SHORT COMMUNICATION

G protein activation's correlation with the dynamics of GLP-1R peptide agonist engagement

Nicole Watson

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ABSTRACT

The Glucagon-like Peptide-1 Receptor (GLP-1R) has a wide range of physiological functions and is a well-established therapeutic target for metabolic diseases. Despite recent progress in elucidating the structure of the GLP-1R, a full mechanistic understanding of how various peptides create dramatic variations in G protein-mediated signalling is still absent. To investigate the mechanism and implications of GLP-1R binding to four peptide agonists, we used cryo-electron microscopy, molecular dynamics simulations, receptor mutagenesis, and pharmacological experiments. These findings show that differences in peptide N-terminal contacts and dynamics with the GLP-1R transmembrane domain are linked to variances in G protein allosteric coupling.

Key Words: *Glucagon-like Peptide-1 Receptor, G protein-mediated signalling*

INTRODUCTION

The Glucagon-like Peptide-1 Receptor (GLP-1R) is found in numerous tissues and is responsible for mediating the activity of the gastrointestinal peptide hormone Glucagon-like Peptide-1 (GLP-1). GLP-1 has a number of physiological actions that are beneficial in the treatment of type 2 diabetes and obesity, including insulin secretion modulation, stomach emptying delaying, hunger suppression, and carbohydrate metabolism regulation. Several endogenous agonists, such as GLP-1, oxyntomodulin, and glucagon, activate the GLP-1R, and a number of exogenous peptide agonists are licenced or in clinical research for the treatment of type 2 diabetes and/or obesity. The GLP-1R is a G Protein-coupled Receptor (GPCR) of class B1 that is activated by heterotrimeric G proteins1. The receptor is pleiotropically connected to several transducers, including other G protein subtypes and regulatory proteins. It is primarily related to the stimulatory G protein Gs to elevate cAMP levels within the cell, but it also binds to other G protein subtypes and regulatory proteins [1]. Other GLP-1R agonists, when compared to GLP-1, can have varied efficacies within a single signalling pathway, as well as preferential signalling towards certain pathways over others. When GLP-1R agonists are tested across several signalling pathways, these behaviours result in biassed agonism, which is typical. However, the molecular foundation for how individual agonists can cause significant pharmacological differences is still unknown.

RESULTS

The GLP-1R:Gs complex bound by oxyntomodulin and exendin-4 was determined using cryo-EM

Using proven methods, the cryo-EM structures of exendin-4- and oxyntomodulin-bound GLP-1R-Gs complexes were determined. Purified complexes were vitrified and photographed using single-particle cryo-EM on a 300 kV Titan Krios with (oxyntomodulin) or without a Volta Phase Plate (VPP). At the gold standard FSC 0.143, these datasets produced consensus maps with global resolutions of 3.3 (oxyntomodulin) and 3.7 respectively. The -helical domain (AHD) of the G subunit was masked out during the refining since there was only little density. The best resolution was reported within the G protein and receptor TMD, similar to the prior peptide-bound: GLP-1R:Gs complex structures, with lesser resolution in the extracellular half of the receptors, including the ECD, indicating more flexibility

Editorial Office, Journal of Pharmacology and Medicinal Chemistry, Switzerland

Correspondence: Nicole Watson, Editorial Office, Journal of Pharmacology and Medicinal Chemistry, Switzerland, E-mail medpharma@scholarlypub.org

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in these regions. With the exception of the Extracellular Loop (ECL) 1 and Intracellular Loop (ICL) 3, which were not modelled, the cryo-EM map for the oxyntomodulin-bound complex enabled robust modelling and confident assignment of most of the side-chain rotamers for oxyntomodulin, the G protein, and the receptor TMD [2]. The ECD was less well defined, but the protein backbone could be factored into the density from the start. Although the overall resolution of the exendin-4-bound complex was lower, robust modelling into the map was possible for the bulk of the peptide, the G protein, and the receptor TMD. ECL1, ECL3, and ICL3 were not modelled since the density was less well defined, indicating that these domains had more flexibility. Because the ECD of the exendin-4-bound map had low resolution, confident modelling was impossible; as a result, the ECD was rigid body fitted to the density, followed by MD refinement of the backbone [3].

General characteristics of GLP-1R:Gs complexes coupled to peptide

The GLP-1R:Gs complexes bound by oxyntomodulin and exendin-4 demonstrated important properties of active state class B1 GPCRs that are comparable with the general features of the active state GLP-1R previously identified when bound by other agonists. This includes an upwards and clockwise rotation of the ECD relative to the TMD, reorganisation of the extracellular TM regions to accommodate peptide engagement, and rearrangement of a conserved central polar network to stabilise a sharp kink within the centre of TM6, which facilitates the large outward movement of this TM at the intracellular face that is required to accommodate G protein binding, as compared to the inactive GLP-1R [4]. Exendin-4 and oxyntomodulin adopted a continuous alpha helix, similar to previous peptide-binding GLP-1R structures with their C-terminus bound within the ECD and their N-terminus bound deep within the TMD, generating extensive contacts with residues within TM1, TM2, TM3, TM5, TM6, TM7, and ECL2. The oxyntomodulin-bound structure is very similar to the previously determined high-resolution GLP-1-bound GLP-1R (PDB 6X18) with just a minor variation in the position of the ECD relative to the bundle. Furthermore, side-chain rotamers within the TMD cavity were similar, while the strength and nature of their interactions with attached peptides differed.

Exendin-4 contacts the GLP-1R in a similar way, with the ECD assuming a conformation that is identical to the GLP-1-bound receptor. The exendin-4 bound cavity is more open within the TMD, owing to a more outward placement of TM1, while TM2, TM4-ECL2-TM5, and TM7 are similarly placed further from the bundle's centre. In comparison to the GLP-1-bound and oxyntomodulin-bound complexes, the exendin-4 and exendin-P5 bound (PDB 6B3J) structures indicated similar ECD locations and more similarities in backbone orientations for TMs. Due to a more outer placement of TM7 in the presence of exendin-P5, the TMD-binding cavity is even more open in the presence of exendin-P5 [5]. While the top of TM6 and ECL3 could not be confidently modelled for the exendin-4 complex, the portion of TM6 and TM7 that could be modelled, as well as the weak density corresponding to ECL3, supports a backbone conformation more similar to GLP-1 and oxyntomodulin bound receptors, rather than exendin-P5, though it is likely that this region is more conformationally dynamic.

Individual peptide dynamic interactions within the GLP-1R binding site are revealed by cryo-EM structures, molecular dynamics, and mutagenesis

Atomic modelling of static consensus cryo-EM maps showed particular details about oxyntomodulin and exendin-4 interactions with the GLP-1R. Supplementary Table 1 shows the interactions of the peptide N-termini with the TMD, and shows the interactions of the peptide N-termini with the TMD. The N-terminus of GLP-1, exendin-4, and oxyntomodulin is substantially conserved, and as a result, a large number of receptor interactions are conserved as well, whereas these are more divergent when compared to exendin-P5. We used receptor mutagenesis to investigate the relative importance of consensus structure interactions for receptor binding and activation. Each residue within the TMD that formed an interaction with any of the four peptides in the static cryo-EM structures was mutated to alanine (except A3686.57, which was mutated to glycine), and the binding affinity and cAMP signalling of each peptide were assessed. pIC50 values and transduction ratios that evaluate signalling effectiveness were determined using concentration-response curves. These were compared between mutant and wild-type receptors to see how the mutation affected each peptide's affinity and signalling, and the results were mapped onto cryo-EM structures. The effects on exendin-4 were similar to those on GLP-1 when comparing the global mutagenesis profile, with a strong positive correlation identified for the mutagenesis data for both affinity and cAMP generation, however the effect on cAMP signalling was generally weaker for exendin-4. While the TMD conformation of oxyntomodulin-bound GLP-1R in the static cryo-EM structure was more comparable to that of GLP-1, the effect of mutagenesis was more divergent, with several mutations differentially altering oxyntomodulin affinity and/or signalling data relative to GLP-1. Nonetheless, there was still a substantial weak positive correlation across all mutant datasets and comparable to exendin-4, mutations affecting both peptides generally had a stronger influence on GLP-1 than oxyntomodulin signalling. Exendin-P5 mutagenesis, on the other hand, was substantially different from the other peptides, with few mutations changing exendin-P5 affinity and only a modest, but significant, association with the effect of mutations on GLP-1 in cAMP signalling experiments. Furthermore, when evaluating the overall effect of mutations, there was no association between oxyntomodulin and exendin-P5 mutagenesis, however there were a few mutations that had similar effects on both peptides' signaling [6-9]. Even where interactions in the static consensus structures were similar, the effect of mutagenesis of residues comprising the TM-binding pocket diverged, confirming that static visualisation of complexes is insufficient to fully understand binding and activation mechanisms, and suggesting that the dynamics of peptide-receptor engagement likely play a critical role. As a result, we used Molecular Dynamics (MD) simulations to investigate the stability and dynamics of each receptor complex in a simulated POPC lipid environment. Prior to starting the simulations, regions that were not modelled in the cryo-EM maps due to low resolution were initially simulated and the receptor complex energy was minimised.

The allosteric effect of G proteins on agonist affinity and G protein structure is linked to the dynamics of peptide–TMD interactions

The dramatic difference in the necessity for persistent TMD connections for exendin-P5 affinity versus GLP-1, oxyntomodulin, and exendin-4 raises critical issues about peptide binding and receptor activation molecular pathways. To investigate this, researchers used wildtype and CRISPR-engineered HEK293 cells with all G subtypes depleted (all G HEK293)34 to see how the attached G protein affected the affinity of each agonist. The capacity of each peptide to prevent binding of the fluorescent probe ROX-Ex4 to the GLP-1R N-terminally tagged with nanoluciferase was determined using a NanoBRET membrane competition binding assay (Nluc) [10-11].

DISCUSSION

When experimentally determined GLP-1R structures are combined with structure–function investigations and simulations of receptor dynamics, new insights into how different agonists interact and activate the receptor emerge. While GLP-1R peptide agonists bind to both the ECD and the TMD, the roles of the interactions with each domain vary [12-13]. In functional and structure–function research, GLP-1, oxyntomodulin, and the therapeutically employed mimic exendin-4 are among the most widely investigated GLP-1R peptide agonists. Exendin-4 and oxyntomodulin-occupied receptors are more dynamic, which may be related to the more transitory nature of their interactions with the TMD found in MD simulations. Oxyntomodulin is a biassed agonist, forming different and more dynamic interactions with the GLP-1R, notably with residues at the base of the peptide binding cavity, which are positioned above the conserved central polar network, which is critical for receptor activation. While the profile of exendin-4-mediated signalling is more similar to that of GLP-1, structural and dynamic data reveal discrepancies in receptor interaction between the two peptides. Exendin-4 has more transitory interactions with residues at the base of the peptide binding cavity than GLP-1, similar to oxyntomodulin, however the pattern of interactions with the polar core is essentially constant. GPCRs' peptide and G protein binding sites are allosterically connected, allowing information to be sent from peptide binding to G protein coupling. The use of highly conserved class B1 GPCR residues for information transmission through the GLP-1R TM bundle was discovered through network and community analysis [14-15]. While the TM bundle contacts were conserved when the four peptides were engaged, there were variances in how each peptide used different networks to enhance G protein coupling. G proteins can allosterically modify ligand affinity for GPCRs thanks to this information transmission across the TM bundle.

METHODS

Insect cell expression- Human DNGs46, His6-tagged human G1 and G2, and HA-signal peptide-FLAG-3C-GLP-1R-3C-8HIS13 were expressed in Tni insect cells (Expression systems) using baculovirus as previously described. Cell cultures were grown to a density of 4 million cells/ml in ESF 921 serum-free media (Expression Systems) and then infected with three different baculoviruses at a ratio of 2:2:1 for GLP-1R, DNGs, and G12. 60 hours after infection, the culture was harvested by centrifugation, and the cell pellet was kept at 80 °C.

Complex Purification- The cell pellet was frozen in a solution containing 20 mM HEPES pH 7.4, 50 mM NaCl, 5 mM CaCls, and 2 mM MgCl2, as well as Roche's cOmplete Protease Inhibitor Cocktail tablets and benzonase. The complex was formed by adding 10 M exendin-4 or 50 M oxyntomodulin (China Peptides), Nb35–His (10 g/mL), and apyrase (25 mU/mL, NEB) to a solution at room temperature for 1 hour. 0.5 percent (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace) supplemented with 0.03 percent (w/v) cholesteryl hemisuccinate was used to solubilize the complex from the membrane for 1 hour at 4 °C. The insoluble material was removed by centrifugation at 30,000 g for 30 minutes, and the solubilized complex was immobilised by batch binding to anti-FLAG affinity resin M1 in the presence of 5 mM CaCl2.

SDS–PAGE and Western blot analysis- Precast gradient TGX gels (Bio-Rad) were used for SDS–PAGE. Instant Blue was used to stain the gels, or they were immediately transferred to a PVDF membrane (Bio-Rad) at 100 V for 1 hour. Two primary antibodies were used to probe the proteins on the PVDF membrane: a rabbit anti-Gs C-18 antibody (cat. no. sc-383, Santa Cruz) for the Gs subunit and a mouse penta-His antibody (cat. no. 34660, QIAGEN) for the His tags. Secondary antibodies, 680RD goat anti-mouse and 800CW goat anti-rabbit, were used to incubate the membrane after it had been washed (LI-COR).

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