Peptide binding at the GLP-1 receptor

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Abstract

The receptor for GLP-1 [glucagon-like peptide-1-(7–36)-amide] is a member of the ‘Family B’ of GPCRs (G-protein-coupled receptors) comprising an extracellular N-terminal domain containing six conserved cysteine residues (the N-domain) and a core domain (or J-domain) comprising the seven transmembrane helices and interconnecting loop regions. According to the two-domain model for peptide binding, the N-domain is primarily responsible for providing most of the peptide binding energy, whereas the core domain is responsible for binding the N-terminal region of the peptide agonists and transmitting the signal to the intracellular G-protein. Two interesting differences between the binding properties of two GLP-1 receptor agonists, GLP-1 and EX-4 (exendin-4), can be observed. First, while GLP-1 requires its full length to maintain high affinity, the eight N-terminal residues of EX-4 can be removed with little reduction in affinity. Secondly, EX-4 (but not GLP-1) can bind to the fully isolated N-domain of the receptor with an affinity matching that of the full-length receptor. In order to better understand these differences, we have studied the interaction between combinations of full-length or truncated ligands with full-length or truncated receptors.

Introduction

In order to better understand how GLP-1 [glucagon-like peptide-1-(7–36)-amide] and its analogues bind and activate the GLP-1R (GLP-1 receptor), our group have spent several years dissecting the molecular details of the peptide–receptor interaction. This paper reviews our laboratory’s work to date.

GLP-1 is a 30-amino-acid peptide hormone secreted into the bloodstream in response to the ingestion of food. It potentiates insulin secretion by pancreatic β-cells in a glucose-dependent manner and has also been shown to stimulate transcription of the proinsulin gene, stimulate insulin biosynthesis, slow down gastric emptying and reduce food intake [1]. As a result of such biological actions, GLP-1 and its analogues have received much attention as potential tools for the treatment of Type 2 diabetes. Indeed, the US Food and Drug Administration (FDA) has recently approved one such analogue (exenatide, trade name Byetta [2]) as an adjunctive therapy to oral diabetes medications. To date there are no non-peptidic GLP-1 analogues suitable for therapeutic use, and this remains an important goal for many pharmaceutical companies.

Exenatide is the name for the synthetic version of the peptide EX-4 (exendin-4), a 39-amino-acid peptide secreted by the salivary glands of the Gila monster (Heloderma suspectum). EX-4 is a potent agonist at GLP-1R, but it possesses a much longer half-life owing to its resistance to N-terminal cleavage by the protease DPP-IV (dipeptidyl peptidase IV). While N-terminal truncation of GLP-1 greatly reduces its affinity for GLP-1R, N-terminally truncated analogues of EX-4 maintain high receptor affinity. Indeed, the removal of the first eight residues yields a high-affinity antagonist, EX-4-(9–39) [3].

The receptor for GLP-1 is a member of the ‘Family B’ of GPCRs (G-protein-coupled receptors) and hence comprises a characteristic extracellular N-terminal domain containing six conserved cysteine residues (the N-domain) and a core domain comprising the 7TMs (seven transmembrane helices) and interconnecting loop regions. According to the two-domain model for peptide binding (see, e.g., [4–6]), the N-domain is primarily responsible for providing most of the peptide binding energy, whereas the core domain is responsible for binding the N-terminal region of the peptide agonists and transmitting the signal to the intracellular G-protein.

The N-domain

The three-dimensional structure of most of the N-domain of a Family B GPCR, the mouse CRF-2β (corticotropin-releasing factor 2β) receptor, has been determined by NMR, opening up the possibility of a detailed analysis of peptide binding at this domain [7]. The structure revealed that the N-domain represents an example of a scr (short consensus repeat) fold and that the most essential features that stabilize this fold (e.g. a buried salt bridge, three disulfide bonds and two buried tryptophan residues) are conserved across the entire Family B GPCR class. The disulfide connectivity in the NMR structure is compatible with that observed in earlier analyses of the isolated N-domain of GLP-1R [10], reinforcing the view that this NMR structure could be used as a template to build homology models of the domain of GLP-1R. Nevertheless, since the NMR structure lacks the first 19–20 amino acids of...
the mature receptor, it cannot enable us to interpret the role of this region in recognizing ligands, a region which we find to be involved in peptide binding.

Using an *Escherichia coli* expression system, we have refolded the isolated N-domain of GLP-1R from inclusion bodies [8]. The expressed domain, constituting residues Ala21–Leu144 from GLP-1R, binds GLP-1 poorly as expected from the previous work [9,10]. However, surprisingly, we discovered that the isolated N-domain binds exendin peptides with affinity equivalent to the full-length receptor [8], suggesting that the two peptides may have rather different binding mechanisms. In order to remove the possibility that these differences were due to the bacterial expression system and the *in vitro* refolding protocol, we have also analysed a membrane-tethered version of the N-domain expressed (with its signal peptide) in HEK-293 (human embryonic kidney) cells [6]. As with the N-domain expressed in bacteria, we observed that the membrane-tethered N-domain in the mammalian system had greatly reduced GLP-1 affinity but unaltered EX-4-(9–39) affinity compared with the full-length receptor.

The disulfide connectivity of the refolded N-domain of GLP-1R has been determined [10]. In order to discern whether any of the disulfide bonds were an artefact of refolding in the absence of the core domain, and hence unnecessary for EX-4-(9–39) binding, we prepared three double mutants and expressed proteins in which each putative disulfide was removed (C46A/C71A, C62A/C104A or C85A/C126A). By preparing a double cysteine-to-alanine mutant in each case, we aimed to reduce the chances of any spurious disulfide bond formation taking place due to a non-partnered cysteine residue, which would have been likely had single cysteine-to-alanine mutants been refolded in the optimized *in vitro* conditions. The three double mutants were expressed, refolded and analysed alongside the non-mutated control protein, as described in [8]. The protein preparations had equivalent purity and the single protein bands were indistinguishable from each other on SDS/polyacrylamide gels. Nevertheless, while high-affinity 125I-EX-4-(9–39) binding was obtained for the non-mutated Ala21–Leu144 N-domain preparation, the three double mutants showed no detectable binding, suggesting that each of the three disulfide bonds is critical for obtaining a functional protein domain [11].

In order to determine whether the N- and C-terminal regions of the N-domain were required for EX-4-(9–39) binding, truncated versions of the original Ala21–Leu144 N-domain were prepared in order to express proteins constituting residues Ala21–Ser136, Ala21–Glu127, Gly22–Glu127, Pro23–Glu127, Arg24–Glu127, Pro25–Glu127, Ser33–Leu144, Gly22–Leu144 and Ser33–Ser136. Figure 1(A) shows a cartoon of the N-domain with some of the truncation points highlighted. These truncations were expressed, refolded and analysed alongside the non-truncated Ala21–Leu144 control protein. On the basis of SDS/polyacrylamide gels, the truncated protein preparations were each obtained in equivalent quantity and purity, but had, as expected, differing apparent molecular masses according to the extent of the truncation. High-affinity 125I-EX-4-(9–39) binding was obtained for the non-truncated Ala21–Leu144 N-domain and for the C-terminal truncations Ala21–Ser136 and Ala21–Glu127, demonstrating that residues 128–144 were not required for high-affinity antagonist binding. The Ala21–Glu127 truncation was characterized in more detail and was shown to have similar properties to the membrane-tethered version of the N-domain, with low GLP-1 affinity and high EX-4-(9–39) affinity which was dependent on the presence of the tryptophan-cage motif (Table 1).
High specific binding was also obtained for the Gly²²–Glu¹²⁷, Pro³¹–Gly¹²⁷, Arg²¹–Glu¹²⁷ and Pro²⁵–Glu¹²⁷ N-domains, suggesting that residues 21–25 were not essential for high-affinity EX-4-(9–39) binding. However, no specific binding could be detected for the remaining proteins. Hence, since Pro³⁵–Glu¹²⁷ and Ala²¹–Leu¹⁴⁴ bind the antagonist while Ser³³–Leu¹⁴⁴ cannot, it suggests that the removal of one or more residues between Pro³⁵ and Leu³² is responsible for abolishing the high-affinity EX-4-(9–39) binding [11]. Figure 1B shows the smallest domain for which we were able to demonstrate high-affinity EX-4-(9–39) binding and highlights the extreme N-terminal region required for high-affinity EX-4-(9–39) binding. This region was analysed further in the full-length receptor by a mutation scan in which each residue between Arg³³ and Leu³² was individually mutated to tryptophan. The mutant receptors were expressed in HEK-293 cells and analysed by radioligand binding, and, from this screen, Leu³² was identified as the only residue involved in EX-4-(9–39) binding. Compared with the wild-type receptor (pIC₅₀ 8.8 ± 0.01, n = 3), the mutant L32W had a 10-fold reduction in EX-4 affinity (pIC₅₀ 7.8 ± 0.01, n = 3), while that of L32A was reduced 30-fold (pIC₅₀ 7.3 ± 0.01, n = 3) [11].

### Table 1 | pIC₅₀ values (means ± S.E.M.; n = 3) for three peptide ligands at the isolated N-domain Ala²¹–Leu¹⁴⁴ refolded from E. coli inclusion bodies [11] and the membrane-tethered N-domain (rNT–TM1) expressed in HEK-293 cells [6]

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Domain ...</th>
<th>pIC₅₀ value</th>
<th>nNT–TM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>EX-4-(9–39)</td>
<td>Ala²¹–Leu¹⁴⁴</td>
<td>7.9 ± 0.1</td>
<td>7.9 ± 0.1</td>
</tr>
<tr>
<td>EX-4-(9–30)</td>
<td>Ala²¹–Leu¹⁴⁴</td>
<td>6.4 ± 0.1</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Ala²¹–Leu¹⁴⁴</td>
<td>6.4 ± 0.2</td>
<td>6.1 ± 0.1</td>
</tr>
</tbody>
</table>

Model
Our model for peptide binding at GLP-1R is compatible with models for peptide–receptor binding at other Family B GPCRs (see, e.g. [4,5]). We define two peptide–receptor interactions N and H, common to GLP-1 and EX-4, with an additional Ex interaction unique to EX-4 and its N-terminally truncated analogues (Figure 2). We propose that the N interaction is between the N-terminus of the peptide and the receptor core domain, where it provides interactions that contribute to both affinity and activity. The N interaction formed by GLP-1 is clearly of a greater magnitude to that formed by EX-4, suggesting that additional peptide–receptor interactions are made with the N-terminal region of the natural hormone. The H interaction, which provides in the region of 80% of the total binding energy of GLP-1 and EX-4, occurs between the receptor’s N-terminal domain and the helical region of the peptide, in particular with the face of the helix composed of the conserved residues (EX-4...
The interactions H, N and Ex are depicted. N describes an interaction between the N-terminal region of the peptide and the core domain of GLP-1R, this interaction is stronger for GLP-1 than for EX-4. H describes the interaction between the helical region of the peptides and the N-domain of the receptor; this accounts for approx. 80% of the binding energy for both peptides. Ex describes an interaction absent from GLP-1 that accounts for EX-4’s N-independent affinity and its ability to bind with high affinity to the isolated N-domain. It is dependent, either directly or indirectly, upon the nine-residue C-terminal extension which forms the tryptophan-cage.

Figure 2 | Cartoons of GLP-1R binding to (A) GLP-1 and (B) EX-4

The interactions H, N and Ex are depicted. N describes an interaction between the N-terminal region of the peptide and the core domain of GLP-1R; this interaction is stronger for GLP-1 than for EX-4. H describes the interaction between the helical region of the peptides and the N-domain of the receptor; this accounts for approx. 80% of the binding energy for both peptides. Ex describes an interaction absent from GLP-1 that accounts for EX-4’s N-independent affinity and its ability to bind with high affinity to the isolated N-domain. It is dependent, either directly or indirectly, upon the nine-residue C-terminal extension which forms the tryptophan-cage.

Site-directed mutagenesis

Although site-directed mutagenesis is useful for the identification of ligand binding residues, it often does not yield information about which part of the ligand is involved in interacting with the mutated residue. However, the overlapping yet distinct binding properties of GLP-1 and EX-4 enable a more useful approach to be taken. Since high-affinity binding for GLP-1 depends upon its N-terminal sequence, it would be expected that the disruption of the N interaction would lead to a marked reduction in GLP-1 affinity. In addition, according to the peptide-binding model described above, a receptor residue that is involved in the N interaction should be in the core domain and hence should not interact with EX-4(9–39). This provides the opportunity of using a very clear screening assay for identifying receptor residues involved in the N interaction between GLP-1 and GLP-1R, since their mutation should specifically reduce GLP-1 affinity without reducing that of EX-4(9–39). Likewise, the mutation of core domain residues that interact with the N-terminal region of GLP-1 should not reduce GLP-1(15–36) affinity, since it binds to the N-domain. Indeed, removal of the N interaction of GLP-1 should reduce its affinity to that of its truncated analogue. We have successfully used such an approach to identify a number of residues in (or close to) the first and second extracellular loops which, when substituted, result in a mutant receptor with unaltered EX-4(9–39) and GLP-1(15–36) affinity, but with GLP-1 affinity reduced to that of GLP-1(15–36) [13–15]. Some of our current work involves attempting the converse, i.e., the identification of residues that are involved in the Ex interaction by mutating residues in the N-domain and looking for mutant receptors that have reduced EX-4(9–39) affinity but unaltered affinity for GLP-1 and EX-4(1–30).

Summary

The receptor for GLP-1 is an important target for potential drugs for the treatment of Type 2 diabetes. It is a typical Family B GPCR and hence binds peptide ligands according to the two-stage model found in many other family members. However, despite their similarity, the peptide agonists GLP-1 and EX-4 bind to GLP-1R in different modes, with EX-4 relying more on its interaction with the N-domain, while GLP-1 utilizes greater interactions with the core domain.

References


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