FEBS Letters 589 (2015) 588–597

FEBS Letters

journal homepage: www.FEBSLetters.org

N-glycosylation and disulfide bonding affects GPRC6A receptor expression, function, and dimerization

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ARTICLE INFO

Article history:
Received 19 August 2014
Revised 28 December 2014
Accepted 14 January 2015
Available online 21 January 2015

Edited by Judit Ovádi

Keywords:
Post-translational modification (PTM)
N-linked glycosylation
Disulfide
G protein-coupled receptor (GPCR)
GPRC6A
Signal transduction
Dimerization

ABSTRACT

Investigation of post-translational modifications of receptor proteins is important for our understanding of receptor pharmacology and disease physiology. However, our knowledge about post-translational modifications of class C G protein-coupled receptors and how these modifications regulate expression and function is very limited. Herein, we show that the nutrient-sensing class C G protein-coupled receptor GPRC6A carries seven *N*-glycans and that one of these sites modulates surface expression whereas mutation of another site affects receptor function. GPRC6A has been speculated to form covalently linked dimers through cysteine disulfide linkage in the extracellular amino-terminal domain and here we show that GPRC6A indeed is a homodimer and that a disulfide bridge between the C131 residues is formed.

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1. Introduction

The G protein-coupled receptor class C, group 6, member A (GPRC6A) belongs to the class C of G protein-coupled receptors (GPCRs), which are characterized by large amino terminal extracellular domains (ECDs) [49,54], comprised of a Venus-flytrap (VFT) domain and a cysteine-rich domain (CRD). The VFT domain is responsible for ligand binding whereas the CRD is important for signal transfer to the transmembrane domain [18,24,32,55]. The GPRC6A receptor is activated by a promiscuous range of basic and small aliphatic L-α-amino acids, in addition to calcium that either directly activates or co-activates the receptor, leading to a G_q-coupled response and production of inositol phosphate messenger molecules [5,20,23,39,41,56]. Other ligands as well as pathways like G_s and G_i have been proposed [12,34,35,39,40,41,42], however we have been unable to confirm this [20]. The physiological function of the receptor has so far only been characterized via knockout mice, which have shown interesting phenotypes like diet-induced obesity, feminization, glucose intolerance, and insulin resistance identifying this receptor as a potential drug target in metabolic disorders [6,7,38].

To better understand the pharmacology and regulatory aspects of GPCRs much research is focused on the role of post-translational modifications (PTMs) and oligomerisation of these receptors. PTMs regulate many essential processes within the cell, preparing the synthesized protein for folding, trafficking, etc. by adding e.g. oligosaccharides (glycans), phosphorylation, ubiquitination, and palmitoylation at specific residues. Even small errors in the regulation of these PTMs may result in misfolded and/or dysfunctional proteins. *N*-glycosylation is one of the most common types of glycosylation, resulting from the attachment of a sugar moiety to an asparagine (Asn, N) placed in the consensus sequence: N-X-S/T, X ≠ P. The role of glycosylation is not well understood for GPCRs, however research has clearly shown the importance of *N*-glycans in surface targeting and second messenger production [9,15,16,25,26,29,30,31,57]. Characterization of both rat and mouse GPRC6A has revealed that the receptor is *N*-glycosylated

Abbreviations: BSA, bovine serum albumin; CRD, cysteine-rich domain; dFBS, dialyzed fetal bovine serum; DPBS, Dulbecco's Phosphate-Buffered Saline; DTT, dithiothreitol; ECD, extracellular domain; ELISA, Enzyme-Linked Immunosorbent Assay; ER, endoplasmic reticulum; EtOH, ethanol; GPCR, G protein-coupled receptor; GPRC6A, G protein-coupled receptor family C, group 6, member A; HA, hemagglutinin; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; HTRF®, homogenous time-resolved Förster resonance energy transfer; IP_1, D-myo-inositol monophosphate; Opti-MEM, improved Minimal Essential medium; rGPRC6A, rat GPRC6A; RT, room temperature; TBS, Tris buffered saline; VFT, Venus flytrap; WT, wild-type; 7TM, seven transmembrane

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http://dx.doi.org/10.1016/j.febslet.2015.01.019

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in both HEK-293 cells and a transformed cell line variant tsA [23,56]. However the specific glycosylation pattern is still unknown as well as the role of this PTM on receptor pharmacology.

Another important factor for receptor regulation and pharmacology is subunit stoichiometry. Class C receptors are regarded as dimers or for the $GABA_B$ receptor even tetramers [3,8,11,13]. Dimer- or oligomerization of receptors can be mediated via covalent disulfide bridges [3,45,48,53] or via non-covalent association of the lipophilic transmembrane domains [4,10,17,22,33], interactions between the extracellular domains [1] or C-terminus interactions [28,36]. These interactions can affect the pharmacological diversity, signal transduction, ontogeny, internalization and ligand-promoted regulation of GPCRs emphasizing the importance of this phenomenon [52]. mGluR1, mGluR2, and mGluR5 form homodimer disulfide bridges in positions C140, C121 and C129, respectively [13,44,46]. The CaSR, which is the closest homolog to GPRC6A, has also been shown to form homodimers by bridging the cysteine residues C129 and C131 [23,45]. However, disruption of these disulfide bridges seems not to interrupt the dimerization questioning the role of the cysteine linkage [44,45]. Evidence based on Western blots showing bands of dimer size, which are converted into monomer bands under reducing conditions with addition of dithiothretiol (DTT) suggest that GPRC6A also forms a homodimer [23,56]. We therefore sat out to investigate if GPRC6A is a dimer, if such a disulfide bridge is present, and if it affects the dimerization of GPRC6A. Collectively, we wished to identify specific *N*-glycosylation and disulfide bridge residues, with the aim of increasing our understanding of the regulation and pharmacology of the GPRC6A receptor.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich unless otherwise specified.

2.1. GPRC6A constructs

Upon alignment with other class C GPCRs two cysteine residues possibly involved in dimer disulfide linkage were identified. The cysteine residues were mutated to alanine, alone and together, resulting in a total of three constructs; C122A, C131A and C122A + C131A made by Genscript (Piscataway, NJ, USA) (Fig. 1). Nine asparagine residues were predicted to carry in vivo glycosylations by the use of NetNGlyc 1.0 Server at www.cbs.dtu.dk and these were mutated to glutamine; N86Q, N121Q, N259Q, N332Q, N378Q, N452Q, N555Q, N567Q and N733Q by Genscript (Piscataway, NJ, USA) (Fig. 1 and Table 1). The previously described N-terminally c-myc-tagged rat GPRC6A receptor inserted into a pEGFP-N1 vector was used as template for the introduced mutations [56]. The N-terminally hemagglutinin (HA)-tagged rGPRC6A receptor was generated by replacing the c-myc-epitope with an HA-epitope in the c-myc-rGPRC6A-pEGFP-N1 construct as previously described for the $GABA_B$ receptors [36]. N-terminally HA-tagged $5\text{-}HT_{2A}$ receptor ($HA\text{-}5\text{-}HT_{2A}$) was purchased from the Missouri S&T cDNA resource center, USA.

2.2. Transfection

Transient transfection of HEK-293T cells was performed in white 96-well plates (Corning, Corning, NY, USA) for Enzyme-Linked Immunosorbent Assay (ELISA), white 96-well plates (Falcon, Corning, NY, USA) for dimerization assay and clear 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) for functional signaling assays, whereas transfection in 60 mm dishes was performed for the Western blot experiments. For transfection in 96-well plates a DNA:Lipofectamine 2000 ratio (Life Technologies, Nærum, Denmark) of 0.5 μg:0.25 μl/well was used. Plates were coated with poly-D-lysine before addition of DNA and cells. For each well 0.5 μg of DNA was prepared and diluted in 25 μl Opti-MEM. Lipofectamine 2000 was diluted in 25 μl Opti-Mem and then mixed with the DNA solutions and left at room temperature (RT) for 20 min. Plates were washed with 100 μl/well of Dulbecco's phosphate buffered saline (DPBS) cat. No. 14190169 (Life Technologies, Nærum, Denmark) and 50 μl of DNA complexes were added to each well. 12,500 HEK-293T cells suspended in Dulbecco's Modified eagle medium (DMEM) cat. No. 31966047 (Life Technologies, Nærum, Denmark) + 10% dialyzed fetal bovine serum (dFBS) (Life Technologies, Nærum, Denmark) were added to each well. For Western blotting experiments 500,000 HEK-293T cells were seeded in a 60 mm culture dish. After overnight incubation 4 μg of DNA and 7.5 μl Lipofectamine 2000 were used to transfect cells as described by the manufacturer. Cells were used 24–48 h post transfection.

2.3. Quantification of receptor expression

Total and cell membrane receptor expression levels were determined by ELISA utilizing a c-myc epitope fused to the N-terminal of rGPRC6A [56]. The $GABA_B$ system was applied as control for correct cell surface expression. Cells were treated as follows: Media was removed and 50 μl/well of DPBS + 4% paraformaldehyde was added and incubated for 5 min at RT. The wells were washed twice with DPBS + 1 mM $CaCl_2$. In wells where the total amount of c-myc-tagged receptor were to be determined, 50 μl of 0.1% Triton-X in DPBS was added and incubated at RT for 5 min. The Triton-treated wells were further washed two times with DPBS. All wells were blocked with 100 μl/well 3% skim milk in DPBS for at least 30 min. After blocking, 75 μl/well of 1:1000 anti-c-myc antibody (Life Technologies, Nærum, Denmark) or 1:1000 anti-HA antibody (Nordic Biosite, Copenhagen, Denmark) were added and incubated at RT for 45 min. Following antibody incubation, wells were washed twice with DPBS + 1 mM $CaCl_2$ before 75 μl of 1:1500 peroxidase labeled horse anti-mouse IgG (Vector Laboratories Inc., Burligame, CA, USA) was added and also incubated for 45 min. Wells were then washed four times with 100 μl/well of blocking solution followed by another four washes with 100 μl/well of DPBS + 1 mM $CaCl_2$. 70 μl of DPBS + 1 mM $CaCl_2$ was added to each well and prior to plate reading, 10 μl/well of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was added. The chemiluminescence was measured immediately after substrate addition on an EnSpire microplate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

2.4. Functional inositol monophosphate accumulation assay

Activation of the GPRC6A receptor was determined by an inositol monophosphate accumulation assay. Cells were washed for 2×2 h with Wash Buffer (Hank's balanced salt solution (HBSS) cat. No. 14175129 (Life Technologies, Nærum, Denmark) + 20 mM HEPES + 1 mg/ml bovine serum albumin (BSA) pH 7.4) prior to ligand stimulation. L-ornithine was diluted in ligand buffer (HBSS + 20 mM HEPES + 40 mM LiCl + 1 mM $CaCl_2$ and 1 mM $MgCl_2$ pH 7.4) and cells were stimulated for 1 h at 37 °C. Stimulation buffer was then removed and cells were lysed by adding 30 μl of Conjugation & Lysis buffer (Cisbio Bioassays, Codolet, France) to each well and incubated at RT for at least 30 min. 10 μl of cell lysis were transferred to a 384-well plate and 10 μl of the detection solution composed of 2.5% anti-IP_1 cryptate Tb and 2.5% IP_1-d2 (IP-One HTRF® kit, Cisbio, Codolet, France) dissolved in assay buffer (HBSS + 20 mM HEPES + 1 mM $CaCl_2$ and 1 mM $MgCl_2$) were added

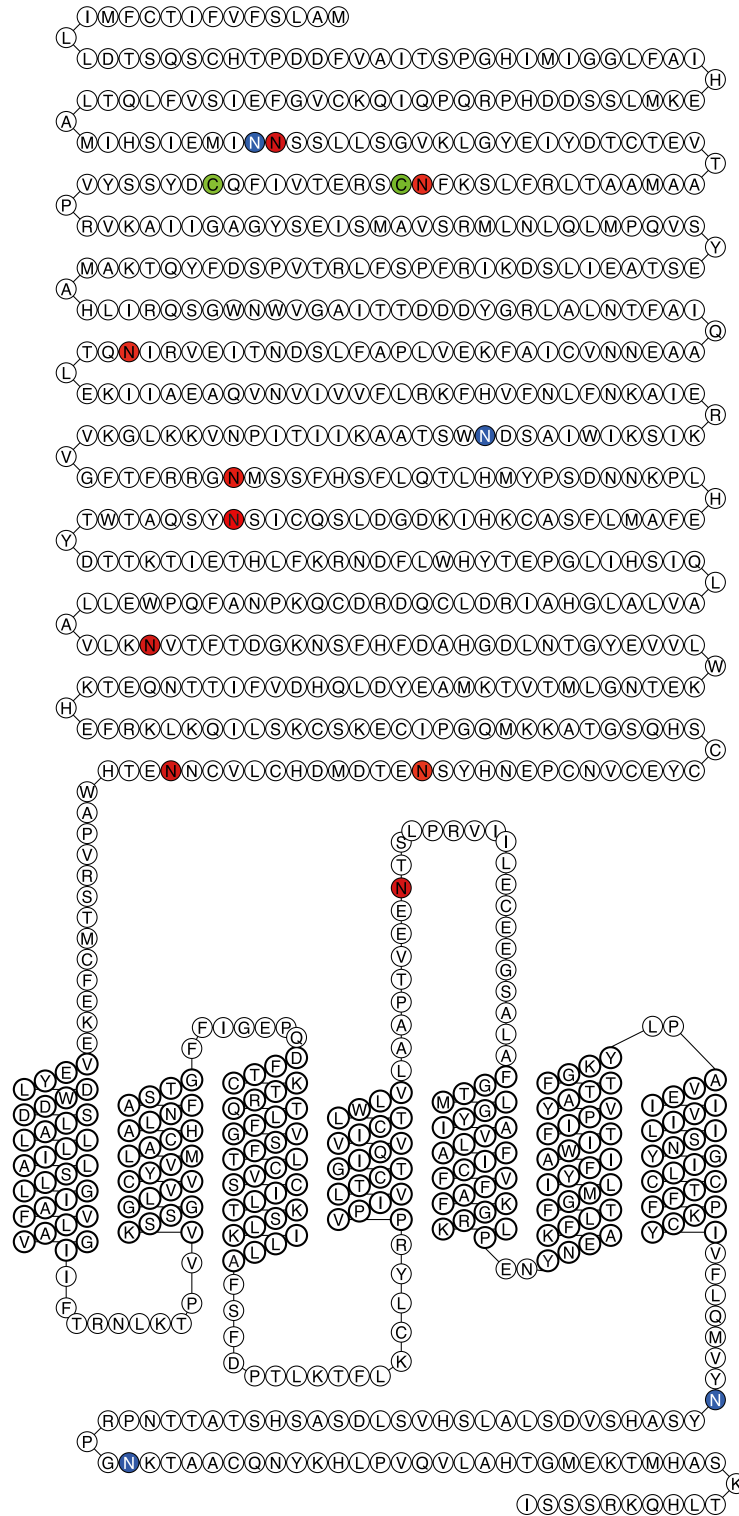

Fig. 1. Schematic representation of the rat GPRC6A showing locations of putative *N*-linked glycosylation sites as well as possible disulfide bridge residues. Each amino acid residue is represented by a circle. Asparagine residues predicted to be glycosylated are colored in red (N86, N121, N259, N332, N378, N452, N555, N567, and N733). Residues marked in blue have not been investigated due to a negative prediction of in vivo glycosylation (N85 and N304) or because they are positioned in the intracellular part of the receptor (N857 and N889). Possible conserved cysteines are in green (C122 and C131).

to each well. After 1 h incubation at RT away from light the signals at 615 and 665 nm were measured on an EnVision platereader after excitation at 340 nm (PerkinElmer Life and Analytical Sciences, Waltham, MA). The FRET ratios (665 nm/615 nm) were then converted to the concentration of IP_1 by use of a provided standard.

2.5. Dimerization assay by homogenous time-resolved Förster resonance energy transfer (HTRF®)

HTRF® experiments were carried out using a combination of a Tb^{3+}-labeled anti-HA antibody, as the donor fluorophore, and a

Table 1
Prediction of *N*-glycosylation of rGPRC6A using the NetNGlyc 1.0 Server. Potential threshold = 0.5.

Position	Motif	Potential	Result
85	NNSS	0.4166	–
86	NSSL	0.5433	+
121	NCSR	0.6523	+
259	NQTL	0.5910	+
304	NWST	0.3219	– –
332	NMSS	0.7583	+++
378	NYSQ	0.5581	+
452	NVTF	0.5899	+
555	NETD	0.7071	++
567	NETH	0.5682	+
733	NTSL	0.5944	+
857	NYSA	0.5162	+
889	NKTA	0.5512	+

d2 labeled anti-c-myc antibody as the acceptor fluorophore from Cisbio Bioassays (Codolet, France) as previously applied [8,27]. Cells were, after transfection, washed once in cold Wash Buffer and incubated with the fluorophore-coupled antibodies (1 nM anti-HA-Tb and 50 nM anti-c-myc-d2) diluted in Wash Buffer for 25 h at 4 °C. The HTRF® signal was measured at 665 nm after excitation at 340 nm (Fig. 4A) on an EnVision Multilabel Reader (PerkinElmer, Waltham, MA).

2.6. Membrane preparation

Cells were kept on ice and washed once with ice-cold DPBS. 800–1000 μl of RIPA Lysis buffer (50 mM TrisHCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate and 0.1% SDS) were added. For reducing conditions the RIPA buffer was supplied with 1 mM DTT and 0.5% v/v β-mercaptoethanol. Cell lysates were transferred to tubes which were incubated for 2 h at 4 °C on a rotator. Tubes were then centrifuged at full speed for 30 min at 4 °C in a tabletop centrifuge. Supernatant was transferred to a fresh tube and protein concentration was determined by performing a Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Copenhagen, Denmark) according to the manufacturer's protocol.

2.7. Western blotting

Protein samples were mixed with 4× loading buffer (NuPage LDS Sample buffer) and 40 μg of protein were loaded onto a NuPAGE NOVEX 3–8% Tris–acetate gel (Life Technologies, Nærum, Denmark). Samples were run for 75 min at 200 V in 1× NuPAGE Tris–acetate SDS Running Buffer (Life Technologies, Nærum, Denmark). Proteins were transferred to Amersham Hybond-ECL membranes (GE Healthcare, Brøndby, Denmark) for 90 min at 30 V in ice-cold Tris–glycine buffer (25 mM Tris and 192 mM glycine) with 15% ethanol (EtOH). Membranes were blocked for at least 20 min in 5% skim milk in DPBS before adding 1:1000 anti-c-myc antibody (Life Technologies, Nærum, Denmark) and were then incubated at RT for 1 h or at 4 °C overnight in a wet chamber. Membranes were then washed four times in 1× Tris Buffered saline with 0.2% Tween-20 (TBST) and incubated with 1:15,000 secondary peroxidase labeled horse anti-mouse IgG (Vector Laboratories Inc., Burligame, CA, USA). Following four times washing in TBST the membranes were developed using Amersham ECL Prime Western Blotting Detection reagent (GE Healthcare, Brøndby, Denmark). Pictures were taken using the FluorChem HD2 System (ProteinSimple, Santa Clara, CA, USA). Exposure times were optimized for the individual experiments.

2.8. Deglycosylation

Wild type GPRC6A protein samples were treated with PNGase F to remove *N*-glycans. Approximately 40 μg of protein were added 2 μl of PNGase F corresponding to one unit and incubated at 37 °C for 2 h.

2.9. Data analysis

Data obtained were analyzed using GraphPad Prism v. 5 (GraphPad Software, San Diego, CA) and ImageJ v. 1.48 (Wayne Rasband, National Institutes of Health, USA). Statistical analysis (one-way analysis of variance (ANOVA) followed by Dunnett's test) was performed where appropriate and is indicated in the figure captions. Statistical significance was determined at the following levels: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Identification of N-linked glycosylation sites on rGPRC6A

To verify rGPRC6A receptor glycosylation in HEK-293T cells, we transfected cells and treated with PNGaseF. The results showed a marked downward shift in molecular weight for both monomeric and oligomeric bands compared to control, suggesting the presence of *N*-linked oligosaccharides on rGPRC6A (Fig. 2).

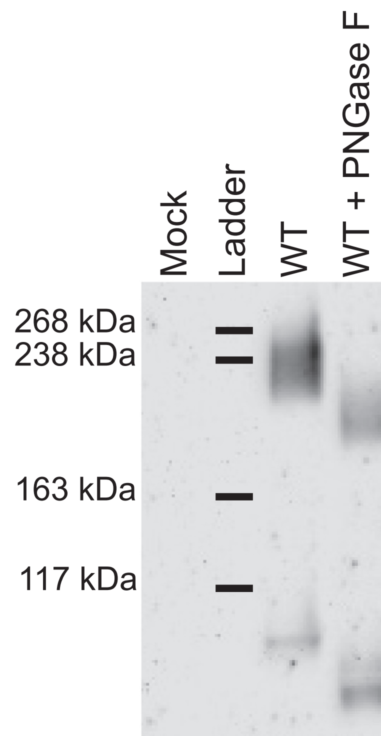

Fig. 2. Rat GPRC6A is glycosylated. Membranes from HEK-293T cells transiently transfected with c-myc-tagged rGPRC6A receptor (WT) or empty plasmid (mock) were detected with anti-c-myc antibody and visualized with anti-mouse HRP-conjugate and ECL reagent. Mock-transfected cells did not show any significant immunoreactivity. Cells transfected with WT receptor show a dense band at ∼230kDa and a smaller band at ∼100kDa, representing dimeric and monomeric forms, respectively. Treatment with the deglycosidase PNGase F resulted in lower migrating bands (both dimeric and monomeric) of the receptor. The blot is a representative of two independent experiments.

In order to identify the possible in vivo *N*-glycosylation sites (N-X-S/T) we used the NetNGlyc 1.0 prediction server (prediction server at Center for Biological Sequence Analysis, Technical University of Denmark). Eleven of 13 possible sites were returned with a positive score suggesting *N*-glycosylation (Table 1). The two residues predicted not to carry any *N*-glycans were not investigated further as well as the two sites (N857 and N898) that were localized intracellularly. The remaining nine Asn residues were mutated to glutamine (Gln, Q) and analyzed on SDS–PAGE. Membrane preparations from HEK-293T cells, transiently transfected with either wild-type (WT) or mutant rGPRC6A receptors, showed subtle but clear migration differences (Fig. 3A). The bands at ~220kDa represent receptor dimers/oligomers whereas the two bands seen around 100kDa are monomeric forms of the receptor corresponding to

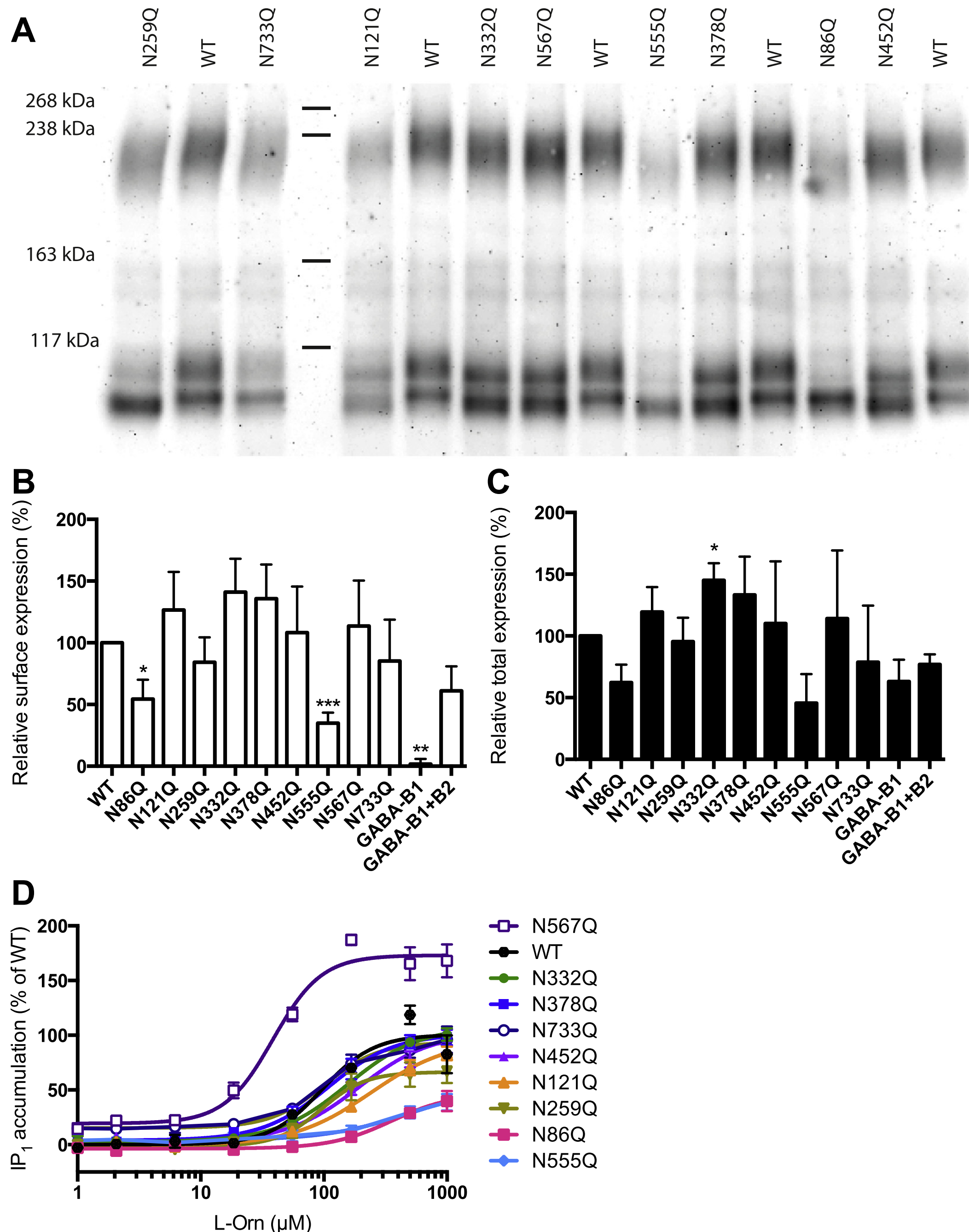

Fig. 3. Effects of mutation of putative *N*-glycosylation sites in rGPRC6A. (A) 40 µg of protein from membrane extracts of transiently transfected HEK-293T cells were loaded and fractioned on a 3–8% Tris–acetate gel by SDS–PAGE. Western blotting was done using an anti-c-myc antibody followed by anti-mouse HRP-conjugate and ECL reagent. For each lane a major band at ~220kDa is seen corresponding to multimer receptors. For all receptors except N86Q and N555Q, two bands are seen at ~90 and ~110kDa corresponding to core and fully glycosylated monomeric receptor forms. For N86Q and N555Q only the lower band is seen. The blot is representative of five independent experiments. (B and C) ELISA was used to determine surface and total expression for wild-type (WT) and indicated mutants of rGPRC6A. White bars show surface expression and black bars show total expression. Data are presented as means ± S.E.M. of at least four independent experiments performed in triplicates and are normalized to WT. Significant differences from WT were calculated by a one-way ANOVA followed by Dunnett's post-test (*$P < 0.05$, ***$P < 0.001$). (D) HEK-293T cells transiently transfected with indicated constructs 24 h prior to experiments where they were stimulated with L-Orn for 1 h. IP_1 accumulation was detected by the IP-One HTRF® assay from Cisbio (Codolet, France). Data are normalized to wild-type (WT) IP_1 accumulation. Data are means ± S.D. of a single representative experiment out of 5 individual repeats performed in triplicates.

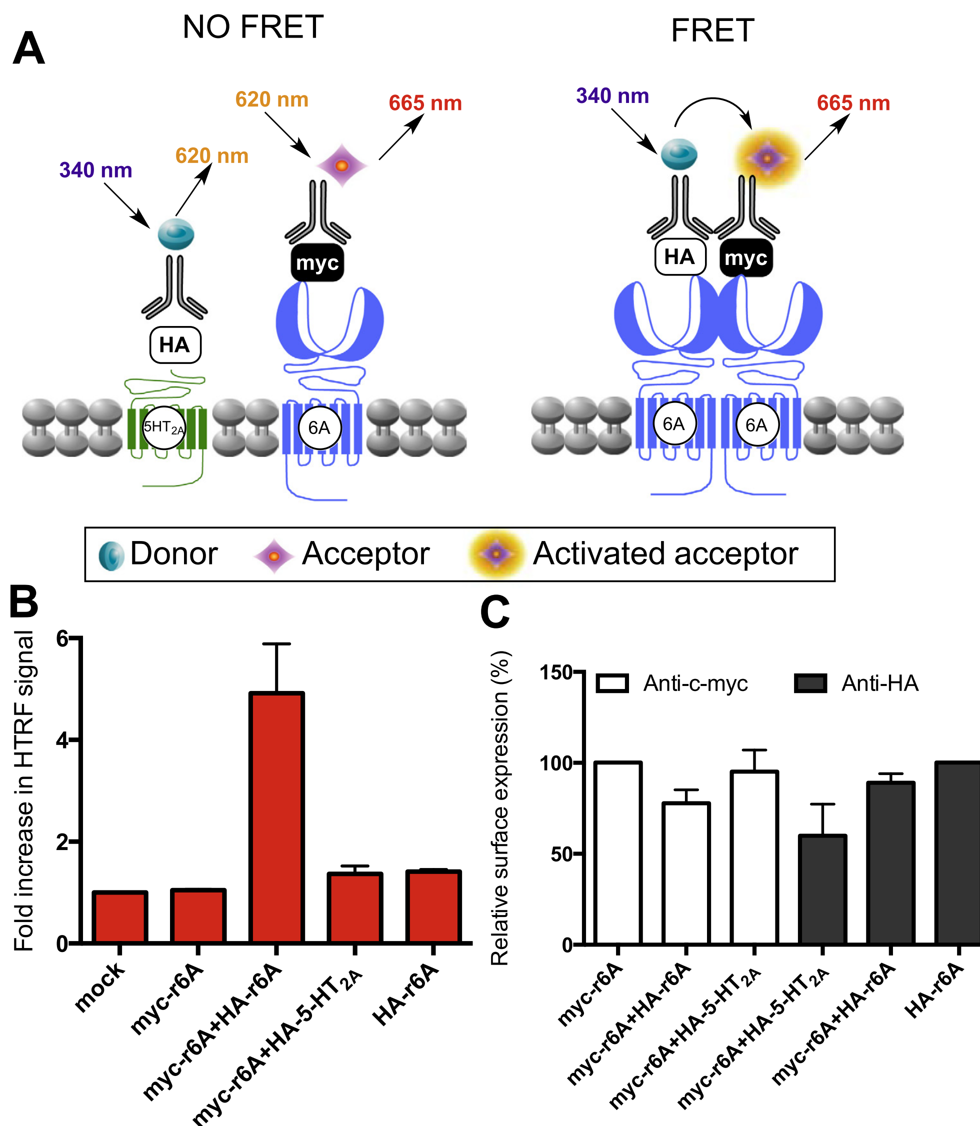

Fig. 4. Detection of rGPRC6A dimers on the cell surface. (A) Principle of the HTRF® protein–protein interaction system. Two receptor subunits carry different epitope tags at the N-termini and are incubated with antibodies conjugated with donor and acceptor flourophores. This allows detection of a FRET signal if the respective subunits are in close proximity. 6A: GPRC6A. (B) Transiently transfected HEK-293T cells were labeled with a combination of anti-HA-Tb and anti-c-myc-d2 antibodies for 25 h and the HTRF® signal was measured as described in Section 2. Upon co-transfection of HA and c-myc labeled rGPRC6A a FRET signal is generated demonstrating dimerization of rGPRC6A. Data are presented as means ± S.E.M. of at three independent experiments performed in triplicates and are normalized to mock. (C) ELISA was used to determine surface expression for the indicated constructs. White bars are detection of c-myc-tagged constructs, whereas gray bars represent HA-tagged constructs. Data are presented as means ± S.E.M. of at three independent experiments performed in triplicates and are normalized to c-myc-rGPRC6A or HA-rGPRC6A.

the calculated weight of (non-glycosylated) rGPRC6A of 104kDa. We believe that the two bands with a size of approximately 100kDa probably represent the fully and the core glycosylated forms of the monomeric receptor. N86Q and N555Q interestingly only display the lower band. Bands from N86Q and N733Q mutants migrates like WT, whereas the N121Q, N259Q, N332Q, N378Q, N452Q, N555Q and N567Q mutants all migrate with a ~5kDa lower weight compared to WT indicating the loss of an *N*-glycan. These data suggest a total of seven *N*-glycosylations on rGPRC6A.

3.2. Mutation of putative N-linked glycosylation sites on rGPRC6A affects receptor expression

Next we investigated if the mutations had an effect on receptor expression. Cells were transiently transfected with the indicated c-myc-rGPRC6A receptor constructs and ELISA was used to analyze surface and total GPRC6A receptor expression. Only the N86Q and N555Q mutants showed an altered expression level compared to WT, which was reduced approximately 50% for both surface and total expression (Fig. 3B and C). For N555Q this could be due to loss of the *N*-glycan whereas mutation of N86 did not alter migration of the receptor. The reduced expression of the N86Q mutant can therefore not be explained by a changed glycosylation pattern.

3.3. Mutation of putative N-linked glycosylation sites on rGPRC6A affects receptor function

Receptor function is often linked with expression and we therefore assayed the functional responses of the different mutants using the IPOne accumulation assay that measures G_q-activation. Most mutants showed similar activation profiles as the wild-type receptor, except N86Q and N555Q, which had significantly reduced

activity at the maximum concentration of agonist (Fig. 3D). The lower expression levels of these two mutants and the loss of glycosylation could indicate that intracellular trapping/degradation causes this reduction. Interestingly, we observed that the N567Q mutant increased the maximal response by more than 20% compared to wild-type (Table 2), which is not caused by an increased surface expression (Fig. 3B and C).

3.4. Identification of cysteine residue involved in rGPRC6A dimer formation

To determine if rGPRC6A is indeed a dimer an HTRF® assay was conducted using an anti-HA antibody labeled with a donor fluorophore and an anti-c-myc labeled acceptor fluorophore (Fig. 4A).

Table 2
L-Orn signaling parameter estimates for rGPRC6A Asn mutant receptors. IP_1 accumulation was detected by the IP-One HTRF® assay from Cisbio (Codolet, France). No statistical significant differences compared to WT were observed.

	EC_{50} (μM)	pEC_{50} ± S.E.M.	Max ± S.E.M. (%)	Max response/surface expression
WT	200	3.70 ± 0.19	100	100
N86Q	386	3.41 ± 0.09	40 ± 8.3	74
N121Q	289	3.54 ± 0.07	72 ± 16	57
N259Q	122	3.91 ± 0.09	59 ± 8.8	70
N332Q	205	3.69 ± 0.19	67 ± 13	47
N378Q	156	3.81 ± 0.10	69 ± 11	50
N452Q	177	3.75 ± 0.07	83 ± 29	77
N555Q	338	3.47 ± 0.07	34 ± 13	97
N567Q	141	3.85 ± 0.25	123 ± 17	108
N733Q	294	3.53 ± 0.16	62 ± 11	73

The results clearly show that rGPRC6A is capable of homodimerizing as co-expression of HA- and c-myc-tagged rGPRC6A receptors generates a FRET signal, whereas co-expression of c-myc-rGPRC6A with the unrelated HA-tagged 5-HT_{2A} receptor did not generate a FRET signal (Fig. 4B). These experiments were performed on intact cells demonstrating that the rGPRC6A receptors dimerize on the cell surface. In parallel surface expression was determined for all constructs demonstrating similar expression levels across the different receptor constructs (Fig. 4C). Next we wished to investigate if the dimerization was mediated via a disulfide bridge. An alignment of rGPRC6A with other class C receptors (CaSR and mGluR1–8) revealed two cysteines potentially involved in such a disulfide bridge; C122 and C131 (Fig. 5A). Membrane preparations from HEK-293T cells transfected with wild-type, C122A, C131A, or C122A + C131A receptors were immunoblotted. The C122A mutant displayed a band-pattern similar to wild-type receptor, but interestingly we found distinct differences for both the C131A mutant and the double mutant (Fig. 5B). For the C131A and the C122A + C131A mutant the receptor dimer band was negligible whereas the density of the monomeric bands were increased. These data clearly show that when mutating C131 into an alanine, the receptor is unable to preserve the dimeric structure upon Western blotting probably due to loss of the disulfide bridge formation.

3.5. Mutation of cysteine residues on rGPRC6A affects receptor expression

We next investigated the surface and total expression of the cysteine mutants by ELISA. We found that both surface and total expression levels of the C131A and especially the C122A + C131A

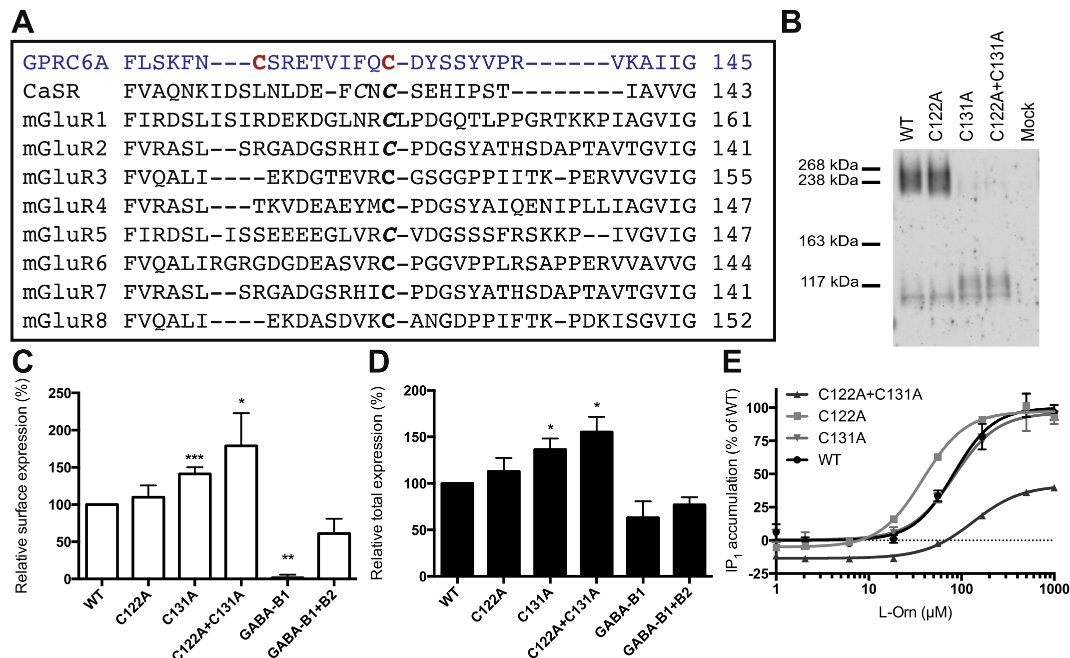

Fig. 5. Effects of mutation of putative VFT disulfide sites in rGPRC6A. (A) Alignment of rat GPRC6A with other family C receptors. Alignment of the loop region of rat GPRC6A (blue), the calcium-sensing receptor (CaSR) and the eight metabotropic glutamate receptors (mGluR1–8), which has previously been shown to harbor disulfide bridges in CaSR and mGluRs (shown in italic). The conserved cysteines among the receptors are marked in bold. GPRC6A contains two cysteines which, based on the alignment, could be involved in formation of disulfide bridges (red). Alignment performed using MEGA v.6.06 with default settings [51]. (B) Mutation of cysteine residues results in loss of dimer formation for rGPRC6A. Membranes from transiently transfected HEK-293T cells were blotted with anti-c-myc antibody. Wild-type (WT) receptor shows a dense band at ~230kDa and a smaller band at ~100kDa, as previously shown. Mutation of C122A did not affect receptor composition, however when mutating C131A, either alone or with C122A, the upper dimer band was markedly reduced whereas the lower monomeric band was intensified. Control cells were blank. Blot is representative for three repeated experiments. (C and D) ELISA was used to determine surface and total expression for wild-type (WT) and indicated rGPRC6A mutants. White bars are surface expression, whereas black bars show total expression. Data are presented as means ± S.E.M. of at least four independent experiments performed in triplicates and are normalized to WT. Significant differences from WT were calculated by a one-way ANOVA followed by Dunnett's post-test ($^{*}P < 0.05$, $^{***}P < 0.001$). (E) HEK-293T cells transiently transfected with indicated constructs were stimulated with L-Orn for 1 h. IP_1 accumulation was detected by the IP-One HTRF® assay from Cisbio Bioassays (Codolet, France). Data are normalized to WT IP_1 accumulation. Data are means ± S.D. of a single representative experiment performed in triplicates.

Table 3
L-Orn signaling parameter estimates for rGPRC6A Cys mutant receptors. IP_1 accumulation was detected by the IP-One HTRF® assay from Cisbio (Codolet, France). No statistical significant differences compared to WT were observed.

	EC_{50} (μM)	pEC_{50} ± S.E.M.	Max ± S.E.M. (%)	Max response/ surface expression
WT	90	4.04 ± 0.05	100	100
C122A	55	4.26 ± 0.09	113 ± 6.8	103
C131A	92	4.04 ± 0.11	90 ± 14	64
C122A + C131A	181	3.74 ± 0.20	43 ± 17	24

mutants were increased compared to WT (Fig. 5C and D). Our data therefore suggest that the disulfide bridge formed from C131 does not affect the ability of the receptor to reach the cell surface.

3.6. Mutation of cysteine residues on rGPRC6A affects receptor function

It is widely known that receptor oligomerization can affect activation and signaling of GPCRs. Since we have shown that C131 is important for rGPRC6A receptor dimerization, we wanted to investigate the role of this disulfide bridge in receptor signaling. Transiently transfected cells were stimulated with L-Orn as described earlier and IP_1 accumulation was measured. The maximal responses of the two single mutants, C122A and C131A, were not markedly different from wild-type (113% ± 6.8 and 90% ± 14, respectively), whereas L-Orn had a slightly increased potency at the C122A mutant (EC_{50} of 55 μM compared to 90 μM for wild-type, Fig. 5E and Table 3). The double C122A + C131A mutant had a much lower activation level with a maximum response of 43 ± 17% and a ~twofold lower potency (EC_{50} of 181 μM) compared to wild-type (Fig. 5E and Table 3).

4. Discussion

Studies of GPCRs have shown that their PTMs affect important regulatory aspects such as: protein synthesis, trafficking and signaling. In this study we have investigated the role of *N*-glycosylation and disulfide bridging in rat GPRC6A via analyses of surface expression, receptor activation and receptor size/migration on SDS–PAGE. We found that seven asparagines (N121Q, N259Q, N332Q, N378Q, N452Q, N555Q and N567Q) located in the ECD carry *N*-glycans, all of which are located in the VFT domain except N555 and N567, which are located in the CRD. Glycosylation of the NXS/T consensus site occur with an average frequency of ~67% in vivo [2], which correlates nicely with our observation of seven out of the nine proposed glycosylation sites to be modified. In order to understand why residues N86 and N733 were not modified we found that conservation of N85, but not N86 is seen between class C GPCRs. N733 is located in the second extracellular loop and probably not glycosylated because it would induce a constraint on the flexibility of the seven transmembrane (7TM) domain.

When we analyzed the different mutants by SDS–PAGE we found that N86Q and N555Q mutants had lost one of the monomeric band when compared to the remaining mutants and wild-type receptor. We speculate that the missing band corresponds to the fully glycosylated form of the receptor, whereas the remaining band corresponds to the core glycosylated form. This has previously been observed for other receptors such as the delta opioid receptor [37]. Core glycosylation takes place in the endoplasmic reticulum (ER) whereas fully glycosylated and mature proteins will have had to go through the Golgi apparatus [50]. Since mutants N86Q and N555Q cannot become fully glycosylated this could explain their lack of surface expression and functional response. We identified N555 as a direct target for *N*-glycosylation, which suggest that modification of this residue is important for surface expression and functional response. N86 that did not carry an *N*-glycan and the lack of expression may be caused by folding difficulties due to the mutation itself or via disturbance of other nearby PTMs causing a more unstable protein product.

In addition, we found that mutation of residue N567 resulted in an increased functional response. N567 is located in the end of the CRD just before the 7TM domain. It is possible that glycosylation of this residue impede the flexibility of this domain, thereby restraining signal transmission from the VFT to the 7TM domain. However N555, which is also glycosylated, did not show the same effect on activation even though it is also located in the CRD. Mutation of the remaining four glycosylated residues did not alter cell surface expression or functionality markedly.

Alignment of GPRC6A sequences from various species showed a conserved putative *N*-glycosylation pattern in most species, of note the seven detected *N*-glycosylation sites in rat are fully conserved in human, gorilla and mouse (alignment not shown). Future studies will show if these sites are also *N*-glycosylated in these species of GPRC6A.

The closest homolog of GPRC6A, the CaSR, has been shown to carry eight *N*-glycans at residues N-90, -130, -261, -287, -446, -468, -488, and -541 of which several affect surface expression whereas none of them significantly affect receptor signaling [43]. The eight sites are all located outside the CRD, which may explain why receptor function is not altered upon mutation. Interestingly, the study by Ray et al. [43] also showed that when mutating several of these residues, glycosylation of three additional putative sites could be seen, indicating that the process is highly dynamic and that glycosylation of one site might affect the glycosylation of other sites. Furthermore, introduction of glycosylation sites into the $GABA_B$ receptor has demonstrated that glycosylation can also modulate dimerization [47] and oligomerization [8]. It is therefore important to keep in mind that rGPRC6A glycosylation in vivo can differ from our findings.

It is evident that class C GPCRs are constitutive dimers at the cell surface, yet this has never been shown experimentally for GPRC6A although bands of dimeric weight have been shown on Western blots [56]. Here we show for the first time that GPRC6A is able to form dimers. The time-resolved FRET studies demonstrate that the receptor dimerizes at the cell surface of intact cells (Fig. 4B) and the Western blot studies demonstrate that the receptor dimer is covalently linked via a disulfide bridge (Fig. 5B). Conserved disulfide bridges linking the VFT domains have been shown to be involved in the dimerization of this class of receptors, however for mGluR5 and CaSR it has also been shown that additional non-covalent interactions are involved in the dimerization [46,58]. For CaSR it was even shown on Western blots under non-reducing conditions that a large fraction of the receptor was still present on the cell surface in the form of a dimer even after mutating C129 and C131 [58]. Our data clearly demonstrate that mutation of C131 results in loss of the dimeric form of the receptor under non-reducing conditions upon Western blotting. C122A did not change the dimer-monomer ratio, hence we suggest that only C131 is involved in disulfide linkage of the rGPRC6A receptor. The physiological role of the conserved cysteine residues for class C receptors remain puzzling, since the receptors still can form dimers at the cell surface and maintain functionality upon mutation [19]. Studies on solubilized CaSR have shown that agonist treatment increases the amount of dimeric receptors, suggesting a dimer-stabilizing action of the agonists [53]. The agonist treatment also protected the receptor from the action of reducing agents and suggests the presence of conformationally sensitive disulfide bridges [53]. This might lead to speculations that the

disulfide bridges help fine-tune stability and/or conformation of the receptors, however more research is needed to clarify this.

Surface and total expression of C122A and C131A mutants were not impaired in agreement with what has been seen for CaSR mutants [14,58] and the double mutant even showed higher expression. This suggests that the disulfide bridge in rGPRC6A is not required for surface expression of the receptor; rather it looks like expression is increased when the disulfide bridge is absent. Functional analysis showed that C131A had a similar pharmacological profile as WT, while L-Orn was slightly more potent on the C122A mutant compared to the WT receptor. We have previously shown, that the corresponding loop region in CaSR, where the cysteines involved in disulfide bridge formation are located (A116-P136), is very sensitive to activation inducement by mutation [21]. This may also be the case for GPRC6A and hence explain why the C122A mutant responds more potently to L-Orn stimulation. Mutating both residues lowered the response to ~40% of wild-type, indicating that the double mutant might impair proper folding of the ECD. Studies have previously shown increased ligand sensitivity for CaSR lacking the intermolecular cysteine linkers, suggesting that a disulfide bridge might constrain the receptor in the inactive form [45]. However, when mutating one of the cysteines in CaSR (C129 or C131) this did not alter the functional response level. Since the GPRC6A mutant C131A showed a similar functional profile to wild-type this indicates that disulfide linkage is not required for correct receptor signaling. Conservation of C131 was verified through all GPRC6A species except guinea pig and long-tailed chinchilla, supporting the idea that the human receptor also forms a disulfide-linked dimer on the cell surface.

In conclusion, we have demonstrated that the rat GPRC6A receptor is *N*-glycosylated at seven extracellular Asn residues, forms a disulfide-linked homodimer via C131, and that some of these residues regulate expression and functional signaling of the receptor.

Acknowledgments

This work was supported by grants from the Danish Council for Independent Research|Medical Sciences and the Lundbeck Foundation. M.Sc. Stine Engesgaard Jacobsen is greatly acknowledged for generating the HA-rGPRC6A construct, Dr. Damien Maurel is acknowledged for fruitful discussions on the dimerization HTRF® assay setup, and M.Sc. Guillaume Blanc is acknowledged for discussions on Western blot assay setup.

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