Conformational locking of GpHR TMDs by exoloop antibodies

The antibodies against the computationally designed mimic of the Glycoprotein hormone receptor transmembrane domain provide insights into receptor activation and suppress the constitutively activated receptor mutants

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*Running Title: Conformational locking of GpHR TMDs by exoloop antibodies

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Background: Mechanism of glycoprotein hormone receptor activation is not clearly understood.

Results: Antibodies against computationally designed TMD mimic bind TSHR/LHR/FSHR and inhibit hormone independent and dependent receptor activation without affecting respective hormone binding

Conclusions: Conformational alterations in transmembrane helices leading to receptor activation are dependent on changes in hinge-exoloop engagements

Significance: Antibodies against novel TMD mimic have therapeutic potential against gain-of-function diseases and provide insights into receptor activation

SUMMARY

The exloops of glycoprotein hormone receptors (GpHRs) transduce the signal generated by the ligand-ectodomain interactions to the transmembrane helices, either through direct hormonal contact and/or by modulating the inter-domain interactions between the hinge region (HinR) and the transmembrane domain (TMD). The ligand induced conformational alterations in the HinRs and the inter-helical loops of LHR/FSHR/TSHR were mapped using exoloop specific antibodies generated against a mini-TMD protein designed to mimic the native exoloop conformations which was created by joining the TSHR exoloops constrained through helical tethers and library derived linkers. The antibody against the mini-TMD specifically recognized all three GpHRs and inhibited the basal and hormone stimulated cAMP production without affecting hormone binding. Interestingly, binding of the antibody to all three receptors was abolished by prior incubation of the receptors with the respective hormones suggesting that the exloops are buried in the hormone-receptor complexes. The antibody also suppressed the high basal activities of gain-of-function mutations in the HinRs, exloops and TMDs such as those involved in precocious puberty and thyroid toxic adenomas. Using the antibody and point/deletion/chimeric receptor mutants, we demonstrate that changes in the HinR-exoloop interactions play important role in receptor activation. Computational analysis suggests that the mini-TMD antibodies act by conformationally locking the transmembrane helices by means of restraining the exloops and the juxta-membrane regions. Using GpHRs as a model, we describe a novel computational approach of generating soluble TMD mimics that can be used to explain the role of exloops
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The class A rhodopsin type receptors form the largest subset of the GPCR superfamily bearing the canonical heptahelical serpentine domain and a common mode of activation through the heterotrimeric G-proteins. The ligand binding sites in these receptors primarily lie buried inside transmembrane helices (TMH) (opsin, odorant receptors) or in the juxta-membrane regions (neuropeptides, small endogenous ligands) (1) with the notable exception of the glycoprotein hormone (GpH) receptor (GpHR) family comprising of Thyroid Stimulating Hormone receptor (TSHR), Follicle Stimulating Hormone receptor (FSHR) and Luteinizing Hormone receptor (LHR). The specific binding of the respective ligands, Thyroid Stimulating hormone (TSH), Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) /human Chorionic Gonadotropin (hCG) to these receptors takes place at the large extracellular domains (ECD) containing tandem repeats of nine or more Leucine Rich Repeats (LRR) flanked by the cysteine box (Cb) motifs (2) and the signals thus generated are transmitted to the distally situated transmembrane domains (TMD), a process still not well understood.

Various models have been proposed to explain the mechanism of signal transmission between these two distinct regions. A model for receptor activation, mainly derived directly from the crystal structure of FSH-FSHR ECD, suggests receptor activation occurs through direct interactions of the loops 1 and 3 of the common α-subunit of the hormones with the TMD and the Extracellular loops (ECL) after the determinant loops of the β-subunits of hormones make initial contacts with the ECLs and the Extracellular loops (ECL) after the determinant loops of the β-subunits of hormones make initial contacts with the LRRs (2). This model of receptor activation has been challenged by Moyle et al, who envisaged additional contacts between the ECD and ECLs to be critical for receptor activation. These multipoint interactions are thought to occur between the N-terminal ECD and the ECLs through the β-loop region of the LRR (3). On the contrary, it has also been reported that the C-terminal region of the ECD makes extensive contacts with the ECLs 1 and 2 and lies parallel to the concave surface of the LRR domain (4).

Difficulty in ascertaining the correct model stems from the unavailability of the structural information on the C-terminal region of the ECD called the Hinge Region (HinR). Initially thought to be a structural scaffold, HinR was assumed to act as a flexible hinge facilitating contacts between the hormone and the TMD (5). However, the recent mutation based evidence (6) and our earlier studies on the agonistic antibodies against the FSHR HinR (7) suggest that the HinR may be involved in hormone dependent, as well as, independent activation of the receptor. Moreover, presence of activating mutations at the conserved motifs in the Cysteine box-2/3 (Cb-2/3) of HinR and the combined effect of such mutations with those present in the exoloops have helped in development of an alternate model of receptor activation where the HinR acts as a “tethered inverse agonist” constraining the receptor in an inactive state which is reversed by hormone binding resulting in its activation (8).

A major difficulty in deriving a holistic view of the receptor activation process is the inability to demonstrate direct interactions between the hormone and the ECLs and/or HinR. Moreover, the models do not take into account unique attributes of each member of GpHR family such as the relatively higher basal cAMP production of TSHR compared to LHR or FSHR and the variations in interactions between each receptor component. Although the co-operativity between ECLs during receptor activation is well documented (9), role of individual loops or change in their spatio-geometric arrangement during receptor activation is not clearly understood. Mutational studies provide only transitional information on these highly dynamic interactions.

Antibodies are the ideal tools to monitor such activation related conformational changes during ligand-receptor interaction. For example, ability of ECL specific antibodies of rhodopsin (10) and CCR5 receptors (11) to distinguish between the conformations of the loops in inactive and active states of the receptors highlights their suitability to study the ECLs of GpHRs. Unfortunately, there have not been many reports on antibodies against the exoloops of GpHRs which recognize the native conformations of the loops as they exist in the wild type receptor. Inherent difficulties in obtaining soluble TMD for raising antibodies and...
loss of conformational information in the ECL peptide specific antibodies are the primary causes of such lacunae.

We have, therefore, used a novel approach of designing a recombinant mini-TMD protein where TSHR ECLs are computationally joined to ICLs through the library derived linkers and helical tethers, thus preserving the natural spatio-geometric arrangement of the ECLs in the native TMD of the receptor. This approach circumvents the difficulties in generation of a soluble TMD while maintaining the relative arrangements of the ECLs with respect to each other. Binding and functional studies with antibodies against such a protein provide novel insights into the role of ECLs in GpHR activation.

**EXPERIMENTAL PROCEDURES**

Modeling of the transmembrane domain – A bipartite strategy was employed to model TMDs, the first step being to model the individual helices and loops and then create a composite model by joining ECLs/Intracellular Loops (ICLs) with the modeled TMH. The scheme of the strategy used is shown in Supplemental Fig S1.

A multi-template approach was employed to model [using Modeller 9.07 (12)] the individual helices of TSHR TMD in order to incorporate structural features of GPCRs that are distributed over different crystal structures and may not be represented by a single template (13). The human β2-adrenergic receptor (PDB ID. 2RH1) served as the optimum template for TMHs 2, 3 and 6 as it conforms to the absence of the glycine bend or disulfide Bridge in the helix 2, the absence of a second disulfide bridge between TMH6 and ECL3 and an insertion in TMH3. The turkey β1 adrenergic receptor (PDB ID. 2VT4) was the template of choice for TMH1 owing to the absence of glycine-glycine or proline motifs, whereas the squid rhodopsin (PDB ID. 2Z73) was used for modeling TMH4 and TMH7. Bovine rhodopsin (PDB ID. 1U19), similar to TSHR, does not contain an intra ECL2 disulfide bridge with TMH5 and hence was used as a template for modeling the same. Regular helix extension was carried out for TMH2 and TMH5 in the absence of the characteristic structural bulges caused by consecutive threonines in TMH2 or proline in TMH5 of squid rhodopsin.

Rotational and translational symmetry operations on the modeled TMH were carried out to simulate a membrane bound GPCR by implicitly adding a 30 Å membrane and setting shift angles to 15° from -6° to +9°, and rotation to -50°. The structural alignments of the modeled TMHs and their respective templates are shown in Fig. 1A.

The ECLs and the ICLs were modeled by first searching the database of *Loops In Membrane Proteins* (LIMP) (14) for loops with sequences and gap tolerance similar to the beginning and ends of each TMH. These candidate loops were joined to the modeled TMH by a loop closing algorithm implemented in cyclic coordinate descent (CCD) module of Rosetta 3.1 (15), and the loop conformations were optimized by systematic conformational sampling of the loop backbone followed by energy minimization (16). Several TSHR specific loop constraints were implemented during loop optimization. Firstly, the β-sheet like hairpin structure of ECL2 was maintained between the transmembrane helices based on the rhodopsin structure and those proposed in case of the CCR5 chemokine receptor (17). Secondly, the ICL3 conformations were adopted from the NMR structure of the rhodopsin cytosolic loop peptide complex (18) and lastly, the placement of ECLs 1 and 3 at TMH periphery was carried out according to the previously reported mutational data (19).

Molecular Dynamics (MD) simulations were performed on the composite model of TSHR TMH and ECLs/ICLs after being typed with implicit membrane solvation under the CHARMM27 force field [INSIGHTII-2000, (20)]. The solvent in each minimized system was equilibrated for 10 ps while gradually heating the model from 0 to 300K in 25 ps followed by a second equilibrium phase of 25 ps under a constant pressure and finally subjected to a one nanosecond (ns) isothermal, constant volume MD simulations. Additional restrain parameters such as a disulfide bridge patch between the residues C494 and C569 were applied during MD simulations and the distance restraints were imposed for maintenance of the α-helical regions in TMD.

Similar procedure was also adopted for modeling the transmembrane domains of LHR.
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Rationale, design, assembly, expression and purification of the mini-TMD protein - The mini-TMD protein was synthetically created by joining the ECLs to the ICLs by tethering them with the juxta-membrane elements adjacent to the membrane interface as shown in Fig. 1B. The ECL1 (S479-T490) was connected to ECL2 (G559-P577) using the ICL2 (T524-I533) as the linker. A471-H478, part of the TMH2 was used to tether the N-terminus of the ECL1 whereas the first three residues of TMH 3 (P492-C494) were joined to the last two residues of TMH3 (W520-Y521) to connect the ECL1 to the ICL2. The ICL2 was in turn connected to the ECL2 by helix elements formed by the first five residues (R534-A538) and the last six residues (A553-V558) of the TMH4. The ECL2 was similarly connected to the ECL3 (N650-S657) by the ICL3 (V608-D617) as a linker where the helix segments in the TMH5 (A579-V584) acted as the N-terminal tether whereas A647-N650 in the TMH6 acted as the C-terminal tether. The helix element in the TMH 7 (S659-V664) was used to stabilize the C-terminal end of the mini-TMD. The optimum length of the above mentioned helical tethers used for joining ECLs to ICLs was determined by growing the helical chain by successive addition of N-terminal and C-terminal residues of the helical segment and concomitant prediction of the secondary structure conformation using iterative Tasser simulations (21).

The assembled sequence was modeled on the energy minimized TSHR TMD structure to identify the regions of distorted geometry and potential steric clashes. Dihedral violations were observed at I26 corresponding to the residue I523 belonging to the C-terminal tether of ECL2. Helix discontinuity was also observed for A522 and T524 in the same region. An in silico mutagenic scan was undertaken for these two residues which would enable regular helix extension in this region and based on these scans, both residues were replaced by threonine and alanine respectively. The main chain bond angle violations were also observed for L578 where an extended coil was noticed in place of the expected β-turn. This might arise due to the presence of proline (P577) in the PLA motif and a search of the pre-computed secondary structure assignments for different crystal structures (22) revealed that substituting L578 with glycine may restore the correct secondary structure.

The corrected mini-TMD protein sequence was reverse translated to obtain the encoding DNA sequence which was codon optimized for expression in E. coli. The resultant sequence was synthetically assembled by a modified two-step multiplex PCR (23) where the multiple overlapping primers yielded an amplified mixture of annealing combinations. This mixture was subsequently used as the template for assembling the entire full length gene by the primers specific for the 5’ and 3’ ends corresponding to the N-terminal and the C-terminal regions of the designed protein, respectively.

The mini-TMD encoding sequence was cloned into pPROexHtA vector and expressed in E. coli as a His-tagged protein and purified using Ni-NTA chromatography under native conditions.

Circular Dichroism spectra of the mini-TMD protein – Circular Dichroism (CD) spectra of the mini TMD were recorded between 195 and 250 nm using Jasco J810 Polarimeter at the protein concentration of 10 µM at 25°C in 10 mM phosphate buffer, pH 7.4 with the scan setting set to 1 nM wavelength pitch, 10 nm/min scanning speed in continuous mode and 3 accumulations per measurement. The protein was reduced with 1 mM DTT and 20 µM of 2-mercaptoethanol for 3h and the CD spectra were recorded again and the background was subtracted from observed ellipticities and converted into mean molar ellipticity per residue. The relative secondary structures were calculated using SELCON3, CDSSTR and CONTINLL included in the CDpro spectra analysis package using a reference set of 43 proteins. A weighted average of the outputs of the three programs was considered reliable for estimating the secondary structure content of the protein.

TSHR/LHR/FSHR wild type and mutant constructs - Stable cell lines expressing WT TSHR, LHR and FSHR were created in HEK293 background and characterized as described previously (7,24,25).

LRR and ECD deleted mutants and the activating mutations of TSHR and LHR used in the present study were created as described earlier for FSHR (7). Briefly, modified pcDNA3.1-MycHis vectors (Invitrogen) were created.
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containing the signal peptides of TSHR/LHR/FSHR (pCDNA3.1-SP) and the DNA fragments encoding the receptors without their LRRs (TSHR HinTMD, aa 261-764, LHR HinTMD, aa 265-699 and FSHR HinTMD, aa 260-695) and without their entire ECDs (TSHR TMD, aa 355-699 and FSHR TMD, aa 367-695) of GpHRs were cloned downstream of the respective cognate signal peptides to ensure proper translocation to the cell surface.

The hinge region activating mutations S281I, gain-of-function mutation in TSHR ECL 1/2/3 - I486T, I568F and V656F, and the inactivating mutation D410N were introduced into TSHR wild type background using a two-step PCR based mutagenesis (26). The LHR hinge region mutation S277Q corresponding to the TSHR S281I mutant, and the activating mutations in the LHR TMD at TMH6 (D578Y) were similarly created.

The TSHR/LHR chimeric receptor (TSH-LHR-6) and the LHR TMH3 activating mutant, L457R were kind gifts of Profs. Basil Rapoport (Cedars-Sinai Medical Center, UCLA.) and Deborah Segaloff (University of Iowa, USA) respectively.

Generation and characterization of the mini-TMD antibodies – Antibodies against the mini-TMD protein were generated as described earlier (27). Briefly, the protein (500 µg) emulsified with Freund’s complete adjuvant was administered to adult rabbit subcutaneously at multiple sites. The treatment was repeated 21 days later followed by another injection in Freund’s incomplete adjuvant after another 21 days. The subsequent saline boosters (200 µg) were administered at intervals of 30 days and the animal bled after 10 days. IgGs were purified from the antisera using Protein G chromatography. The antibodies, referred to hereafter as the mini-TMD antibodies, were characterized for specific binding to its cognate antigen fragment in ELISA and to all three GpHRs using immunoblotting and Flow cytometry.

Transfection Experiments - HEK 293 cells seeded into 6-well (~ 10^6 cells/well/2 ml), 24-well (~3X10^5 cells/well/500 µl) or 48-well (~10^5 cells/well/250 µl) plates were transiently transfected with WT or mutated GpHR constructs (3.2 µg of the plasmid DNA/ml of the plating medium) using Lipofectamine 2000 reagent as per the manufacturer’s protocol (Invitrogen) and the transgene expression studies were carried out 48 h later. In each experiment, parallel plates were transfected simultaneously to determine the ligand binding to the intact cells and membrane preparations, flow cytometric analysis and cAMP production as described elsewhere (24).

Receptor binding and in vitro response - Binding characteristics of the WT and mutant receptors were investigated as described earlier (7). Briefly, the membrane preparations obtained from the cell lines expressing TSHR/LHR/FSHR (approximately 50 µg/ml) were incubated with respective radio-iodinated hormones (~ 0.14 nM of ^125^I-hormone, specific activity of the tracer ~ 0.26 µCi/fmol) at 37°C for 1 h in a reaction volume of 200 µl. At the end of the incubation, PEG8000 was added (final concentration 2.5%) at 4°C and the hormone-receptor complex was separated by centrifugation at 5,000g for 20 minutes and discarding the supernatant. The pellet was counted for radioactivity in the Perkin Elmer γ-counter. The non-specific binding was determined by adding excess unlabeled hormone (0.5 µg/tube). Each binding experiment was carried out in duplicates and repeated at least two times.

Whenever the effect of antibodies on hormone-receptor interactions was investigated, the receptor preparations were pre-incubated first with different concentrations of the antibodies for 1 h at 37°C followed by addition of the labeled hormone and continuing incubation for one more hour. The bound hormone-receptor complex was determined as described above.

Similarly, to determine the effect of antibodies on hormone response, 10^5 cells/well (stable cell line or transiently transfected) were plated in a 48-well plate and 24 h later incubated with fresh medium containing 1 mM phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine for 30 min at 37°C (100 µl) followed by incubation with the mini-TMD antibodies or preimmune IgG (NRIgG) for 1 h and finally with a fixed or increasing concentrations of the hormones for 15 min at 37°C (100 µl) and cAMP produced was determined by RIA (28). Effect of antibodies on hormone independent receptor activation was investigated by omitting the hormones.

Flow cytometric analysis of receptor mutants - Flow cytometric analysis was performed to quantify the cell-surface expression of different
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receptor mutants and measure the relative accessibility of ECLs to the mini-TMD antibodies using the protocol described previously (24,29). Briefly, HEK293 cells (approximately, 5X10⁵), transfected with different GpHR constructs were detached by treatments with Ca²⁺/Mg²⁺ free PBS containing 1 mM EDTA and EGTA and washed with PBS followed by incubation with the mini-TMD antibodies or NRlG in PBS containing 5% FBS on ice for 1 h. The cells were washed twice with the same buffer and incubated on ice for 1 h with a 1:500 dilution of FITC-conjugated secondary antibody (Sigma Chemical Co, USA) and the cell surface binding of the antibodies was assessed using the FACSCANTO II (Becton-Dickinson, Franklin Lakes, NJ, USA), flow cytometer. The ratio of the normalized binding of the control antibody (Specific against either LRRs or the HinR) to the WT receptor (Relative mean fluorescence intensity-WT, RMF₁WT) and the mutant (Relative mean fluorescence intensity-mutant, RMF₁MUT) indicates the relative cell surface expression of a given mutant (Rₑ). A similar ratio determined using the mini-TMD antibodies, instead of the control antibody, provided information on the relative accessibility (Rₐ) of ECLs in receptor mutants and allowed comparison of mini-TMD antibody binding across different mutants.

RESULTS

Molecular Modeling of TSHR TMD and the mini-TMD proteins.

TMD- The templates used in modeling of TSHR transmembrane helices have been mostly derived from the crystal structures in the inactive state of the receptors. For example, the Turkey β₂-adrenergic receptor (β-AR) that served as a template for TMH1 was crystalized with the antagonist cyanopindolol (PDB ID- 2VT4), while the crystal structure of human β-AR (PDB ID- 2RH1) complexed with the inverse agonist carazolol served as the template for TMHs 2, 3 and 6. Similarly, the squid rhodopsin crystallized in its 11-cis conformation representing the receptor in the basal state (PDB ID-2Z73) served as the template for TMHs 4 and 7. Therefore, the TMD model derived from these templates can be assumed to represent the inactive “off” state of the receptor. The resting state of TSHR can be further corroborated by stabilization of both potential and total energy profiles of the modeled TSHR TMD in the first 50 ps of MD. Moreover, presence of an ionic lock between R3.50 (Ballesteros Weinstein numbering, aa:519 in TSHR, aa:R464 in LHR) in the highly conserved E/DRY motif in TMH3 and D6.30 at the D/E motif at the cytoplasmic face of TMH6 (TSHR aa:D619, hLHR aa:D564) indicates attainment of a minimized structure with intact functional inter-helical interactions (Fig. 1C). In spite of high variability of the extra/intra cellular loops of TSHR, the signature sequence matched well with the previous mutational studies indicating reliable modeling. For example, the residues such as K660 that have been proposed to be a toggle switch between the activated and inactive states through its interaction with ECL2 makes multiple ionic interactions with D573 in ECL2 further validating the modeling procedure (30). However, the receptor specific attributes were also noticed in the residues of the juxta-membrane region such as TSHR Y643 that depicted a 12º turn towards ECL3 as compared to a phenylalanine at similar position for LHR.

Mini-TMD- The mini-TMD protein, modeled on TSHR TMD, retained these conserved interactions and also maintained the relative positions of the juxta-membrane residues with respect to the membrane (Fig. 1D). The stereochemical validations of the mini-TMD revealed G-factor scores to be -0.28, with 93% residues in the allowed region of the Ramachandran plot (data not shown) confirming the robustness of the modeling procedure and a probable reason for its high solubility in spite of its chimeric nature. The model of the mini-TMD protein displayed α-carbon RMSD of 0.3 Å in the helical segments and 1.1 Å for ECLs when compared to TMD suggesting stabilization of the loop conformation through helical tethers.

The presence of actual α-helical segments in the mini-TMD protein was demonstrated by the far UV CD spectroscopy of the native and the reduced forms of the protein. As shown in Fig. 2A, this protein showed 51.4% and 50.9% α-helicity in presence and absence of the reducing agent respectively indicating the presence of helical tethers introduced for stabilization of the ECLs. A comparison of the experimentally derived and a theoretical CD spectra computed from the MD...
simulated structure of the mini-TMD protein using the online CD analysis tool DichroCalc (31), showed a close correlation between the model and the purified protein (Fig.2B) further demonstrating likelihood of similar secondary characteristics of the mini-TMD protein as predicted. The helical content of the computed spectra was found to be 63% as compared to the experimentally derived helical content of 52%. Interestingly, decrease in the β-turn content from 10% to 6% due to treatment with reducing agents is intriguing, as the ECL2 of TSHR was shown to possess a β-hairpin like motif stabilized via a disulfide bridge between C569 (ECL2) and C494 (TMH3) (32). Maintenance of the disulfide bridge in the soluble mini-TMD protein is expected to provide a loop conformation similar to that of the full-length TMD.

Generation of the mini-TMD antibodies: Specific interactions with ECLs of other members of GpHR family-

The soluble mini-TMD protein was loaded onto Ni²⁺–NTA Sepharose column in 20mM Na phosphate buffer containing 300mM NaCl and 5mM imidazole, pH 7.4 and eluted with increasing concentration of imidazole (Fig. 3A). The purity of the mini-TMD protein eluted with 300mM imidazole (peak 2) was ascertained by SDS PAGE and western blot analysis with anti His-tag antibody (Fig. 3B). The antibodies raised against the mini-TMD protein could bind to the cognate antigen in ELISA (data not shown), as well as, to the full-length TSHR in immunoblot analysis (Fig. 3C). Interestingly, the mini-TMD antibodies also recognized FSHR and LHR in immunobloting (Fig. 3C) and flow cytometric analyses (Fig. 4A(ii)) indicating cross-reactivity to other two GpHRs. Binding of the mini-TMD antibodies to LHR and FSHR was found to be 93% and 70% respectively to that observed with TSHR which may be attributed to 66% and 60% sequence identity (80% and 72% of sequence similarity) the mini-TMD protein shares with LHR and FSHR ECLs respectively (Fig. 4B). However, the antibody did not cross react with HEK293 cells transfected with D2-Dopamine receptor or M4-muscarinic receptor (data not shown) clearly indicating the specificity of the mini-TMD antibodies.

Effect of the mini-TMD antibodies on hormone action-

The mini-TMD antibodies exhibited different effects on hormone binding and response. The antibodies had no effect on binding of ¹²⁵I-hFSH/TSH to the respective receptors when they were added prior to addition of the hormone while a marginal decrease was observed in case of hCG binding to LH receptor (Fig. 5A). In contrast, pre-incubation of cells with the antibodies inhibited hormone response in a dose dependent manner in case of all three receptors (Fig. 5B). Further, the dose response curves for the hormones (hTSH and hCG) exhibited decrease in Rₘₚₓ without any changes in Eₐₚ₀ in presence of the mini-TMD antibodies, clearly indicating that the antibodies exhibit non-competitive antagonism (Figs. 5C and 5D).

Effect of hormone binding on receptor-antibody interactions-

The non-competitive antagonism exhibited by the mini-TMD antibodies indicated that the antibodies either inhibit ECLs/TMH from attaining a conformation required for hormonal activation, or they interfere in the critical secondary contacts between the hormone and ECD and/or ECLs. While the first possibility may be ruled out by the oligoclonal nature of the antibody, precluding specificity to any given loop conformation, the second possibility will result in loss of access of the ECLs to the antibodies in the preformed hormone–receptor complex. Hence, the cells expressing TSHR/ LHR/FSHR were first incubated with or without respective hormones (10nM) at 4 °C followed by incubation with the saturating concentration of the mini-TMD antibodies (20µg/ml) and determining the antibody binding by flow cytometry. As shown in Fig. 4A(ii), pre-incubation with the hormones significantly decreased the subsequent antibody binding to all three receptors indicating loss of epitopes (ECLs) post hormone binding.

Effect of the mini-TMD antibodies on mutant GpHR receptors: differential interactions of GpHR HinRs with their ECLs-

The above experiment as well as, the previously proposed contact between hormone α-L1 and L3 loops and the ECLs (33) emphasize the critical role played by the loops in mediating the hormone stimulated response. Role of ECLs in
maintenance of the basal or ligand independent receptor activation is not clearly understood. The contribution of the HinR in maintaining the basal cAMP production in GpHRs is particularly important through the selective interactions with the critical residues in the ECLs (34). To provide a physical basis for the HinR-ECL interactions, effects of the mini-TMD antibodies on cAMP production by the Hin point and truncation mutants were investigated and accessibility of their ECLs to the mini-TMD antibodies was monitored by flow cytometry.

**Binding of the mini-TMD antibodies to GpHR truncated mutants** - WT (WT), LRR deleted (HinTMD) or ECD deleted (TMD) mutants of all three GpHRs (schematically shown in Fig. 6A) were transfected in HEK293 cells and binding of the mini-TMD antibodies or respective hinge specific antibodies was monitored by flow cytometry (Fig. 6B). The ratio of RMFIs for the mutant and WT receptors (RMFIMUT/RMFICTM) using the HinR antibodies was indicative of the relative cell surface expression of the mutant receptors (Re) while the ratio of RMFIs for WT and mutated receptors using the mini-TMD antibodies indicated the relative accessibility of ECLs in the mutant receptors (Ra).

As seen in Fig. 7A, while Re values of TSHR and FSHR HinTMD mutants were comparable (69% and 71% of WT expression, respectively), expression of LHR HinTMD was 40% of LHR WT expression. Accessibility of ECLs of TSHR/FSHR HinTMD to the antibodies was proportionate to their cell surface expression suggesting binding of antibodies was similar in WT and LRR deleted mutants of TSHR/FSHR and hence the relative orientation of the hinge with respect to ECLs did not differ substantially in the LRR deleted and WT receptors. On the other hand, the relative accessibility of LHR HinTMD to the mini-TMD antibodies was considerably higher. This relatively higher binding of the mini-TMD antibodies to LHR HinTMD as compared to FSHR or TSHR HinTMD suggests that LHR hinge has a more open conformation with respect to the ECLs and has a lesser constraint on the ECLs.

More interestingly, binding of the mini-TMD antibodies was highest to LHR TMD followed by FSHR and TSHR TMDs indicating that though considerable sequence identity exists among the ECLs of the three receptors, their relative conformations are different pointing out to a very important role of TMH in maintaining ECL conformations. However, Re values for the ECD deleted mutants were not available in the absence of appropriate normalizing antibodies such as the HinR antibodies used for LRR deleted mutants.

**Effect of the mini-TMD antibodies on the basal cAMP levels of the receptor truncated mutants** - As seen in Fig. 7B, the WT basal activities of GpHRs in terms of hormone independent cAMP production vary widely with TSHR being the ‘noisiest’ receptor followed by FSHR and LHR. Moreover, as reported previously, removal of ECD further increased cAMP production for both TSHR and FSHR (7,35). This high basal cAMP level in TSHR/FSHR TMD mutants decreased by introduction of HinR in the HinTMD mutants suggesting that HinRs act as a tethered inverse agonist to the TMD, a direct evidence for their roles in maintaining the basal, as well as, hormone independent receptor activation (7,24).

Interestingly, the higher basal cAMP production did not occur on removal of LHR ECD, indicating a lack of built-in inverse agonism of LHR ECD. Similar observations regarding the intrinsic low basal cAMP production of LHR have also been made with LHR truncated mutants expressed as a HA-Tag Vasopressin receptor fusion protein (36).

The high basal cAMP production activities of TSHR WT and TSHR/FSHR TMD mutants decreased in presence of the mini-TMD antibodies. The effect of antibodies was significantly more in case of the TMD mutants compared to WT indicating that HinR probably modifies the accessibility of ECL to antibodies. However, the antibodies had a marginal effect on the basal activities of LHR or FSHR WT.

**Effect of the mini-TMD antibodies on HinR and ECL activating and inactivating mutations** - Several residues in HinR of GpHRs have been implicated in maintenance of the basal cAMP levels of which the serine in the highly conserved “YPHCCCAF” motif seems to be most critical. Mutations in this residue (TSHR: S281. LHR: S277, FSHR: S273) lead to constitutive activation of the receptor as a result of a conformational shift from a “closed” constrained receptor state to an “open” unconstrained one (37). The accessibility
of ECLs in such mutants to the mini-TMD antibodies would provide a clear picture on the spatial orientation of the HinR with respect to the ECLs during the activation process. TSHR and LHR serine mutants (TSHR: S281I, LHR: S277Q) displayed Re values of 0.48 and 0.42 respectively (48% and 42% of WT expression) computed using the respective HinR antisera as control antibodies clearly suggesting reasonable surface expression (Figs. 8A(i) and 8B(i)). Interestingly, as shown in Figs. 8A(ii) and 8B(ii), the mini-TMD antibodies showed higher binding to both TSHR (S281I) and LHR (S277Q) mutants as compared to the WT receptor (Ra values of 1.6 and 0.85 respectively), indicating higher accessibility of the ECLs in these mutants. Intriguingly, binding of the antibodies to the mutant receptor with inactivating mutation D410N at the C-terminal end of the TSHR ECD was found to be considerably higher than either the WT or the HinR activating mutations \([R_e=2.12, \text{surface expression = 63% of WT expression (}R_e=0.63)].\)

We also created three activating mutations residing in each of the ECLs of TSHR (ECL1: I486F, ECL2: I586T, ECL3: V656F) and binding of the mini-TMD antibodies was investigated. All three mutants displayed 65%-75% relative surface expression compared to the WT receptor. However, binding of the mini-TMD antibodies (and hence the relative accessibilities) varied among the three mutants with I486F (Ra/Re=3.4) showing the highest binding followed by V656F (Ra/Re=1.7) and finally, I568T (Ra/Re=1.6) (Fig. 9A (i)).

More interestingly, the high basal cAMP production of both HinR, as well as, ECL activating mutations was dampened in presence of the mini-TMD antibodies (Figs. 8C&D, 9A(ii)). In addition, the hormone stimulated response of the serine mutants of LHR and TSHR was also inhibited in presence of the mini-TMD antibodies, although the degree of inhibition of ‘expression normalized’ response in case of the mutant receptor was lower (46% for LHR, 37% for TSHR) than those of the WT receptors which was 68% and 72% for TSHR and LHR respectively (Figs. 8C&8D). Inhibition of the hormone stimulated response of the ECL mutants was not carried out due to the poor stimulation of these receptors making analysis unreliable.

**Effect of the mini-TMD antibodies on TSRR-LHR chimeric receptors** - The higher accessibility of ECLs to mini-TMD antibodies in LHR HinTMD as compared to TSHR HinTMD suggests that the interactions of the LHR HinR with its ECLs were less stringent as compared to those of TSHR. Binding of mini-TMD antibodies to the chimeric receptor where the HinR of TSHR has been replaced by LHR (TSH-LHR-6) was investigated. Expression of this mutant on the cell surface, as demonstrated by binding of the TSHR-LRR specific antibodies (Fig.10A(i)), was marginally lower than the WT receptor (86%, R_e=0.86). Interestingly, binding of the mini-TMD antibodies to the chimeric receptor (Fig.10A(ii)) was found to be higher (200%) than to TSHR WT receptor suggesting the presence of LHR HinR influences the accessibility of the ECLs to these antibodies (Fig.10A(iii)). As in case of LHR, the mutant exhibited much lower basal cAMP production rate than TSHR WT. Response of the chimeric receptor to TSH was also inhibited in presence of the mini-TMD antibodies suggesting LRR independent action of these antibodies (Fig. 10B).

**Effect of the mini-TMD antibodies on LHR TMH activating mutations: Clues to the mechanism of inhibition of mini-TMD antibodies** - Effect of mini-TMD antibodies on the constitutively active mutants of LH receptor caused by mutations in TMH leading to precocious puberty was next investigated. Expression of activating mutants L457R present in TMH3 and D578Y present in TMH6 on the cell surface was confirmed by flow cytometry with LHR Hinge antibodies. As seen in Fig. 9B, the mutants exhibited high basal cAMP production and poor response to hCG. The mini-TMD antibodies were able to decrease the constitutive activities of both mutant receptors, inhibition of D578Y (Fig. 9B(i)) being much greater than that of L457R (Fig. 9B(ii)).

**DISCUSSION**

Importance of the extracellular loops of GpHR in signal transduction is evident from several somatic and germ line mutations that cause abnormal receptor function (38). However, the precise mechanism of the signal transduction has not been elucidated. In the present study, the antibodies recognizing GpHR ECLs in their native
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forms have been used to understand the signaling mechanism of GpHRs. The antibodies raised against TSHR-TMD inhibited the basal and hormone stimulated cAMP production by all three GpHR without affecting the hormone binding. The antibodies also suppressed the high basal activities of gain-of-function mutations in the HinRs, exoloops and TMDs such as those involved in precocious puberty and thyroid toxic adenomas. Thus, these antibodies, while providing interesting insights into receptor activation, also provide interesting therapeutic strategy for treating conditions such as precocious puberty and thyroid adenomas.

The method of designing a mini-TMD protein used as an antigen not only overcame issues such as solubility and poor expression, but allowed retention of a degree of the native conformation of the TMD as demonstrated by the near native helical content in the CD spectra of the protein. More importantly, retention of the β turn motif in the ECL2 and disulfide bridge between ECL2 and TMH3 ensured generation of conformation specific antibodies against the protein. The main goal of this study was to provide answers to the following pertinent questions, a) the spatial organization of the loops in the absence and presence of the hormone, b) the interaction (or lack thereof) of the HinR with the ECLs, c) a comparative analysis of ECLs of the three GpHRs during hormone dependent and independent activation and d) provide a holistic view of ECLs as a functional receptor unit and not just determine few critical residues involved in the signal transduction process.

Spatial organization of the loops in the resting and hormone stimulated receptor: Hormonal contact with ECLs causes the ECLs to become buried inside the TMH-

The interactions between the α-subunit and ECLs or hinge-ECL following the initial hormone binding to LRRs are probably the critical events in hormonal activation of the receptors. Preferential photo-affinity labeling of the α subunit of hCG by the HinR and exoloop 2 peptide (39), inhibition of hormone response by the α-subunit specific antibodies without any effect on hormone binding (40) and loss of bioactivity in the mutants of the hormones with mutations in the α-subunit (41) indicate such a possibility. Ability of the mini-TMD antibodies to inhibit hormone stimulated response in all the three receptors without affecting the respective hormone binding strongly supports this hypothesis and also indicates a common mechanism of GpHR activation. Activation of the receptor may occur through the contact of the L1 and L3 loops of the α-subunit with the residues in the hydrophobic core of the juxta-membrane domain after displacement of the second or third extracellular loop (2) or the α-subunit may disengage the HinR from ECL 2 leading to TMH relaxation and receptor activation (42). Both conditions may lead to removal of the loops from their hydrophobic environment and should result in higher exposure and hence, better accessibility of these epitopes to the mini-TMD antibodies. On the contrary, the hormone–bound-receptors showed lower binding to the antibodies. This cannot be attributed to internalization of the receptor as the flow cytometry experiments were performed on ice. Moreover, no decrease in binding of the control antibodies to other regions (LRR 1-3 specific antibodies) was observed. These data suggest that the exoloops are buried deep inside the hormone-receptor complex and the tips of the α subunit may be in contact with ECLs while making additional contact with the TMH residues. This could also possibly explain the contact between ECL and TMH residues and how double mutant I568V/I640L (ECL2/TMH6) suppresses the increased basal activity exhibited by I568V alone (32). The lack of inhibition of hormone binding by the antibodies also seems to be contrary to the isolated reports of the ECLs being part of the primary hormone binding site (43,44), although the evidence from mutational studies of ECL residues suggested a compromise in signaling, but not in hormone binding (45,46)

The HinR–ECL interaction as the basis of basal cAMP production: weaker interactions of Hinge-ECL in LHR as compared to TSH/FSH receptors-

The low basal of cAMP production of LHR or FSHR as compared to TSH (Fig. 8C&D) is a hallmark of the physiological regulation by these receptors(47). Even with the remarkable similarity in the receptor architecture, the mechanistic basis of such differential basal receptor activation has been not well elucidated. Removal of the ECD from TSHR further increased the relatively high
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basal cAMP production, similar to that reported earlier (48). This increase has been attributed to the tethered inverse agonistic effect of the TSHR ECD where the ECD keeps the TMD in an inactive state (49) and this inverse agonism has been narrowed down to the HinR of TSHR where specific contacts between the residues of HinR and ECLs are primarily responsible for the hinge-TMD constraint (50). Supporting this is our observation that high basal cAMP production of TSHR TMD is dampened by the HinR in the TSHR HinTMD mutants (Fig. 8C&D). In an earlier study on FSHR (7), we found that deletions in the segment of amino acids 290 to 331 in the HinR resulted in an increase in the basal cAMP production. These data suggest a common role for the HinR in regulating FSHR and TSHR activities. On the other hand, neither the WT LHR nor ECD deleted mutants show a high degree of basal cAMP production possibly due to different interactions of the LHR HinR with its ECLs (36). This possibility was investigated by determining the relative accessibilities of the TMDs and HinTMDs of all three receptors to the antibodies. LHR HinTMD showed a relative higher antibody binding suggesting that the HinR of LHR interacts comparatively weakly with its ECLs as compared to those of TSHR or FSHR and hence LHR TMD was more accessible to antibodies than its counterparts in TSHR or FSHR. Moreover, even with a higher degree of binding, LHR TMD mutants were relatively less affected by the mini-TMD antibodies as compared to the significant inhibition of TSHR and FSHR TMDs in terms of basal cAMP production indicating that ECLs of LHR acted in a manner unlike the other two members of GpHR family. A further evidence for a differential involvement of the HinR of LHR is provided with the chimeric TSHR-LHR-6 mutant that responds to TSH, but does not exhibit high basal activity of TSHR. The relatively higher binding of the mini-TMD antibodies to TSH-LHR-6 as compared to the TSHR WT also suggests that presence of LHR HinR not only decreases the interaction of HinR with the ECLs, but also directly correlates with the basal activation of the receptor.

Both Activating and inactivating mutations in GpHRS cause the HinR to become disengaged from ECLs

Transient changes in HinR-ECL interactions in response to the hormone are thought to be mediated by the highly conserved motifs in Cb-2 and Cb-3, especially, through the conserved serine in the “YPHCCAF” motif as mutations at this serine for all the three receptors cause constitutive activation of the receptor (51). This has been attributed to the loss of packing in the aromatic environment at the ECL-TMH interface (52) and it may be hypothesized that the serine mutants in this region undergo a spatial reorganization with respect to ECLs. Both TSHR-S281 and LHR-S277 mutants are characterized by higher binding of the mini-TMD antibodies as compared to the WT counterpart suggesting that these mutant receptors are less constrained than WT. More interestingly, binding of the mini-TMD antibodies to ECL mutants varied for each loop with I486F (ECL1) displaying the highest degree of binding followed by V656F (ECL3) and then by I586T (ECL2). The molecular modeling and mutagenesis studies have shown the β-turn of ECL2 to lie on the plane of the TMD interface and would hence be expected to have lower accessibility to the mini-TMD antibodies. On the other hand, I486 in ECL1 has been known to form the hydrophobic core of ECLs and interacts directly with S281 (52). Hence, one would expect a similar increase in antibody binding to I486F mutant as observed with S281.

In contrast to the discussion above that disengagement of the HinR from the ECL is a necessary and sufficient event for increase in basal cAMP, we find that the mini-TMD antibodies display an even higher binding to the inactivating mutation D410N as compared to S281 or WT receptors. This mutation is characterized by extremely low cAMP basal levels but is capable of stimulation by TSH. Location of D410N is said to be in proximity with ECL3 and ECL1 (30) and mutations in both these ECLs are characterized by higher binding to the mini-TMD antibodies. This would suggest that the hinge-ECL interactions are maintained through equilibrium of pro-active and pro-inactive conformational states and the active or inactive state of the receptor is a function of position of the perturbation of interaction between these two domains. Simple disengagement of hinge may no longer be considered the only event responsible for maintenance of basal receptor activation.
The mechanism of action of mini-TMD antibodies: an experimental and theoretical consideration into the inhibitory effect of the mini-TMD antibodies

As shown here, the mini-TMD antibodies could decrease the basal cAMP production by both WT receptor and activating mutations and inhibit the hormone stimulated responses of all the three GpHRs. The pan-receptor effects of the mini-TMD antibodies cannot be explained simply by invoking their effects in altering the HinR-ECL interaction as the constitutively activating mutation in the ECD cannot bypass disruption of signal transduction in the serpentine domain (8). This would indicate that the mini-TMD antibodies must affect critical inter-helical interaction as well. Ability of these antibodies to inhibit the high basal activity of the LHR activating mutations L457R (TMH 3) and D578Y (TMH 6) indicates this possibility. Previous reports suggest that L457 and D578 lie in close proximity and introduction of a positively charged amino acid at the position 457 generates an attractive effect on D578 thus inducing perturbations in TMH3-TMH6/7 interaction patterns (53). Whether the mini-TMD antibodies influence such helix arrangement through their interactions with the ECLs was investigated by comparing the deviation of the mean structure after MD of the WT and mutant receptors. For this purpose, LHR TMD was modeled using the method adopted for TSHR TMD and the L457R mutant was incorporated using MODELLER and the side chain conformations were refined through the chiprotor algorithm refinement. MD simulations were performed on the WT and mutant receptors under identical conditions and the mean structure was extracted from a production run of the last 800 ps. The structural alignment of the WT and L457R simulated structures reveal significant Cα deviations with the ICL 2 tethers deviating by 6.7 Å and the main chain RMSD for ECL 1 was found to be 3.8 Å (Fig. 11B). More interestingly, in the WT receptor the OD1 atom of D578 was found to be hydrogen bonded to HD22 atom of N619, whereas in L457R this interaction is replaced by hydrogen bonding between HH12 atom of R457 and OD1 atom of N619 with an additional hydrogen bond between HH11 of R457 with OD2 with D578 (Fig. 11A). Mean side chain movement for D578 was found to be 2.3 Å and this along with changes in ICL2 can explain the stabilization and high basal activity of the mutant receptor.

Simulation of antibody binding to the receptor exoloops was carried out by keeping the centroid of the ECLs under a relatively stiff harmonic potential (force constant 3000 pN/Å) with the residues of the ECLs under a spring constant of 65 pN. This setup allowed the TMD to adapt to the enforced antibody binding, e.g., by rotations, intramolecular conformational motions or as experienced during induced fit, as observed in typical antigen-antibody AFM experiments (54). MD simulations of L457R mutant in presence or absence of the above constraint yielded surprising results. Not only did the tripartite hydrogen bonding of the R457, D578 and N619 was lost, R457 (Fig. 11A(iii)) showed a negative trajectory where the helix 3 deviated 3.1 Å from the mean Cα of the unconstrained mutant model. A plot of all the Cα RMSD of the mean trajectory of TMH residues in the constrained and unconstrained mutant receptor using the unconstrained WT receptor as reference showed a large reversal of the simulated trajectories (>1 Å) in TMH 3 and TMH 6 with smaller perturbations in the juxta-membranal regions of ECL2 and TMH5 and cytoplasmic face of ICL3 and TMH7 (Fig. 11C). The inter helical network of hydrogen bond between L457, D578 and N619 has been shown to be highly conserved in all the three GpHR members as shown through the rearrangement of carboxylate oxygen of TSHR N674 (N619 in LHR) with D633 (D578 in LHR) resulting in a switch between the activated and inactivated states (55). Control of such inter-helical molecular switch by modulating the ECLs has already been exploited in designing small molecule agonist for TSHR and LHR (56). The above data taken together would suggest that antibody or small molecules binding to ECLs or the exoplasmic face of the TMH can affect global TMD conformation.

Significance of the mini-TMD antibodies: applicability as a therapeutic strategy or a tool for biophysical studies of the TMD

Activating mutations in TSHR typically cause thyroid cancer and precocious puberty in case of LHR. S281I and I486F are typical germline mutations that cause toxic adenomas whereas I568T and V656F are somatic mutations that cause...
hot nodules and adenomas respectively. L457R in LHR causes Leydig cell hyperplasia and the corresponding mutation in TSHR (L512R) has been shown to be involved in thyroid nodule formation. Therapeutic strategies such as EGFR specific monoclonal antibody for cancer treatment are unavailable in such cases and surgical removal of the thyroid or testis remains the sole alternative. The present study has shown that antibodies against TMD can be the right therapeutic tools for these conditions.

In addition, ability of the mini-TMD antibodies to stabilize TMHs offers a powerful tool for crystallography of GpHRs. Antibody fragments that recognize the native protein conformations have been shown to facilitate crystallization of other membrane proteins by increasing the polar surface area for protein-protein contacts and by restricting the flexibility of mobile domains. The mini-TMD antibodies seem to be an ideal tool for purification of the stabilized TMD.

In conclusion, we report a novel strategy of developing specific antibodies against the exoloops of GpHRs. We have used these antibodies to demonstrate the differential interactions of the HinR of LHR as compared to those of TSHR and FSHR. In addition, we have provided a preliminary evidence of a possible cooperative translocation of the ECLs and the α-subunit into the hydrophobic core of TMH and provided a physical basis of the mechanism of ECL control of the TMHs. A theoretical model is presented to explain the inhibitory effect of the mini-TMD antibodies on hormone binding and basal activity of WT and mutant receptors.

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FOOTNOTES
1The abbreviations used are: hCG, human Chorionic gonadotropin ; FSH, Follicle Stimulating Hormone, TSH, Thyroid Stimulating Hormone, TSHR, Thyroid stimulating hormone receptor; LHR, Luteinizing hormone receptor; FSHR, Follicle stimulating Hormone receptor; GpHR, Glycoprotein Hormone Receptors; ECD, Extracellular domain; LRR, Leucine Rich Repeat; TMD, 7-Transmembrane Domain; TMH, Trans-membrane helix; ECL, Extracellular loop; ICL, Intracellular Loops; aa, Amino acids; WT, Wild Type; CAM, Constitutively activating mutations; Cb-2/3, Cysteine Box-2/3; NRIGG, Normal Rabbit IgG (pre-immune).
FIGURE LEGENDS

FIGURE 1. Modeling and design of GpHR mini-TMD.
A. Orthogonal views of the alignment of the ECL/ICL deleted model of TMD and TMH templates used for homology modeling. The colored helices denote TMHs derived from the templates used in comparative modeling the TMD (vide experimental procedures), whereas the gray helices represent TMHs derived from the model of TSHR TMD. The numbers in parenthesis denote the Cα RMSD values of the given TMH with its corresponding template.
B. Sequence alignment of TSHR TMD and mini-TMD. Green, blue and yellow arrows indicate TMH, ECLs and ICLs respectively. The Mini-TMD residues bearing homology to TMD are shown in black whereas the non-identical residues and gaps are shown in red.
C. Molecular modeling of TSHR TMD with the TMHs shown in green and the ECLs in blue. The highly conserved ionic bridge present in Class A GPCRs between D/E motif in TMH6 (D519 in TSHR) and R3.50 (Ballesteros Weinstein numbering) in TMH3 (D619 in TSHR), as well as, the signature GpHR residue (Vide text) K660 in TMH7 are shown in red.
D. Design of mini-TMD protein based on the TSHR TMD model. The green residues indicate the helical tethers and synthetic linkers. The ECLs are represented in blue and the computational corrections introduced to maintain the spatio-geometric restraints are shown in brown. The probable disulfide bridge between C24 and C59, corresponding to C494 and C569 of TSHR TMD is shown in yellow.

FIGURE 2. Secondary structure characterization of the mini-TMD protein using far-UV CD spectroscopy. A. Representative CD spectra of the purified mini-TMD protein was recorded in presence (marked by X) or absence (open circles) of the reducing agent and are presented as [θ]mean, mean residue ellipticity. The percentage α-helical and β-sheet contents were calculated as described in the experimental procedures. B. Comparison of the theoretical (solid lines) and the experimental (dotted line) CD spectra of mini-TMD protein. The theoretical spectra was computed from the average structure extracted from the molecular dynamic simulation cascade of the modeled mini-TMD protein using the CD analysis tool DichroCalc (31) and plotted against the recorded spectra. Aromatic or side chain – side chain transition parameters were not selected during the computation.

FIGURE 3. A. Purification of the mini-TMD protein using Ni²⁺-NTA IMAC chromatography. The peaks 1 and 2 show the proteins eluted with 100 mM and 300 mM imidazole respectively. B. SDS-PAGE and western blot analyses of the proteins obtained from the Peak 1 and 2 of the Ni²⁺-NTA chromatography using the anti-His Tag monoclonal antibody (GE BioSciences). C. Specificity of the mini-TMD antibody. The membrane preparations obtained from cell lines expressing TSHR/LHR/FSHR, TSHR TMD or HEK293 cells were solubilized with 1.2% Digitonin and electrophoresed on 10% (left panel) or 7.5% (right) SDS, transferred to PVDF membrane and probed with the mini-TMD antibodies. TSHR ECL protein was used as a positive control.

FIGURE 4. Binding of the mini-TMD antibodies to receptor and Hormone-receptor complex.
A(i). Flow cytometric analysis of binding of the mini-TMD antibodies (20 μg/ml) to HEK293 cells expressing LHR (left panel), TSHR (middle panel) or FSHR (right panel).
A(ii). HEK293 cells expressing LHR (left panel), TSHR (middle panel) or FSHR (right panel) were previously saturated with their respective hormones (10 nM) and binding of the mini TMD antibodies (20μg/ml) was monitored by flow cytometry (Gray histograms). In both Fig A(i) and A(ii), the white
histograms indicate binding of the mini TMD antibodies to the mock transfected HEK293 cells. The broken and unbroken lines designate the median fluorescence intensity of the grey histograms and black histograms respectively measured in the same experiment.

**B. Multiple sequence alignment of the transmembrane domains of the three glycoprotein hormone receptors**

**FIGURE 5.** Effect mini-TMD antibodies on hormone-receptor interactions.

A. Increasing concentrations of the mini-TMD antibodies were pre-incubated with HEK293-TSHR/LHR/FSHR membrane preparations (5μg) at 37°C followed by addition of respective radioiodinated hormones and the receptor bound hormone was determined: (A(i)) hCG-LHR, (A(ii)) TSH-TSHR and (A(iii)) hFSH-FSHR.

B. Effect of the mini-TMD antibodies on hormone stimulated response was determined by pre-incubating the cells expressing the individual receptors with increasing concentrations of the mini-TMD antibodies or NRIgG for 1 h at 37°C followed by incubation with 5nM of the respective hormones for 15 minutes at 37°C and determining cAMP produced by RIA: B(i). LHR, B (ii) TSHR, B(iii) FSHR.

C and D. The cells expressing (C) LHR and (D) TSHR were incubated with the mini-TMD antibodies (1μg/ml) for 1 h followed by addition of increasing concentration of the respective hormone for 15 minutes and cAMP produced was determined by RIA.

**FIGURE 6.** Relative binding of the mini-TMD antibodies to GpHR deletion mutants

A. Schematic representation of GpHR deletion mutants. The putative residues marking the start of a given domain are mentioned for each receptor; T-TSHR, L-LHR, F-FSHR.

B. HEK293 cells transiently transfected with the full length WT receptor or HinTMD and TMD deletion mutants were incubated with either NRIgG (open histograms) or 1μg/ml of the mini-TMD antibodies (grey histograms) and the antibody binding was determined by flow cytometry. The surface expression of each mutant was ascertained using the respective HinR specific antibodies (10µg/ml) shown as black histogram.

**FIGURE 7.** Effect of the mini-TMD antibodies on GpHR deletion mutants

A. The ratio of RMFI for mutant/WT was determined using the respective HinR specific antibodies to estimate the relative expression of the mutant on cell surface and designated as $R_e$ (24). Similar ratios were determined for the mini TMD antibodies to determine the relative accessibility of the epitope in mutants and designated as $R_a$. $R_a/R_e > 1.2$ indicate higher binding of the mini-TMD antibodies to the mutant as compared to the WT. Each bar represents RMFI ratios of three experimental replicates repeated thrice.

B. The WT and deletion mutants (HinTMD and TMD) of all the three GpHRs were transiently transfected and their basal cAMP production was measured in presence of NRIgG or the mini-TMD antibodies (10µg/ml) after 1 h incubation and cAMP produced was normalized to their cell surface expression (as determined in Fig 7A). The statistical significance was compared to the respective pre-immune IgG control and is denoted by the $P$-value calculated from the two-tailed unpaired t-test.

**FIGURE 8.** Effect of the mini-TMD antibodies on the HinR activating and inactivating mutations of TSHR and LHR.

A&B. Flow cytometric analysis of HEK293 cells transfected with the WT or different HinR mutants of LHR (panel A) or TSHR (Panel B) using the HinR specific antibodies (10µg/ml) and the mini-TMD antibodies (1μg/ml). Accessibility of epitopes to the mini-TMD antibodies to the HinR mutants was estimated by $R_a$ of the mutants with respect to the WT (A(ii)) LHR or (B(ii)) TSHR as mentioned in the legends of Fig.7A.
C&D. The hormone stimulated and the basal cAMP production of (C) LHR CAM S277Q and (D) TSHR HinR mutants S281I and D410N was measured in presence of NRIgG or the mini-TMD antibodies (10μg/ml).

FIGURE 9. Effect of the mini-TMD antibodies on the activating mutations of TSHR ECLs and LHR TMD, Flow cytometric analysis of HEK293 cells transfected with the WT or TSHR ECL CAMs (A(i)) using the mini-TMD antibodies (1μg/ml) and the RMFI values so obtained were normalized to those using the HinR specific antibodies. Basal cAMP production of (A(ii)) TSHR ECL CAM, (B(i)) LHR TMD CAM D578Y and (B(ii)) L457R was estimated in presence of 10μg/ml of pre-immune IgG (gray bars) or mini-TMD IgG (black bars).

FIGURE 10. Effect of the mini-TMD antibodies on TSHR-LHR chimeric mutant.
A. Binding of antibodies to TSHR WT (A(i)) and HinR chimeric mutant TSH-LHR-6 (A(ii)) was monitored by flow cytometry using the TSHR LRR 1-6 specific IgG (black bars) and the mini-TMD antibodies (gray bars). The MFIs of each antibody for the WT and TSH-LHR-6 receptor, as derived in Fig A(i) and A(ii), was normalized to pre-immune IgG (NRIgG, white bars) and expressed as RMFI (A(iii)). Numbers in parentheses over each bar represent the ratio of RMFI of TSH-LHR-6 of a given antibody to those obtained with the WT receptor.
B. Hormone stimulated and basal cAMP production of LHR WT, TSHR WT and TSH-LHR-6 was measured in presence of NRIgG or the mini-TMD antibodies (10μg/ml).

FIGURE 11. Computational analysis of the mechanism of action of the mini-TMD antibodies.
A. Relative spatial orientation of (A(i)) L457, D578, N619 in the modeled LHR WT TMD; (A(ii)) R457, D578, N619 in the modeled mutant LHR L457R and (A(iii)) R457, D578, N619 in ECL –juxtamembrane domain constrained LHR L457R mutant, simulating binding of the mini-TMD antibodies. Hydrogen bonds are shown as dotted lines.
B. MD simulations were carried on the modeled structures of the wild type and LHR457 mutants. TMH3, TMH6 and TMH7 derived from the MD simulated model were superimposed on each other. Similar analysis was carried out by constraining the ECLs to simulate an antibody-bound condition. (B(i)) The relative changes in the helix orientations of TMH3, TMH6 and TMH7 in the WT (shown as white helices) and LHR L457R mutant (black helices) and (B(ii)) the same shown in the mini-TMD antibody bound simulated condition. Changes in the positions of L457 in WT and R457 in mutant are shown as white and black sticks.
C. Ca-backbone RMSDs were calculated for all residues of the LHR L457R mutant with respect to the WT receptor after performing MD simulation on each model. Gray lines indicate MD simulations under constrained ECLs (mimicking antibody bound condition) whereas the black lines indicate no constrain on the ECLs (simulating a free unbound receptor). RMSD calculations were not carried out for ECLs or ICLs and are marked by gaps in the plot. Reversal of the trajectory may be observed for TMH3, TMH6 and TMH7.
Figure 1

Conformational locking of GpHR TMDs by exoloop antibodies

A

Helix 2 (0.8 Å)
Helix 6 (0.8 Å)
Helix 4 (0.8 Å)
Helix 7 (1.26 Å)
Helix 3 (1.2 Å)
Helix 5 (0.9 Å)
Helix 1 (1.9 Å)

90°

B

TSHR TMD
YTFKRTLVWFVSLALALLCNYTVVLLYTLTSHYKNYPFVLFMCNLAFADCFMCMVYLLLIASTV
420
1ICL
ICL1
TMH2
TMH1
440
460
60
ASV
3
TSHR miniTMD
DLTVSEYVNAHIDQWGDPNATGFFTVFASLHVLTV1TLERWATTFAMRLDRK120
90
2ICL
ICL2
TMH3
TMH4
520
520
37
TSHR miniTMD
SLYTVSYYHA1IDQWGDPGCWYTAFAMLRDRK
370
1ICL
ICL1
TMH4
TMH5
940
940
180
TSHR TMD
RLRHAACAIMVGIWVCCFLALLPLVQISYAKVSCIIPMDTETPLALAYIVFVLTNIVA
1230
1ICL
ICL1
TMH5
TMH6
240
TSHR miniTMD
FYIVCCCTYKIITVRPHQYNPGDKTIKKARMAVLITDFICMFAISFYALSATLNKPL
300
1ICL
ICL1
TMH7
TMH8
690
690
74
TSHR TMD
IYTVNSKIELLVLFYPPLNSCNPFLYAFTKAFQIDFVIFLLSSFQICBKQAQQAYGQRVP
1000
1ICL
ICL1
TMH7
TMH8
690
690
102
TSHR miniTMD
KNSTDIPQKVTHDORMQGLHNMEDVIELIENSHTPKQGG1ISKEYMQTVL351
381
102

C

ECL1

ECL2

ECL3

K660

D619

R519

D

ECL2

C494

ECL3

C569

ECL1
Figure 2

Conformational locking of GpHR TMDs by exoloop antibodies
Figure 3
Conformational locking of GpHR TMDs by exoloop antibodies

Figure 4

A (i) Vector control (88) Mini-TMD IgG on LHR (1130)
(ii) Vector control (88) Mini-TMD IgG on NCG-LHR complex (197)

B

|   | TMH1 | TMH2 | TMH3 | TMH4 | TMH5 | TMH6 |
|---|------|------|------|------|------|------|
| nTSHR | <br> PCE Did MGYKPRLR1VWFVYLALL0NVFVLIL<br> TMH1 | <br> ICL1 | <br> TMH2 | <br> ICL1 | <br> TMH3 | <br> ICL1 |
| LTSHR | <br> PCE Did MGYDPRLR1VWFVYLALL0NVFVLIL<br> TMH1 | <br> ICL1 | <br> TMH2 | <br> ICL1 | <br> TMH3 | <br> ICL1 |
| nTSHR | <br> PCE Did MGYNPLRL1VWFISLAIATGNIVIL<br> TMH1 | <br> ICL1 | <br> TMH2 | <br> ICL1 | <br> TMH3 | <br> ICL1 |
| LTSHR | <br> PCE Did MGYDPRLR1VWFVYLALL0NVFVLIL<br> TMH1 | <br> ICL1 | <br> TMH2 | <br> ICL1 | <br> TMH3 | <br> ICL1 |
| Conservation | <br> ECL1 | <br> TMH4 | <br> TMH5 | <br> TMH6 | <br> TMH7 | <br> TMH8 |

Vector control (88) Mini-TMD IgG on TSH-TSHR complex (165)

Vector control (88) Mini-TMD IgG on FSH-FSHR complex (123)
Figure 5

A (i) Conformational locking of GpHR TMDs by exoloop antibodies

B (i) LHR

C D LHR TSHR

23
Figure 6

A

| WT          | T:21/L:26/F:22 | T:261/L:265/F:260 | T:413/L:354/F:358 | T:764/L:699/F:695 |
|-------------|----------------|-------------------|-------------------|-------------------|
| SP          | LRRD           | HinR              | TMD               |                   |
| HinTMD (ΔLRRD) | SP           | HinR              | TMD               |                   |
| TMD (ΔECD)  | SP             | TMD               |                   |                   |

B

Graphs showing fluorescence-FITC histograms for different conditions and antibodies.
Figure 7

A

B
Conformational locking of GpHR TMDs by exoloop antibodies

Figure 8

A. (i) LHR HiR IgG
   - Vector control
   - LHR S277Q
   - LHR WT

   (ii) Mini-TMD IgG
   - Vector control
   - LHR S277Q
   - LHR WT

B. (i) TSHR HiR IgG
   - Vector control
   - TSHR D410N
   - TSHR S281I
   - TSHR WT

   (ii) Mini-TMD IgG
   - Vector control
   - TSHR D410N
   - TSHR S281I
   - TSHR WT

C. Bar graph showing expression normalized cAMP (pmol/10^6 cells/minute)
   - 1 nM hCG
   - LHR WT
   - LHR S277Q

D. Bar graph showing expression normalized cAMP (pmol/10^6 cells/minute)
   - 1 nM hCG
   - 1 nM hTSH
   - LHR WT
   - TSHR WT
   - TSHR S281I
   - TSHR D410N

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**Conformational locking of GpHR TMDs by exoloop antibodies**

### Figure 9

#### (i)

![Graph A (i)](image)

- **A**
  - **Wt**
  - **I486F**
  - **I568T**
  - **V656F**

#### (ii)

![Graph A (ii)](image)

- **Expression Normalized cAMP pmols/10^6 cells/minute (Mean ± S.D. n=3)**

#### B

![Graph B](image)

- **Expression Normalized cAMP pmols/10^5 cells/minute (Mean ± S.D. n=3)**
  - **WT**
  - **D578Y**
  - **WT**
  - **L457R**
Figure 10

A (i) (ii)

B

Expression Normalized
CAMP pmols/10^5 cells/minute (Mean ± S.D., n=3)

TSHR WT TSH-LHR-6

1 nM hCG 1 nM hTSH 1 nM hTSH

LHR WT TSHR WT TSH-LHR-6

Buffer Buffer Buffer

Ra/Re=2 (1.72/0.86) (0.86)

TSHR WT TSH-LHR-6

NR IgG miniTMD IgG

TSHR WT 1-6 IgG

1 nM hCG 1 nM hTSH 1 nM hTSH

Buffer Buffer Buffer

NR IgG

TSHR WT 1-6 IgG

1 nM hCG 1 nM hTSH 1 nM hTSH

Buffer Buffer Buffer

Conformational locking of GpHR TMDs by exoloop antibodies
Figure 11

Conformational locking of GpHR TMDs by exoloop antibodies
The antibodies against the computationally designed mimic of the Glycoprotein hormone receptor transmembrane domain provide insights into receptor activation and suppress the constitutively activated receptor mutants

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