Structure/function studies of the first extracellular loop of the glucagon-like peptide-1 receptor

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Introduction

Glucagon-like peptide-1 (GLP-1) is a 30 amino-acid hormone mainly secreted by intestinal cells. It is the most potent stimulator of insulin release in response to food intake so far described. GLP-1 inhibits postpandrial gastric emptying and acid secretion, and also appears to play a key role in the central regulation of appetite. Since its diverse but synergic effects collectively alleviate the Type 2 diabetic phenotype, the study of the hormone and its specific receptor has attracted great interest for the design of novel antidiabetic agents.

The glucagon-like peptide-1 receptor (GLP-1R) belongs to the Family B of G protein-coupled receptors (GPCRs). One of the distinguishing features of this subclass of GPCRs is a relatively large extracellular N-terminus that incorporates three disulphide bridges, thus forming a globular domain. Although the peptide has major points of interaction in this domain, experimental evidence suggests that this isolated fragment is insufficient for high-affinity binding and additional contact sites have also been identified in the extracellular loops and outer portion of transmembrane helices of the GLP-1 R and other members of this family. Upon activation, the receptor couples with G_S, initiating the sequential stimulation of adenylate cyclase and protein kinase A.

The aim of our study is to identify specific hormone interaction points and/or activation determinants located in the extracellular loops of the receptor. Using a site-directed mutagenesis approach, pairs of residues have been systematically mutated to alanine (or valine in place of a native Ala), in order to remove functional side groups without likely perturbing the tertiary structure of the region. Once a double mutation shows a functional effect, then the subsequent single mutations are performed to determine the individual residue responsible for it.

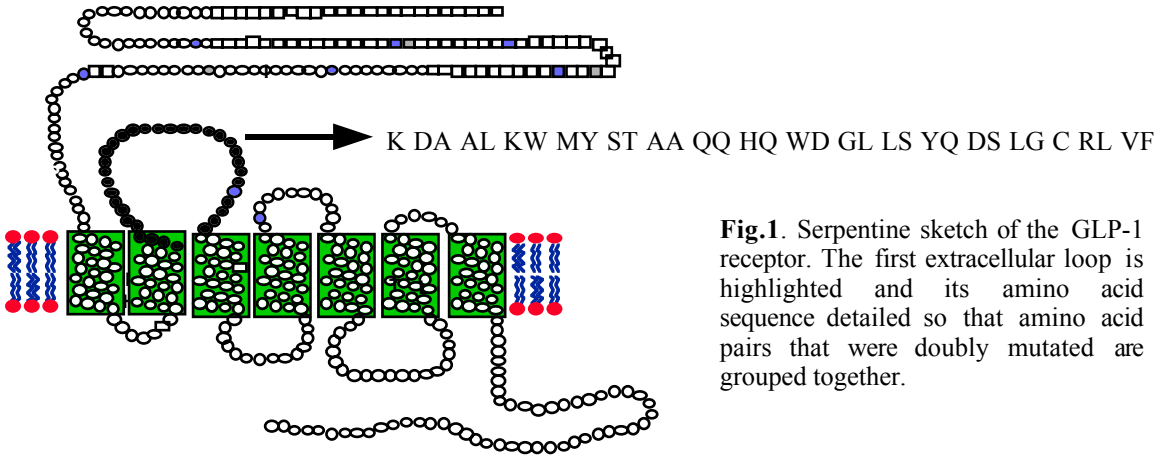

Fig.1. Serpentine sketch of the GLP-1 receptor. The first extracellular loop is highlighted and its amino acid sequence detailed so that amino acid pairs that were doubly mutated are grouped together.

Methodology

Residues spanning the first extracellular loop of the GLP-1R have been mutated to alanine in pairs, with the exception of Lys and Cys, which have been individually changed (**Fig.1**). In this way, the following mutant receptors were prepared: K:A, DA:AV, AL:VA, KW:MY, ST:AA, AA:VV, QQ:AA, HQ:AA, WD:AA, GL:AA, LS:AA, YQ:AA, DS:AA, LG:AA,

C:A, RL:AA and VF:AA. The substitutions have been made by site-directed mutagenesis (QuickChange^{TM}, Stratagene), with the template wild-type GLP-1R in the pcDNA3 vector. The wild-type and mutant GLP-1 receptors have been stably transfected into HEK-293 cells (SuperFect® reagent; Invitrogen) and selection of transfected cells is maintained by the addition of the G418 antibiotic.

The effect of the substitutions on ligand binding affinity has been assessed through homologous competitive binding assays, determining [^{125}I]GLP-1(7-36)amide binding to either transfected cells or membrane preparations of such cells. The ability of the receptor to be activated is determined by estimating the cAMP accumulated after GLP-1(7-36)amide stimulation. Briefly, the method utilises a preincubation step with [^{3}H]-adenine prior to agonist addition for a few minutes and acidic lysis of the cells. The [^{3}H]-cAMP produced is purified through Dowex and alumina columns by cation exchange chromatography. A standard amount of [adenine-U-^{14}C]-cAMP is also added to all columns to assess individual column efficiency. The GraphPad Prism^{TM} data analysis program is used to calculate IC_{50} and EC_{50} values, as well as the significance of results compared to wild-type parameters. B_{max} values, a reflection of receptor surface expression, are also determined with the combination of a protein assay on membranes and the binding data derived from them.

Definition of the GLP-1R binding pocket

Both IC_{50} and EC_{50} values for the wild-type GLP-1R are found to be approximately 1 nM in this system. All double mutants appear to display affinity and activation parameters in a close range within these values, except the K:A, DA:AV, MY:AA, C:A and RL:AA mutant receptors, which show reduced affinity and, hence, low EC_{50}. Surface expression of all receptors seems not to be significantly different to the wild-type GLP-1R.

Based on this finding, new mutants were designed. In order to confirm that the charged residues Asp and Arg were responsible for the effect detected with their corresponding double mutant receptor, single substitutions of these two residues were made. Additionally, Asp was also mutated to Asn to assess the role of its charge. The D:A and R:A mutant receptors provided the expected results, showing low functionality. Interestingly, the D:N mutant possesses normal affinity and activation profiles, indicating that the polar side-chain of Asp, but not the negative charge itself, is involved in binding GLP-1. The importance of the charged residues Lys, Asp and Arg is not surprising. They are almost totally conserved in Family B GPCRs and, in fact, experimental evidence proving they contribute to high-affinity binding exits for the GLP-1R and other related receptors. Thus, they are postulated to interact with oppositely charged residues in the GLP-1, possibly amino acids also highly conserved across the related ligands.

Similarly, the conserved Cys residue forms a putative disulphide bond with another conserved Cys in the second extracellular loop of the GLP-1R. Although this pairing has not been directly shown in this family of receptors, its crucial role in maintaining tertiary structure has been widely demonstrated in the Family A of GPCRs.

On the contrary, the results obtained from the study of the Met and Tyr residues were unanticipated and complex. The double substitution of these amino acids resulted in undetectable GLP-1 binding and a remarkably reduced EC_{50}. However, the single MY:AY and MY:MA mutant receptors exhibit a virtually wild-type behaviour. In an attempt to

elucidate the implication of the side chains and functional groups of the Met and Tyr, the MY pair has been mutated to a range of amino acids intermediate between the native residue and alanine. Consequently, the mutant receptors MY:AF, MY:AV and MY:VA were made. These three more subtle changes produce mutant receptors with a 10-100 fold decreased affinity and activation ability, which are in-between the former double and single mutant receptors (*e.g.* **Fig. 2**), while cell surface expression is not reduced for any of them. The significance of differences between these mutants still needs to be further assessed before safe conclusions can be drawn. Notably, Tyr (or Phe) is a relatively highly conserved residue across Family B, whereas Met is specific for the GLP-1R. Thus, putative ligand interactions would be likely to occur with conserved and specific amino acids in the hormone, respectively.

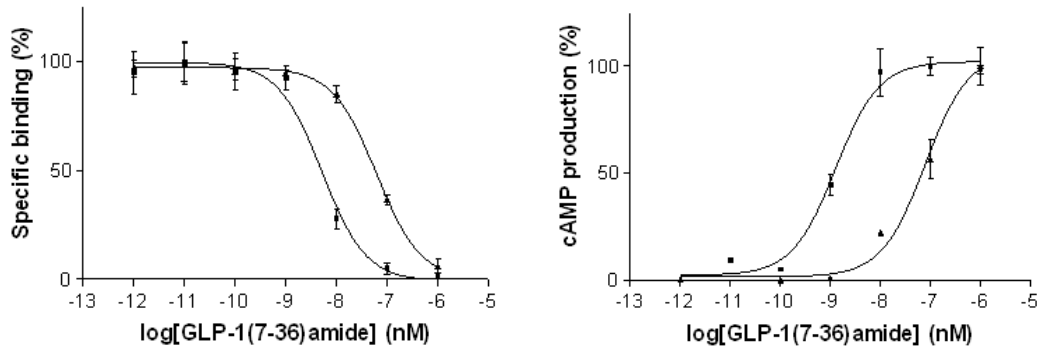

Fig.2. Binding and activation profiles obtained for the wild-type (■) and MY:VA (▲) receptors.

At present, work to define exactly the implication of the Met and Tyr residues in hormone binding is in progress. New substitute residues have been introduced at these positions and their functional effects will be determined. Moreover, a similar double alanine scan is being carried out in the remaining extracellular loops to identify additional determinants involved in GLP-1 binding and/or receptor activation. Hopefully, our work will improve our understanding of how the GLP-1R functions and will contribute to the design of bioactive agonists for the receptor.

Funding
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