Crystal Structure of Glucagon-Like Peptide-1 in Complex with the Extracellular Domain of the Glucagon-Like Peptide-1 Receptor

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Running title: Crystal Structure of Receptor-bound GLP-1

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Glucagon-like peptide-1 (GLP-1) is a peptide hormone produced by posttranslational processing of proglucagon in the intestinal L-cells (1). GLP-1 is an incretin that potentiates the synthesis and release of insulin from pancreatic β-cells in a glucose-dependent manner (2). GLP-1 is a kinked but continuous α-helix from Thr13 to Val33 when bound to the extracellular domain. We supplemented the crystal structure with site-directed mutagenesis in order to link the structural information of the isolated extracellular domain with the binding properties of the full length receptor. The data support the existence of differences in the binding modes of GLP-1 and exendin-4 on the full length GLP-1 receptor.
ECD of the human type-1 CRF receptor (CRFR1) (15), the human type-1 PACAP receptor (PAC1-R) (16), the human GLP receptor (GIP-R) (17), the human GLP-1R (18) and the human type-1 PTH receptor (PTH-1R) (19). The ECDs of class B receptors have a common structure – the secretin recognition fold – which is stabilised by three conserved disulphide bonds and five conserved residues (Asp^{67}, Trp^{72}, Pro^{86}, Gly^{108} and Trp^{110} in GLP-1R). The receptor-bound ligands are primarily in α-helical conformation, and the C-terminal part of the ligands binds the ECD in agreement with the two-domain binding mechanism. Several receptor models have been proposed for full length class B receptors (20-23). However, the orientation of the ECD relative to the TM-domain is uncertain.

Exendin-4 is a 39 amino acid peptide, which was originally isolated from the venom of the lizard Heloderma suspectum (24). GLP-1 and exendin-4 are 50% identical, and exendin-4 is a full agonist with similar affinity and potency for the full length GLP-1R (25). However, structure-activity studies have demonstrated interesting differences between the binding modes of GLP-1 and exendin-4: 1) The isolated ECD binds exendin-4 with high affinity (IC_{50} of 6 nM) and GLP-1 with low affinity (IC_{50} > 500 nM) (26;27), 2) GLP-1 binding is more sensitive to site-directed mutagenesis of the TM-domain compared to exendin-4 binding (28-30) and 3) GLP-1 is much more sensitive to N-terminal truncation than exendin-4 (31). N-terminally truncated exendin-4 variants maintain high affinity but are unable to activate GLP-1R i.e. competitive antagonist, whereas N-terminal truncation of GLP-1 severely affects both binding and activation (31). Clearly, the interaction between the N-terminal part of GLP-1 and the TM-domain is critical for binding and activation of GLP-1R.

Exendin-4(9-39) is a truncated form of exendin-4, and a competitive antagonist which maintains high affinity for GLP-1R through interactions with the ECD (IC_{50} value of 6 nM for the isolated ECD) (27;32). We recently solved the crystal structure of the GLP-1R ECD in complex with exendin-4(9-39) (18). Exendin-4(9-39) is α-helical in the ECD-bound conformation except for the C-terminal segment – the so-called Trp-cage (33;34). In solution, the helical propensity of exendin-4 is higher than that of GLP-1 (27;33). Biophysical studies showed a positive correlation between α-helical propensity in solution and affinity for the GLP-1R ECD (27). Moreover, charged residues of exendin-4 interact with the ECD in a manner not possible for GLP-1 (18). Hence, the high affinity of exendin-4 for the ECD may be a combination of high helical propensity in solution and unique receptor interactions.

Here we report the crystal structure of the GLP-1R ECD in complex with its endogenous agonist, GLP-1. We supplemented the crystal structure with site-directed mutagenesis in order to link the structural information of the isolated ECD with the binding properties of the full length receptor.

**EXPERIMENTAL PROCEDURES**

**Protein and Peptide Preparation** - The GLP-1R ECD was prepared as previously described (27). Briefly, N-terminal His\_6-tagged ECD was expressed in E. coli inclusion bodies, isolated as inclusion body protein, solubilised in guanidine-HCl and dithiothreitol (DTT), dialysed against guanidine-HCl to remove the DTT, and refolded using L-Arg and a 1:5 molar ratio of reduced and oxidized glutathione. The refolded ECD was purified by hydrophobic interaction chromatography and size exclusion chromatography in 10mM Tris-HCl pH 7.5, 0.1 M Na\_2SO\_4, 2% glycerol. The His\_6-tag was removed by thrombin cleavage. The purified GLP-1R ECD consisted of four amino acids, Gly-Ser-His-Met, of the linker attached to the N-terminus of ECD (Arg^{24}-Tyr^{145}), M_w = 14723 g/mol after removal of the His\_6-tag. Native GLP-1(7-37)-OH was synthesized as previously described (12).

**Purification and Crystallisation of the GLP-1-bound extracellular domain** - The purified GLP-1R ECD was concentrated to 1.2 mg/ml, mixed with 3-fold molar excess of GLP-1(7-37) (dissolved in 50 mM Tris-HCl pH 7.5) and incubated over night at 4°C. The GLP-1-bound ECD was purified by size exclusion chromatography on a Superdex75 column in 10 mM Tris-HCl pH 7.5 at a flow rate of 0.3 ml/min and characterised by SDS-PAGE (supplemental data Fig. S1). The complex was concentrated to 4 mg/ml and crystallised by hanging drop vapour diffusion. The crystallisation conditions were initially identified using the Crystal Screen from Hampton Research and subsequently optimised to 0.1 M N-(2-acetamido) iminodiacetic acid (ADA), pH 6.9, 14 vol-% (+/-)2-methyl-2,4-pentanediol (MPD) and 9 mM n-decyl-β-D-
Single crystals were flashed cooled in liquid N\textsubscript{2} using 30% glycerol in the cryo solution.

**Data collection and Structure Determination**

Diffraction data were collected from a single crystal using beamline 1911-3 at MAX-lab (Lund, Sweden). The data were integrated and scaled using XDS (35). The crystals belonged to space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with the unit cell dimensions a = 35.7 Å, b = 42.7 Å and c = 95.1 Å. The phases and electron density map were obtained by molecular replacement using Phaser running in the CCP4 programme interface with one complex in the asymmetric unit. Refinement was done using COOT (36) and REFMAC5 (37). Well-defined electron density was obtained for GLP-1 residues Thr\textsuperscript{115}-Val\textsuperscript{135}, and for ECD residues Val\textsuperscript{30}-Glu\textsuperscript{128}. Poor density was observed for His\textsuperscript{7*}-Glu\textsuperscript{9*} and Arg\textsuperscript{36*}-Thr\textsuperscript{29-Glu\textsuperscript{128}}, but it should be noted that the conformation of Lys\textsuperscript{34*} of GLP-1 is very uncertain, and because of the poor density we have chosen to show Asn\textsuperscript{115} as Gly. The final GLP-1-bound ECD structure has 116 residues in the CCP4 programme interface with one complex in the asymmetric unit. Refinement was done using COOT (36) and REFMAC5 (37).

**Receptor Constructs**

The cDNA encoding the human GLP-1R was originally obtained from Dr. B. Thorens (9) and subcloned into the mammalian expression vector pcDNA3.1/v5-His-TOPO\textsuperscript{®} (Invitrogen). The presence of the C-terminal His\textsubscript{6}-tag was previously shown not to influence the functional response of the receptor (12). Site-directed mutagenesis of GLP-1R was done using QuikChange\textsuperscript{TM} (Stratagene). Plasmid DNA was generated using the NucleoBond\textsuperscript{®} Xtra Maxi Plus kit (Macherey-Nagel), and the desired mutations were confirmed by dideoxynucleotide sequencing.

**Cell culture and receptor expression**

Human Embryonic Kidney (HEK) 293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (BioWhittaker) supplemented with 10 vol-% fetal bovine serum and 1 vol-% penicillin/streptomycin (100 U/ml) in T175-flasks. HEK293 cells were transiently transfected with 21 µg GLP-1R DNA using the FuGene\textsuperscript{™} transfection reagent (Roche), harvested 24 hours after transfection and used directly in functional experiments or plasma membrane preparations as previously described (12).

**Functional Assay**

Transiently transfected HEK293 cells expressing wild-type GLP-1R or mutant receptors were harvested and resuspended in assay buffer (Flashplate\textsuperscript{®}, Perkin Elmer) to a cell density of 2.4 x 10\textsuperscript{6} cells/ml. GLP-1(1-37)-acid and exendin-4 were diluted in PBS with 0.02 vol-% Tween-20. Cells in assay buffer (50 µl) and GLP-1 or exendin-4 (50 µl) were mixed in 96-well FlashPlates\textsuperscript{®} (Perkin Elmer), gently agitated for 5 minutes and incubated for 25 minutes at room temperature. The resulting intracellular level of cAMP was measured according to supplier’s manual and analysed by non-linear regression/sigmoidal dose-response fitting using Prism 5.0\textsuperscript{®} (GraphPad Software, Inc.).

**Receptor Binding Assay**

Freshly thawed plasma membrane preparations from transiently transfected HEK293 cells expressing GLP-1R (20 µg protein/well) were pulled through a 25-gauge needle three times and diluted in assay buffer (50 mM HEPES, 5 mM MgCl\textsubscript{2}, 5 mM EGTA, 0.005 vol-% Tween-20, pH 7.4). GLP-1 and exendin-4 were diluted in assay buffer. The concentration range was 1 pM to 100 nM for GLP-1 and exendin-4. \textsuperscript{125}I-GLP-1 (7-36)-amide (2.2 Ci/µmol) was dissolved in assay buffer and added at 50,000 cpm per well to a final concentration of 50 pM. Nonspecific binding was determined with 1 µM GLP-1. Membrane-preparation and radioligand were mixed in 96-well 0.65 µm filter plates (Millipore) with either diluted GLP-1 or exendin-4 and incubated at 1 hour at 37°C. Subsequently, bound and unbound radioligand were separated by vacuum filtration (Millipore vacuum manifold). The filters were washed twice in 100 µl cold assay buffer and left to dry. Data were analysed by non-linear regression and the expression level (B\textsubscript{max}) was calculated using Prism 5.0\textsuperscript{®} (GraphPad Software, Inc.).
RESULTS AND DISCUSSION

Purification, Crystallisation and Structure Determination. The GLP-1R ECD was expressed in E.coli inclusion bodies, refolded and purified as previously described (27). A complex of GLP-1 and the ECD was purified by size exclusion chromatography (supplemental data Fig. S1). The purified complex was characterised by SDS-PAGE (supplemental data Fig. S1), concentrated and crystallised by hanging drop vapour diffusion. Diffraction data were collected from a single crystal using the beamline 1911-3 at MAX-lab (Lund, Sweden) and the structure of GLP-1 in complex with the GLP-1R ECD was solved to 2.1 Å resolution by molecular replacement (Fig. 1A). We removed exendin-(9-39) from the structure of the exendin-(9-39)-ECD complex (Protein Data Bank code 3C59) and used the apo-form of GLP-1R ECD as the search model for the molecular replacement. GLP-1 was then built into the model, its position was unambiguous due to good electron density for most of the ligand. Data collection and refinement statistics are summarised in Table 1. Throughout the text, GLP-1 and exendin-4 residues are designated with * and **, respectively. Exendin-4 is numbered 1-39 and GLP-1 is numbered 7-37, with * and **, respectively. Exendin-4 residues are designated with * and **, respectively. Exendin-4 is numbered 1-39 and GLP-1 is numbered 7-37.

Structure of the GLP-1R extracellular domain. The crystal structure of the ECD in the GLP-1-bound form shown here is very similar to the exendin-(9-39)-bound form shown previously (rmsd of 0.79 Å for Cα atoms of the ECD) (18). The ligand binding sites are identical, which is not surprising given the competitive binding of GLP-1 and exendin-4 for the full length GLP-1R (38), so the role of Trp31* is less pronounced. The α-helical conformation is further stabilised by intramolecular interactions between Glu16**, Glu17**, Arg20**, Glu24** and Lys27** on the hydrophilic face (18). In addition, Arg20** and Lys 27** interact with Glu128 and Glu127 of the ECD, respectively (18). The corresponding intramolecular stabilisation is not possible in GLP-1 due to a less favourable alignment of oppositely charged residues (Fig. 1B), and Glu127 of the ECD is not involved in binding of GLP-1 (Fig. 2A).

The hydrophilic face of GLP-1, which interacts with the ECD is defined by Ala 24*, Ala25*, Phe28*, Ile29*, Leu 32* and Val 33* (Fig. 2B). The importance of Phe28*, Ile29* and Leu 32* in GLP-1 binding has previously been demonstrated by Ala-scanning of GLP-1 (38). Substitution of Phe28* with Ala had the most severe effect on GLP-1 affinity in the Ala-scan (IC50 value increased by 1300 fold) and indeed Phe28* is centrally positioned in the ligand-receptor interface emphasising the importance of this hydrophobic ligand-receptor interaction. The Ile29*-Ala and Leu 32*-Ala substitutions also reduced GLP-1 affinity significantly (IC50 value increased by 93 and 17 fold respectively) (38). Trp31* is also on the hydrophilic face of GLP-1 but is rather solvent exposed and does not interact with the ECD (Fig. 2B). Trp31* is conserved in the glucagon peptide family (GLP-1, exendin-4, glucagon, GIP and GLP-2, Fig. 1B), which implies a unique role of this residue. However, substitution of Trp31* with Ala only reduced the binding affinity of GLP-1 slightly at the full length GLP-1R (38), so the role of Trp31* in receptor binding is unclear. Val33* is the final residue in the α-helix of GLP-1 and it is the final residue in the C-terminal of GLP-1 which interacts with the ECD (Fig. 2A and C). The side chain of Val33* makes hydrophobic contacts with Tyr69 and Leu 123 and the backbone carbonyl of Val33* interacts through a hydrogen bond with one of the terminal
of Leu123 is flipped towards Arg121, which again its side chain away from GLP-1. The side chain of Arg102 and by decreasing the distance between core of the ECD by rotating the guanidine group 1 specific conformations affect the conserved four residues in (or close to) the binding pocket (Fig. 3A). A water molecule is coordinated by the terminal nitrogen of Arg121 interacts with a water closing of this cavity is assisted by a side chain exendin-4(9-39)-bound structure (Fig. 3A). The functional consequences of the ligand-specific conformational differences are not known.

*Site-directed mutagenesis of the GLP-1R- In order to link the structural information of the isolated ECD with the binding and functional properties of the full length receptor, we targeted the ligand binding site of the ECD by site-directed mutagenesis. The mutants were characterised by their ability to bind GLP-1 and exendin-4, using the agonist 125I-GLP-1 tracer, and by their ability to stimulate cAMP production in response to GLP-1 and exendin-4 (Table 2). The main objective was to search for mutations with differential effect on GLP-1 and exendin-4. We initially focused on Glu127 which showed an obvious conformational difference in the two ligand-bound structures (Fig. 3A) – Glu127 interacts directly with exendin-4(9-39) but not with GLP-1 (18). The ECD structures suggest that the hydrogen-bonding potential of Glu127 is important for exendin-4 binding, but not for binding of GLP-1, and this is supported by the site-directed mutagenesis data (Table 2). Mutation of Glu127 to Ala reduced the affinity for exendin-4 but not for GLP-1 (6.8 versus 1.7 fold, respectively). Glu128 interacts with a positively charged residue in both GLP-1 and exendin-4, which may explain why the Glu128-Ala substitution did not have a differential effect on the binding affinity of GLP-1 and exendin-4. The differential effect of the Glu127-Ala mutation on GLP-1 and exendin-4 binding is rather small compared to the differential affinity of the isolated ECD shown previously (27). Clearly, the superior helical propensity of exendin-4 contributes strongly to its high affinity for the ECD. Several interactions are conserved in the two ECD structures, and mutagenesis of the implicated receptor residues was not expected to have differential effects on ligand binding (Table 2). Surprisingly the Leu32-Ala mutation reduced both the affinity and potency of exendin-4 relative to GLP-1 (7.1 and 9.5 fold, respectively, Table 2 and Fig. 4), demonstrating a ligand-specific effect of the Leu32-Ala mutation. Neither the potency nor the affinity of GLP-1 was
affected by the Leu\textsuperscript{32}-Ala mutation (Fig. 4) and the expression level of the receptor was similar to wild-type GLP-1R, which confirmed the structural integrity of this receptor mutant. Leu\textsuperscript{32} is the first residue in the \(\alpha\)-helix of the GLP-1R ECD and it defines the border of the hydrophobic binding cavity by interacting with Ala\textsuperscript{24}, Ala\textsuperscript{25} and Phe\textsuperscript{28} (Fig. 2B). The results suggest that Leu\textsuperscript{32} is important for the binding of exendin-4, but not for the binding of GLP-1 to GLP-1R. It is difficult to give a structural explanation of the ligand-specific effect of the Leu\textsuperscript{32}-Ala mutation by comparing the two ligand-bound forms of the ECD, because the structural differences in this region are quite subtle. Nevertheless, the ligand-specific effect of the Leu\textsuperscript{32}-Ala mutation supports the existence of differences in the binding modes of GLP-1 and exendin-4 to the full length GLP-1R. Clearly the two-domain binding mechanism of the full length GLP-1R is more complex than binding of the isolated ECD.

*The conformation of GLP-1: receptor-bound and in solution*- GLP-1 is highly flexible in aqueous buffers whereas in trifluoroethanol (TFE) a single-stranded \(\alpha\)-helix forms (Thr\textsuperscript{13} to Lys\textsuperscript{34}) with a less defined \(\alpha\)-helical region around Gly\textsuperscript{22}, as demonstrated by NMR spectroscopy (33;39). Structure-activity studies of GLP-1 showed that side chain to side chain cyclisation by lactam bridge formation of residues 16 to 20 and 18 to 22 were well tolerated. Cyclisation of residues 11 to 15 improved potency for GLP-1R compared to the linear counterpart (40;41). These data support the existence of an \(\alpha\)-helical conformation in the N-terminal part of GLP-1 when bound to the full length GLP-1R. It is interesting that in the ECD-bound structure, the Thr\textsuperscript{13}-Glu\textsuperscript{21} segment of GLP-1 adopts an \(\alpha\)-helical conformation even though this segment does not interact with the ECD. The C-terminal segment of GLP-1 (Ala\textsuperscript{24}-Val\textsuperscript{35}) is stabilised in a specific \(\alpha\)-helical conformation through binding to the ECD. This may subsequently stabilise an \(\alpha\)-helical conformation in the N-terminal part of the ligand (Thr\textsuperscript{13}-Glu\textsuperscript{21}). This hypothesis is supported by the solution structure of GLP-1 in different concentrations of TFE (39). GLP-1 is a random coil in pure water, but adding TFE enables the C-terminal segment of GLP-1 to adopt an \(\alpha\)-helical conformation. The C-terminal \(\alpha\)-helix is gradually extended towards the N-terminal of the peptide with increasing concentrations of TFE (39), and it seems possible that a similar mechanism is initiated upon binding of GLP-1 to the ECD. It was recently proposed that \(\alpha\)-helix formation of the ligand upon binding to the ECD is an important step in the activation of class B GPCRs (23). As shown in Fig. 5A, the \(\alpha\)-helix of GLP-1 has a central distortion of the backbone around Gly\textsuperscript{22} which is not observed in the exendin-4(9-39)-bound structure. The distortion is also observed in the NMR structures of GLP-1 in solution (39). However, we cannot exclude that the kink observed in the crystal structure is a result of crystal packing between the N-terminal part of GLP-1 (Gly\textsuperscript{10}-Glu\textsuperscript{21}) and symmetry related ECDs rather than a functionally important characteristic of GLP-1 (Fig. 5B-5D).

Substitution of Gly\textsuperscript{22} with Ala was previously shown not to affect the functionality or the binding affinity of GLP-1, which suggests that flexibility around Gly\textsuperscript{22} is not required for binding to or activation of GLP-1R (38). Interestingly, Leu\textsuperscript{32} of the ECD is positioned right next to the kink of GLP-1 (Fig. 5A). Thus, we have demonstrated a ligand specific effect of the Leu\textsuperscript{32}-Ala mutation and shown that ECD-bound GLP-1 has a kink right next to Leu\textsuperscript{32} whereas ECD-bound exendin-4(9-39) is straight. This may be a coincidence but it is tempting to speculate that there is a connection between the structural difference of the ligands (kinked or straight helix) and the differential effect of the Leu\textsuperscript{32}-Ala mutant on binding of the ligands.

A three-dimensional model of GLP-1R was recently published (21). From the NMR structure of GLP-1 in TFE (pdb code 1D0R, (39)), it was suggested that GLP-1 might assume one of two forms when bound to GLP-1R: A slightly kinked \(\alpha\)-helix or an L-shaped \(\alpha\)-helix, and the authors concluded that the L-shaped \(\alpha\)-helix conformation of GLP-1 seemed more reasonable (21). The crystal structure presented here is more compatible with the kinked conformation of GLP-1.

The structure of His\textsuperscript{7}-Gly\textsuperscript{10} was not determined in this study probably due to the inherent flexibility in this part of GLP-1 and other peptide ligands for class B receptors (33;39;42;43). The only structural evidence showing a unique conformation comes from a structural study of PACAP(1-21) comparing micelle- and receptor-bound states. Residues 1-7 of PACAP(1-21) adopt a specific \(\beta\)-coil structure upon receptor binding followed by an \(\alpha\)-helical structure of residues 8-21 (44). The relevance of the receptor-bound PACAP(1-21) structure for GLP-1 in particular is supported by a previous study.
of chimeric PACAP/GLP-1 peptides (45). Substituting five residues from the N-terminus of GLP-1 with those of PACAP (three non-conserved, Fig. 1B) had no effect on either the affinity or potency for GLP-1R. Thus, on the basis of both sequence homology and structure-activity, GLP-1 would be expected to adopt a similar conformation upon binding to the GLP-1R TM-domain.

Conclusion- The crystal structure presented here shows the molecular details of GLP-1 binding to the GLP-1R ECD – an essential step in the two-domain binding mechanism of GLP-1R and class B GPCRs in general. Collectively, results from structural characterisation of GLP-1 in solution, structure-activity analyses of GLP-1 analogous and the crystal structure of GLP-1 bound to the GLP-1R ECD presented here suggest that GLP-1 is a continuous α-helix from Thr$^{13*}$ to Val$^{33*}$ when bound to the full length GLP-1R. This is important information for the design of peptide therapeutics targeting GLP-1R. GLP-1 and exendin-4 share the same binding site of the GLP-1R ECD, but the ligand-specific effects on the ECD structure and the ligand-specific effects of receptor mutagenesis support the existence of differences in the binding modes of GLP-1 and exendin-4 to the full length GLP-1R. The nature of these differences as well as the active conformation of peptide agonists (kinked or not) and a better understanding of the two domain binding mechanism await structural characterisation of the full length GLP-1R.

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FOOTNOTES

2 The abbreviations used are: GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; 7TM, seven transmembrane; GPCR, G protein-coupled receptor; GLP-2, glucagon-like peptide-2; GIP, glucose-dependent insulinotropic polypeptide; PACAP, pituitary adenylyl cyclase-activating polypeptide; VIP, vasoactive intestinal polypeptide; CRF, corticotrophin-releasing factor; PTH, parathyroid hormone; ECD, extracellular N-terminal domain; TM-domain, extracellular loops and transmembrane α-helices; CRFR1, type-1 CRF receptor; PAC1-R, type-1 PACAP receptor; GIP-R, GIP receptor; PTH-1R, type-1 PTH receptor; GluR, glucagon receptor; ECL, extracellular loop.
Crystal Structure of Receptor-bound GLP-1

**LEGENDS**

Table 1. Data collection and refinement statistics
The data set was collected from a single crystal. Values in parentheses are for the highest resolution shell (2.2-2.1 Å). Rmsd, root mean square deviation.

Table 2. Functional and binding experiments with GLP-1R mutants
EC_{50} and IC_{50}-values are given in pM and nM, respectively, and expression levels are given in fmol/mg total protein. Data represent the mean ± SEM of three or more independent experiments performed in duplicates. The EC_{50} or IC_{50}-values of GLP-1 and exendin-4 are compared to the wild-type GLP-1R using the unpaired t-test, * P < 0.05, ** P < 0.01, no * means no significant difference. Numbers in parentheses equal the relative difference between EC_{50} or IC_{50}-values of GLP-1 and exendin-4 at each mutant compared to the wild-type receptor. ND, not detectable, possibly due to no expression or no binding.

Figure 1. Structure of the GLP-1-bound ECD of the GLP-1R
A, Stereoview of GLP-1 (blue) bound to the ECD of the GLP-1R (α-helix in black, β-strands in red and loops in gray). Disulphide bridges are shown as orange sticks. Residues Cys^{62}-Asp^{67} (β_1) and Ala^{70}-Gly^{75} (β_2) constitute the first region of antiparallel β-sheets, and the second region is comprised of residues Gly^{78}-Ser^{84} (β_3) and His^{90}-Thr^{105} (β_3), which is shown in red. Our final structure contains GLP-1 residues Gly^{103}-Gly^{35}. The residues that interact with GLP-1R ECD lie within Ala^{24*} and Val^{33*}, which are shown as sticks. B, Sequence alignment of GLP-1, exendin-4, GIP, GLP-2, glucagon and PACAP(1-27). Fully conserved residues are highlighted in yellow, and partially conserved residues are highlighted in green. The residues of GLP-1 and exendin-4 that interact with GLP-1R ECD are coloured blue. The underlined residues symbolise residues of GLP-1 in α-helical conformation when bound to the ECD. Residue number 1 of exendin-4 corresponds to residue number 7 of GLP-1.

Figure 2. Interactions between GLP-1 and GLP-1R ECD
A, Ribbon diagram of GLP-1 and its hydrophilic interactions with GLP-1R ECD. GLP-1 is coloured in marine, and residues Gln^{23*}, Lys^{26*}, Glu^{27*}, Trp^{31*} and Val^{33*} are illustrated as sticks. Receptor residues Arg^{121}, Leu^{123}, Glu^{127} and Glu^{128} are shown as sticks. The surface of the hydrophilic binding cavity of ECD is illustrated in gray. B, Ribbon diagram of GLP-1 and its hydrophobic interactions with GLP-1R ECD. GLP-1 residues Ala^{24*}, Gly^{27*}, Phe^{28*}, Trp^{31*} and Leu^{32*} are illustrated as sticks, and so are ECD residues Leu^{32}, Trp^{39}, Asp^{67} and Arg^{121}. The surface of the hydrophobic binding cavity of ECD is illustrated in gray. C, Ribbon diagram illustrating a common motif found in the GLP-1R ECD and in the GIP-R ECD. The side chain of Arg^{121} interacts with the backbone carbonyls of Asp^{67} and Leu^{32*} through a water molecule. GLP-1 residues Leu^{32*} and Val^{33*} are illustrated as sticks, and so are ECD residues Asp^{67} and Arg^{121}.

Figure 3. Differences between the GLP-1- and exendin-4(9-39)-bound structure of ECD
Ribbon diagrams showing significant differences in side chain conformations between the GLP-1-bound structure and the exendin-4(9-39)-bound structure of GLP-1R ECD. Receptor- and ligand residues are highlighted in blue for the GLP-1-bound structure and in orange for the exendin-4(9-39)-bound structure. Water molecules in orange are present only in the exendin-4(9-39)-bound structure. A, One diverging residue, Val^{33*} of GLP-1 and Lys^{27*} of exendin-4(9-39), causes a shift in the conformations of four residues namely Glu^{127}, Leu^{123}, Arg^{121} and Pro^{119}. B, The GLP-1-specific conformations affect the conserved core of the ECD by rotating the guanidine group of Arg^{102} and by decreasing the distance between Asp^{67} and Arg^{102} compared to the exendin-4(9-39)-bound structure without affecting the relative position of Trp^{52} and Trp^{102}.

Figure 4. Functional- and binding properties of the Leu^{32}-Ala GLP-1R mutant
Upper panel, Stimulation of cAMP production by transiently transfected HEK293 cells expressing the Leu^{32}-Ala mutant by GLP-1 (squares, A) and exendin-4 (circles, B). Dashed dose-response curves...
represent cAMP production by GLP-1 and exendin-4 at the wild-type GLP-1R, respectively. **Lower panel.** Competition binding assay on plasma membranes from transiently transfected HEK293 cells expressing the Leu$^{32}$-Ala mutant. GLP-1 binding curves are presented with squares (C) and exendin-4 curves with circles (D). Dashed binding curves represent $^{125}$I-GLP-1 displacement by GLP-1 and exendin-4 at the wild-type GLP-1R. Data are normalised according to $^{125}$I-GLP-1 binding, and correspond to three independent experiments performed in duplicates.

**Figure 5. Crystal packing**
A, Superposition of ECD-bound GLP-1 (blue) and exendin-4(9-39) (cyan). GLP-1 residue Gly$^{22*}$ denotes a kink in the $\alpha$-helix, which is situated in close proximity to Leu$^{32}$ of the ECD. B, Crystal packing involving symmetry related complex molecules C, Ribbon diagram of GLP-1 (blue) and its interactions with the ligand of a symmetry related molecule. Residues Tyr$^{19*}$, Gln$^{23*}$ and Glu$^{27*}$ are shown as sticks, and the surface of the GLP-1R ECD is shown in gray. The packing of complex molecules allows Tyr$^{19*}$ to interact with Gln$^{23*}$ (3 Å) and Glu$^{27*}$ (2.5 Å) in a symmetry related ligand molecule. D, Interactions between GLP-1 and residues of symmetry related ECD molecules. GLP-1 residues Thr$^{11*}$, Thr$^{13*}$, Ser$^{14*}$ and Glu$^{21*}$ are shown as sticks. The backbone carbonyl of Thr$^{11*}$ could form a weak hydrogen bond (3.2 Å) to the backbone amide of Gln$^{112}$, the backbone amide of Thr$^{13*}$ could form a hydrogen bond (2.9 Å) to the backbone carbonyl of Gln$^{112}$ and the backbone amide of Ser$^{14*}$ may form a hydrogen bond (3 Å) to the side chain of Asp$^{114}$. The side chain of Glu$^{21*}$ forms a hydrogen bond to the backbone amide of Phe$^{80}$ (2.6 Å).
TABLE 1

| Space group      | P2₁2₁2₁          |
|------------------|-------------------|
| Unit cell dimensions (Å) |                  |
| a                | 35.7              |
| b                | 42.7              |
| c                | 95.1              |
| **Data collection** |                  |
| Wavelength (Å)   | 1.0               |
| Resolution range (Å) | 95.1-2.1 (2.2-2.1) |
| Total reflections | 73811             |
| Unique reflections | 10348            |
| Completeness (%) | 97.9              |
| I/σ(I)           | 14.0 (5.3)        |
| R_sym            | 11.1 (44.9)       |
| **Refinement statistics** |         |
| Number of non-hydrogen atoms | 1109          |
| Resolution (Å)   | 95.1-2.1 (2.155-2.100) |
| Total reflections | 8786             |
| Reflections in test set | 429            |
| R_work           | 0.181 (0.187)     |
| R_free           | 0.226 (0.255)     |
| Average B factors | 13.6             |
| **rmsd**         |                   |
| Bond lengths (Å) | 0.02              |
| Bond angles (°)  | 1.7               |
### TABLE 2

| Mutant | EC\textsubscript{50} (pM) | IC\textsubscript{50} (nM) | Expression level (fmol/mg) |
|--------|-----------------|-----------------|---------------------|
| WT     |     |                  | 5.5 ± 0.16          |
| L32A   | 12 ± 2.7 (1.1) | 52 ± 24 (9.5)   | 1.1 ± 0.17 (1.1) 5.4 ± 1.5* (7.1) 6.0 ± 0.40 |
| T35A   | 30 ± 7.4 (2.7) | 23 ± 12 (4.2)   | 3.1 ± 0.80* (3.1) 0.44 ± 0.1** (0.6) 0.38 ± 0.01 |
| V36A   | 57 ± 32 (5.2)  | 36 ± 20 (6.5)   | 2.8 ± 0.86 (2.8) 0.98 ± 0.34 (1.3) 5.7 ± 0.28 |
| E68A   | 15 ± 2.4 (1.4) | 9.5 ± 4.2 (1.7) | 1.9 ± 0.90 (1.9) 0.48 ± 0.07 (0.6) 5.7 ± 0.07 |
| Y69A   | ND              | ND              | ND                  |
| Y88A   | ND              | ND              | ND                  |
| L89A   | ND              | ND              | ND                  |
| P90A   | 55 ± 12* (5.0) | 30 ± 7.3* (5.5) | 2.8 ± 1.6 (2.8) 1.6 ± 0.28 (2.1) 5.6 ± 0.22 |
| R121A  | 51 ± 13* (4.6) | 44 ± 10* (8.0)  | 2.3 ± 1.1 (2.3) 1.2 ± 0.02 (1.6) 5.7 ± 0.37 |
| L123A  | 17 ± 4.6 (1.5) | 9.5 ± 1.9 (1.7) | 1.1 ± 0.38 (1.1) 0.33 ± 0.04 (0.4) 2.6 ± 0.09 |
| E127A  | 13 ± 4.2 (1.2) | 11 ± 0.45* (2.0) | 1.7 ± 0.76 (1.7) 5.2 ± 1.8 (6.8) 5.6 ± 0.46 |
| E127Q  | 12 ± 3.3 (1.1) | 6.8 ± 1.4 (1.2) | 1.1 ± 0.27 (1.1) 0.82 ± 0.15 (1.1) 6.11 ± 0.21 |
| E128A  | 28 ± 6.2 (2.5) | 25 ± 6.7* (4.5) | 2.7 ± 0.91 (2.7) 1.8 ± 0.48 (2.4) 6.0 ± 0.49 |
| E128Q  | 10 ± 3.0 (0.9) | 8.0 ± 3.3 (1.5) | 0.66 ± 0.11 (0.7) 0.45 ± 0.1 (0.6) 5.2 ± 0.12 |
FIGURE 1

A

B

GLP-1(7-37) HAEGETFTSDVSSYLEGQAAKEIAYMVKGRG
Exendin-4 HEGFTFTSDLKQMEEEAVIDFIEW1KNGGPPSGAPPSS
GIP YAEGETFISDYIAMDKIHQQDEVNLALLAQAQLGGKKNWWKHNTQ
GLP-2 PHADEGTFTSDYISKLAMNLAARDPFWFVVIQTKITD
Glucagon HSQGTFTSDY5KYLD5RQQDEVQVIQNT
PACAP(1-27) HSDGIFTDSY5R5YRKQMAVKKYYLAAVL

1 5 10 15 20 25 30 35
Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor
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