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Research Article

Molecular and structural effects of inverse agonistic mutations on signaling of the thyrotropin receptor – a basally active GPCR

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Abstract. Several mutations that decrease the basal signaling activity of G-protein coupled receptors (GPCRs) with pathogenic implications are known. Here we study the molecular mechanisms responsible for this phenotype and investigate how basal and further activated receptor conformations are interrelated. In the basally active thyroid stimulating hormone receptor (TSHR) we combined spatially-distant mutations with opposing effects on basal activity in double-mutations and characterized mutant basal and TSH induced signaling. Mutations lowering basal activity always have a suppressive influence on TSH-induced signaling and on constitutively activating mutations (CAMs). Our results suggest that the conformation of a basally 'silenced' GPCR might impair its intrinsic capacity for signaling compared to the wild-type. Striking differences in conformation and intramolecular interactions between TSHR models built using the crystal structures of inactive rhodopsin and partially active opsin help illuminate the molecular details underlying mutations decreasing basal activity.

Keywords. Inverse agonism, signal transduction, activation mechanism, endocrinology, glycoprotein hormone receptors, TSHR, LHR, LHCGR, FSHR.

Introduction

Nearly 20% of all G-protein coupled receptors (GPCRs) are reported to permanently activate G-proteins in the basal state, independent from ligand induced stimulation [1–5]. The distribution of GPCR/G-protein subtype specificity is the same for receptors with or without constitutive activity (Gαi: ~45%, Gαq: ~33%, and Gαs: ~22%) [2], indicating that ligand-independent basal activity is not dependent on a particular G-protein subtype or GPCR specificity. The ligand-independent basal-signaling activity of GPCRs is responsible for the maintenance of a basal level of physiological function and is fundamental to physiological, medical and pharmacological research [6–8].

Pathogenic mutations which modify the basal activity of GPCRs have been reported [1, 9–12]. Furthermore, relationships between the basally active conformation and other signaling properties such as ligand binding efficacy or G-protein selectivity have been demonstrated in several monomeric or dimeric GPCRs, such as viral chemokine, cannabinoid, melanocortin and opioid receptors [3, 7, 12, 13]. Therefore, ligand-independent G-protein activation is of relevance to physiology and consequently pharmaceutical research [8].

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A large number of mutations regulating the basal signaling activity (up or down) of GPCRs are known [3, 11, 14, 15]. In contrast to constitutively activating mutations (CAMs), side-chain substitutions which decrease or abolish the basal signaling activity we term inverse agonistic mutations (IAMs). Inverse agonistic mutations differ from other inactivating mutations (e.g. loss of ligand induced stimulation due to decreased cell surface expression) by shifting the conformation of the wild-type basal active state to a basally non-active receptor state. IAMs are helpful in revealing detailed insights into the signal transduction mechanisms of GPCRs [14, 16, 17]. Furthermore, IAMs have been used to design stabilized conformers of the β1A-adrenergic receptor for crystallization studies of this GPCR [18].

In the subfamily of glycoprotein hormone receptors (GPHRs), the thyroid stimulating hormone receptor (TSHR) has the highest level of basal signaling activity compared to the homologous lutropin receptor (LHCGR) and the follicle stimulating hormone receptor (FSHR) [19, 20] and is simultaneously able to activate different G-protein subtypes [21]. The stimulation of the TSHR is initiated by TSH, but can also be mediated by CAMs (www.ssfa-gphr.de; http://innere.uniklinikum-leipzig.de/tsh) and to a certain extent by the deletion of epitopes [22–24].

In contrast to these activating events, an opposing mutation-phenotype was identified in the TSHR which is characterized by an impaired basal Gαs mediated signaling activity [20, 25–30]. Surprisingly, utilizing a GPHR mutation database (www.ssfa-gphr.de) reveals that the locations of published IAMs are distributed all over the structure of the TSHR. IAMs were observed in the serpentine domain as well as in the intra- and extracellular regions, indicating that there are likely to be different intra- or intermolecular mechanisms leading to this common decrease in basal signaling activity. Some of these mutations are suggested to have a modifying influence on the intramolecular interaction between the extracellular region and the serpentine domain [28] or interactions between specific amino acids in the transmembrane region [27, 30–32] as well as directly affecting interactions between the receptor and the G-protein [33, 34].

Therefore, in the current study we investigate whether a silenced (non-active) basal receptor conformation can influence the activation mechanisms caused by CAMs or induced by TSH. Our aim was to gain insight into the intrinsic signaling properties of GPCRs and the molecular relationship between basal activity and induced activation. We designed and tested IAMs in combination with CAMs in double mutants of the TSHR (Fig. 1). To prevent direct interaction between two mutated side-chains, we selected spatially distant mutations in the extracellular and transmembrane region (as defined by homology models). We characterized the effect of these double mutants on the level of basal and TSH-induced signaling activity and observed a strong suppression of mutation-induced constitutive activation and TSH-induced activation in these double mutated receptors. Additionally, we investigated the molecular and structural basis of inactive and basally active receptor states by comparison between homology models of TSHR based on the inactive rhodopsin [35] and (partially) activated opsin [36] X-ray structures.

We demonstrate that a receptor conformation with a decreased or nullified basal signaling activity also strongly reduces the receptors intrinsic capability for further activation. Our study suggests that the achievement of an active conformation (particularly caused by CAMs) is very likely dependent on a basally active conformation. The results presented here provide more detailed insight into the relationship between the conformations of different activity states and structural-functional prerequisites for the maintenance of activated GPCR conformations.

Materials and methods

Site-directed Mutagenesis. TSHR mutations were constructed by PCR mutagenesis using the human TSHR-pcDNA3.1(-)/hygro as template as previously described [37]. 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 mutated TSHRs. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Life technologies, Paisley, UK) at 37 °C in a humidified 5% CO_2 incubator. Cells were transiently transfected in 24-well plates (0.5×10^5 cells per well) with 300 ng DNA per well using the GeneJammer® Transfection Reagent (Stratagene, Amsterdam, NL).

FACS Analyses. The TSHR 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 PBS and transferred into Falcon 2054 tubes. Cells were washed once with PBS and then incubated at 4 °C for 1 h with a 1: 400 dilution of a mouse anti human TSHR antibody (2C11, 10 mg/l, Serotec Ltd., Oxford, UK) in the same buffer. Cells were washed twice and incubated at 4 °C

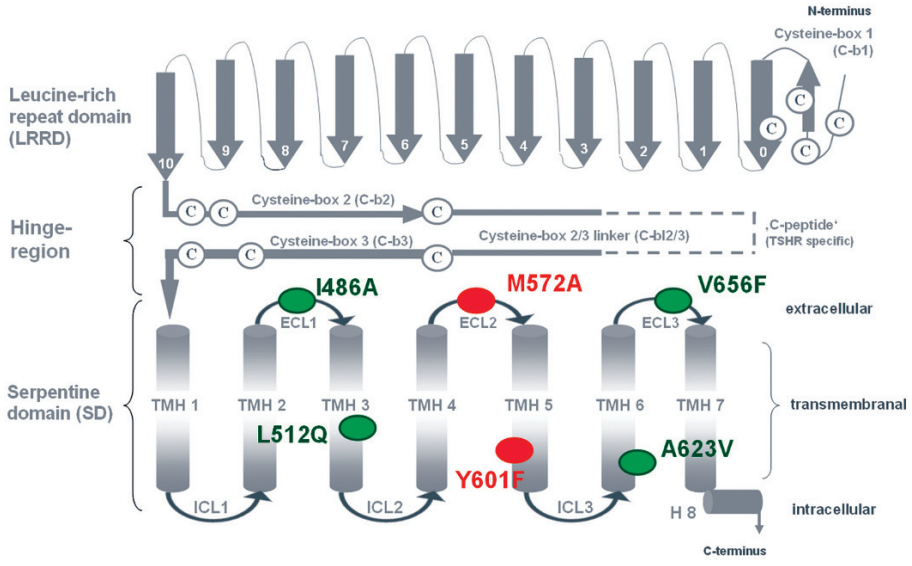

Figure 1. Localization of IAMs and CAMs selected for double mutations. The location of mutations that we combined in the present study to produce double mutations are represented on a scheme of the TSHR: red – inverse agonistic mutations (IAM) with abolished basal activity, green – constitutively activating mutations (CAM). The selection of spatially distant single mutations prevents a direct side-chain interaction in double mutants (which always feature an IAM and a CAM).

for 1 h with a 1 : 200 dilution of fluorescein-conjugated F(ab′)2 rabbit anti mouse IgG (Serotec). Before FACS analysis (FACscan Becton Dickinson and Co., Franklin Lakes, NJ, USA) cells were washed twice and then fixed with 1 % paraformaldehyde. Receptor expression was determined by the mean fluorescence intensity (MFI). All values for the wt TSHR and mutated receptors were subtracted with the value obtained by MOCK transfected cells. The wt TSHR was set at 100 % and receptor expression of the mutations was calculated according to this. The percentage of signal positive cells corresponds to transfection efficiency, which was approximately 50–60 % of viable cells for each mutation.

cAMP Accumulation Assay. For cAMP assays cells were grown and transfected in 24-well plates. Forty eight hours after transfection, cells were incubated in the absence or presence of 100 mU/ml bTSH (Sigma Chemical Co.) in serum free medium supplemented with 1mM IBMX (Sigma) for one hour. Reactions were terminated by aspiration of the medium. The cells were washed once with ice cold PBS and then lysed by 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. All values for the wt TSHR and mutated receptors were subtracted with the value obtained by MOCK transfected cells.

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 FACS. COS-7 cells were transiently transfected in 24-well plates with increasing concentrations of wt or mutated TSHR plasmid DNA (50; 100; 150; 200; 250 and 300 ng per well). The total DNA amount for the determination of constitutive activity was kept constant by cotransfection with empty vector to the amount of the highest DNA concentration of 300 ng per well. For determination of cell surface expression of the transfected constructs see "FACS Analyses". Basal cAMP formation as a function of receptor expression was analyzed according to Ballesteros et al. [38] using the linear regression module of GraphPad Prism 4 for Windows. All values for the wt TSHR and mutated receptors were subtracted with the value obtained by MOCK transfected cells.

Structural Bioinformatics and Molecular Modeling. Until recently, the only available GPCR structures, the β2-adrenergic receptor (β2-AR) (PDB entry codes: 2RH1 and 2R4R [39–41]) and bovine rhodopsin (PDB entry codes: 1F88 [35], 2I35 [42], 2J4Y [43]), for building homology models of GPCRs contain an inverse agonist as ligand which changes the GPCR conformation from the natural ligand-unoccupied receptor conformation. The recently published structure of opsin [36] (PDB entry code: 3CAP) lacks the inverse agonistic ligand, retinal, and consequently represents structural changes likely due to a partially active receptor state. We generated a homology model using the opsin structure as a template for the basally-active TSHR. For comparison between this basally active TSHR model and inactive TSHR models, we generated homology models of the inactive TSHR based on the structure of rhodopsin.

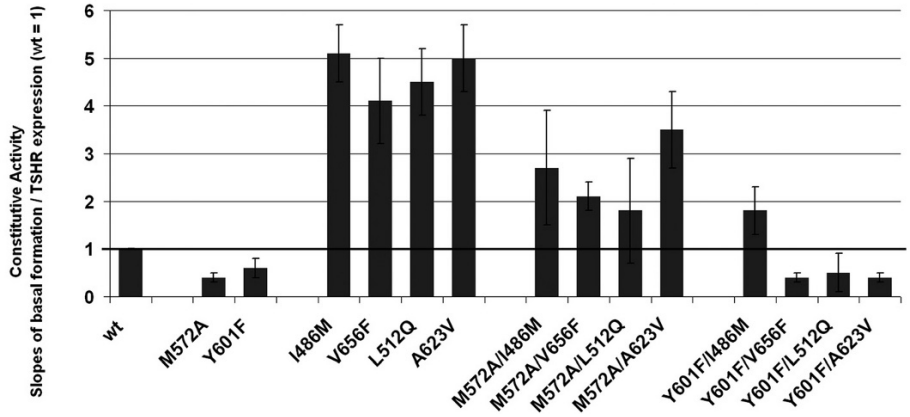

Figure 2. Basal signaling activity (slopes) of the wild-type TSHR and single/double mutations. This diagram shows the slopes obtained after linear regression analysis of experimentally determined activity data for the single and double mutants. Two single mutations (M572A and Y601F$^{5.58}$) were characterized by a decreased basal signaling activity in contrast to the constitutive activation by the other single mutations. The double mutants containing the inverse agonistic mutations (IAMs) and CAMs displayed decreased ligand independent signaling. Mutation Y601F$^{5.58}$ located at TMH5 close to the intracellular site showed the strongest suppressive influence on the CAMs in comparison to those double mutants with IAM M572A in the ECL2.

Several receptor-specific corrections in the homology models were made based on sequence alignments. In opsin and rhodopsin interactions of the side-chains of two consecutive threonines with the helical backbone of the preceding residues causes a structural bulge in TMH2. In the TSHR, no consecutive threonines exist in TMH2, which suggests the presence of a regular α-helix, extending to position 2.71 (Y481 in the TSHR). At TMH5, a minor change of orientation (10 to 15 degrees twist) of the N-terminal half of TMH5 was generated due to the lack of a proline compared to opsin/rhodopsin. Gaps of missing residues in the loops of the template structure were closed by the 'Loop Search' tool implemented in Sybyl 7.35 (Tripos Inc., St. Louis, Missouri, 63144, USA).

To facilitate the comparison of different GPCRs we used the Ballesteros-Weinstein nomenclature [44]. This position identifier scheme uses a highly conserved residue in each transmembrane helix as a common reference for all family A GPCRs. For example, the highly conserved N in TMH1 is defined as 1.50 and the highly conserved P from the NPxxY motif of TMH7 is defined as 7.50.

Results

The IAM, M572A, located on extracellular loop 2 (ECL2) and a second IAM, Y601F, in transmembrane helix 5 (TMH5) were combined with CAMs I486M in ECL1 [45], L512Q$^{3.43}$ in TMH3 [46], A623V$^{6.34}$ in TMH6 [47] and V656F in ECL3 [48]. Each IAM/CAM pair was located on the TSHR structure such that direct, mutual interaction was prevented (Fig. 1). The inverse agonistic mutation, M572A, was identified by an alanine scan of amino acids in the ECL2 [49]. The second residue with inverse agonistic characteristics when substituted, Tyrosine 601 (Y5.58) in TMH5, is highly conserved in GPCR family 1. Detailed site-directed mutagenesis studies have revealed that this tyrosine has a key role in Gαs- and Gαq-mediated signaling of the TSHR [25]. Functional characteristics of the mutated TSHR were studied by transient expression in COS-7 cells. The effects of the mutations on cell surface expression, basal and TSH stimulated cAMP accumulation are summarized in Table 1.

Cell surface expression. All of the tested single IAMs and CAMs showed a level of cell surface expression comparable to wild-type (set at 100%), ranging between ~70 to ~110% (Table 1). Therefore, the suppressed basal-activity observed in the IAMs is not due to a decreased number of cell surface expressed receptors.

Those double mutants with Y601F tend to have similar cell surface expression to wild-type with the exception of Y601F/I486M, which was reduced to 45%. Double mutants with M572A have slightly decreased cell surface expression levels, ranging between ~50 and ~76% compared to wild-type TSHR (Table 1).

Basal cAMP accumulation. The levels of basal cAMP accumulation for the single CAMs were between 3.7 and 4.1 fold compared to wild-type (set at 1) (Table 1), which was confirmed by linear regression analyses where the CAMs displayed slopes in a range between 4.1 and 5.1 (Fig. 2).

Table 1. Functional characterization of double-mutants containing constitutively activating mutations and mutations decreasing the level of basal activity. COS-7 cells were transfected with the wt TSH receptor or mutated TSH receptors. The TSH receptor cell surface expression was quantified on a FACS flow cytometer. Due to 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 were determined after stimulation with 100 mU/ml bovine TSH. Constitutive activity (CA) is expressed as the slope of basal cAMP formation plotted as a function of receptor expression determined by FACS analyses. Slopes were calculated by linear regression analysis. Data are given as mean ± SD of three independent experiments, each carried out in duplicate. The pcDNA3.1 vector was used as a control.

Mutation type/ Location	Transfected construct	Cell surface expression	cAMP accumulation (fold over TSHR wt basal)		
		FACS % of TSHR wt	basal	TSH stimulated	slope
	TSHR wt	100	1.0	14.5 ± 1.0	1.0
IAMs					
ECL2	M572A	86.4 ± 1.9	0.8 ± 0.0	7.0 ± 1.0	0.4 ± 0.1
TMH5	Y601F	111.5 ± 5.2	0.6 ± 0.1	3.8 ± 0.2	0.6 ± 0.2
CAMs					
ECL1	I486M	71.0 ± 1.5	3.7 ± 0.1	9.5 ± 0.5	5.1 ± 0.6
ECL3	V656F	93.5 ± 5.7	4.1 ± 0.5	10.8 ± 0.9	4.1 ± 0.9
TMH3	L512Q	106.4 ± 5.2	3.7 ± 0.7	11.5 ± 1.0	4.5 ± 0.7
TMH6	A623V	100.7 ± 1.9	3.8 ± 0.3	16.2 ± 2.2	5.0 ± 0.7
IAMs/CAMs					
ECL2/ECL1	M572A/I486M	50.9 ± 4.8	1.3 ± 0.0	7.3 ± 0.7	2.7 ± 1.2
ECL2/ECL3	M572A/V656F	75.9 ± 0.9	1.9 ± 0.8	5.7 ± 0.8	2.1 ± 0.3
ECL2/TMH3	M572A/L512Q	56.9 ± 6.2	2.7 ± 0.2	11.6 ± 0.6	1.8 ± 1.1
ECL2/TMH6	M572A/A623V	61.7 ± 3.8	2.3 ± 0.3	11.7 ± 1.4	3.5 ± 0.8
TMH5/ECL1	Y601F/I486M	45.6 ± 2.4	0.8 ± 0.1	5.1 ± 0.7	1.8 ± 0.5
TMH5/ECL3	Y601F/V656F	93.9 ± 0.2	0.6 ± 0.2	2.8 ± 0.3	0.4 ± 0.1
TMH5/TMH3	Y601F/L512Q	94.9 ± 3.1	0.6 ± 0.1	1.9 ± 0.1	0.5 ± 0.4
TMH5/TMH6	Y601F/A623V	92.7 ± 7.5	0.6 ± 0.2	3.6 ± 0.4	0.4 ± 0.1

In contrast, the single IAM, M572A (ECL2), was characterized by a slightly decreased level of basal cAMP activity, but determination of the basal activity independently of the cell surface expression revealed a slope of 0.4 compared with the wt TSHR (set at 1) (Table 1, Fig. 2). Similar results were measured for IAM Y601F (TMH5). Y601F showed a basal activity of 0.6 fold and also a reduced slope of 0.6 (Table 1, Fig. 2). Compared to the respective single CAMs, the double mutants M572A/I486M and M572A/V656F exhibited with 1.3 and 1.9 fold respectively, a basal activity similar to that of the wild-type. Mutations M572A/L512Q$^{3.43}$ and M572A/A623V$^{6.34}$ also showed reduced basal activity to about 50 % compared with the respective single mutations L512Q$^{3.43}$ and A623V$^{6.34}$ (Table 1). This decrease in basal activity mediated by IAM M572A was confirmed by the linear regression analyses, which showed for all mutants a lower slope as compared with the single CAMs (Table1, Fig. 2).

A similar result was observed for the IAM, Y601F$^{5.58}$; however, it has a greater suppressive influence on the signaling capacity of the TSHR than M572A, both for single and double mutants. For all combinations having Y601F$^{5.58}$ the basal-activity level was reduced to that of the single Y601F$^{5.58}$ mutation, which is reflected by slopes below that of the wt TSHR. Only the double mutation Y601F$^{5.58}$/I486M was characterized by a slope of 1.8 (Table 1, Fig. 2).

TSH induced cAMP accumulation. The single IAMs, M572A and Y601F$^{5.58}$, reduced TSH- induced maximum signaling activity to 50 % and 30 % respectively, compared to wild-type. The reduced TSH-induced signaling observed for Y601F$^{5.58}$ is particularly striking considering that it has a comparable cell surface expression level to wild-type (Table 1). The single CAMs have similar or slightly decreased TSH mediated signaling activity compared to the wild-type (Table 1).

IAM, M572A, has a stronger negative influence on extracellular located CAMs (I486M and V656F) than on those located in the transmembrane-helix region, reducing the signal to 50 % compared to wild-type

(Table 1). The strongest reduction in signaling induced by TSH occurs in the double mutants with IAM Y601F$^{5.58}$ with a reduction to 30 % (Table 1).

Molecular homology models of the TSHR. Two different TSHR serpentine domain models were generated (Fig. 3). The first model is based on the new opsin structure and is therefore most likely to represent a (partially) active TSHR (Fig. 3A,B). The second model is based on the inactive rhodopsin structure which originally contained an inverse agonistic ligand (Fig. 3C). Comparison of the two TSHR models reveals that several wild-type amino acids have different side-chain orientations and interactions close to where IAMs are observed. A most striking difference in the two TSHR homology models in terms of our IAM data is the differently oriented side-chain of the highly conserved Y601$^{5.58}$ (corresponding to Y223 in rhodopsin/opsin). Whereas in the rhodopsin based model the side-chain of Y601$^{5.58}$ is oriented towards TMH6 (Fig. 3C), in the opsin based TSHR model it is oriented to the core of the receptor towards TMH3 (Fig. 3B). As observed in the opsin structure, Y601$^{5.58}$ is within hydrogen-bonding distance (< 3 Å between donor and acceptor atoms) of the highly conserved R519$^{3.50}$ of the DRY motif in TMH3 and the highly conserved Y678$^{7.53}$ of the NPXXY motif in TMH7. Mutation of Y601$^{5.58}$ to Phe would disrupt any hydrogen bond interactions formed by the side-chain, and consequently may alter the interaction-interface between the transmembrane helices TMH5/TMH6 and TMH3 or TMH7. In turn, this may result in a different arrangement of these helices in the wild-type and mutant, similar to that observed in the active and non-active conformations of the receptor (as shown in the superimposition of the two models in Fig. 3D). The observations we made in the rhodopsin based inactive TSHR models are similarly observed in a model we previously built for TSHR, based on the β2-adrenergic receptor [50] (results not shown).

Discussion

Inverse agonistic mutations in the intracellular region of the TSHR influence interactions with G-proteins. To our knowledge, the first clue for an interrelation between a basally active conformation and the capability for mutations to induce constitutive activation of the TSHR was published in 1998 [51]. In this study two single side-chain substitutions were combined *in vitro* to a double mutation and functionally tested. One mutation was located in the intracellular loop 2 (R528H) and the second in the extracellular N-terminal domain (S281N). Mutation S281N [23] was known to induce a constitutive Gαs mediated signaling activity of the TSHR. In the above mentioned *in vitro* study it was shown that, in the double mutant, the constitutive activity induced by S281N was reduced by the R528H mutation to a level under that of wild-type basal activity. Likewise, the single intracellular substitution R528H showed an impaired basal signaling activity compared to the wild-type. Therefore, it is hypothesized that in the double-mutant TSHR, the decreased level of basal activity caused by mutation R528H has a strong negative influence on CAM S281N. The molecular mechanism underlying this decreased G-protein activation might be related to conformational changes in the TSHR ICL2 structure, partially preventing the G-protein activation or leading to a partial disruption of the G-protein recognition pattern on the cytoplasmatic surface. Interestingly, at the corresponding position of R528 in the TSHR, the homologous LHCGR has a histidine (H473). Similarly to the R528H TSHR mutant, the LHCGR is characterized by a lower level of basal signaling activity compared to the wild-type TSHR.
Mutagenesis studies of the intracellular region of the TSHR [33, 34] have identified a large number of mutations which decrease the basal signaling activity (Table 2 and Fig. 3A). Most of them are located directly in ICL2 and at the junctions between TMH5/ICL3 and ICL3/TMH6. Therefore, we would expect that these wild-type residues would be directly involved in the intermolecular interaction with G-protein in the basal state of the receptor. Recent chimeric mutagenesis studies in the TSHR and the LHCGR support this observation [20]. This study revealed that a single point mutation in the ICL2 of the TSHR led to a decreased basal signaling activity, moving it towards the level observed in the less active LHCGR.

Basally active receptors are different from silenced receptors in intermolecular interactions. The basally active conformation of several GPCRs might also be related to observed promiscuity for different ligands and G-protein subtypes. Findings in viral chemokine receptors hint that a relationship exists between the basally active state and promiscuous G-protein coupling. Viral chemokine receptors are characterized by a high level of basal signaling activity whereas other chemokine receptors are not [12]. Additionally, viral chemokine receptors bind promiscuously to a broad spectrum of chemokines and couple to a variety of different G-protein subtypes. This is in contrast to other chemokine receptors without basal signaling activity, which specifically couple to one G-protein subtype and chemokine ligand.

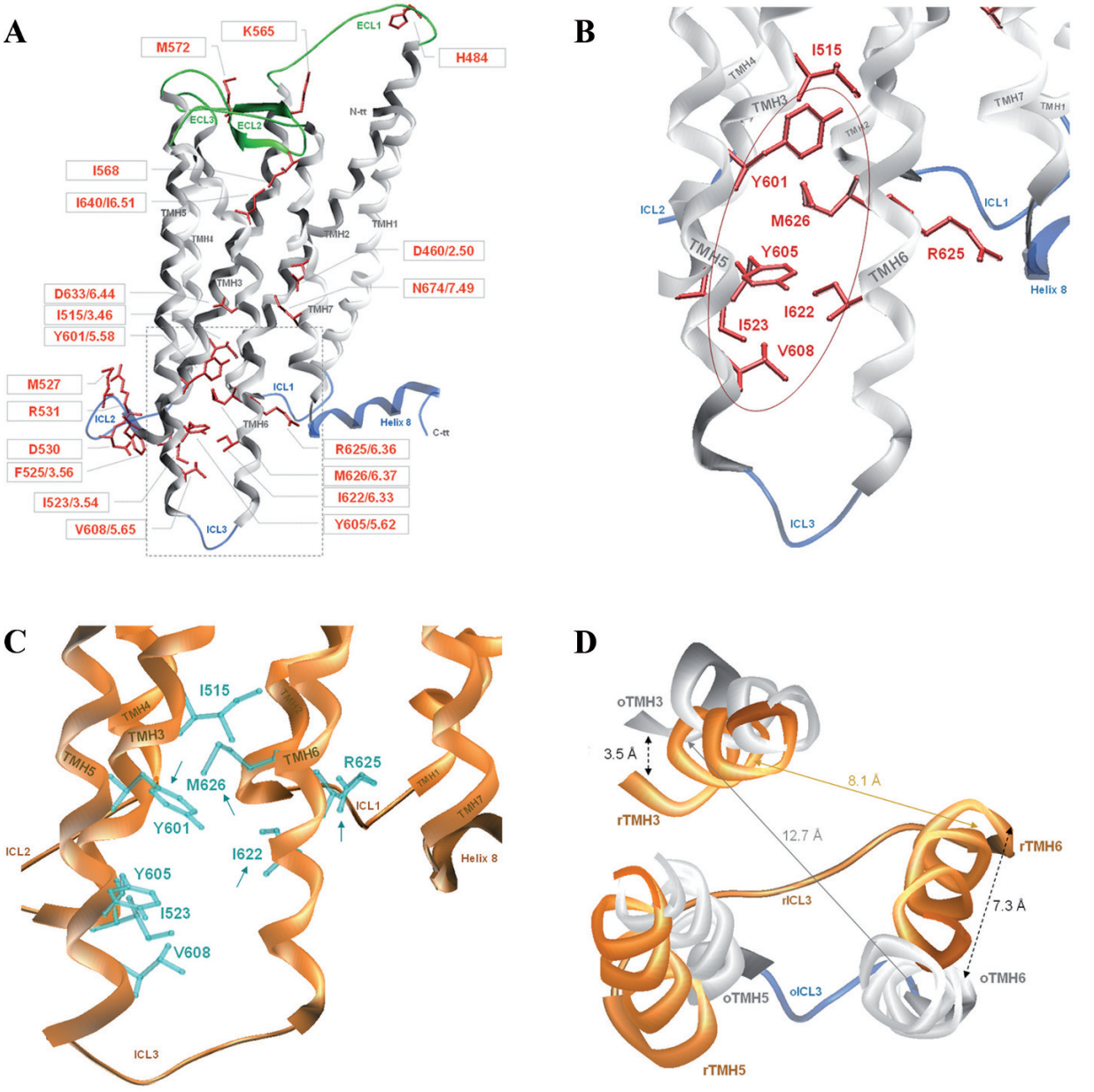

Figure 3. Amino acid positions of inverse agonistic mutations: Comparison of side-chain orientations at two different TSHR models based on an (partially) activated and an inactive receptor state. (*A*) The wild-type amino acid positions of reported (Table 2) inverse agonistic mutations (IAMs, red) are distributed over the serpentine structure of TSHR, in the extracellular loops (ECL1–3: green), transmembrane helices (TMH1–7: grey) and intracellular loops (ICL1–3: blue). The homology model is based on the (partially active) opsin crystal structure. Interestingly, few of these IAMs are located at highly conserved positions in GPCR family 1, for example D2.50 and Y5.58. Transmembrane positions are indicated by their specific TSHR numbering and their general numbering according Ballesteros/Weinstein numbering for family 1 GPCRs as well. The dashed box indicates the TMH5 and TMH6 fragments, which are compared in more detail for the TSHR models based on the (partially active) opsin structure (Fig. 3B) and the (inactive) rhodopsin structure (Fig. 3C). (*B*) Detailed view of the opsin based TSHR homology model (gray). Positions of IAMs (red) occur in a cluster of tight hydrophobic and aromatic interactions between TMH5 and TMH6 as well as between TMH3 and TMH5 (oval). These helix-interfaces are most probably involved in the maintenance of an (partially) active receptor conformation because mutation of each of these wild-type amino acids lead to an abolished basal signaling activity, most likely by disruption of stabilizing contacts. (*C*) Detailed view of the rhodopsin based TSHR model (orange) for the identical positions of inverse agonistic mutations. The tight hydrophobic cluster between TMH5, TMH6 andTMH3 observed in the opsin based model is not present. Different side-chain orientations (arrows) of several residues are observed and are likely to be related to the different spatial arrangement between TMH5, 6 and 3 (see D). (*D*) Superimpositions of opsin based (grey) and rhodopsin (orange) based TSHR models (view from the extracellular side on helices 3, 5, and 6 and intracellular loop 3. One major difference is the intracellular arrangement of these helices. The spatial differences between activated and inactive TSHR models are indicated by arrows. The 'more open' TMH6 conformation at the intracellular site in the opsin based TSHR model is stabilized by interaction to TMH5 and TMH7 (Fig. 3B) and might be involved in the mediation of a G-protein accessible surface. rTMH: helices based on rhodopsin, oTMH: helices based on opsin.

Table 2. Previously reported mutations decreasing the level of basal signaling activity of the TSHR. In this table 27 mutations at 20 different positions of the TSHR were extracted from a GPHR resource (www.fmp-berlin.de/ssfa). All of them decrease the level of basal activity down to 60 % of the wt and have a cell surface expression level up to 60 % compared to wild-type. Therefore, these mutations are most likely directly influencing the basal conformation of the TSHR, which mediates a permanent signaling activity despite a reduced expression. This special type of mutation is inverse agonistic due to negative modulation of the intrinsic signaling capability and thus is called inverse agonistic mutation (IAM).

Localization	Wild-type	Ballesteros-Weinstein	Mutation	Citation
C-b3	C390		S	[28]
TMH2	D460	2.50	N	[73]
ECL1	H484		A	[37]
TMH3	I515	3.46	M	[29]
	I523	3.54	A	[33]
	F525	3.56	A,K	[33]
ICL2	M527		A	[33]
	D530		A K	[33]
	R531		A	[33]
ECL2	S567		A	[30]
	I568		L	[30]
	M572		A	[30]
TMH5	Y601	5.58	A,D,F,S,W	[25]
	Y605	5.62	A	[25] [34]
	V608	5.65	A	[34]
TMH6	I622	6.33	A,D	[34]
	R625	6.36	A	[73]
	M626	6.37	L	[29]
	D633	6.44	K	[32]
	I640	6.51	L	[30]
TMH7	N674	7.49	A	[26]

Similar observations were made in the GPHRs. Most of the TSHR mutants that reduce basal cAMP accumulation compared to the wild-type TSHR also strongly reduce hormone induced IP accumulation that occurs in the wild-type TSHR [49]. Furthermore, hFSHR mutations in the transmembrane region result in constitutive activation and, unlike the wild-type receptor, these constitutively activated receptors can bind to three different glycoprotein-hormones (FSH, TSH, hCG) at the extracellular N-terminal site [52, 53]. This hormone promiscuity must be related to abolition of the extracellularly encoded hormone specificity and is most likely caused by structural differences between the basally non-active conformation and the partially activated (by a CAM) receptor.

Inverse agonistic mutation in the extracellular region of the TSHR. Recently, it was shown by Ho and co-workers [28] that a single mutation of the TSHR in the extracellular region (C390S) decreases the basal signaling activity and suppresses the signaling activity of CAMs located in the ECLs and the transmembrane region to 50–70 % of that observed for the single CAMs. Our findings for IAMs suggest that a mutant conformation with a decreased basal signaling activity causes a general suppression of activity in CAMs and TSH induced conformations, with the data for C390S further supporting our hypothesis.

In a previous study it was observed that specific chimeric substitution of amino acids from the hinge-region (the region in between the LRRD and the serpentine domain) of the LHCGR into the hinge-region of flyLGR2 induces constitutive activation [54]. Furthermore, a second chimeric substitution of amino acids from the LHCGR ECL2 into the ECL of the activated flyLGR2 (containing the chimeric hinge region) 'rescued' the increased basal signaling activity almost to the level of wt flyLGR2. This finding led to the proposal of a stabilizing interaction between the ECL2 and specific parts of the hinge-region which helps to constrain the wild-type conformation of the GPHRs or LGRs [54].

In contrast, other recent mutagenesis data for the ECL2 of the homologous TSHR have shown that slight modifications of one amino acid in ECL2 (at several positions) lead to a decreased basal signaling activity. It is strongly proposed that the ECL2 is involved in the regulation of basal activity, most likely by interaction with amino acids in the transmembrane helices such as TMH6 [30, 55]. The data we present here for M572A in ECL2 show clearly that at least one specific amino acid substitution in the ECL2 (M572A) has a suppressive influence on basal and mutation induced signaling activity. With regard to the LHCGR/flyLGR2 chimera, there are several different amino acids substituted in the ECL2 of the flyLGR2. Therefore, we suggest, that the impaired constitutive signaling activity observed in the chimeric flyLGR2 is more likely related to silencing modifications in the receptor structure introduced by mutagenesis of the ECL2 (like in the TSHR), than to stabilizing interactions between ECL2 and the hinge-region.

Regulation of the basal signaling activity at specific transmembrane components of GPCRs. The key involvement of particular transmembrane helices in the regulation of basal activity is supported by elegant studies in the thyrotropin releasing hormone receptors type 1 and 2 (TRHR1) and (TRHR2). In these studies the authors investigated molecular differences between the TRHR1 (has no measurable basal signaling activity) and the TRHR2 (has a significant basal

signaling activity) [17]. They found that the flexibility of the highly conserved tryptophan, W6.48, in TMH6 (in the GPCR family 1) together with TMH5 is responsible for this functional difference between TRHR1 and TRHR2. By combining computational methods and mutagenesis studies it was shown that the higher flexibility of TMH5 and W6.48 in TMH6 of the TRHR2 are correlated with the permanent basal signaling activity of this receptor compared with the basally non-active TRHR1.

The sensitive interplay between movement of TMH5 and TMH6 relative to TMH3 observed in TRHR2 is similar to that observed for amino acid Y601 (Y5.58) in the TSHR. Since each possible mutation of Y601 leads to a shift in the intrinsic signaling capacity of the TSHR it is likely that Y601 has a role in maintaining the active conformation [25, 56]. Furthermore, we provide a deeper insight into the regulation mechanism and molecular basis of this sensitive transmembrane-helix arrangement by comparison between homology models of TSHR based on the inactive rhodopsin and the (partially) active opsin structures.

The structural template of opsin is suitable to study partially active conformations of GPCRs. The previously available GPCR structures of bovine rhodopsin [35, 42, 43], squid rhodopsin [57], human β2-adrenergic receptor [39–41] and turkey β1-adrenergic receptor [18] all contain an inverse agonistic ligand and thus represent the inactive receptor state. In contrast, the recently published partially activated bovine opsin X-ray structure [36] lacks an inverse agonistic ligand. Several divergent structural properties of the opsin structure compared to the rhodopsin and the β2-adrenergic receptor are attributed to it being in a partially activated receptor state.

Apart from intracellular extensions of TMH5 and the outwardly tilted TMH6, a further striking difference in the partially active opsin structure concerns the side-chain of tyrosine Y5.58 that interacts via a hydrogen bond with arginine R3.50 of the highly conserved DRY motif in TMH3. Since Y5.58 corresponds to Y601 in the TSHR, we tested whether this also occurs in the basally active TSHR. Indeed, in the opsin based TSHR model the corresponding side-chain $Y601^{5.58}$ is sufficiently close to form a hydrogen bond with $R519^{3.50}$ of the ERW motif in TMH3 or with the highly conserved $Y678^{7.53}$ of the NPXXY motif in TMH7, all of which are highly conserved in GPCR family 1. In the opsin based TSHR model these three residues are very likely to be interacting with each other, although this is not observed in the rhodopsin or β-adrenergic based TSHR models (for reasons of clarity not shown). This strongly indicates that the highly conserved tyrosine in TMH5 is involved in the stabilization of (partially) activated receptor conformations.

Moreover, although amino acid positions of observed IAMs are distributed over the whole serpentine domain structure, our model reveals clusters of IAM sensitive residues, such as between TMH2/TMH7 ($D460^{2.50}$, $N674^{7.49}$) or in ICL2 (M527, D530, R531) (Fig. 3A). The most dense clusters of IAM-sensitive positions involve tight hydrophobic interactions between TMH5 and TMH6 as well as between TMH3 and TMH5 (red in Fig. 3A,B). $Y601^{5.58}$ is located in the center of this cluster. These hydrophobic and aromatic helix-interfaces probably help to maintain a partially active conformation of the TSHR as mutation of each of these wild-type amino acids abolishes basal signaling activity, probably by disruption of stabilizing contacts within this cluster.

In contrast, the TSHR model based on the inactive rhodopsin template shows significant differences in the side-chain orientations of $Y601^{5.58}$, $I622^{6.33}$ $R625^{6.36}$ and $M626^{6.37}$ (arrows on Fig. 3C). This leads to disruption of the hydrophobic contacts between TMH5/TMH6 and TMH5–6/TMH3 and subsequently to a different transmembrane and intracellular arrangement of helices 3, 5 and 6 in the inactive and partially active TSHR models (Fig. 3D). Such differences in the helical arrangement between non-active and activated states fit with observations in other GPCRs (reviewed in [58]).

In conclusion, molecular TSHR homology models based on the new opsin structure are likely to represent a partially active TSHR and are more consistent with mutation data influencing the basally active TSHR than models based on an inactive rhodopsin template. Therefore, we propose that the new structural template of opsin is most suitable for studying (partially) active conformations of GPCRs.

Medical and physiological implications

Several reports underline the importance and pathogenic consequences of IAMs in GPCRs. Srinivasan et al. [7] demonstrated the essential role of constitutive GPCR signaling in normal human physiology by showing that the basal activity of MC4R is essential for body weight regulation in humans. Mutations that reduce the basal activity of GPCRs have been demonstrated to be associated with obesity [7, 59], familial short stature [60, 61] and loss of TSHR function [62]. Silencing of the TSHR appears to be related to the most aggressive form of thyroid cancer [63]. Clearly, the loss of basal activity induced by IAMs not only has implications for signal transduction in GPCRs but also for disease. The causal mechanisms

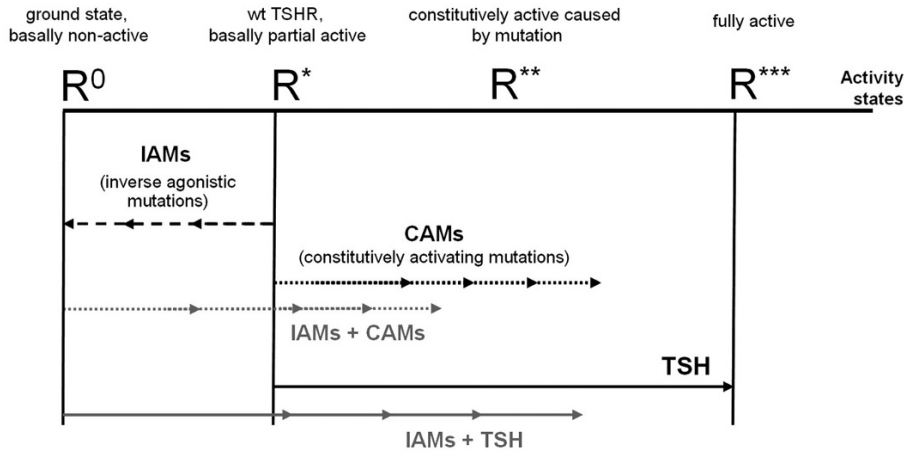

Figure 4. Scheme showing an assignment of mutation phenotypes to an activation state scale for the TSHR. The native wild-type state of the TSHR with partial basal signaling is indicated as R*. Inverse agonistic mutations abolishing the partial activity to an activity of zero reposition the state back to R0. Constitutively activating mutations (CAMs) activate the TSHR beyond the basal activity (R*) up to a higher but still almost partial activity state R** of the receptor. TSH can fully activate TSHR, which is considered as state R***. In our study we found that the signaling activity caused by CAM or TSH induction (both normally starting from R*) is strongly reduced if an inverse agonist mutation is simultaneously involved and thus activity starts from R0 of the activation scale. This suggests that the basally active conformation is an important requirement for the general signaling-capability of the TSHR.

leading to "loss-of-function" caused by these pathogenic IAMs might be distinct to that caused by other classes of inactivating mutations [64]. Further research is required to determine the molecular mechanisms underlying IAMs as well as their role in pathological states.

Numerous diseases are known to be caused by elevated basal activity of GPCRs, such as hyperthyroidism [10, 65, 66], ovarian hyperstimulation syndrome [67–70] and congenital stationary night blindness [71]. Our study has implications for the treatment of these types of diseases, which require the development of drugs that help to lower the basal activity of the mutant proteins. Therefore, a more detailed understanding of conformation(s) caused by IAMs would provide a useful starting point for the structure-based design of small molecules acting as inverse agonists.

In summary, we have investigated the influence of IAMs causing decreased basal signaling activity on the intrinsic signaling-capability of the TSHR. Based on our data and in agreement with findings for other GPCRs, we conclude that the shift from a basally active to a basally non-active conformation has a dominant or strongly negative influence on the intrinsic signaling capabilities of these receptors. This is reflected by a reduction of the ligand independent basal, mutation induced, and/or the ligand mediated signaling. Based on our findings as well as insights from previously published IAM data, we propose that a "silenced" basal conformation resulting from an IAM generally prevents the full-activation that is capable by the wild-type (which has a basally pre-activated conformation). It is the diminished capacity to produce the adequate response by the ligand and not the loss of ability to respond to a ligand which is affected. In other words, if activation starts from a non-active basal state (i.e. the basal activity is reduced) this will cause a decreased level of hormone induced activation. Conversely, it can be assumed that receptor activation starting from a pre-activated basal state can facilitate higher (by CAMs) or full (by hormone) activation (Fig. 4).

This feature has also been observed for combinations of different types of small molecules. Conformational change induced by the agonist, norepinephrine, is reversed by the inverse agonist, yohimbine in the α2A-adrenergic receptor [72]. Furthermore, these authors also demonstrated that binding of an inverse agonist to a constitutively activated mutant receptor also induced conformational changes that were accompanied by inverse agonism. These kinetic results for ligands are in clear agreement with our observations, thus suggesting that the conformational switches induced by inverse agonists, partial or full agonists (whether small molecule, mutation induced or native ligand) proceed by comparable distinct conformational states.

We utilized here for the first time the recently available opsin X-ray structure [36] to study the different conformations of the inactive and partial active state of GPCRs. In contrast to an inactive rhodopsin based model, the opsin based homology models for TSHR likely represent a partially active state. Our experimental findings and the IAMs reported by others clearly support opsin as a structural

template for the partially activated state(s) of GPCRs. The TSHR model of an activated conformation provides much more consistent molecular details about wild-type amino acid interactions where IAMs are observed than the model based on a structural template of an inactive receptor state. Wild-type amino acids having IAM-phenotypes when mutated are likely to be involved with stabilizing the partially activated state by forming side-chain interactions, for example as seen between TMH3/5/6 (Fig. 3A,B).

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