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Distinct Regions in the C-Terminus Required for GLP-1R Cell Surface Expression, Activity and Internalisation

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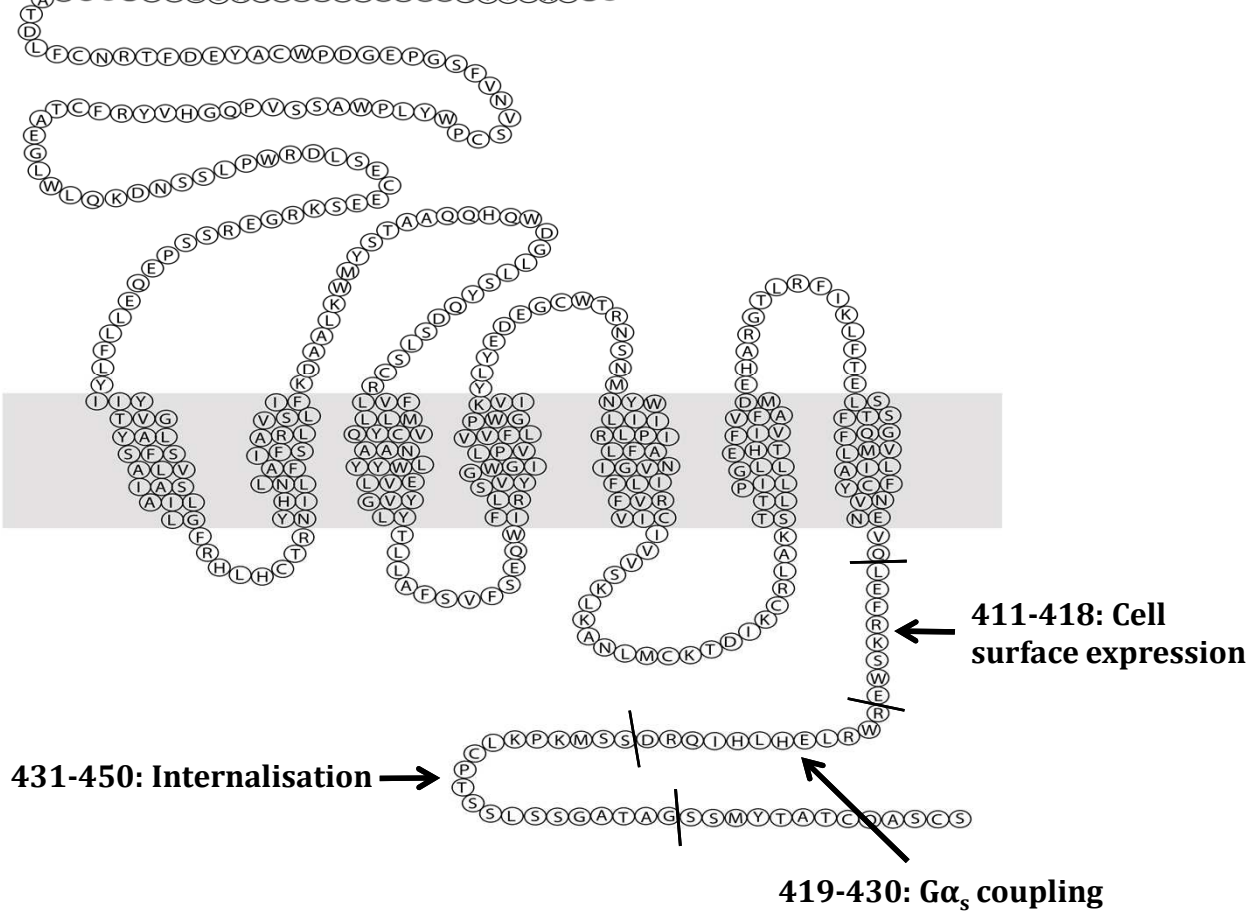
411-418: Cell surface expression
431-450: Internalisation
419-430: Gα$_s$ coupling

Expression, Activity and Internalisation

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Abbreviated title: Functional analysis of GLP-1R C-terminus

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N-terminus controls GLP-1R biosynthetic trafficking to the cell surface but the C-terminus involvement in that trafficking is unknown. The aim of this study was to identify distinct regions within the C-terminal domain required for human GLP-1R (hGLP-1R) cell surface expression, activity and internalisation using a number of C-terminal deletions and site-directed mutations. The results of this study revealed that the residues 411-418 within the C-terminal domain of the hGLP-1R are critical in targeting the newly synthesised receptor to the plasma membrane. The residues 419-430 are important for cAMP producing activity of the receptor, most likely by coupling to $G\alpha_s$. However, the residues 431-450 within the C-terminus are essential for agonist-induced hGLP-1R internalisation. In conclusion, these findings demonstrate the hGLP-1R has distinct regions within the C-terminal domain required for its cell surface expression, activity and agonist-induced internalisation.

Keywords:

Glucagon like peptide-1 (GLP-1); GLP-1 receptor (GLP-1R); G protein coupled receptor (GPCR); biosynthetic trafficking; C-terminus; diabetes.

ARF1, ADP-ribosylation factor 1; AT_2R, angiotensin II receptor; BSA, bovine serum albumin; Ca^{2+}, calcium; $CaCl_2$, calcium chloride; cAMP, cyclic adenosine monophosphate; D1R, dopamine D1 receptor; DABCO, 1,4-diazabicyclo[2.2.2.]octane; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, dulbecco's modified eagle medium; DTT, dithiothreitol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FL, full length; FSM, full serum medium; GFP, green fluorescent protein; GLP-1, glucagon like peptide-1; GLP-1R, GLP-1 receptor; GPCR, G-protein coupled receptors; GRK, GPCR kinase; HCl, hydrochloric acid; HEK293, human embryonic kidney 293; hGLP-1R, human GLP-1R; HRP, horseradish peroxidise; IgG, immunoglobulin G; LL, dileucine; M_3R, M_3 muscarinic receptor; MGC, mammalian gene collection; $MgCl_2$, magnesium chloride; mGluR, metabotropic glutamate receptor; NP40, nonidet P40; NaCl, sodium chloride; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; PLC, phospholipase C; PLD, phospholipase D; PMSF, phenylmethanesulfonylfluoride; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulphate; SFM, serum free medium; TBS, tris buffered saline; TBS-T, TBS-0.1% tween20; TM, transmembrane; VSVG, vesicular stomatitis virus glycoprotein; WT, wild type

2 diabetes [1, 2]. GLP-1 in vivo half-life is very short (~1.5min) due to its rapid proteolytic degradation by dipeptidyl peptidase-IV (DPP-IV) and therefore long acting GLP-1 analogues such as liraglutide, which have resistance to DPP-IV proteolysis, are developed for the treatment [3]. The GLP-1R, which is a member of the family B G-protein coupled receptors (GPCRs), functions at the cell surface by coupling to $G\alpha_s$ and $G\alpha_q$ pathways and causing extracellular signal-regulated kinase (ERK) phosphorylation [2, 4]. Agonist-induced GLP-1R internalisation plays an important role in glucose induced insulin secretion [5]. The GLP-1R internalises with similar kinetics in response to GLP-1 and liraglutide stimulation [6]. However, the rate of GLP-1R recycling in cells treated with liraglutide is slower than that in cells treated with GLP-1 [6]. Inhibition of the $G\alpha_q$ pathway, but not the $G\alpha_s$ pathway, has recently been shown to significantly reduce agonist-induced GLP-1R internalisation [7]. In the same study, chemical inhibitors of the $G\alpha_q$ pathway also shown to suppress GLP-1-induced ERK phosphorylation, indicating that ERK phosphorylation acts downstream of the $G\alpha_q$ pathway in GLP-1R internalisation [7].

The extracellular N-terminal domain in many GPCRs is important for their biosynthetic trafficking and maturation [2, 8]. Like other family B GPCRs, the GLP-1R contains an N-terminal signal peptide (SP) and undergoes N-linked glycosylation, which are important for the newly synthesised receptor cell surface expression [9-11]. We have recently demonstrated the importance of SP cleavage, N-linked glycosylation and the hydrophobic region after the SP (HRASP) within the N-terminus of the GLP-1R for its biosynthetic trafficking to the plasma membrane [12]. The intracellular C-terminal domain of GPCRs plays a critical role in agonist-induced internalisation, desensitisation, down regulation and arrestin signalling of the receptors [13]. Furthermore, the C-terminal region is required for some GPCRs trafficking from the endoplasmic reticulum (ER) to the plasma membrane [8]. The C-terminal domain of GPCRs is also known to interact with several intracellular proteins involved in the internalisation of the receptor to intracellular signalling pathways [2, 8].

transmembrane (TM) 7 that associates with a number of intracellular proteins [14]. Additionally, many GPCRs possess a PDZ binding site at the very end of the C-terminal domain, which interacts with the PDZ domain of proteins required for biosynthetic trafficking of the receptor [2, 15].

Agonist-induced GPCR internalisation is predominantly mediated by GPCR kinases (GRKs), arrestins and clathrin coated pits. GRKs phosphorylate agonist-activated GPCRs to facilitate the recruitment of arrestins, which target the GPCRs to clathrin-coated pits for rapid internalisation [16]. In addition to its role in trafficking of newly synthesised GPCRs, the C-terminal domain can interact with intracellular proteins involved in the internalisation of the receptor [14]. The tyrosine motif (YXXΦ) within the C-terminus has been shown to associate with clathrin [17]. The β_3-adrenoceptor (AR) contains a PXXP motif within the C-terminal domain that interacts with Src, which results in the activation of ERK [18]. Further, a NPXXY motif within TM7 close to the C-terminal domain of the serotonin 5-hydroxytryptamine 2a ($5\text{-}HT_{2a}$) receptor interacts with ADP-ribosylation factor 1 (ARF1) small GTPase and couples to phospholipase D (PLD) in a heterotrimeric G-protein-independent manner [19].

A dileucine (LL) motif within the C-terminal domain of some GPCRs has been shown to promote some GPCRs internalisation by binding to adapter proteins [20]. GPCRs, such as the neurokinin 1, β_2-AR and the angiotensin II receptor (AT_2R), require the conserved aromatic residue tyrosine in the C-terminal domain for their internalisation [21-23]. A mutation of Ser^{344} within the C-terminal domain of the δ-opioid receptor (DOR) prevents protein kinase C (PKC) dependent phosphorylation required for internalisation of the receptor [24]. The GLP-1R contains three serine doublets at positions $Ser^{441,442}$, $Ser^{444,445}$ and $Ser^{451,452}$ and their phosphorylation is also important for internalisation of the receptor [25]. Further, phosphorylation of some serine doublets within the C-terminal domain of the GLP-1R is mediated by PKC [26].Moreover, the mutation of Glu^{408}, Val^{409}, Gln^{410}, which are conserved among family B GPCRs, in the C-terminus of GLP-1R has been shown to affect GLP-1R agonist binding and activity [27].

present within the C-terminus of GLP-1R. Therefore, the aim of this study was to establish the importance of residues and regions within the C-terminal domain of the human GLP-1R (hGLP-1R) for the receptor biosynthetic trafficking to cell surface, activity and internalisation using a number of C-terminal deletion and site-directed mutants. It was determined that residues 411-418 of the hGLP-1R in the C-terminus are critical in targeting the receptor to the plasma membrane. Residues 419-430 within the C-terminal domain are important for the activity of the receptor (as assessed by cAMP production), most likely for coupling to $G\alpha_s$. Further, residues 431-450 within the C-terminus are essential for agonist-induced hGLP-1R internalisation.

2. Materials and methods

2. 1. Materials

The primary antibodies used were rabbit anti-vesicular stomatitis virus glycoprotein (VSVG) (ab34774, Abcam Biochemicals), mouse anti-green fluorescent protein (GFP) (11814460001, Roche), mouse anti-hGLP-1R (MAB2814, R&D Systems), mouse anti-hGLP-1R (sc-390773, Santa Cruz), rabbit anti-phospho ERK1/2 (pERK1/2) (9102, New England Biolabs) and rabbit anti-ERK1/2 (9102, New England Biolabs). The Cy3-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody (715-165-150, Jackson Laboratories) was used for immunofluorescence. The horseradish peroxidase (HRP)-conjugated anti-mouse (NA933) and anti-rabbit (NA934) IgG (GE Healthcare) secondary antibodies were used for immunoblotting. Enhanced chemiluminescence (ECL) select reagent was obtained from GE Healthcare. Liraglutide (GLP-1 analogue) was from Novo Nordisk. All other chemicals were from Sigma unless otherwise stated.

as described previously [7] for expression as the N-terminus VSVG-tagged (after the SP) and the C-terminus GFP-tagged fusion protein in mammalian cells (SP-VSVG-hGLP-1RΔN23-GFP). The SP-VSVG-hGLP-1RΔN23 with no GFP-tag and its C-terminal deletion constructs were generated by PCR using sequence specific primers containing *Eco*RI restriction site (5' primer), *Sal*I restriction site and stop codon (3' primer) and SP-VSVG-hGLP-1RΔN23-GFP plasmid as the template, and cloning into the same sites of pEGFP-N1. The E408A,V409A,Q410A mutation within the hGLP-1R was generated using Quickchange II XL site-directed mutagenesis kit (Stratagene) and SP-VSVG-hGLP-1RΔN23-GFP plasmid as the template [28]. The mutants with internal deletions (Δ) within the C-terminus of hGLP-1R were generated using Q5 site directed mutagenesis kit (New England Biolabs) and SP-VSVG-hGLP-1RΔN23-GFP plasmid as the template. The mutations, deletions and right reading frames were confirmed by DNA sequencing of the constructs.

2. 2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained at 37^{o}C in a 5% CO_2 humidified environment in Dulbecco's modified Eagle medium (DMEM; serum free medium [SFM]) supplemented with 10% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (full serum medium [FSM]). Cells were transiently transfected for 48 h using JetPrime transfection reagent (Polyplus; 2 µl/µg DNA) according to the manufacturer's instructions.

2. 3. Enzyme linked immunosorbent assay (ELISA)

This is carried out as described previously with unpermeabilised cells to quantify cell surface expression [7]. Briefly, HEK293 cells expressing the hGLP-1R were serum starved for 1 h and then stimulated without or with 100 nM liraglutide for 1 h at 37^{o}C/5% CO_2. Cells were then fixed with 4% paraformaldehyde (PFA) for 5 min and non-specific binding sites blocked with 1% bovine serum albumin (BSA) made in Tris buffered saline (TBS) (1% BSA/TBS) for 45 min. Cells were incubated

stopped by adding an equal volume of 2 M sulphuric acid. The optical density was read at 450 nm using a plate reader.

2. 4. Immunofluorescence

Intracellular localisation of hGLP-1R expression was assessed by immunofluorescence as described previously [7]. Briefly, cells were serum starved for 1 h, incubated with the anti-hGLP-1R mouse antibody (diluted 1:5000) in 1% BSA/SFM for 1 h at 4^{o}C and then stimulated without or with 100 nM liraglutide for 1 h at 37^{o}C/5% CO_2. Cells were then fixed with 4% PFA for 30 min. Cells were permeabilised with 0.2% Triton X-100 made in phosphate buffered saline (PBS) for 10 min, blocked in blocking buffer (1% BSA made in wash buffer [0.1% Triton X-100 in PBS]) for 30 min and then incubated with the Cy3-conjugated anti-mouse antibody (diluted 1:200 in blocking buffer) for 1 h. Cells were then washed 3 times with wash buffer and incubated with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, 1 mg/ml) diluted 1:2000 in PBS to stain nucleus. Coverslips were mounted on glass microscopic slides using mounting solution (0.1 M Tris-HCl, pH 8.5, 10% Mowiol 50% glycerol) containing 2.5% DABCO (1,4 diazabicyclo (2.2.2) octane). Immunofluorescence staining was visualised using a Zeiss LSM710 confocal microscope fitted with a 63x oil immersion lens.

2. 5. cAMP assay

Cells grown in 24-well plates were serum starved for 1 h and then stimulated without or with 100 nM liraglutide for 1 h at 37^{o}C/5% CO_2 in the presence of 0.25 mM phosphodiesterase inhibitor Ro201724. Cells were lysed and cAMP levels in the cell lysates were estimated using the cAMP direct immunoassay kit (Abcam) [7].

ethylenediaminetetraacetic acid [EDTA], 1% nonyl phenoxypolyethoxylethanol [NP40], 0.1% sodium dodecyl sulphate [SDS], 0.5% sodium deoxycholate and 150 mM NaCl) with 1% mammalian protease inhibitors. The cell lysate was mixed with 50% 3x SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer (75 mM Tris-HCl, pH 6.8 containing 3% SDS, 30% glycerol, 0.003% bromophenol blue and 0.3 M dithiothreitol [DTT]) and incubated at room temperature for 1 h. These cell lysates used to detect hGLP-1R expression by immunoblotting using the anti-GFP and anti-VSVG antibodies.

For assessing ERK1/2 phosphorylation, HEK293 cells expressing the hGLP-1R were serum starved for 1 h and then stimulated without or with 100 nM liraglutide for 5 min at 37^{o}C/5% CO_2. Cells were then lysed in ice cold modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 10 mM $MgCl_2$, 0.1% SDS; 0.5% sodium deoxycholate, 1% TritonX-100 and 5% glycerol) with 1% mammalian protease inhibitors. The cell lysate was mixed with 25% 5x SDS-PAGE sample loading buffer (125 mM Tris-HCl, pH 6.8 containing 5% SDS, 50% glycerol, 0.005% bromophenol blue and 5% β-mercaptoethanol) and heated at 100^{o}C for 5 min. These cell lysates were used to detect phosphorylated ERK and total ERK by immunoblotting using the anti-pERK1/2 and anti-ERK1/2 antibodies.

2. 7. Immunoblotting

This was carried out as described previously [29, 30]. Briefly, proteins were separated in a SDS-PAGE gel by electrophoresis and transferred onto polyvinylidene fluoride (PDVF) membrane. Membranes were blocked with TBST (TBS with 0.1% tween 20) containing 5% milk powder (blocking buffer) for 1 h at room temperature. Membranes were immunoblotted with either the anti-GLP-1R mouse antibody (diluted 1:500 in blocking buffer) or anti-GFP mouse antibody (diluted 1:500 in blocking buffer) to assess protein expression levels or the anti-pERK1/2 rabbit antibody (diluted 1:1000 in blocking buffer) to assess ERK1/2 phosphorylation for overnight at 4^{o}C. Membranes were

probed with the anti-hGLP-1R mouse antibody or anti-GFP mouse antibody were stripped by using Western blot stripping buffer (Thermo Scientific) and re-probed with the anti-VSVG rabbit antibody (diluted 1:1000 in blocking buffer) to assess protein expression levels. Blots probed with the anti-pERK1/2 rabbit antibody were stripped and re-probed with the anti-ERK1/2 rabbit antibody (diluted 1:1000 in blocking buffer) to assess total ERK1/2. The HRP-conjugated anti-rabbit secondary antibody (diluted 1:2500 in blocking buffer) was used as described above.

2. 8. Tunicamycin treatment

This was carried out as described previously [7, 11] Briefly, cells were treated with 5 µg/ml tunicamycin at the time of transfection. After 48 h of transfection, cells were lysed and subjected to immunoblotting.

2. 9. Data analysis

Data were analysed using the GraphPad Prism program**.** All data are presented as means ± standard error of the mean (SEM) of three independent experiments. Statistical analysis between multiple groups were determined by the Bonferroni post-test after one-way analysis of variance (ANOVA), where $p>0.05$ was considered as statistically not significant (n.s.). Statistical analysis between multiple groups normalised to a control group was analysed by the Dunnett's post-test after one-way ANOVA. Confocal images shown in the figures are representative of 190-200 transfected cells from three different experiments. Similarly, immunoblotting data shown in the figures are representative of three independent experiments.

The importance of the C-terminus for hGLP-1R cell surface expression was determined using a number of C-terminal deletion mutants, which contain the VSVG-epitope tag at the N-terminus after the SP (Figure 1). However, three deletion mutations, Δ411-418, Δ419-430 and Δ431-450, and a site-directed mutant (E408A,V409A,Q410A), which has previously been shown to affect agonist binding to the hGLP-1R [27], contain an additional GFP-tag at the C-terminus. We have shown previously that the cAMP producing activity of the SP-VSVG-GFP construct (which contains both VSVG and GFP tags) is similar to that of the hGLP-1R with no tag or either of the VSVG-tag or GFP-tag, indicating that the attachment of the VSVG and GFP tags to the hGLP-1R had no effect on the activity of the receptor [12].

Lysates of HEK293 cells expressing some of the C-terminal mutants of hGLP-1R showed a doublet of same intensity in immunoblotting (~55 kDa and ~35 kDa in size) with both the anti-hGLP-1R and anti-VSVG antibodies, demonstrating no alterations in hGLP1-R protein expression by these mutations. The high molecular weight band in the doublet has previously been shown as the mature form of the receptor whereas the low molecular weight band represents the immature form of the receptor [12, 31]. However, the N410 construct only showed a single band, which corresponds to the lower molecular weight band of the doublet, with both the antibodies, demonstrating its existing mainly as the immature form of the receptor (Figure 2). It is important to note that immunoblotting with the anti-VSVG antibody produces a non-specific band at ~35 kDa and the anti-hGLP-1R antibody produces a non-specific band at ~55 kDa, which are also present in lanes loaded with the lysate of untransfected HEK293 cells (Figure 2). HEK293 cells do not express GLP-1R [32, 33] and therefore ~55kDa produced by the anti-hGLP-1R antibody in untransfected HEK293 cells lysates loaded lanes was considered as a non-specific band. The lower molecular weight (~35 kDa) band of SP-VSVG (full length [FL]) produced by the anti-VSVG antibody co-migrated with the non-specific band. Additionally, the GFP-tagged wild type (SP-VSVG-GFP), Δ419-430 and Δ431-450 constructs all showed a doublet (~70 kDa and ~85 kDa in size) when the lysates of HEK293 cells transfected

immature form of the receptor (Figure 2).

To determine the effect of the C-terminal deletion and site-directed mutations on hGLP-1R cell surface expression, these mutants were expressed in HEK293 cells and analysed for their cell surface expression by ELISA (Figure 3A) and immunofluorescence (Figure 3B). The FL (463aa) and the N450, N443, N440 and N430 deletion mutants showed identical cell surface expression when assessed by ELISA (100%, 96.6 ± 2.2%, 97.1 ± 1.7%, 93.5 ± 3.7%, 97.0 ± 1.5%, $p>0.05$, respectively, Figure 3A). However, the mutant with the entire C-terminal domain deletion (N410) showed no cell surface expression (0.1 ± 0.1%, $p<0.001$), demonstrating that the C-terminal domain is required for hGLP-1R biosynthetic trafficking to the cell surface. These results also demonstrate that the last 33aa within the C-terminus are not required for cell surface expression of the hGLP-1R. Therefore, these results suggest that the residues 411-430 are most likely involved in the receptor's cell surface expression. Next, the effect of internal deletions made within (Δ411-418, Δ419-430) and outside (Δ431-450) of the 411-430 residues were used to assess the exact region within the C-terminus that is required for targeting newly synthesised the hGLP-1R to cell surface. The Δ411-418 deletion abolished hGLP-1R cell surface expression (0.7 ± 0.7%, $p<0.001$). However, the Δ419-430 and Δ431-450 mutants cell surface expressions were similar to that of the SP-VSVG-GFP WT control (81.6 ± 6.1% and 97.9 ± 3.7%, $p>0.05$, respectively, of the control). These results demonstrate that the 411-418 region of the hGLP-1R is critical for cell surface expression of the receptor. The E408A,V409A,Q410A mutation, which has previously been shown to affect GLP-1R agonist binding and activity [27], also largely abolished hGLP-1R cell surface expression (9.7 ± 9.7%, $p<0.001$) when assessed by ELISA. The ELISA results were also confirmed by immunofluorescence (Figure 3B) where cell surface expression was seen for the N450, N443, N440, N430, Δ419-430 and Δ431-450 deletion mutants, which was assessed by cell surface staining of the receptor with the anti-hGLP-1R antibody. However, N410, Δ411-418 and E408A,V409A,Q410A mutants showed no cell surface expression (Figure 3B). The reduction in cell surface expression of these mutants was not due to

3. 2. The C-terminal domain mutations have no effect on hGLP-1R N-glycosylation

The hGLP-1R that is targeted to the cell surface (mature hGLP-1R) is *N*-glycosylated [9-11]. The hGLP-1R C-terminal deletion or site-directed mutants were assessed for their *N*-linked glycosylation to establish whether these mutations affect hGLP-1R cell surface expression by altering its N-glycosylation. For this purpose, cells expressing the C-terminal deletion mutants or E408A,V409A,Q410A mutant were treated without or with tunicamycin, an *N*-linked glycosylation inhibitor, and analysed their expression (band pattern) by immunoblotting using either the anti-VSVG or anti-GFP antibodies. Immunoblotting of the lysates of HEK293 cells expressing VSVG-SP (FL) showed the characteristic doublet at ~55 kDa and ~35 kDa (Figure 4). Treatment with tunicamycin, altered this pattern and instead a single band at ~27 kDa was seen. This shift is used as readout to assess hGLP-1R *N*-linked glycosylation. The C-terminal deletion mutants (N450, N443, N440 and N430) of the hGLP-1R that express at the cell surface also showed a shift in the band pattern when treated with tunicamycin, demonstrating that these deletions are also glycosylated like the FL. However, the N410 deletion mutant, which do not express at cell surface, showed a single band at ~35 kDa in immunoblotting and a shift in that band mobility when treated with tunicamycin. This suggested that the mutant is N-glycosylated to some extent and therefore loss of this mutant cell surface expression may not be due to absence of *N*-linked glycosylation.

The SP-VSVG-GFP WT showed a doublet (~70 kDa and ~85 kDa) in immunoblotting (Figure 4). Treatment with tunicamycin altered this band pattern, two bands at ~60 kDa and ~65 kDa were observed. Like the WT, the Δ419-430 and Δ431-450 deletion constructs showed the double band pattern that shifted to ~60 kDa and ~65 kDa when treated with tunicamycin, demonstrating that the deletions have no effect on *N*-linked glycosylation. The E408A,V409A,Q410A and Δ411-418 mutants that did not target to the cell surface only showed a single band at ~70 kDa and a shift in that band mobility when treated with tunicamycin, indicating that these mutants are still *N*-glycosylated to some extent. Taken together, these results demonstrate that all hGLP-1R C-terminal mutants are *N*-

3. 3. Effect of the C-terminal mutations on the hGLP-1R activity

The GLP-1R is a $G\alpha_s$ coupled GPCR and therefore the effect of C-terminal mutations on the receptor activity was assessed by measuring cAMP produced in cells expressing these mutants stimulated without and with 100 nM liraglutide (Figure 5). All constructs expressing cells showed basal cAMP levels between 0.4 ± 0.04 and 0.6 ± 0.1 pmol/well ($p>0.05$), indicating that the mutants expression has no effect on basal cAMP levels. Deleting up to 33aa from the end of the C-terminal domain (WT, N450, N443, N440 and N430) of the hGLP-1R had no significant effect on agonist-induced maximal cAMP production (3.4 ± 0.2, 3.8 ± 0.4, 3.3 ± 0.4, 3.3 ± 0.04 and 3.3 ± 0.4 pmol/well, $p>0.05$, respectively). However, deleting the entire C-terminal domain (residues 411-463 [N410 construct]) prevents the receptor expression at the cell surface and therefore the cells expressing this mutant showed no agonist-induced cAMP production 0.4 ± 0.1 pmol/well, $p<0.001$). We then assessed the effect of internal deletions made in the region of 411-430 (Δ411-418 and Δ419-430) on agonist-stimulated cAMP production. The hGLP-1R deletion Δ411-418 does not express at the cell surface and therefore as expected no agonist stimulated cAMP production was observed in cells expressing this mutant (0.6 ± 0.1 pmol/well, $p<0.001$). However, the 419-430 deletion within the C-terminal domain of the hGLP-1R had also almost completely abolished cAMP production (0.8 ± 0.06 pmol/well, $p<0.001$) even though this deletion still allowed targeting of hGLP-1R to the cell surface. Further, the Δ431-450 mutant showed maximal cAMP production (3.5 ± 0.5 pmol/well, $p>0.05$) similar to that of the SP-VSVG-GFP WT control (3.5 $\underline{+}$ 0.3 pmol/well). Additionally, the E408A,V409A,Q410A mutant of the hGLP-1R showed very low cAMP production (0.6 ± 0.2 pmol/well, $p<0.001$), which is expected as this mutant does not target to the cell surface. Taken together, these results indicate that the mutants of hGLP-1R with negligible cell surface expression show very low levels of ligand induced cAMP but those levels are similar to that of wild-type when normalised to cell surface expression of the mutants. This suggests that ligand-induced maximal

3. 4. Effect of the C-terminal mutations on agonist-induced hGLP-1R internalisation and ERK1/2 phosphorylation

The C-terminal deleted and site-directed mutants of the hGLP-1R that show cell surface expression were assessed for their effect on agonist-induced hGLP-1R internalisation by ELISA (Figure 6A) and immunofluorescence (Figure 6B). Deleting 13aa (N450) from the end of the C-terminal domain had no effect on agonist-induced internalisation (100.0 ± 1.0%, $p>0.05$). However, the N443, N440 and N430 mutants all showed a significant reduction in agonist-induced internalisation compared to the FL (79.5 ± 4.7% [$p<0.01$], 57.1 ± 2.4% and 31.5 ± 5.8% [$p<0.001$], respectively). This demonstrated that residues 430-450 are most likely to be involved in hGLP-1R internalisation. This was confirmed by using the hGLP-1R internal deletion mutants, Δ419-430 and Δ431-450. The Δ431-450 deletion significantly reduced agonist-induced internalisation of the hGLP-1R, as only 22.9 ± 5.3% ($p<0.001$) of the receptor expressed at the cell surface was internalised. However, the Δ419-430 deletion had no effect on agonist-induced hGLP-1R internalisation (111.9 ± 7.1%, $p>0.05$). These results were confirmed by immunofluorescence (Figure 6B).

Upon activation by agonist binding, the GLP-1R is known to cause ERK1/2 phosphorylation [34]. We have recently shown that the agonist-induced hGLP-1R internalisation is mediated by the $G\alpha_q$ pathway and the phosphorylated ERK acts downstream of the $G\alpha_q$ pathway in hGLP-1R internalisation [7]. Therefore, the C-terminal deletion mutants were assessed for their effect on agonist-induced ERK1/2 phosphorylation (Figure 7A-B). The N410, Δ411-418 and E408A,V409A,Q410A mutants, which show no cell surface expression, did not induce ERK1/2 phosphorylation (4.0 ± 1.5%, 6.7 ± 2.7% and 10.3 ± 0.6%, $p<0.001$, respectively). The hGLP-1R C-terminal deletion mutants, N450, N443, N440 and N430, ERK1/2 phosphorylation inducing ability correlated with their agonist-induced internalisation. Deletion of 13aa (N450) from the C-terminus of hGLP-1R had no effect on agonist-induced ERK1/2 phosphorylation (103.6 ± 2.4%). However, the hGLP-1R mediated ERK1/2 phosphorylation reduced with increasing deletion from the C-terminus of

internalisation of the receptor, deletion of these residues (Δ431-450) also reduced ERK1/2 phosphorylation mediated by the hGLP-1R (20.7 ± 3.1% [$p<0.001$]). Taken together, these results demonstrate that residues 431-450 are essential for hGLP-1R internalisation and ERK1/2 phosphorylation.

4. Discussion

The C-terminal domain of many GPCRs plays a critical role in the receptor trafficking, agonist-induced internalisation, desensitisation, down regulation and arrestin signalling [13]. In this study, several deletion and site-directed mutants of the hGLP-1R were generated to identify the distinct regions within the C-terminal domain required for hGLP-1R biosynthetic trafficking, its $G\alpha_s$ coupling (cAMP producing activity) and internalisation. Additionally, an E408A,V409A,Q410A mutant was generated and assessed for its effect on hGLP-1R cell surface expression, as this mutation had previously been shown to inhibit cAMP production of the hGLP-1R [27].

The expression of GPCRs at the cell surface is essential for their functional response. Reduced cell surface expression of the GLP-1R in pancreatic β-cells has been shown to contribute to the impaired incretin effects in type 2 diabetes [35, 36]. Therefore, the mechanisms underlying newly synthesised GPCR targeting to the cell surface is of high importance. GPCRs are synthesised in the ER and transported to the Golgi before being trafficked to the plasma membrane, which is tightly regulated [8]. Some GPCRs require specific motifs within the C-terminal domain to target to endosomes, the Golgi and plasma membrane, but this specificity is not clear for many GPCRs [14, 37-40]. Using a number of C-terminal deletion mutants of the hGLP-1R, this study determined residues 411-418 are critical for newly synthesised hGLP-1R cell surface expression. The membrane proximal region of the C-terminal domain is important for the biosynthetic trafficking of many GPCRs such as α_{2B}-AR, AT_2R type 1A and dopamine receptor 1 (D1R) to the plasma membrane [41]. The membrane

[9-11]. However, the hGLP-1R with residues 411-418 deletion is still N-glycosylated to some extent but not targeted to the cell surface. It is therefore possible that this deletion prevents biosynthetic trafficking of the glycosylated hGLP-1R to the plasma membrane but the mechanism underlying this requires further investigation. Residues 411-418 of hGLP-1R form part of the helix-8 region, which has been demonstrated to stabilise the receptor at the cell surface and associates with a number of intracellular proteins [14]. Therefore it is possible that the deletion of residues 411-418 of hGLP-1R may affect stability of the receptor. It is also possible that this mutation may affect trafficking of the hGLP-1R from the Golgi/ER to the plasma membrane or interfere with further processing within ER and/or the Golgi. In this study, the E408A,V409A,Q410A mutation within TM7 (closest to the C-terminal domain) of the GLP-1R has been shown to affect cell surface expression of the receptor. This triple mutation has previously been shown to reduce dramatically agonist binding and cAMP production [27]. Consisting with the previous study, our study also demonstrated that the triple mutant does not induce cAMP production because its cell surface expression is drastically reduced. Glu^{408}, Val^{409}, Gln^{410} in hGLP-1R are adjacent to the membrane proximal region of the C-terminus and form part of the putative helix-8 region. Mutating these residues may therefore cause a conformational change within the C-terminus and thereby reduce the receptor's localisation at the cell surface and/or reduce access to residues 411-418, which are required for biosynthetic targeting of the receptor to the plasma membrane.

The C-terminal domain of GPCRs is also known to interact with intracellular proteins to activate intracellular signalling pathways [20, 43]. The C-terminal domain of the β_3-AR interacts with Src, which results in the activation of ERK [18]. Additionally, the βγ subunit of the heterotrimeric G-protein and Ca^{2+}/calmodulin bind to the C-terminal domain of the metabotropic glutamate receptor (mGluR) 7a and 7b and regulate P and Q type Ca^{2+} channels [44]. In this study, the deletion of residues 419-430 of the hGLP-1R has shown to impair agonist-induced cAMP production, suggesting that this region is directly or indirectly involved in coupling the receptor to $G\alpha_s$. It is also possible that

differences would not be identified with the saturating agonist concentration used in this study. However, a previous study suggested that deleting residues 419-435 of the hGLP-1R decreases the agonist-induced cAMP production [27]. Taken together, the results of our study and a previous study [27] indicate that 419-430 residues within the C-terminus of GLP-1R are important for cAMP stimulation. Like the GLP-1R, a mutant of mGluR1α lacking the C-terminus has been shown to be defective in stimulating cAMP production through the $G\alpha_s$ pathway [45]. The intracellular loop three (IC_3) of the GLP-1R has also been shown to be important for the $G\alpha_s$ coupling [46]. Therefore it is possible that, in addition to the IC_3, the residues 419-430 within the C-terminus are critical for $G\alpha_s$ coupling of the GLP-1R.

The internalisation of GPCRs from the cell surface after agonist stimulation is required to dampen the biological response, to recycle and resensitise the receptor through dephosphorylation, or to propagate signals through novel signalling pathways [47]. Upon agonist stimulation, the internalised GLP-1R has been shown to co-localise with adenylyl cyclase (AC) on endosomes and stimulate insulin secretion from pancreatic β-cells demonstrating the importance of hGLP-1R internalisation for insulin secretion [5]. Therefore, agonist-induced internalisation of the hGLP-1R into intracellular compartments of the cell is important for regulation of the receptor's activity [48]. The phosphorylation of serine/threonine residues within the C-terminal domain is critical for the internalisation and desensitisation of many GPCRs [25, 49, 50]. The hGLP-1R has previously been shown to require four serine phosphorylation sites. $Ser^{431,432}$, $Ser^{441,442}$, $Ser^{444,445}$ and $Ser^{451,452}$, within the C-terminus for internalisation and desensitisation of the receptor [25, 26, 51]. Here, a series of C-terminal deletion mutants were used to identify the distinct region(s) within the C-terminus required for hGLP-1R internalisation. This study showed that the region between 431-450, which contains serine doublets $Ser^{431,432}$, $Ser^{441,442}$ and $Ser^{444,445}$, of the hGLP-1R is required for internalisation of the receptor. This is consistent with a previous report, which demonstrated the mutation of the serine doublet, $Ser^{451,452}$, had little effect on hGLP-1R internalisation [25]. Additionally, a separate study

previous studies ruled out the requirement of β-arrestin-1 for hGLP-1R internalisation [7, 51]. However, the involvement of the phosphorylation of serine doublets $Ser^{441,442}$ and $Ser^{444,445}$ in GLP-1R internalisation need to be confirmed by using phosphomimetic and alanine mutations of the serine doublets. The bradykinin B2 receptor with alanine mutations to serine/threonine residues within the C-terminal domain has been shown to be deficient in arrestin binding and internalisation of the receptor [53]. Further, phosphorylation of Ser^{355}, Ser^{356} and Ser^{366} within the C-terminus domain of the β_2-AR by GRK2 has been shown to be required for internalisation of the receptor [54]. In this study, GLP-1-induced ERK1/2 phosphorylation was also used as readout for hGLP-1R internalisation. This is because the ERK1/2 phosphorylation, downstream of the $G\alpha_q$ pathway, has recently been shown to be important for agonist-induced hGLP-1R internalisation [7]. Interestingly, residues 419-430 of the hGLP-1R were important for stimulation of cAMP production with no negative effect on its internalisation, which supports the our recent observations that the GLP-1R does not require cAMP production for internalisation of the receptor [7].

In this study, cAMP production and receptor internalisation of the hGLP-1R was investigated in response to liraglutide, a long-acting GLP-1 analogue currently being used for treatment of type 2 diabetes [3]. The results from this study confirm that liraglutide and GLP-1 act in a similar manner in coupling the GLP-1R to cAMP production and internalisation. The importance of residues 419-430 of hGLP-1R for liraglutide induced cAMP production was established in this study, which is similar to a previous finding that shown the importance of residues 419-435 of GLP-1R for GLP-1 stimulated cAMP production [27]. Further, $Ser^{441,442}$ and $Ser^{444,445}$ doublets within the C-terminal domain of the GLP-1R have been shown to be important for GLP-1 induced receptor internalisation [25, 26, 51]. In this study, residues 431-450 of GLP-1R, which include these doublets, are shown to be required for liraglutide induced internalisation. Overall, this study identified distinct regions within the C-terminal domain of the hGLP-1R that are critical for cell surface expression (411-418), cAMP production (419-430) and agonist-induced internalisation (431-450) of the receptor

Acknowledgements

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of the C-terminal domain on its cell surface expression, internalisation and activity. Schematic view of the hGLP-1R C-terminal domain deletion and site-directed mutants used in this study.

Figure 2. Effect of the C-terminal domain mutations on hGLP-1R protein expression. Expression of the C-terminal deletion and site-directed mutants of hGLP-1R was assessed by immunoblotting. Lysates of HEK293 cells expressing the C-terminal deleted and site-directed mutants were immunoblotted using the anti-VSVG, anti-GLP-1R and anti-GFP antibodies (*denotes the non-specific band).

Figure 3. Effect of the C-terminal domain mutations on cell surface expression of hGLP-1R. Cell surface expression of hGLP-1R C-terminal domain mutants in HEK293 cells was assessed by ELISA (A) and immunofluorescence (B) using the anti-hGLP-1R antibody. In immunofluorescence, the anti-hGLP-1R antibody staining is shown in red or GFP-tag of the constructs (green) and the anti-hGLP-1R antibody staining (red) overlay is shown in yellow, and nuclear staining with DAPI in blue (scale bars in immunofluorescence images represent 10 μM). Data are mean ± SEM, n=3. Data were analysed by the Dunnett's post-test after one-way ANOVA; values differ from control, n.s. $p>0.05$; ** $p<0.01$.

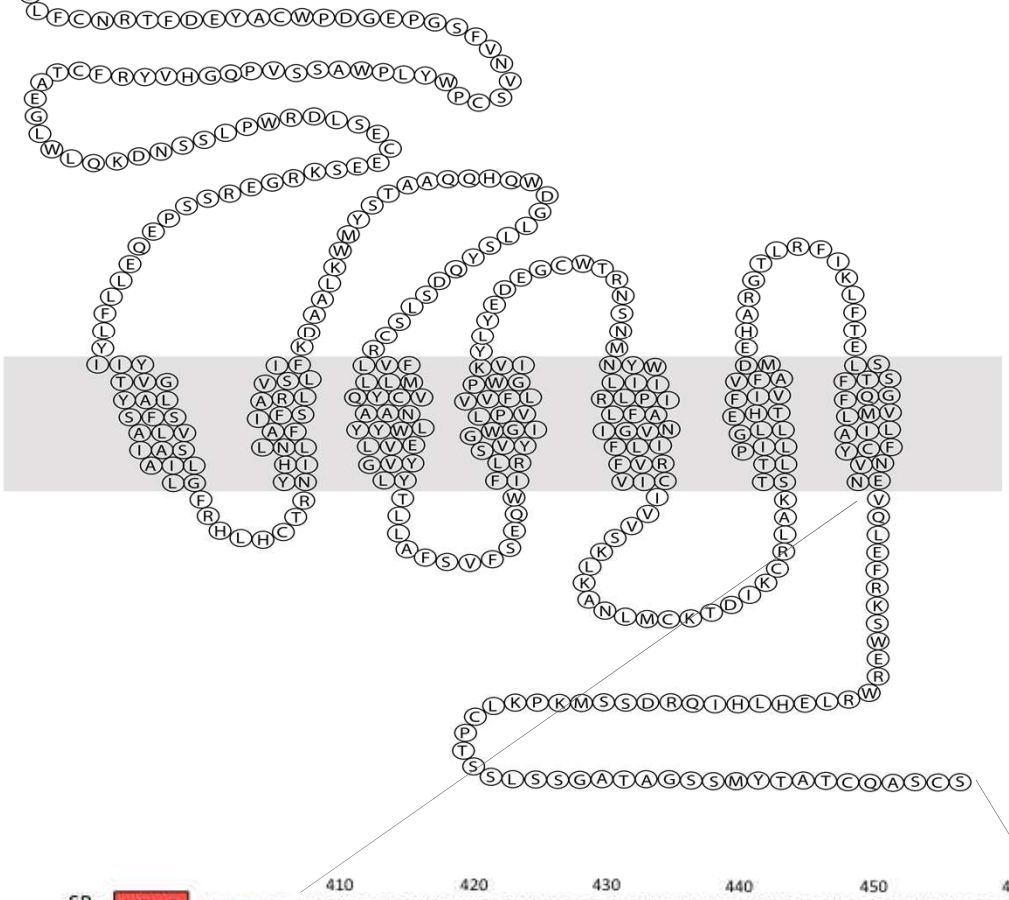
Figure 4. Effect of hGLP-1R C-terminal domain mutations on the receptor *N*-linked glycosylation. HEK293 cells transfected with the C-terminal deletion and site-directed mutants were treated without or with 5 μg/ml tunicamycin for 48 h. The cells were lysed and the cell lysates were immunoblotted with the anti-GFP or anti-VSVG antibodies (*denotes the non-specific band).

Figure 5. Effect of the C-terminal domain mutations on hGLP-1R activity. HEK293 cells expressing the C-terminal deletion and site-directed mutants were stimulated with 100 nM liraglutide

Figure 6. Effect of the C-terminal domain mutations on agonist-induced hGLP-1R internalisation. HEK293 cells expressing the C-terminal deletion and site-directed mutants of hGLP-1R were stimulated with 100nM liraglutide for 60 min and assessed for hGLP-1R internalisation by ELISA (A) and immunofluorescence (B) using the anti-hGLP-1R antibody. In immunofluorescence, the anti-hGLP-1R antibody staining is shown in red or GFP-tag of the constructs (green) and the anti-hGLP-1R antibody staining (red) overlay is shown in yellow, and nuclear staining with DAPI in blue (scale bars in immunofluorescence images represent 10 μM). Data are mean ± SEM, n=3. Data were analysed by the Dunnett's post-test after one-way ANOVA; values differ from control, n.s. $p>0.05$;, ** $p<0.01$.

Figure 7. Effect of hGLP-1R C-terminal domain mutations on the receptor mediated ERK1/2 phosphorylation. HEK293 cells transfected with the C-terminal deletion and site-directed mutant constructs were stimulated with 100 nM liraglutide for 5 min, lysed and ERK1/2 phosphorylation was assessed by immunoblotting (A) and quantified by densitometry and normalised to total ERK1/2 levels (B). The densitometry data is presented as fold phosphorylation (phosphorylated/total) and are ± SEM, n=3. Data were analysed by the Dunnett's post-test after one-way ANOVA; values differ from control, n.s. $p>0.05$; ** $p<0.01$.

Figure 8. Overview of hGLP-1R showing the distinct regions within the C-terminal domain required for its cell surface expression, activity and internalisation as deduced from the present study. The distinct regions within the C-terminal domain of hGLP-1R identified, in this study, for cell surface expression (411-418), activity (419-430) and internalisation (431-450) of the receptor.



				410	420	430	440	450	460
SP-VSVG	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT**SS**L**SS**GATAG**SS**MYTATCQASCS					
N450	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT**SS**L**SS**GATAG					13 aa deletion
N443	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT**SS**L					20 aa deletion
N440	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT					23 aa deletion
N430	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD					33 aa deletion
N410	SP -	VSVG	-hGLP1R-	EVQ					53 aa deletion
SP-VSVG-GFP	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT**SS**L**SS**GATAG**SS**MYTATCQASCS -					GFP
Δ411-418	SP -	VSVG	-hGLP1R-	EVQ - - - - - - - -RWRLEHLHIQRD**SS**MKPKLCPT**SS**L**SS**GATAG**SS**MYTATCQASCS -					GFP
Δ419-430	SP -	VSVG	-hGLP1R-	EVQLEFRKSWE - - - - - - - - - - - - **SS**MKPKLCPT**SS**L**SS**GATAG**SS**MYTATCQASCS -					GFP
Δ431-450	SP -	VSVG	-hGLP1R-	EVQLEFRKSWERWRLEHLHIQRD - **SS**MYTATCQASCS -					GFP
E408A,V409A, Q410A	SP -	VSVG	-hGLP1R-	AAALEFRKSWERWRLEHLHIQRD**SS**MKPKLCPT**SS**L**SS**GATAG**SS**MYTATCQASCS -					GFP

Figure 1

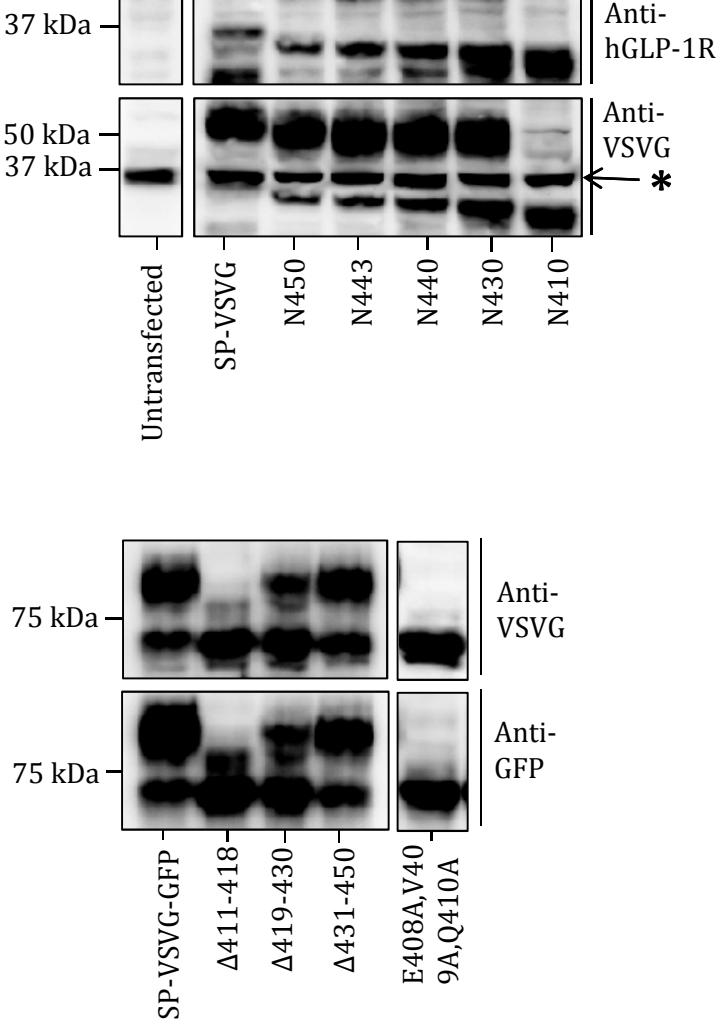
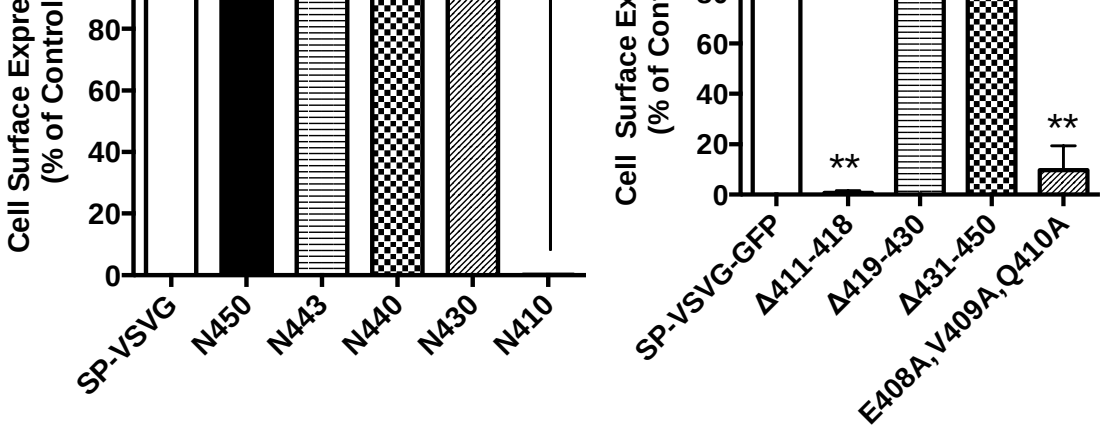

37 kDa
Anti-
hGLP-1R
50 kDa
37 kDa
Anti-
VSVG
*
Untransfected
SP-VSVG
N450
N443
N440
N430
N410
75 kDa
Anti-
VSVG
75 kDa
Anti-
GFP
SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V40
9A,Q410A

Figure 2


Cell Surface Expre
(% of Control
80
60
40
20
0
SP-VSVG
N450
N443
N440
N430
N410
Cell Surface Ex
(% of Con
60
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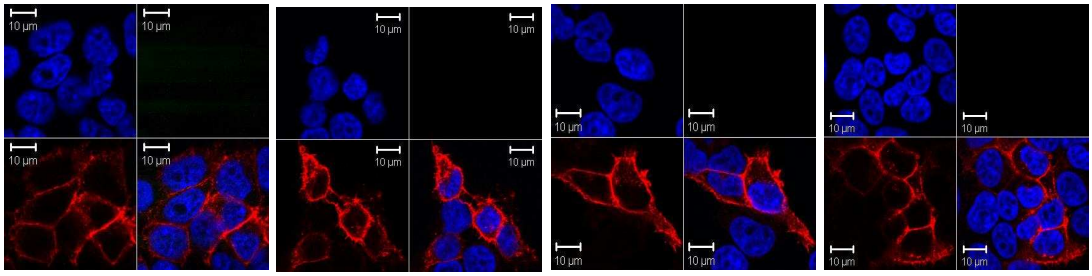
SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V409A,Q410A

B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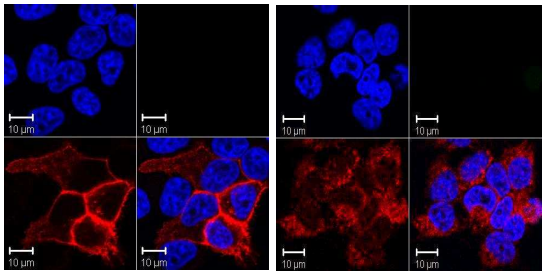
N443
N440


N430
N410

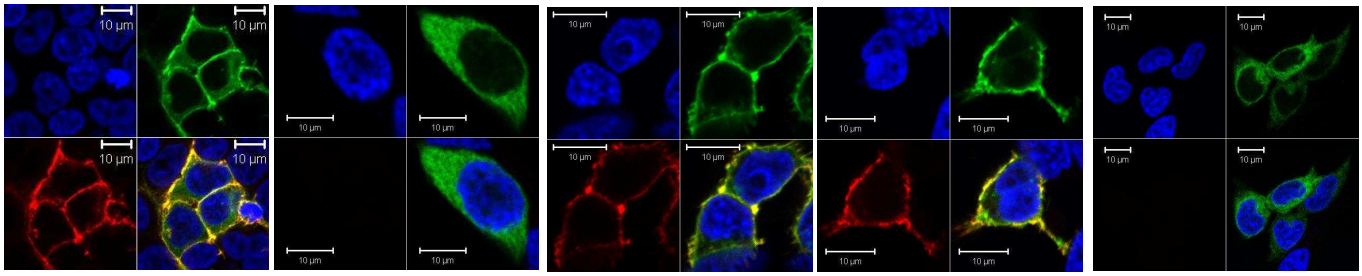

SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V409A,Q
410A

Figure 3

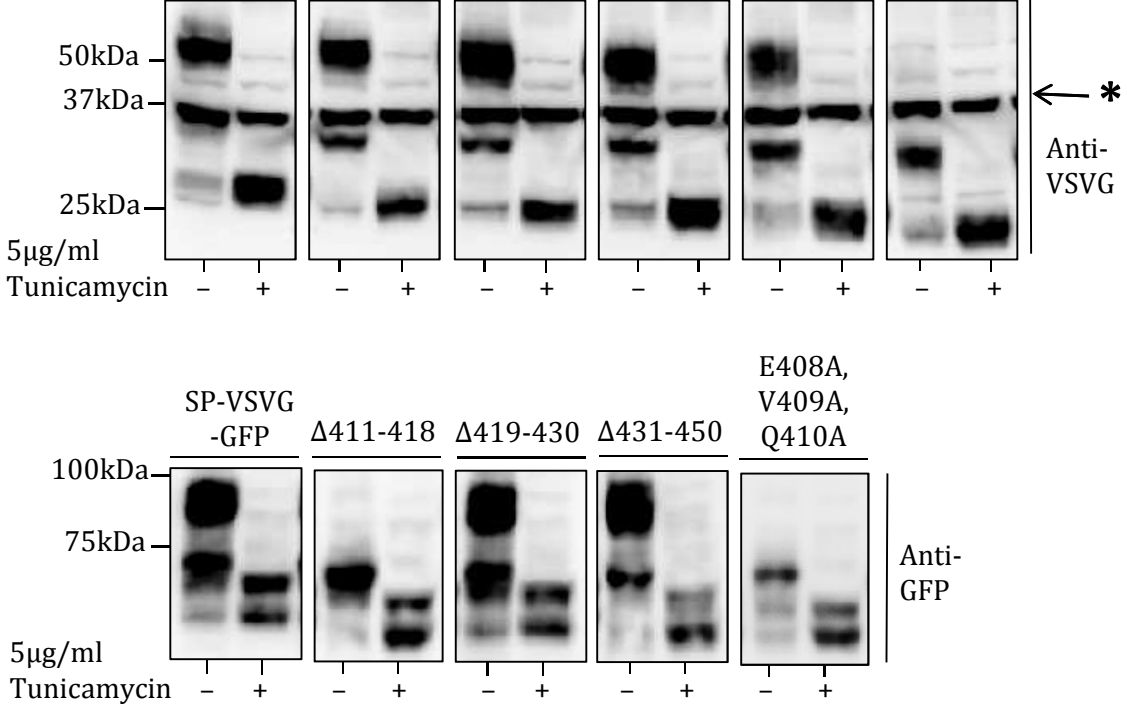
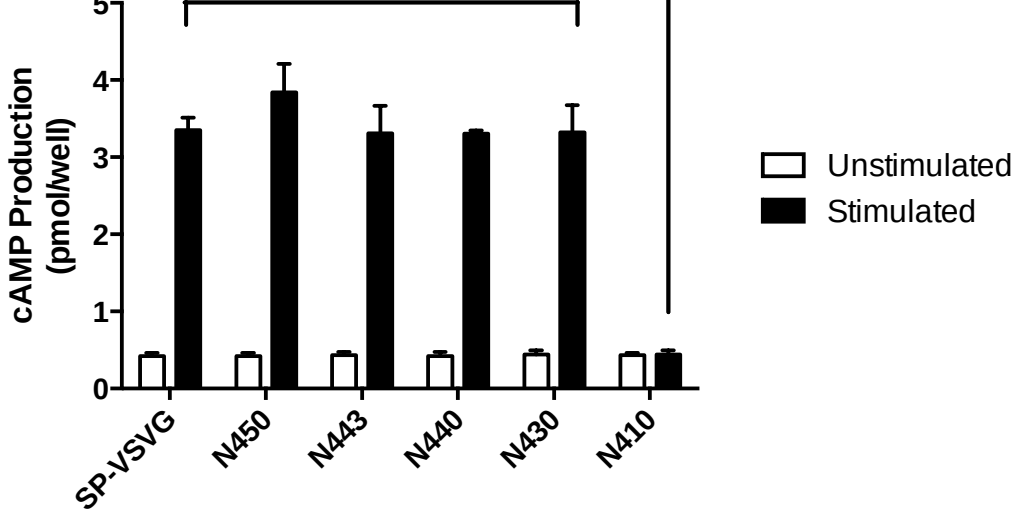

50kDa
37kDa
25kDa
5µg/ml
Tunicamycin
– + – + – + – + – + – +
*
Anti-
VSVG
SP-VSVG
-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,
V409A,
Q410A
100kDa
75kDa
5µg/ml
Tunicamycin
– + – + – + – + – +
Anti-
GFP

Figure 4


cAMP Production (pmol/well)
0
1
2
3
4
5
SP-VSVG
N450
N443
N440
N430
N410
Unstimulated
Stimulated

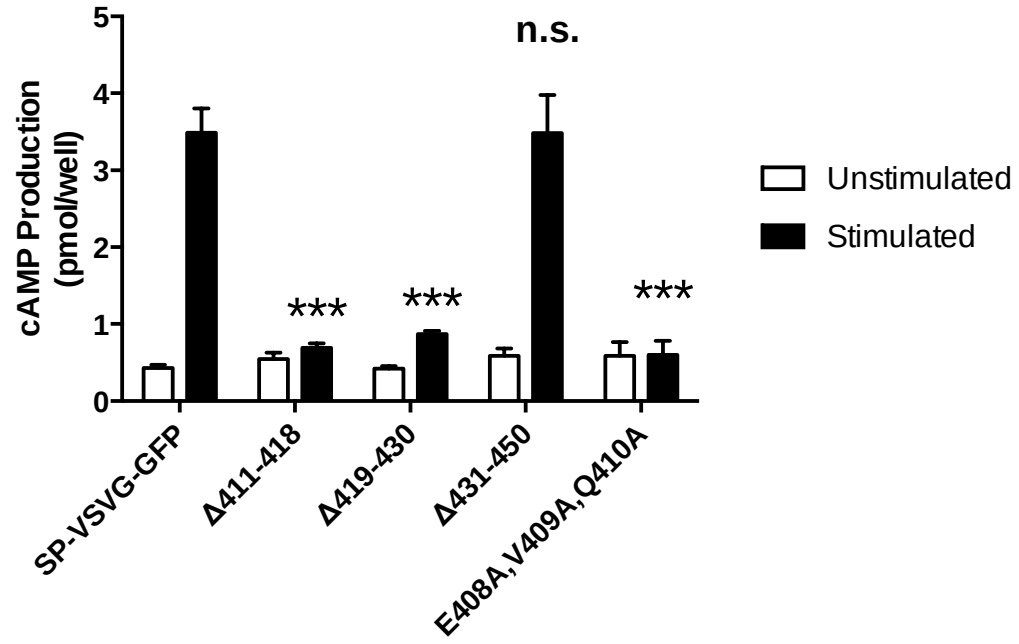
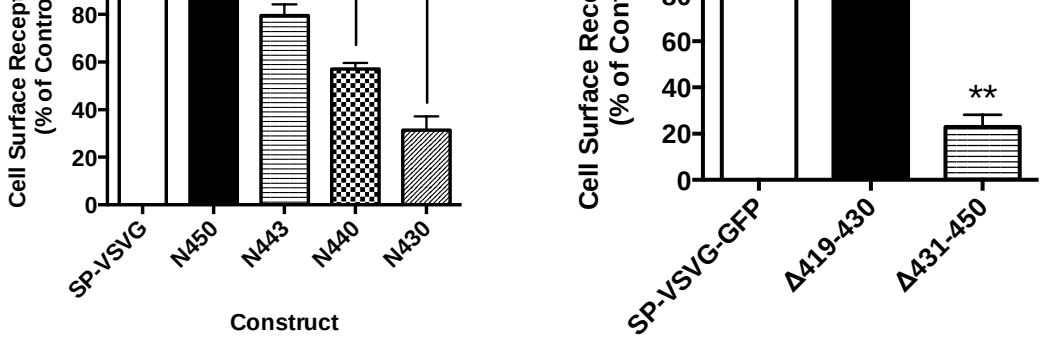

cAMP Production (pmol/well)
0
1
2
3
4
5
n.s.
***
***
***
SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V409A,Q410A
Unstimulated
Stimulated

Figure 5


Cell Surface Recep
(% of Contro
80
60
40
20
0
SP-VSVG
N450
N443
N440
N430
Construct
Cell Surface Rec
(% of Con
60
40
20
0
**
SP-VSVG-GFP
Δ419-430
Δ431-450

B

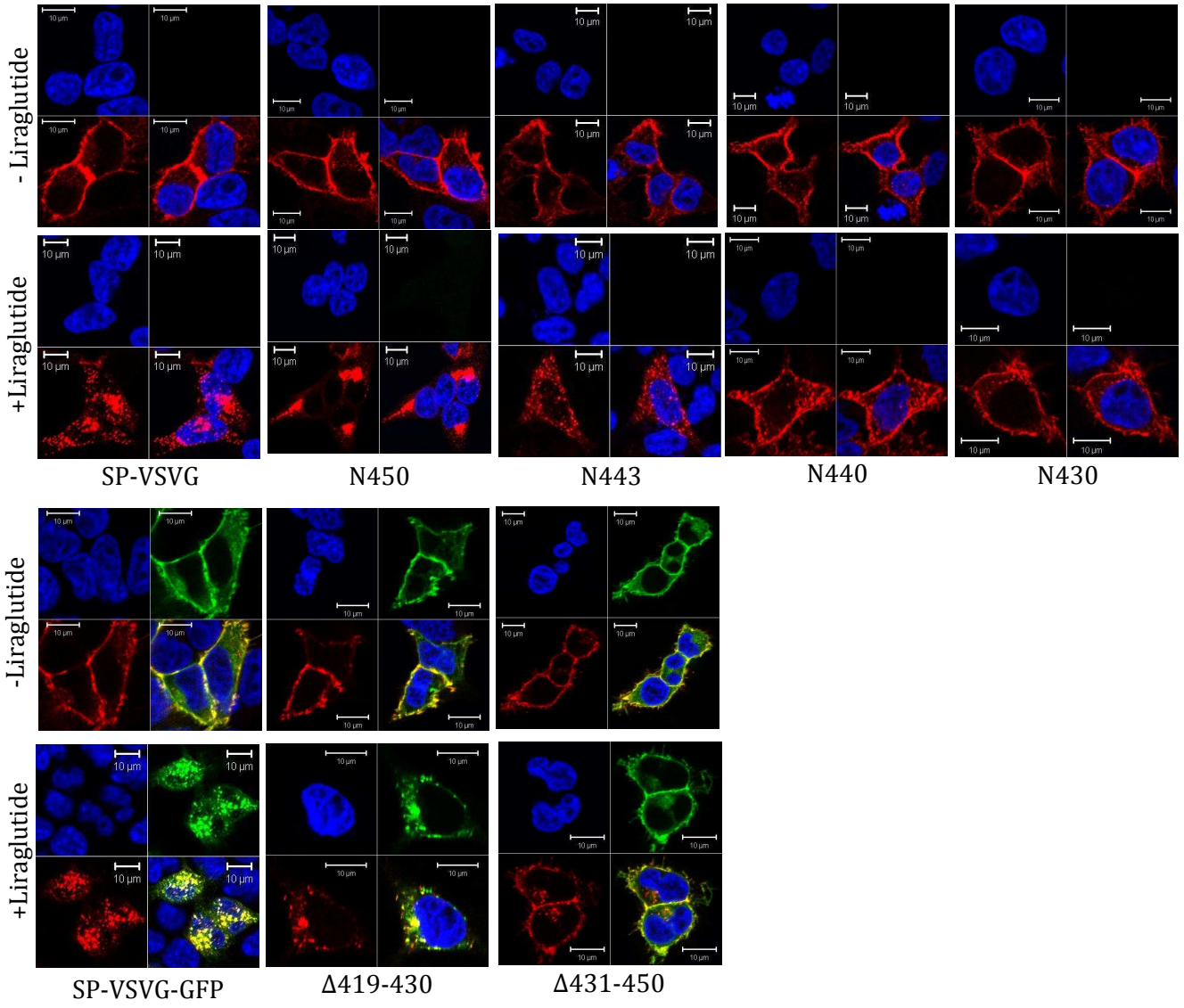

- Liraglutide
+Liraglutide
10 µm
SP-VSVG
N450
N443
N440
N430
-Liraglutide
+Liraglutide
SP-VSVG-GFP
Δ419-430
Δ431-450

Figure 6


-Liraglutide
+Liraglutide
Anti-ERK1/2
Anti-pERK1/2
SP-VSVG
N450
N443
N440
N430
N410
SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V409A
,Q410A

B

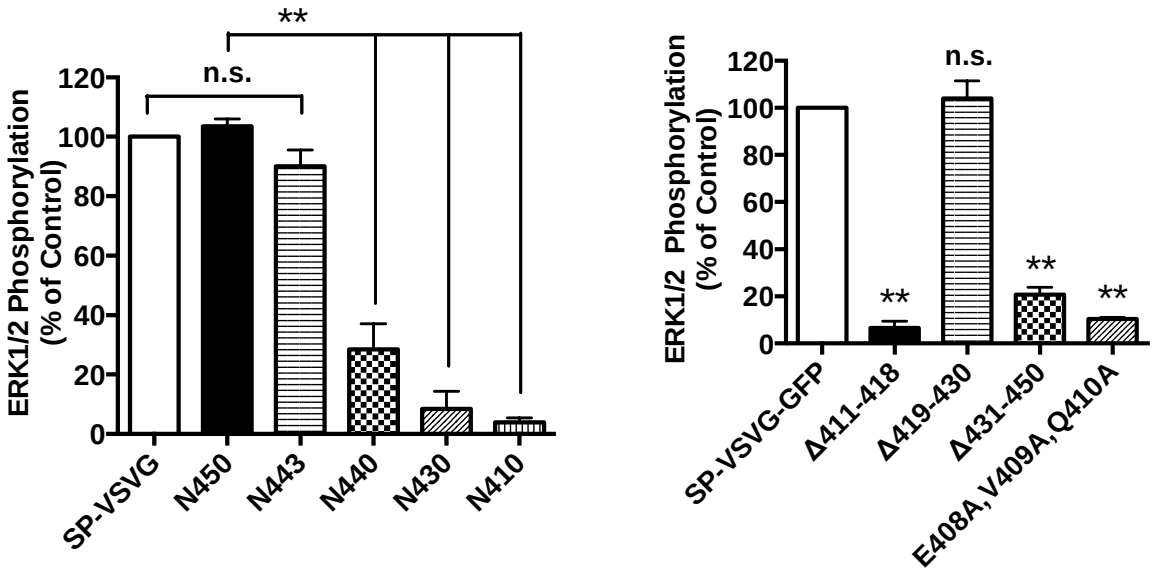

ERK1/2 Phosphorylation
(% of Control)
n.s.
**
SP-VSVG
N450
N443
N440
N430
N410
SP-VSVG-GFP
Δ411-418
Δ419-430
Δ431-450
E408A,V409A,Q410A

Figure 7

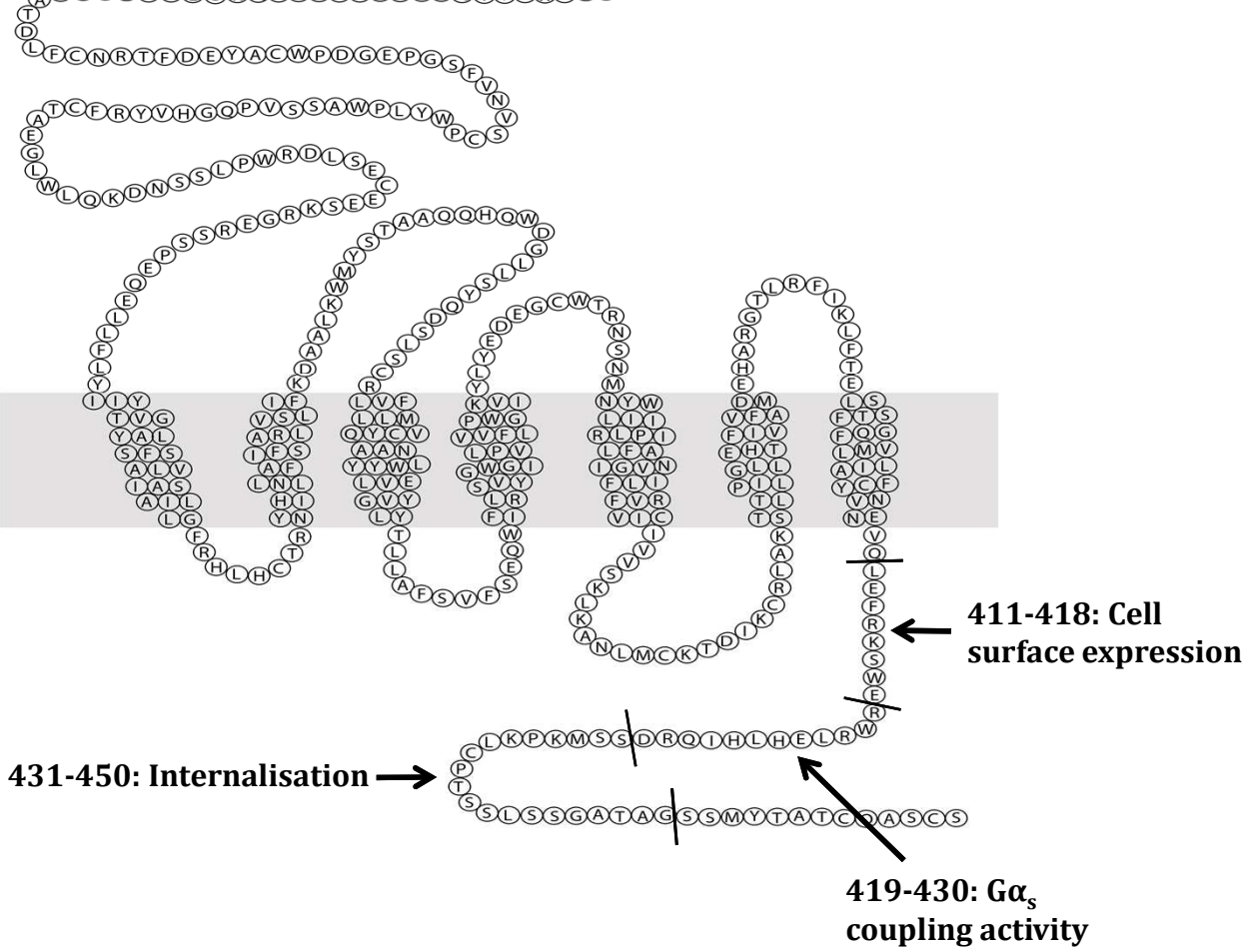

411-418: Cell surface expression
431-450: Internalisation
419-430: Gα$_s$ coupling activity

Figure 8

- Residues 411-418 in the C-terminus are critical for GLP-1R cell surface expression.
- Residues 419-430 within the C-terminus are important for the activity of GLP-1R.
- Residues 431-450 in the C-terminus are essential for GLP-1R internalisation.