FEBS Letters 586 (2012) 810–817

FEBS Letters

journal homepage: www.FEBSLetters.org

Insights into differential modulation of receptor function by hinge region using novel agonistic lutropin receptor and inverse agonistic thyrotropin receptor antibodies

Ritankar Majumdar [1], Reema Railkar [1], Rajan R Dighe *

Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka 560 012, India

ARTICLE INFO

Article history:
Received 26 December 2011
Revised 23 January 2012
Accepted 23 January 2012
Available online 2 February 2012

Edited by Maurice Montal

Keywords:
GPCR
Agonist
Inverse agonist
Receptor activation
Conformational change
Glycoprotein hormone and receptor

ABSTRACT

We report two antibodies, scFv 13B1 and MAb PD1.37, against the hinge regions of LHR and TSHR, respectively, which have similar epitopes but different effects on receptor function. While neither of them affected hormone binding, with marginal effects on hormone response, scFv 13B1 stimulated LHR in a dose-dependent manner, whereas MAb PD1.37 acted as an inverse agonist of TSHR. Moreover, PD1.37 could decrease the basal activity of hinge region CAMs, but had varied effects on those present in ECLs, whereas 13B1 was refractory to any CAMs in LHR. Using truncation mutants and peptide phage display, we compared the differential roles of the hinge region cysteine box-2/3 as well as the exoloops in the activation of these two homologus receptors.

1. Introduction

Although the mechanism of signaling in all three GpHRs follows similar paths, vis-à-vis binding of the hormone to the exodomain LRRs followed by the transduction of signal to the TMD via the hinge region [1], ligand independent receptor activation in these receptors vary widely. On one hand, TSHR is characterized by high level of basal cAMP production and elevated susceptibility to activating mutations [2], negligible basal receptor activation has been observed in the case of LHR [3]. Previous reports of hormone-independent stimulation of FSHR by hinge region antibodies (aa 296–331) [4] and the inverse agonism displayed by TSHR MAb, specific to residues 273 and 274 [5], is indicative of either a unique attribute of individual hinge regions or a dual role played by the interplay between subdomains in the hinge region. Such unique agonistic, inverse agonistic or even antagonistic antibodies against LHR hinge region are not known although truncation studies have revealed that unlike the hinge regions of TSHR and FSHR, LHR hinge does not play role in maintenance of receptor basal activation [3]. Notwithstanding, it is interesting that all three receptors are activated by mutations in conserved patch in the hinge region especially serine in the N-terminal region of Cb-2 subdomain [6]. This would suggest that even with their differences this region might have a common role in receptor activation process.

In order to provide a better understanding of the role of hinge region in LHR and compare its mechanism of action to TSHR, we have isolated a panel of single chain fragment variables (scFvs) against the LHR hinge region from the Tomlinson's I and J human scFv libraries as well as monoclonal antibodies against the TSHR hinge region and investigated their effect on basal and hormone stimulated activity in wild type and constitutively active mutants.

2. Materials and methods

2.1. LHR/TSHR hinge region proteins

The hinge region of LHR (LHR-HinR, aa 265–355) and TSHR (TSHR-HinR aa 261–413) were cloned as N-terminal His-Tag protein, purified using IMAC chromatography (Ni-Sepharose) and their purity ascertained by immunoblot analysis using anti-His-Tag antibodies (Fig. S1).

Abbreviations: GpHR, glycoprotein hormone–receptor; LHR, lutropin receptor; TSHR, thyrotropin receptor; TSH, thyroid stimulating hormone; hCG, human chorionic gonadotropin; LRR, leucine rich repeats; ECL, extracellular loops; MAb, monoclonal antibody; scFv, single chain fragment variable; CAM, constitutively active mutation; aa, amino acid; Cb, cysteine box

* Corresponding author. Fax: +91 080 23600999.
E-mail address: rdighe@mrdg.iisc.ernet.in (R.R Dighe).

[1] Equal author contribution.

0014-5793/$36.00
doi:10.1016/j.febslet.2012.01.052

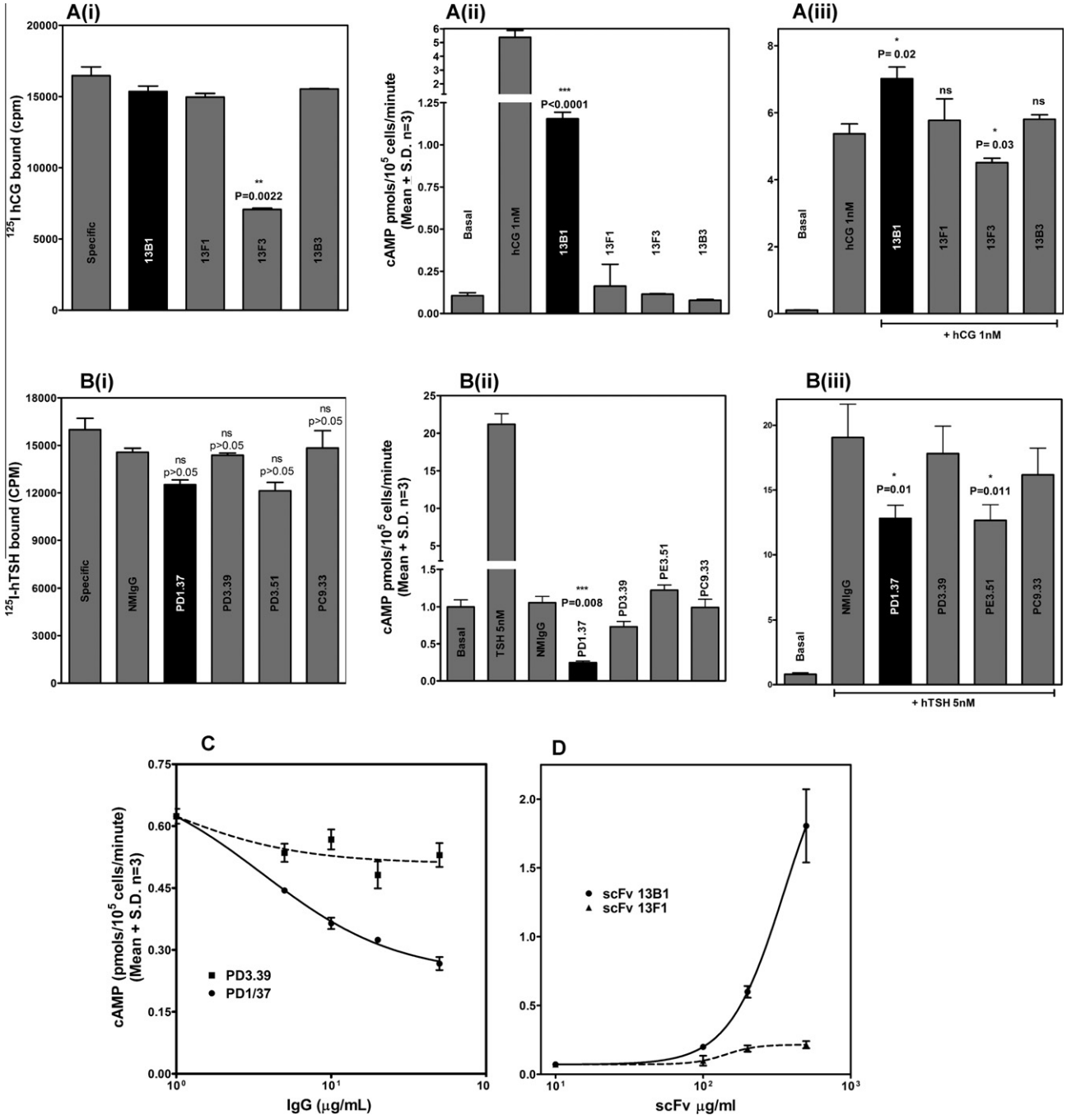

Fig. 1. Effect receptor antibodies on hormone–receptor interactions. Hinge region antibodies (50 μg/ml) were preincubated with HEK293-TSHR/LHR membrane preparation at 37 °C followed by addition of 0.14 nM ^{125}I-hCG/hTSH (specific activity of the tracer – 0.2 μCi/fmol) and bound hCG-LHR (A(i)) or hTSH-TSHR (B(i)) was determined. Effect on basal and hormone stimulated response was determined by preincubating the antibodies (50 μg/ml) with HEK293-LHR or TSHR cells alone (A(ii) and B(ii)), respectively or subsequently followed by addition of (A(iii)) hCG (1nM) & (B(iii)) hTSH (5 nM), respectively and cAMP produced determine by RIA as described by Saha & Majumdar et al. [18]. (C) Dose dependent decrease of cAMP production by HEK293-TSHR cells in presence of increasing concentration of MAb PD1.37 or (D) increase in cAMP production of HEK293-LHR cells in presence of scFv13B1. Neutral antibodies were used as controls for each case. Statistical significance was compared to the control and is denoted by the *P*-value calculated from the two-tailed unpaired *t*-test.

2.2. Generation/selection of receptor antibodies

MAbs against the TSHR-HinR protein were developed according to protocol discussed previously [7]. ScFvs against LHR-HinR protein were selected from Phage human ScFv library (Tomlinson's I + J library) as described previously [8]. Soluble ScFvs were purified by Protein A affinity chromatography. All antibodies thus generated were characterized for specific binding to their cognate antigen fragment, and to their cognate full-length receptor through ELISA and flowcytometry.

2.3. TSHR/LHR WT and mutated constructs

Full length wild type (WT) LHR and TSHR deletion as well as TSHR–LHR chimeric constructs [9] were kind gift of Dr. Prema Narayan and Prof. Basil Rapoport, respectively. TSHR and LHR mutations were created using a single step PCR based mutagenesis protocol [10]. Transient transfection of different TSHR/LHR constructs were carried out simultaneously in parallel plates to determine the ligand binding, cAMP production, and flowcytometric analysis, using Lipofectamine 2000 reagent as per the manufacturer's protocol

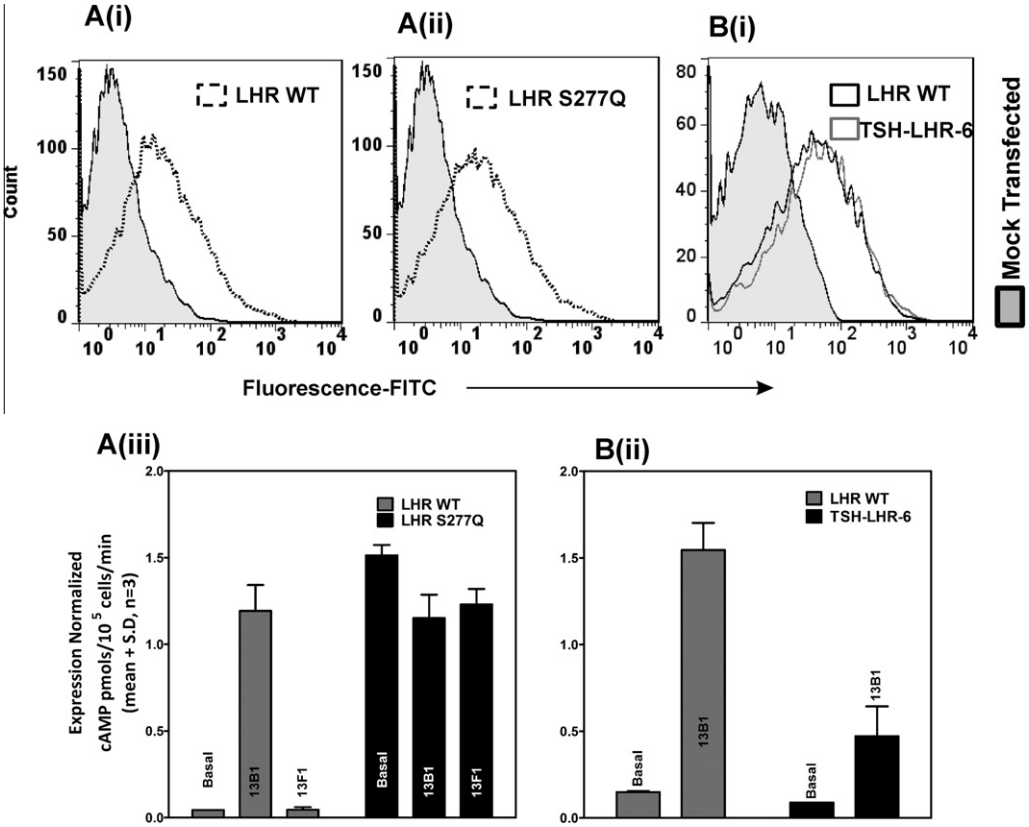

Fig. 2. Effect scFv 13B1 on LHR activating mutation and chimeric mutants. Binding of scFv13B1 to HEK293 cells transfected with the (A(i)) wild type or (A(ii)) S277Q or (B(i)) TSH-LHR-6 constructs was monitored in flow cytometry using protocols discussed in the legends of Fig. S3. Mock transfected cells (shaded histograms) were used as controls. scFv 13B1 (50 μg/ml) was added to HEK293 cells transfected with either (A(iii)) S277Q or (B(ii)) TSH-LHR-6 and total cAMP produced was measured by RIA. cAMP produced was normalized to the surface expression of LHR constructs with respect to wild type as determined by binding of LHR LRR 1–3 specific antibody in flowcytometry (data not shown).

(Invitrogen). Stable cell lines expressing WT TSHR (HEK293-TSHR) or LHR (HEK293-LHR) were similarly created and selected based on their ability to specifically bind radiolabeled hormone and exhibit cAMP production in response to the hormone.

2.4. Receptor binding and in vitro bioassay

The effect of various antibodies on hormone action was investigated by incubating cells (stable cell line or transiently transfected) or their membrane preparations with different dilutions of antibodies prior to the addition of labeled or unlabeled ligand. Measurement of hormone bound receptors or cAMP produced in presence of the antibodies were done as previously described [4].

2.5. Flow cytometric analysis of TSHR/LHR mutants

Cell surface expression of different TSHR/LHR mutants and identity/location of putative epitopes of receptor antibodies was assessed by flowcytometry using standard protocol [11] at low temperature and analyzed in FACSCANTO-II flowcytometer. Antibody binding (median fluorescence intensity – MFI) of a given antibody was normalized to a negative control and expressed as relative MFI (RMFI).

2.6. Peptide phage display

The epitope of scFv 13B1 was identified using the Ph.D.-12 phage-display peptide library (NEB Inc.) according to manufacturer's instructions. The sequences of the dodecapeptides appearing more than twice in the selected phage clones were classified as the consensus sequence, scanned against the full length LHR sequence with non-stringent gap opening and extension penalties (5 and 20, respectively in ClustalW 2.0.12) to determine putative binding site.

3. Results

3.1. Effect of receptor antibodies on hormone binding and receptor activation

Screening of Tomlinson I + J ScFv library yielded ten unique LHR-HinR specific scFvs, as determined by their CDR signatures (Fig. S2), of which four ScFvs with higher binding to LHR-HinR were further characterized. Four out of the nine TSHR MAbs, found to be positive for TSHR-HinR protein binding, were similarly chosen based on their higher binding and specificity to their cognate receptor as determined by ELISA (Figs. S3A and S3B) and flowcytometric analysis (Fig. S3C).

Hormone binding to HEK293-TSHR/LHR membranes or cAMP produced in these cells in response to hormones was measured in presence or absence of receptor antibodies to investigate their effect on hormone action. Out of the four LHR-HinR specific antibodies, only 13F3 could inhibit hormone binding (Fig. 1A(i)) as well as receptor activation (Fig. 1A(iii)), whereas in case of TSHR, marginal or no inhibition of hormone binding was observed (Fig. 1B(i)), although MAb PD1.37 and MAb PE3.51 showed significant inhibition of hormone response (Fig. 1B(iii)). Most interestingly, the MAb PD1.37 in absence of the hormone, could inhibit the basal cAMP production of TSHR (Fig. 1C), whereas scFv 13B1 showed dose dependent increase in LHR stimulation (Fig. 1D). In

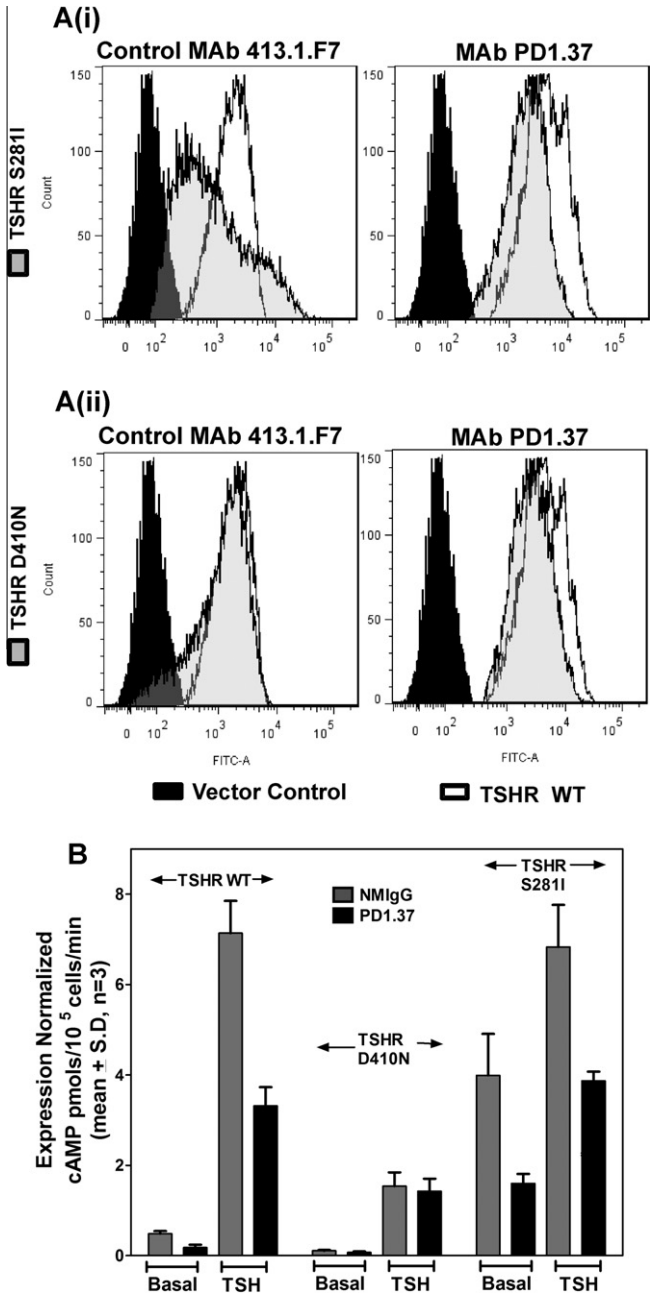

Fig. 3. Effect MAb PD1.37 on TSHR hinge region mutations. Binding of PD1.37 to HEK293 cells transfected with the wild type or (A(i)) S281I or (A(ii)) D410N mutant was monitored by flow cytometry using protocols discussed in the legends of Fig. S3. (B) Effect of PD1.37 (50 μg/ml) on basal and hormone stimulated activation of S281I and D410N mutants. cAMP produced was normalized to the surface expression of TSHR mutants with respect to wild type as determined by binding of TSHR LRR 1–3 MAb 413.1.F7 in flowcytometry, as shown in panel 3A.

addition, scFv 13B1 showed additive stimulation in presence of saturating concentration of hCG (Fig. 1A(iii)), suggesting non-overlap of hormone binding and scFv epitopes.

3.2. Effect of antibodies on receptor mutants

Basal and hormone stimulated response of TSHR/LHR mutants in presence of PD1.37/13B1 and their binding to these mutants was normalized to the surface expression of each mutant with respect to the WT by flowcytometry using a control LRR 1–3 specific TSHR MAb 413.1.F7 or polyclonal antibody specific to LHR LRR 1–3.

Although scFv 13B1 displayed binding to the hinge region CAM S277Q (Fig. 2A(ii)) equivalent to the WT (Fig. 2A(i)) in a flowcytometric analysis, it was not only unable to stimulate the high basal cAMP of the mutant, but displayed a marginal decrease in cAMP basal similar to the neutral antibody 13F1, further suggesting that the epitope of 13B1 may be sensitive to hormone independent changes in LHR-HinR (Fig. 2A(iii)). On the other hand, the scFv could not only bind to chimeric receptor TSH-LHR-6 (replacement of TSHR Hinge by that of LHR) (Fig. 2B(i)), it was also able to stimulate the chimera, although fold stimulation in TSH-LHR-6 was found to be less than that of the WT (Fig. 2B(ii)).

Similar to 13B1, binding of PD1.37 to the LHR S277Q homolog in TSHR (S281I) (Fig. 3A(i)) as well as the inactivating mutation, D410N (Fig. 3A(ii), was found to be comparable to that of the WT. In contrast to the TSHR-HinR mutants, the ECL CAMs, I486F, I568T or V656F, residing in the exoloops 1, 2 and 3 respectively, showed differential binding to PD1.37 (Fig. 4A). In order to quantify the differences in binding, ratios of the normalized binding (RMFI) of each mutant and the WT was considered and it was observed that PD1.37 has a lower binding to I486F and I568T when compared to the control MAb 413.1.F7 but displays higher binding to V656F (Fig. 4B).

Interestingly, unlike 13B1, PD1.37, affected the high basal of S281I and decreased both hormone dependent and independent cAMP production in this mutant, but did not affect the inactivating mutation, D410N (Fig. 3B). However, although PD1.37 displayed higher binding to V656F, it could not decrease the basal cAMP production in this mutant, whereas marked decrease was observed in case of I486F which exhibited comparatively lower MAb binding (Fig. 4C).

3.3. Epitope mapping of receptor antibodies

Binding of PD1.37 to progressive deletion mutants of TSHR was monitored in flowcytometry to establish the epitope of the antibody, as shown in Fig. 5. The binding of PD1.37 to Δ287–366 was slightly lower than that of the WT, whereas considerable loss of binding was observed for Δ287–376 and total loss of binding for Δ287–384. This would suggest that the epitope of PD1.37 could be in the region of residues 366–384 around the Cb-3 of the TSHR-HinR. The epitope of scFv 13B1 was expected to be around the Cb-2 region of LHR due to its insensitivity to critical S277Q mutant. This was confirmed by screening the peptide phage-display library with the purified 13B1. Comparison between the consensus amino acid sequence derived from these phages, (Fig. 6A) and LHR revealed a discontinuous epitope with a strong sequence similarity (>70% similarity) at aa 291–298 in Cb-2 and a second seed sequence at aa 331–334/338–339 in Cb-3 region (Fig. 6B).

4. Discussion

The GpHR hinge regions are thought to physically constrain the exoloops of receptors maintaining them in a low basal state. CAMs, hormonal stimulation, as well as agonistic antibodies disrupt these interactions as the initial events in receptor activation [1]. Interestingly, the role of LHR hinge region in maintenance of basal cAMP has been debated as the removal of its ECD does not result in increase in basal cAMP levels unlike TSHR/FSHR [3]. Moreover, the absence of any previous reports of agonistic LHR-HinR antibodies, unlike those of TSHR/FSHR, further supports the above hypothesis. Isolation of antagonistic (13F3) as well as agonistic (13B1) antibodies against LHR-HinR indicate its role in both hormone binding as well as its ability to affect hormone independent receptor activation. In contrast TSHR-HinR antibodies (PD1.37, PE3.51) inhibited hormone response but had marginal effect on hormone

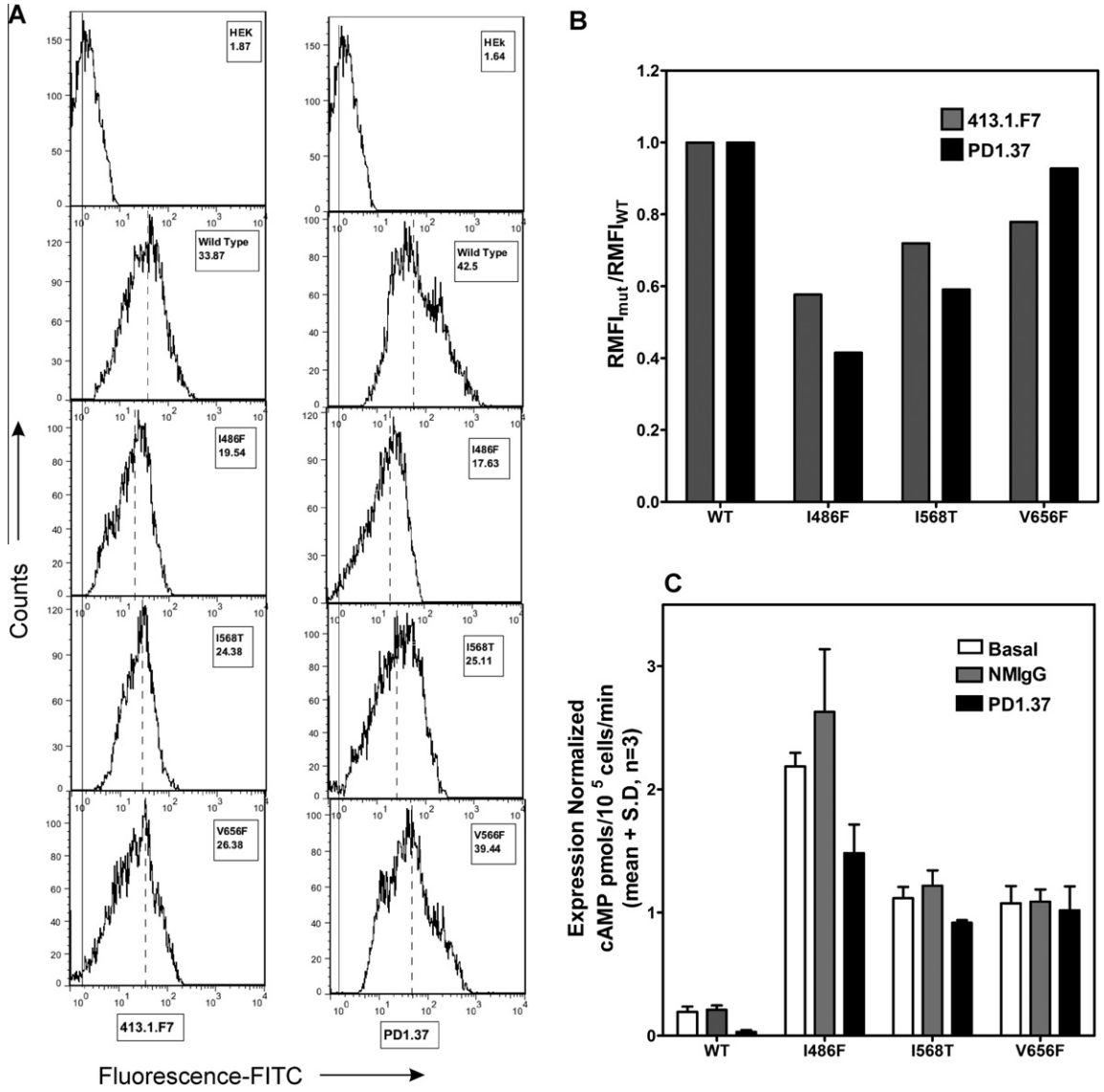

Fig. 4. Effect MAb PD1.37 on basal activity of TSHR ECL CAMs. (A) Flow cytometric analysis of HEK293 cells transfected with the wild type or different ECL mutants with control MAb 413.1.F7 (right panel) or with MAb PD1.37 (left panel). Dotted lines and values in boxes denote MFIs of constructs and the solid line that of the mock transfected control. (B) Ratios of RMFI of each ECL mutant with respect to wild type as determined by the control antibody (gray bar) or PD1.37 (black bar) (C) Effect of PD1.37 on basal activation of ECL mutants. cAMP produced was normalized as described in panel 3B.

binding indicating that TSHR hinge region may not be directly involved in hormone binding, a possibility speculated previously [6].

In addition MAb PD1.37 acted as an inverse agonist by decreasing the basal cAMP production similar to those reported for MAb CS-17 [12] and human MAb 5C9 [13]. However, unlike CS-17 or 5C9, PD1.37 does not compete for the hormone and is purely a hinge region specific antibody. Moreover, CS-17 and 5C9 were shown to decrease the high basal of the hinge region CAM S281I as well as those present in ECLs such as I486F, I568T or V656F. Mechanism of inhibition of basal cAMP production of these CAMs have been thought to be stabilization of the receptor inactive state by suppressing conformational changes in the hinge region of these CAMs [14]. Similar conclusions may also be drawn for PD1.37, as this antibody decreased the hormone dependent and independent activity of S281I, but interestingly did not affect hormone stimulation of the inactivating mutation D410N, probably due to similar conformational selection of the mutant by the antibody. The effect of PD1.37 on exoloop mutants differed markedly from CS-17; PD1.37 displayed lower binding to the first and second exoloop mutants but had higher binding towards the third. This would indicate that the epitope of PD1.37 (aa 366–384) is sensitive to conformational changes caused by CAMs in ECL 1 and 2, whereas activating mutation in ECL 3 causes a reversal of this sensitivity making the antibody more accessible to the receptor. More interestingly, Rapoport and co-workers, based on deletions in TSHR-HinR, have proposed that aa in the vicinity of 371–384 may be involved in inverse agonistic feature of the hinge region [14]. A corollary to this would be a plausible interaction of Cb-3 residues with the exoloop 3 as a negative regulator of hormone independent receptor activation.

Interestingly, unlike PD1.37, the LHR agonistic scFv 13B1 had no effect on S277Q mutant suggesting that its epitope is sensitive to the conformational changes in the Cb-2 region. The additive effect of 13B1 and hormone action suggests that the conformational change in this epitope may be independent of those brought about by the hormone. Such a possibility is further verified by the ability of 13B1 to stimulate the TSH-LHR-6 which responds to TSH but not to hCG.

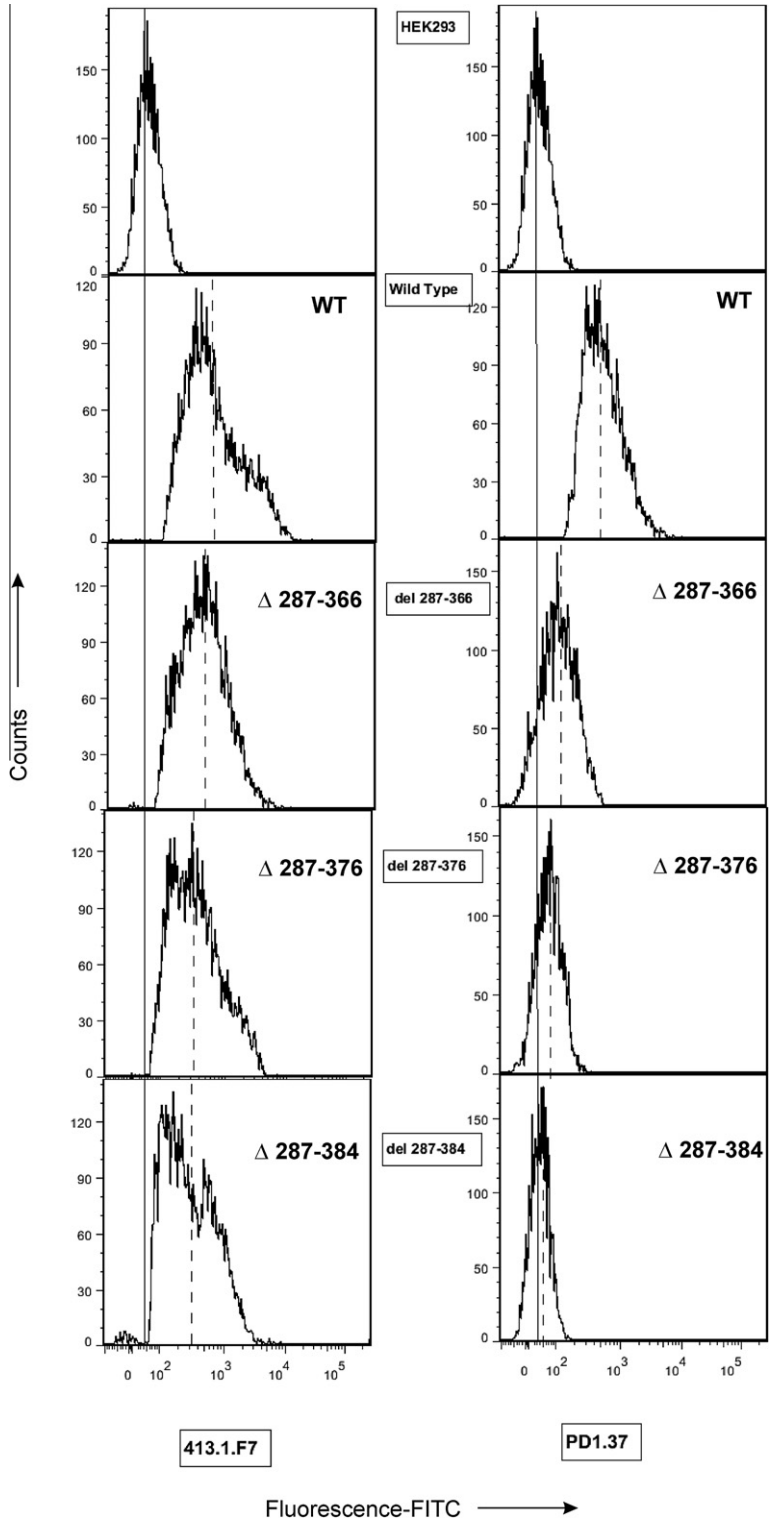

Fig. 5. Epitope mapping of MAb PD1.37. Putative binding site of PD1.37 was mapped by its ability to bind to different TSHR hinge region deletions in flowcytometry. Left panel – control MAb 413.1.F7, Right panel MAb – PD1.37. Dotted lines denote MFIs of constructs and solid line for the mock transfected control.

Interestingly the discontinuous epitope of 13B1 encompassing both Cb-2 and Cb-3 suggests a close spatial proximity of these two subdomains, as represented in Fig. 7. Indeed it has been proposed that the Cb-2 and Cb-3 of GpHRs are disulfide bonded, in close proximity and may constitute conformationally anchored regions [15] and epitope analysis of 13B1 provide tentative experimental evidence of such. Moreover, the Y-E/D-Y motif in Cb-3 that form the second seed sequence of 13B1 epitope is known to be tyrosine sulfated and mutations in these tyrosine residues makes the receptor, signaling and binding sensitive [15,16]. The presence of loss-of-function mutations like C390W, D403N and D410N in TSHR and E354K and C343S in LHR around Cb-3 as opposed to activating mutations in the N-terminal of hinge region hint at a cooperative involvement of the Cb-2 and Cb-3 regions

Fig. 6. Epitope mapping of scFv 13B1 by peptide phage display analysis. (A). Nucleotide sequences deduced from the phage clones selected after three rounds of biopanning. Three groups of sequences were obtained on analyzing the sequence of phage clones and consensus from them. The numbers in the parentheses denote the frequency or ratio of the number of the phage clones with common sequence. (B). Sequence alignment of the translated consensus sequence, derived from the phage clones, with the LHR HinR sequence. Residues marked by (*) denotes identical residues, denoted by (:) marks conserved substitutions and those by (.) denotes semi-conserved substitutions.

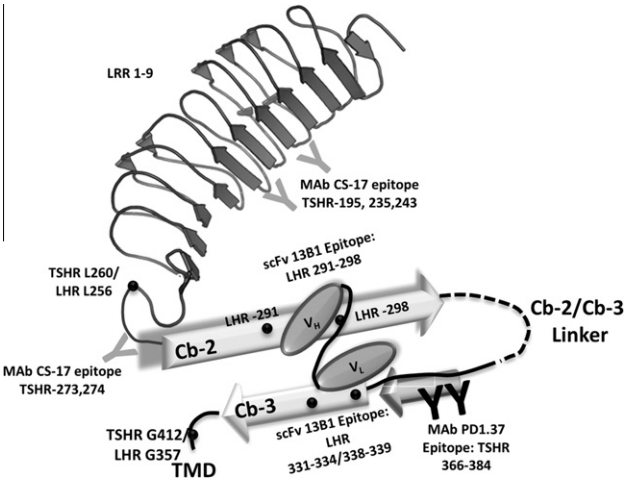

Fig. 7. Schematic representation of the epitopes of scFv 13B1 and MAb PD1.37. A generic model of the glycoprotein hormone receptor exodomain comprising of the N-terminal leucine rich repeats [adapted from TSHR LRR domain-MAb M22 crystal structure (PDB ID: 3GO4)], Cb-2, Cb-3 and Cb-2/3 linker is shown. 50 aa insertion in TSHR is shown in dotted lines. Residues marking the putative beginning and end of the hinge region are shown as gray spheres and residues marking those of the putative epitope of scFv 13B1 by black sphere. Light-gray arrows show Cb-2 and Cb-3 regions and dark-gray arrow the putative epitope of MAb PD1.37. (Image is not to scale). Epitopes of previously characterized inverse agonistic TSHR MAb CS-17 is also mentioned.

which act in concert to maintain the basal cAMP production [17].

In conclusion, we report an inverse agonistic MAb of TSHR that does not compete for hormone binding and preferentially suppresses exodomain CAMs and for the first time, an agonistic scFv of LHR providing, definitive evidence of the role of LHR HinR in hormone independent receptor activation. These antibodies may prove to be valuable tools as therapeutic agents in toxic adenomas arising due to TSHR CAMs as well as provide insight into structure–activity relationship of LHR mutants causing precocious puberty.

Acknowledgements

We thank the FACS facility; IISc for technical assistance. The work presented here was supported by grants from the Department of Biotechnology, New Delhi, India.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2012.01.052.

References

[1] Kleinau, G. and Krause, G. (2009) Thyrotropin and homologous glycoprotein hormone receptors: structural and functional aspects of extracellular signaling mechanisms. Endocr. Rev. 30, 133–151.
[2] Zhang, M., Tong, K.P., Fremont, V., Chen, J., Narayan, P., Puett, D., Weintraub, B.D. and Szkudlinski, M.W. (2000) The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. Endocrinology 141, 3514–3517.
[3] Nurwakagari, P., Breit, A., Hess, C., Salman-Livny, H., Ben-Menahem, D. and Gudermann, T. (2007) A conformational contribution of the luteinizing hormone-receptor ectodomain to receptor activation. J. Mol. Endocrinol. 38, 259–275.
[4] Agrawal, G. and Dighe, R.R. (2009) Critical involvement of the hinge region of the follicle-stimulating hormone receptor in the activation of the receptor. J. Biol. Chem. 284, 2636–2647.
[5] Chen, C.-R., McLachlan, S.M. and Rapoport, B. (2008) Identification of key amino acid residues in a thyrotropin receptor monoclonal antibody epitope provides insight into its inverse agonist and antagonist properties. Endocrinology 149, 3427–3434.
[6] Mueller, S., Jaeschke, H., Günther, R. and Paschke, R. (2010) The hinge region: an important receptor component for GPHR function. Trends Endocrinol. Metab. 21, 111–122.
[7] Dighe, R.R., Murthy, G.S. and Moudgal, N.R. (1990) Two simple and rapid methods to detect monoclonal antibodies with identical epitope specificities in a large population of monoclonal antibodies. J. Immunol. Methods 131, 229–236.
[8] Majumdar, R., Railkar, R. and Dighe, R.R. (2011). Docking and free energy simulations to predict conformational domains involved in hCG–LH receptor interactions using recombinant antibodies. Proteins: Structure, Function, and Bioinformatics
[9] Mizutori, Y., Chen, C.R., Latrofa, F., McLachlan, S.M. and Rapoport, B. (2009) Evidence that shed thyrotropin receptor A subunits drive affinity maturation of autoantibodies causing Graves' disease. J. Clin. Endocrinol. Metab. 94, 927–935.
[10] Papworth, C., Braman, J. and Wright, D.A. (1996) QuikChange site-directed mutagenesis. Strategies 9, 3–4.
[11] Maron, R., Jackson, R.A., Jacobs, S., Eisenbarth, G. and Kahn, C.R. (1984) Analysis of the insulin receptor by anti-receptor antibodies and flow cytometry. Proc. Natl. Acad. Sci. 81, 7446.
[12] Chen, C.-R., McLachlan, S.M. and Rapoport, B. (2007) Suppression of thyrotropin receptor constitutive activity by a monoclonal antibody with inverse agonist activity. Endocrinology 148, 2375–2382.
[13] Sanders, J. et al. (2008) A human monoclonal autoantibody to the thyrotropin receptor with thyroid-stimulating blocking activity. Thyroid 18, 735–746.
[14] Mizutori, Y., Chen, C.-R., McLachlan, S.M. and Rapoport, B. (2008) The thyrotropin receptor hinge region is not simply a scaffold for the leucine-

rich domain but contributes to ligand binding and signal transduction. Mol. Endocrinol. 22, 1171–1182.
[15] Bruysters, M., Verhoef-Post, M. and Themmen, A.P.N. (2008) Asp330 and Tyr331 in the C-terminal cysteine-rich region of the luteinizing hormone receptor are key residues in hormone-induced receptor activation. J. Biol. Chem. 283, 25821–25828.
[16] Bonomi, M., Busnelli, M., Persani, L., Vassart, G. and Costagliola, S. (2006) Structural differences in the hinge region of the glycoprotein hormone receptors: evidence from the sulfated tyrosine residues. Mol. Endocrinol. 20, 3351–3363.
[17] Kreuchwig, A., Kleinau, G., Kreuchwig, F., Worth, C.L. and Krause, G. (2011) Research resource: update and extension of a glycoprotein hormone receptors web application. Mol. Endocrinol. 25, 707–712.
[18] Saha, S., Majumdar, R., Dighe, R.R. and Chakravarty, A.R. (2010) Enhanced photodynamic effect of cobalt (III) dipyridophenazine complex on thyrotropin receptor expressing HEK293 cells. Metallomics 2, 754–765.