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Distinct regions in the C-Terminus required for GLP-1R cell surface expression, activity and internalisation

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ABSTRACT

The glucagon-like peptide-1 (GLP-1) receptor (GLP-1R), an important drug target in the treatment of type 2 diabetes, is a G-protein coupled receptor (GPCR) that mediates insulin secretion by GLP-1. The 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.

1. Introduction

Glucagon like peptide-1 (GLP-1) mediates insulin secretion by acting on the GLP-1 receptor (GLP-1R), making the receptor an important target and of high therapeutic potential in the treatment of type 2 diabetes (Gallwitz, 2010; Thompson and Kanamarlapudi, 2013). GLP-1 in vivo half-life is very short (~1.5 min) 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 (Meier, 2012). 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 (Thompson and Kanamarlapudi, 2013; Montrose-Rafizadeh et al., 1999). Agonist-induced GLP-1R internalisation plays an important role in glucose induced insulin secretion (Kuna et al., 2013). The GLP-1R internalises with similar kinetics in response to GLP-1 and liraglutide stimulation (Roed et al., 2014). However, the rate of GLP-1R recycling in cells treated with liraglutide is slower than that in cells treated with GLP-1 (Roed et al., 2014). 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 (Thompson and Kanamarlapudi, 2015). 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 (Thompson and Kanamarlapudi, 2015).

Abbreviations: 5-HT_{2a}, 5-hydroxytryptamine receptor 2a; aa, amino acid; AC, adenylyl cyclase; AR, adrenoceptor; 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% tween 20; TM, transmembrane; VSVG, vesicular stomatitis virus glycoprotein; WT, wild type.

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The extracellular N-terminal domain in many GPCRs is important for their biosynthetic trafficking and maturation (Thompson and Kanamarlapudi, 2013; Dong et al., 2007). 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 (Huang et al., 2010; Chen et al., 2010; Whitaker et al., 2012). 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 (Thompson and Kanamarlapudi, 2014). The intracellular C-terminal domain of GPCRs plays a critical role in agonist-induced internalisation, desensitisation, down regulation and arrestin signalling of the receptors (Thompson et al., 2008). Furthermore, the C-terminal region is required for some GPCRs trafficking from the endoplasmic reticulum (ER) to the plasma membrane (Dong et al., 2007). 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 (Thompson and Kanamarlapudi, 2013; Dong et al., 2007).

The motifs such as $E(X)_3LL$, $FN(X)_2LL(X)_3L$, $F(X)_3F(X)_3F$, YXXΦ and PPXXFR found within the C-terminus of some GPCRs have been shown to be required for newly synthesised GPCR targeting to the plasma membrane (Dong et al., 2007). Some GPCRs possess a helix-8 located just downstream of transmembrane (TM) 7 that associates with a number of intracellular proteins (Kuramasu et al., 2006). 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 (Thompson and Kanamarlapudi, 2013; Ango et al., 2001).

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 (Gurevich and Gurevich, 2006). 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 (Kuramasu et al., 2006). The tyrosine motif (YXXΦ) within the C-terminus has been shown to associate with clathrin (Bonifacino and Traub, 2003). 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 (Cao et al., 2000). Further, an 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 (Robertson et al., 2003).

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 (Ferguson, 2001). 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 (Bohm et al., 1997b; Thomas et al., 1995; Barak et al., 1994). 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 (Xiang et al., 2001). 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 (Widmann, 1997). Further, phosphorylation of some serine doublets within the C-terminal domain of the GLP-1R is mediated by PKC (Widmann et al., 1996a).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 (Vazquez et al., 2005a).

Some GPCRs require $E(X)_3LL$, $FN(X)_2LL(X)3L$, $F(X)_3F(X)_3F$, YXXΦ, PPXXFR, PXXP, NPXXY and LL motifs within the C-terminal domain for biosynthetic trafficking, interactions with intracellular signalling proteins and internalisation of the receptor (Dong et al., 2007). However, these motifs are not 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.

2.1.1. Plasmids

The cDNA of SP-VSVG-hGLP-1RΔN23, containing the signal peptide (SP, 1–23 amino acids) coding sequence followed by VSVG-epitope coding sequence, was cloned into pEGFP-N1 vector (Clontech), as described previously (Thompson and Kanamarlapudi, 2015) 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 (Kanamarlapudi, 2014). 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 °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