hinge region was identified as highly relevant for receptor activation.\(^23\) Altogether, these results add GPHR to a group of GPCR, containing rhodopsin, protease-activated receptors, and adhesion GPCR, which have their agonists already covalently bound or as an integral part of the receptor protein.\(^24\)

It is currently unknown how this Tethered Agonistic Ligand (termed TALI) is turned into an agonistic conformation and mediates 7TMD activation. Physiologically, binding of GPH triggers the action of TALI; pathophysiologically, missense and deletion mutations in the N-terminus, as well as autoantibody binding, also lead to GPHR activation (reviewed in Kleinau et al\(^3\)), which most likely recruits a similar mechanism. It needs to be determined whether TALI is pre-bound in a binding pocket formed by the 7TMD and isomerizes following GPH binding or whether TALI is simply exposed, and then, binds at the 7TMD for activation.

The low affinity of p10-derived peptides (mM range)\(^21\) is not entirely suitable to address these questions with biophysical or peptide-binding experiments. Furthermore, in peptide studies, the corresponding sequence of p10 is still present in GPHR, which can lead to competition with p10-derived peptides, making it difficult to interpret TALI functions. In addition to several previous mutagenesis studies\(^9,15,17,20,23,25\) and our recent peptide study,\(^21\) we have therefore systematically investigated the TALI region in its natural context by site-directed mutagenesis and structural homology modeling. Thus, we aimed to decipher specific properties of each amino acid residue in this particular region to identify potential intramolecular interaction partners and find a mechanistic explanation of how GPHR transition from a basal to a fully active state is achieved.

2 | MATERIALS AND METHODS

2.1 | DNA constructs and functional assays

Full-length human TSHR and human LHR sequences were cloned into the mammalian expression vector pcDps\(^9,26,27\). A hemagglutinin (HA) epitope and a FLAG epitope were inserted directly downstream of the signal peptide and at the very C-terminus of the GPHR, respectively. All mutant constructs were generated by a PCR-based site-directed mutagenesis and fragment replacement strategy (details provided in Table S1) and confirmed by sequencing.

For functional assays, TSHR and LHR constructs were heterologously expressed in HEK-293T cells upon transient transfection and grown in DMEM supplemented with 10% of fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% of CO\(_2\) in a humidified atmosphere. For the cAMP assay, cells were seeded into 96-well plates (1.5 × 10\(^4\) cells/well) and transfected with 100 ng vector constructs using Lipofectamine (Fisher SCIENTIFIC,
Waltham, USA) according to the manufacturer’s protocol. Wild-type (wt) receptors and empty vectors served as positive and negative controls, respectively. Forty-eight hours after transfection, cells were incubated with DMEM containing 1 mM of 3-isobutyl-methyl-xanthine (IBMX) and indicated concentrations of hormones and E2 (NGC00161870) for 1 hour at 37°C. For cAMP measurements, cells were lysed in 50-100 µL of LI buffer (PerkinElmer Life Sciences, Monza, Italy) and kept frozen at −20°C until measurement. To measure cAMP accumulation, the AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) was used according to the manufacturer’s protocol. The accumulated cAMP was measured in 384-well white OptiPlate microplates (PerkinElmer Life Sciences) with the Multimode Plate Reader EnVision (PerkinElmer Life Sciences).

For direct quantitative measurement of inositol 1-phosphate (IP₁), HEK-293T cells were grown and transfected as described above. To perform the assay, Cisbio’s IP-One Tb kit (Cisbio, Codolet, France) was used. Forty-eight hours after transfection, cells were stimulated for 1 hour at 37°C with 35 µL 1 x IP₁ stimulation buffer (Cisbio) containing the respective reagents. Cells were lysed by adding 30 µL lysis buffer (Cisbio) per well and kept frozen at −20°C until measurement. The accumulated IP₁ was measured according to the manufacturer’s protocol in ProxiPlate-384 Plus microplates (PerkinElmer Life Sciences) with the Multimode Plate Reader EnVision (PerkinElmer Life Sciences).

To estimate the cell surface expression of receptors carrying an N-terminal HA tag, an indirect ELISA was used.²⁸

### 2.2 TSHR model-assembling and homology modeling of structural conformations

A structural homology model of TSHR in a putative hormone- and Gs protein-bound state was generated. We decided to use TSHR models because previous studies already investigated the relevance of determinants in the TALI region²⁰,²⁹ and the p10 region during TSHR activation by autoantibodies³¹ wherein a crystal structure is only available for parts of the N-terminus of TSHR. In the current study, we used the mutagenesis results to refine these TSHR models, specifically with respect to p10 interactions with the extracellular loops (EL). Most additional data of structure-function relationships in the EL are only available for the TSHR because of activating and inactivating mutations found in patients with hyper- and hypothyroidism.³⁰,³¹

The following structural templates from homologous family A GPCR were used: (a) the leucine-rich repeat domain (LRRD, composed of 11 LRR) with the bound hormone was modeled based on a known partial FSHR (extracellular receptor part)-FSH complex structure (PDB entry 4AY9), using the TSHR sequence from Cys24-Asn288, which also included fragments of the hinge region (using the TSHR sequence from Gln289-Ser304 and Ser383-Ile411). (b) For modeling of the TSHR 7TMD in an active state conformation, the available crystal structure of the β₂-adrenergic receptor (ADRB2) in complex with the Gs protein was used (PDB entry 3SN6).³² (c) The EL2 of the µ-opioid receptor (µOR, PDB entry 6DDE)³³ was the template to build the putative TSHR-EL2 structure.

In detail, the EL1 and EL3 lengths of the ADRB2 template were adjusted manually toward the predicted TSHR loop lengths.³³ Further structural modifications of the ADRB2 template were the deletion of the extracellularly fused T4-lysozyme and removal of the bound ligand. Moreover, the intracellular loop 3 (IL3) sequence of the TSHR was added manually to the template, because of the missing IL3 in the ADRB2 structure. The N-terminal parts of the kinked ADRB2 TM2 and TM5 were both manually replaced by a regular α helix, as all GPHR do not have a proline in TM2 and TM5 that causes a kink as observed in various GPCR template structures (eg, Pro2.59 in TM2 and Pro5.50 in TM5 of ADRB2). This procedure was necessary because solved GPCR crystal structures without proline in TM2 do not have any kink or bulge in these helical structures (in detail described in Kleinau et al³¹). The ADRB2 EL2 was substituted with the EL2 of the structurally determined µOR EL2 (PDB entry 6DDE).³³ We predict that the TSHR and the µOR may share a similar conformation and localization of the EL2 relative to the other EL and the 7TMD. In addition to sequence-based similarities, this assumption is supported by the observation that several GPCR with peptidic and protein ligands, such as the neurotensin-1 receptor (PDB entry 4XEE),³⁴ the chemokine receptor CXCR4 (PDB entry 3OE0),³⁵ the nociceptin/orphanin receptor (PDB entry 5DHG),³⁶ and the apelin receptor (PDB entry 5VBL),³⁷ show a similar secondary structure of the EL2 and spatial localization as also present in the recently determined cryo-electron microscopy structure of the µOR active state complex (Figure S1).

The amino acid residues of this chimeric 7TMD receptor-template and the FSHR N-terminus were mutated with the corresponding TSHR amino acid sequences. The Gs protein was kept bound as in the original ADRB2 template. The heterodimeric FSH molecule bound to the FSHR LRRD and hinge region was substituted by a bovine TSH model that was described previously.²⁹

This initial 7TMD model of the TSHR and the N-terminus in complex with TSH were fused at position 411. While the exact orientation and spatial distances between the extracellular part relative to the EL and 7TMD are not precisely known without an experimentally determined 3D-structure of the entire TSHR complex, we supposed a nearly completed TSHR model with relative orientations just an approximation based on the fact, that the transition between the N-terminus and TM1 is linked via a cysteine bridge between
Cys284 (C-terminus of the LRRD) and Cys408 (C-terminus of the hinge region close to TM1). This disulfide bridge is a structural restriction and orients the N-terminus closely to the 7TMD and between the EL.

This assembled complex was minimized by constraining the backbone atoms, followed by molecular dynamics simulation (2ns) and energy minimization of the side chains (constrained backbone atoms) until converging at a termination gradient of 0.05 kcal/mol*Å. The entire TSHR complex was then minimized without any constraint. Next, by constraining the backbones of Gs, TSH, the LRRD, N-terminal parts of the hinge region, and the entire transmembrane helices (the EL and the agonistic unit were unconstrained), a dynamic simulation of 2ns was initiated, followed by energy minimization. In addition, the synthetic small molecule agonist E2 was docked into the generated TSHR model in a binding mode, as suggested previously.38

The homology models were generated with the software Sybyl X2.0 (Certara, NJ, US). The AMBER F99 force field was used for energy minimization and dynamics simulations. For minimization of the TSHR/E2 complex, the Tripos force field was used. Charges of the E2 ligand atoms were computed with Gasteiger-Huckel charges. Structure images were produced using PyMol software (DeLano WL, version 0.99, San Carlos, CA, USA).

3 | RESULTS AND DISCUSSION

Because the TALI-derived peptide p10 (FNPCEDIMGY, amino acid residues 350-359 in LHR and 405-414 in TSHR) activates TSHR and LHR only partially,21 we hypothesized here that after initial hormone binding the TALI region may undergo specific structural changes in the natural context to act as a full (primary) agonist. To address this hypothesis, we substituted every position in the p10 region of the LHR with multiple distinct amino acid residues (Figure 1, Table S1). These substitutions (mainly to Ala, Cys, Asp, His, Lys, Asn, Pro, Ser, and Tyr) were chosen to describe the physicochemical properties which are tolerated or mandatory in terms of receptor expression (ELISA) and signaling (cAMP accumulation assay). The results of the first set of more than 80 LHR mutants are given in Table S2. To investigate the transferability of mutational effects, several LHR mutants with significant changes in signaling were also generated at the corresponding positions in the TSHR (38 mutants) and tested (Table S3). Moreover, we also used the small molecule agonist E2, which activates the TSHR independently from the N-terminus by binding at the 7TMD38 to characterize all TSHR mutants with respect to 7TMD integrity and activation. The results of this systematic mutation screen (summarized in Figure 1) and previously obtained findings in combination with current modeling perspectives are position-wise described and discussed in the following section.

3.1 | Deciphering functional and structural properties of TALI components

Amino acid substitutions at LHR position 350 (F1.23, unifying Ballesteros-Weinstein nomenclature39) revealed that instead of Phe only Tyr and Leu side chains are tolerated for proper expression and signaling, indicating that a hydrophobic and more bulky amino acid residue at this position is mandatory. All other tested amino acid substitutions led to
a significant reduction of cell surface expression and loss of hCG-induced cAMP accumulation (Table S2). These findings are consistent with previous studies showing that Ala substitution of F1.23 in LHR and TSHR leads to inactivation. F1.23Y in TSHR (F405Y) and LHR (F350Y) caused slightly increased basal activity (Tables S2 and S3). In the structural TSHR homology model, the close spatial proximity of the F405 side chain to Y643 in TM6 is suggested (Figure 2), which is also known for constitutive receptor activation by mutation.

Interestingly, activation of TSHR F405L with the agonistic ligand E2 showed signaling properties comparable to the wt TSHR but a nearly doubled cAMP response in the slightly constitutively active mutant (CAM) F405Y (Table S3). The putative binding site of E2 is located spatially below (Figure 2), but adjacent to the postulated localization of the

**FIGURE 2** The TSHR structure model in an active state conformation bound with the Gs protein, TSH, and the synthetic agonist E2. (Middle) The TSHR structure homology model (backbone cartoon) shows the nearly full-length receptor (missing the C-terminus and fragments of the hinge region) in an active state conformation complexed with bovine TSH (surface representation) and the small molecule E2 (magenta sticks). The Gs protein (surface representation of the heterotrimer, alpha-subunit cyan, beta-subunit dark forest green, gamma-subunit blue) is interacting with TSHR according to the orientation supposed by the ADRB2/Gs protein complex. The receptor fragment studied in this current work is highlighted by amino acid side chain representations (sticks) between the extracellular loops (EL). This view is used to highlight the localization of the intramolecular agonist relative to other parts of the receptor such as the extracellular loops (side chains as lines) and intermolecular interaction partner. (Left side) The small molecule compound E2 is bound between the transmembrane helices close to the EL. Four TSHR amino acids involved in this putative binding mode are determinants (mostly aromatic ring systems) of the binding pocket and are highlighted (magenta sticks). The binding pocket is partially shown as inner surface, whereby the intramolecular agonist binding site is also represented with a translucent surface to visualize the spatial discrimination between the E2 binding site and the hinge region/TM1 transition. (Right side) Details of the TSHR intramolecular agonist embedded between the EL. The agonistic intramolecular unit is constituted of side chains between P400 and Y414 at the transition between the hinge region and TM1. Of note, in previous studies mutations at P400, D403, and E404 have been identified to activate the TSHR constitutively, and therefore, should be considered as an extended agonistic unit (TALI region) in combination with the here studied F405-Y414 peptide (p10). In addition, it can be assumed that amino acid S281 (located in the C-terminus of LRRD, helical structure) participates in this structural and functional unit, which is tightly packed by the disulfide bridges between C284 and C408 as well as C283 and C398 (yellow sticks). In this current study, investigated residues at the TSHR are shown with underlined labels. Specific mutations at F405, N406, P407, G413, and Y414 lead to constitutive receptor activation, while variations at positions E409 and D410 are shown to inactivate the receptor. EL residues which may contribute to the activation mechanism in interplay with the agonistic unit are highlighted by lines. These are for instance N650, V656 (EL3), and I486 (EL1), which are known explicitly because constitutive activation can be induced by mutations at these positions, which is complementary to mutants in the intramolecular agonist. In the corresponding Top view, the putative localization of the agonistic unit in the center above the TMD and between the EL is visible. The TALI is embedded tightly and in a sandwich-like manner between extracellular parts and the TMD. H, helix; LRRD, Leucine-rich repeat domain; EL, extracellular loop
bound active TALI. Because of increased signaling activity of E2 in several of the CAM variants, we hypothesize here that E2 efficacy is increased in TSHR conformations induced by constitutively activating mutations. This tendency is supported by analyses of all data at TSHR single and double mutants tested with E2 (G413P, D633H, N406S, P407A, and P407A/G413P in Table S3), which were basally more active than the wt TSHR. In contrast, non-CAM did not show any increased agonistic signaling upon E2 binding (eg, G413D).

All substitutions of N351 (N1.24) in LHR, except for N351P, were tolerated regarding cell surface expression and hCG-induced signaling (Table S2). However, several mutations (Ala, Phe, Ser) resulted in constitutive activity. Similarly, N406F and N406S in TSHR were also characterized as CAM (Table S3). The vulnerability of N1.24 for activating mutations has been demonstrated previously for TSHR20 with very similar activating substitutions found in the current study of LHR.

Based on the fact that some variations in side-chain length and bulkiness led to constitutive activity, we postulate that N1.24 is tightly packed in an area essential for receptor activation and that N1.24 is located at a spatial position highly prone to switching between an inactive and active state conformation. Our structural TSHR model exactly reflects this situation for N406 (Figure 2, top view) by close spatial proximity to the LRRD, the hinge region, and EL2. In addition, a hydrogen bond of the Asn side chain to the backbone of S281 is proposed (Figure 2, top view, dotted black line). This Ser residue is located in the last repeat of the LRRD with a helical secondary structural element and is known from pathogenic mutations and mutagenesis studies to be essential for intramolecular signaling transduction.9,41-43 Moreover, this helix will serve as a pivot during receptor activation after binding of TSH or antibodies.1 In conclusion, modifications at N406 (or N351 in LHR) lead to changes in the core of the agonistic unit that is connected directly via two disulfide bridges (C284/C408, C283/C398) to tightly packed receptor domains (Figure 2). Modifications of N406, interacting partners (eg, S281 in the LRRD) or closely located amino acids (P407, I568 in EL2) constitutively activate both TSHR and LHR. Recently, some of these p10/EL interactions (V656, I568) have also been postulated in an independent TSHR model.44

Mutations at LHR position 352 (P1.25) to Ala, Asp, Asn, and Ser increased cell surface expression compared to the wt LHR (170%-280%, Table S2), which were not observed for the corresponding TSHR variants at this position (Table S3). Any other substitution caused diminished receptor cell surface expression. However, all mutants, even with high cell surface expression levels, showed only a marginal cAMP response to hCG. This indicates that LHR variants at position 352 strongly modify structural receptor properties and cause conformations that are unable to transmit hCG-mediated activation. Notably, the situation is slightly different in TSHR because tested mutations at P407 (Ala, Asp, Asn) revealed moderate surface-expression levels and TSH-induced signaling properties (between 30% and 70%). As already reported,25 constitutive activation of TSHR can be induced by P407 mutations (Ala, Asp) (Table S3).

According to our homology structure model (Figure 2) P407, adjacent to the stabilizing disulfide bridge C284/ C408, is an element that gives structural flexibility to the p10 sequence and is mandatory to orient the p10 region in a well-defined spatial binding site to achieve an active state. Most residues at position P1.25 other than proline induce a different conformation that is incompatible with proper hormone-mediated signaling (Tables S2 and S3, Mueller et al25).

C1.26 (LHR C353, TSHR C408) is essential for LHR and TSHR expression and function since all mutants, except C353N in LHR, are poorly expressed at the cell surface with no response to GPH and E2. This is consistent with previous studies showing that Ala, Arg, and Ser substitution of C353 interferes with proper LHR function.23,45,46 In the TSHR homology model (Figure 2), the highly conserved disulfide bridge between C408 and C284 is a primary constraint and absolutely essential for keeping the different receptor parts (LRRD, hinge region) in close spatial proximity. Any substitution led to an interruption of this structural constraint and, consequently, to a structure unresponsive to TSH-mediated signaling or a trafficking-deficient receptor fold. One should also consider the possibility of newly formed disulfide bonds involving C284 that alter the global receptor structure and lead to intracellular retention of the misfolded receptor protein. Since truncation variants of TSHR lacking the entire N-terminus including C408 (Neumann et al47 and data below) can still be activated by E2, this cysteine does not significantly contribute to structuring the 7TMD.

The positions 354 and 409 in the LHR and TSHR, respectively, require the negatively charged Glu residue because none of the tested substitutions exhibited wt-like properties. Substitution of E1.27 with Asp, Asn, and Gln did not compensate for function, indicating that the Glu side-chain participates in a structurally well-defined interaction(s). As also recently proposed,44 our structural homology model (Figure 2) suggests a potential interaction of E409 with K660 in TM7, which is also known to have an essential functional role in GPHR to achieve an active state.48-50 At this position, a Lys is obligatory and cannot be substituted by any other amino acid without a loss of functionality. The high relevance of E1.27 has already been shown by mutagenesis studies at the TSHR and LHR22,25,49,51 and by a naturally occurring LHR mutation (E1.27K) in a family with hypogonadism.22

None of the substitutions at D1.28 (LHR D355, TSHR D410) revealed wt-like properties indicating that a negatively charged amino acid residue at this position is mandatory for proper GPHR function. One exception is D1.28N that can still be activated to about 60% of the wt (Tables S2 and S3). TSHR
D410N was found in a family with hypothyroidism indicating that the residual function of this mutant TSHR is not sufficient in vivo. In a previous report, the two conserved negatively charged amino acids (E1.27 and D1.28) were studied in LHR, TSHR, and FSHR with regard to side-chain variations and signaling, and the effect of different sodium ion concentrations. This investigation revealed that the functionality of these two residues is buffer-dependent (ionic strength [Na+]1) in LHR but not in TSHR and FSHR. Finally, it can be summarized that most variations at E1.27 and D1.28 lead to an inactive receptor. This is in contrast to adjacent residues (eg, N1.24) where the receptor can be constitutively switched on by specific mutations. Therefore, the two negatively charged amino acid residues are assumed to be part of an “activating component” in the p10 region that is essential to stabilize an active state structure in the process of signal transduction from the N-terminus to the 7TMD upon GPH binding.

All investigated mutants at I1.29 (LHR I356, TSHR I411) were not delivered to the cell surface, which indicates an essential role for proper receptor folding and transport (Tables S2 and S3). Notably, substitution Ile1.29 to Val was previously described as fully functional in rat LHR demonstrating the need for a non-aromatic hydrophobic side chain at this position. At position 357 (M1.30), only M357C preserves full LHR signaling activity, whereas M357L and M357S showed partial signaling activity after hCG binding. In TSHR, Phe, Leu, Ser, and Cys at the corresponding position M412 led to partial signaling activity. In conclusion, both amino acids I1.29 and M1.30, located at the supposed N-terminus of TM1 (Figure 2), are functionally relevant in terms of receptor folding and thereby, function. They are structural elements defining the receptor architecture that is predisposed for activation.

We provide evidence here that G1.31 (LHR G358, TSHR G413) is a very crucial position for activity regulation in both LHR and TSHR. Substitution, especially to proline, resulted in a highly increased constitutive signaling activity in both receptors (Tables S2 and S3). Additionally, LHR G358 substitutions to Ala, Asn, Ser, and Val induced high constitutive activity compared to wt LHR. Ala and Phe substitutions at G1.31 have been reported for the rat LHR with comparable effects on efficacies, but no data on the basal activity of these LHR mutants were presented. In contrast to LHR, G413A in the TSHR was not constitutively active, indicating a small difference between both receptors. Moreover, LHR expression was remarkably increased (up to 200%) due to several mutations at position G358 (eg, to Asn, Pro, Asp), a phenomenon that was not observed for the corresponding TSHR mutants (Tables S2 and S3). Consistent with the above observation that E2 is super-active at some constitutively activated TSHR variants, almost twice the signaling activity of E2 was found with G413V and G413P (Table S3).

Finally, most side-chain substitutions at LHR Y359 (Y1.32), except Y359K, led to a decreased maximum of hCG-induced signaling (~30%-60%) although cell surface expression levels of the mutants were mostly comparable to wt LHR (Table S2). This supposes a role for hormone-induced signaling, as shown previously for LHR Y1.32 mutants to Ala, Asp, and Phe. This observation was also true for most of the investigated mutants at position Y414 in the TSHR. In contrast to LHR Y359G, TSHR Y414G is constitutively active (Table S3). In conclusion, substitutions at position Y1.32 revealed high similarities between both receptors with respect to hormone-induced signaling, but a slight difference regarding the induction of constitutive activation. Our results, therefore, may support again that TSHR is in a higher predisposition to be activated by mutations as any other GPHR, which might be due to the higher level of natural basal activity. According to our TSHR homology model (Figure 2), the aromatic side chain of Y414 is centrally located between Y279 (LRRD) and Y481 (EL1), which are both known to be relevant for signal transduction of TSHR. The same is true for the corresponding tyrosine in the LRRD of LHR.

To summarize this section, our comprehensive mutation screening highlights the positions N1.24 and G1.31 as most sensitive for the increase of GPH-independent signaling activity. N1.24S and G1.31P can be activated further by GPH indicating that these positions are switches that arrange the intramolecular agonist in a conformation that stabilizes a partially active state. These positions together with F1.23 are in interplay with specific amino acid residues of the EL (eg, TSHR I486, I568, V656), the 7TMD (eg, TSHR Y643) and the C-terminal part of LRRD (eg, TSHR S281, LHR S277) (Figure 2) which are also known to act as molecular switches to induce the active state. F1.23, E1.27, D1.28, and Y1.32 are required to stabilize the active state receptor conformation induced by modifications in the p10 region or the full TALI region (including TSHR S281, LHR S277). C1.26 and I1.29 are highly essential for proper folding and function of the GPHR. Therefore, our data support that the extracellular p10 region is essential:

1. to induce a receptor predisposition toward activation (eg, indicated by inactivating mutations at P1.25),
2. to be switched to a fully active state (CAM at D1.21-N1.24), and
3. to stabilize an active state (by E1.27, D1.28, Y1.32).

These multifunctional properties are enabled by specific amino acid side-chain characteristics interacting with other receptor components.

### 3.2 | Interplay of diverse receptor regions to regulate signaling activity

As shown in Figure 3 and Tables S2 and S3, mutations of G1.31 resulted in constitutively active LHR and TSHR. To
explain this finding, we have addressed two possibilities, (a) the mutations of G1.31 directly induce structural rearrangements of the 7TMD, or (b) the mutations of G1.31 switch the conformation of TALI into a conformation that activates the 7TMD. We used the advantages of the double mutations combining G1.31P with further substitutions in- and outside of TALI, either activating (TSHR: S281N, D633H; LHR: S277N, D578H) or inactivating (E1.27K).

E1.27 (TSHR: Glu409; LHR: Glu354) is an important residue which may form a salt bridge to the basic amino acid residue K6.35 (TSHR: K660; LHR: K605) during receptor activation (see above) and transmit the signal from the extracellular to the transmembrane receptor part. Mutations of E1.27 consequently resulted in a loss or reduced functionality of GPHR (Figure 3, Tables S2 and S3). The double mutants LHR E354K/G358P and TSHR E409K/G413P showed significantly reduced basal and GPH-induced activity compared to the respective G1.31P mutants (Tables S2 and S3) although these double mutants were properly expressed at the cell surface (>70% of the wt receptors, Tables S2 and S3). This indicates that activation by G1.31P essentially requires E1.27 to induce constitutive activity and to maintain an active state conformation.

For comparison, we introduced the inactivating mutation E1.27K into the highly constitutively active D6.44 of LHR and TSHR. Surprisingly, LHR E354K/D578H revealed a slightly higher basal activity than D578H alone, but TSHR E409K/D633H showed a significantly decreased constitutive activity (30% of D633H, Tables S2 and S3). However, one must consider the lower cell surface expression of TSHR E409K/D633H, which may cause this difference. Nevertheless, at least in LHR, D6.44H in TM6 does not require the p10 region to shift the 7TMD into its active conformation. Interestingly, despite CAM in 7TMD (eg, D6.44H), the inactivating E409K mutation did not inhibit E2-mediated activation of TSHR, even in combinations with CAM (Figure 3, Tables S4 and S5).

We also observed that the assumed structural changes caused by the mutation of G1.31 (eg, LHR G358P and TSHR G413P) did not induce a fully active receptor conformation because these mutant GPHR can be further activated by the hormones (Figure 3, Tables S2 and S3). Notably, additive effects of combined CAM at different regions in the TSHR indicated multiple signal input to achieve a fully active state. Therefore, we asked whether the combination of G1.31P with other activating mutations in the hinge region, the p10 region and the 7TMD can shift GPHR to fully active receptors. As shown for LHR S277N/G358P and LHR N351S/G358P (Figure 3A), both double mutants are constitutively active and could not be further activated by hCG. The constitutive activities of D578H and G358P/D578H are similarly high and close to the fully active state induced by hCG. Consistent with this, we observed an additive effect between CAM of the TSHR. Most notably, the basal activities of TSHR
S281N/G413P and TSHR G413P/D633H were nearly doubled those of G413P und S281N, and both double mutants were fully active compared to bTSH stimulation (Figure 3B). From these findings, we conclude that GPHR indeed requires multiple local switches in the extracellular region to become fully active.

3.3 Differences and similarities between TSHR and LHR in the activation mechanism

Our study points to several differences between TSHR and LHR:

1. The TSHR can be activated by CAM in the p10 region without the entire N-terminus. This is not the case for the LHR (Figure 4).
2. S277N in LHR is significantly less constitutively active (compared to the hCG-induced maximum in signaling) than the corresponding S281N in the TSHR (Figure 3).
3. Y1.32G is a CAM in TSHR, but not in LHR.
4. D6.44H in the LHR does not require the p10 region to shift the 7TMD into its active conformation (not inhibited by the E354K substitution), however, in TSHR, D6.44H is inhibited by the extracellularly located inactivating E409K variant (Figure 3B; Table S3).
5. Several mutations at the LHR (eg, P352A/D/N/S or G358N/P/D) showed strongly increasing cell surface expression (170%-280%), which has not been observed for corresponding TSHR variants. Of note, LHR D578H was expressed 5-fold higher than the wt LHR (Table S2) as found in a previous study. The M3-LHR D578H is expressed 8-fold higher than the wt LHR.
6. Any mutation at position 352 (P1.25) in LHR showed an only marginal cAMP response to hCG. Mutations at P407 (Ala, Asp, Asn) in TSHR revealed moderate surface-expression levels and bTSH-induced signaling properties (30%-70%). This indicates that the spatial adjustment of the p10 region into an active state in LHR is more sensitive to P1.25 modifications than in TSHR. This should also help to understand why CAM in the p10 region of the N-terminally truncated TSHR variants still cause constitutive activity, but not in the N-terminally truncated LHR.

In summary, the differences between both receptors exist with respect to expression levels and activation by distinct mutations. This might be due to two reasons: (a) the TSHR has a higher basal activity than the LHR, and therefore, is

**FIGURE 4** The G1.31P mutant activates the N terminal truncated TSHR with the p10 region, but not in the corresponding LHR variant. For functional characterization, HEK-293T cells were transiently transfected with the indicated M3-LHR (A) and M3-TSHR (B) with 100 ng/well (96-well plate). The inlet graph in (A) shows the positions of the mutated residues. Basal and E2-induced (only M3-TSHR constructs) cAMP levels in transfected HEK-293T cells were determined as described under Section 2. Empty vector (mock) served as a negative control. Specific cAMP levels were referred to as x-fold over the empty vector. All assay data are given as means ± SEM of three independent experiments, each performed in triplicate. The complete data set is given in Tables S4 and S5.
more prone to further activation. Currently, we cannot rule out the possibility that the found differences are because of higher expression of TSHR. Titrating both GPHR (mutants) to similar cell surface expression levels and comparing their activities may not really clarify this issue because it has been shown that internalized or intracellularly expressed TSHR can still signal.57 (b) Structural differences exist, which have been confirmed directly in a previous study investigating allostery binding sites for drug-like small molecules.58 This study with chimeric TSHR/LHR constructs provided evidence that sequence differences between TSHR and LHR indeed result in significant structural specificities, especially between the extracellular loops and transitions to the 7TMD. This assumption is further supported by chimeric GPHR with swapped N-termini and 7TMD59,60 that are at least less functional compared to the wt receptors. Unfortunately, without structural data from X-ray or cryo-EM studies, we cannot decipher in detail how these differences are manifested because comparative homology models are not appropriate in this case to unravel those fine-tuning differences at the structural level. We postulate that the p10 region of the TSHR is potentially more flexible than in the LHR and that the activation-relevant components (eg, E409) swap closer to their active state interaction partner. This might also lead to higher basal activity, which is indeed nullified in the TSHR variant without the p10 region (M3-TSR p10-Deletion, Table S5). In addition, this hypothesis would help to explain the differences observed for mutations at P1.25, which is most important for hCG-induced LHR activation (impaired signaling by mutation), in contrast to mutations at the corresponding proline in TSHR (decreased TSH signaling). Small flexibility differences in the p10 region of both GPHR may result from different amino acids in close spatial proximity, such as extracellular loops. Those differences were previously specified in detail.21

3.4 Mutations in the p10 region favor a distinct active conformation

TSHR is known to couple not only to Gs, but also to Gq/11 proteins.61 Therefore, we tested the effect of activating mutations in the N-terminus, p10 region and the 7TMD also in IP1 assays as readout for Gq/11 activation. As shown in Supporting Figure S2, TSH induced a strong IP1 accumulation at the wt TSHR, whereas E2 showed a significantly reduced efficacy. Gs protein-activating mutations (eg, S281N, G413P, D633H, and the respective double mutants) displayed no or only marginally elevated basal activity in the IP1 assay but could be stimulated by TSH and E2. The inactivating mutation E409K was also inactive in the IP1 assay. It seems that the activating effect of CAM is usually less pronounced in the Gq/11 signaling pathway, and some CAM can discriminate between Gs- and Gq-protein coupling. These findings are not new and have been observed for many CAM but not for all.52,63 Similarly, several mutations identified in the TSHR selectively impair Gq- but not Gs protein coupling.64-66 The distinct effects of mutants can be explained not only by different conformational states required for the different coupling abilities, but also simply by the lower efficacy of TSHR in coupling to Gq/11—compared to Gs proteins, which is not yet understood in depth. It has been postulated that Gs-mediated cAMP production is initiated by binding of one TSH to one protomer of a putative TSHR homodimer, whereas phosphoinositide signaling requires binding of two TSH molecules to the TSHR homodimer.67 TSHR mutations might affect TSHR homodimerization, and therefore, the ability of the TSHR to activate Gq/11 signaling.

3.5 TALI functions as tethered agonist to induce an active conformational state

Numerous studies investigated the interplay between the extracellular part and the 7TMD and found that the extracellular part of TSHR and LHR acts as a regulatory element in receptor function.9,59,68-70 However, several studies produced contradictory results. For example, after the initial report of TSHR activation by a deletion in the extracellular hinge region71—in the so-called cleavable (“C”) peptide region—another study identified this region as functionally irrelevant.72 Increased constitutive activity of TSHR constructs lacking the N-terminus, some including the p10 region, was reported.15,17 Moreover, specific fragment deletions in the TSHR hinge region also led to increased basal activity,70 which has also been confirmed for the FSHR.73 Therefore, it was generally assumed that the N-terminus of GPHR mediates a constraint on the 7TMD and that a release of this (undefined) constraint causes at least slight ligand-independent receptor activation. This scenario was extended by the suggestion that the TSHR N-terminus not only acts as an intramolecular antagonist, but also additionally switches from an inverse agonist to an agonist.15 Experiments with the LHR have shown that corresponding deletion experiments (deletion of the entire N-terminus up to TM1) did not lead to a constitutively active receptor.9,21,59 However, the role of the N-terminus in constituting an activation-competent conformation and as a key player in hormone- or CAM-induced activation of GPHR was confirmed.9,59,68

In this study, we aimed to clarify whether the sequence flanking the p10 region N-terminally is required for inducing constitutive activity of the mutant receptors. Thus, we N-terminally truncated the LHR and TSHR. To assure proper cell surface expression of N-terminally truncated LHR and TSHR constructs, we fused the HA-tagged N-terminus of the rat muscarinic acetylcholine receptor (M3) directly upstream
of the p10 region (Table S1). Most M3-LHR constructs were expressed moderately compared to the wt receptor (Table S4). In contrast, M3-TSHR constructs were significantly more expressed and had to be diluted (1:20) compared to full-length constructs in transfection setups to allow for ELISA measurements in the suitable assay window (Table S5). However, the cAMP assays for M3-TSHR were performed with similar transfection amounts to allow for comparison with full-length constructs (Tables S3 vs S5).

As shown in Figure 4A and Table S4, none of the activating mutations in the p10 region of the LHR showed constitutive activity when the N-terminus (deletion of positions 1-349) was replaced by the N-terminus of the M3. M3-LHR D578H showed constitutive activity indicating that the 7TMD structure of M3-LHR is correctly arranged. Notably, while constitutive activity induced by mutations of the p10 region occurred only in LHR constructs with the complete N-terminus (Figure 4A), in TSHR, constitutive activation could also be induced by CAM in the p10 region (eg, G413P) without the entire N-terminus (Figure 4B). This finding again is indicative of differences between both receptors.

Next, we asked whether the p10 region participates in the agonistic action of E2. In full-length TSHR, mutation of the individual positions in the p10 region did not specifically influence the agonistic properties of E2 (see Table S3). However, E2-triggered activation of several CAM was higher than of the wt TSHR (Figure 3B). This was also true for TSHR constructs without the extracellular LRRD and hinge region (wt M3-TSHR construct [TSHR from F405-L764]) (Figure 4B). An M3-TSHR construct without p10 (M3-TSHR p10-Deletion) was marginally activated by E2, although the receptor expression level was above 50% (Table S5). This may suggest that the p10 region is required for full recepto
tor activation by this small molecule TSHR ligand or the M3-TSHR p10-Deletion construct is not correctly folded, and therefore, nonfunctional, or the specific sequence of the M3 N-terminus interferes with E2 pharmacodynamics. To address these hypotheses, we introduced the activating mutation D633H into M3-TSHR p10-Deletion. This construct showed a slightly higher basal activity and activation by E2. Previous studies with N-terminally truncated TSHR constructs lacking the p10 region showed that the N-terminus is not required for activation by E2, contrasting with our results for M3-TSHR p10-Deletion. Using the same constructs as in Neumann et al47 (KFLR-TSHR) and, for control purposes, introducing it into the pcDps vector and equipping it with an N terminal HA-tag (Rhod-EcoRI-TSHR p10-Deletion), we could reproduce the data47 that E2-mediated activation does not require the TSHR N-terminus and the p10 region (Table S5). Investigating the reason for the functional differences between the truncation constructs containing the M3 or the rhodopsin N-terminus, we identified that the last three amino acids of the M3 N-terminus were responsible for this functional difference. By exchanging these amino acids (IWQ) with the corresponding amino acids of the N-terminus of rhodopsin (GVV), we reinstalled the increased cell surface expression and E2-induced activation (Table S5). In conclusion, our data clearly demonstrate that the p10 region is not required for E2 activation. Our N-terminal truncation constructs revealed that the p10 region is mandatory to activate GPHR regardless of the nature of the external triggers (hormones, autoantibody, and mutation). Previous data on LHR and the extended data on LHR and TSHR presented here strongly support the exclusive involvement of TALI in GPHR activation. Furthermore, activating deletions in the TSHR N-terminus allow for structural changes or exposition of the p10 region toward the 7TMD, thereby inducing the sequential signal transduction steps. This scenario does not necessarily require a release of inactivating constraints between the N-terminus and the 7TMD for receptor activation, which is consistent with our presented data.

In summary, we experimentally showed that GPHR contains a tethered agonistic ligand TALI that undergoes structural changes upon GPH binding. We not only identified specific structural components in the p10 part of the TALI that stabilize the inactive conformation of TALI, but also establish new ionic and hydrophobic interactions with the extracellular loops of the 7TMD during activation. In addition, we showed that the small molecule agonist E2 directly binds to the 7TMD and does not require TALI for its agonistic action.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
AS and AB performed the experiments. SN helped with the small molecule experiments. GK and PS performed the generation of a receptor model and ligand docking. AS, GK, PS,
AB, and TS analyzed the data. AB, GK, PS, and TS designed the study and wrote the paper with contributions from all authors.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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