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The glucagon-like peptide-1 receptor binding site for the N-terminus of GLP-1 requires polarity at Asp198 rather than negative charge

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Abstract The mutation of Asp198 to Asn in the receptor for glucagon-like peptide-1(7–36)amide (GLP-1) had no effect upon GLP-1 affinity whereas substitution with Ala greatly reduced affinity, demonstrating the importance of polarity rather than negative charge at Asp198. However, the Asp198-Ala mutation had less effect upon the affinity of Exendin-4, a peptide agonist that has been shown previously not to require its N-terminus for high affinity. Moreover, the affinity of a truncated GLP-1 analogue lacking the first eight residues was not affected by the Asp198-Ala mutation, demonstrating that Asp198 is required for maintaining the binding site of the N-terminal region of GLP-1.

Key words: G protein-coupled receptor; Receptor; Agonist; Glucagon-like peptide-1(7–36)amide; Antagonist; Exendin

1. Introduction

Glucagon-like peptide-1(7–36)amide (GLP-1; Fig. 1A) is a 30-residue peptide hormone which plays an essential role in blood glucose homeostasis by augmenting glucose-induced insulin secretion [1]. GLP-1 is a potent 'incretin' which has been shown to increase glucose-dependent insulin secretion [2–4], decrease glucose-dependent glucagon secretion [5,6] and decelerate gastric emptying [7]. In addition, GLP-1 has been shown to reduce food intake in rats [8] and to stimulate pro-insulin gene transcription and biosynthesis in pancreatic β-cells [9,10]. These important actions of GLP-1, particularly upon pancreatic secretion, highlight the GLP-1 receptor as a target for therapeutic agents designed to modulate blood sugar levels. GLP-1 represents a promising approach towards the treatment of hyperglycaemia resulting from diabetes, especially since its actions, unlike those of sulphonylureas, are glucose-dependent and hence do not result in hypoglycaemic side effects [11,12]. However, since the half-life of GLP-1 following subcutaneous injection is very short [13], future research requires the design of physiologically stable GLP-1 receptor agonists.

The venom of the Gila monster *Heloderma suspectum* contains several peptides including a GLP-1 receptor agonist, Exendin-4, a 39-amino acid peptide with 50% sequence identity to GLP-1 (Fig. 1A). Interestingly, this peptide can be truncated by up to eight residues at its N-terminus without significant loss of receptor affinity, although the first two amino acids are essential for efficacy [14–17]. While the N-termini of GLP-1 and Exendin-4 are almost identical, Exendin-4 contains an extra nine amino acids at the C-terminus which have been shown to form a 'Trp cage' by nuclear magnetic resonance (NMR) analysis when in an aqueous environment [18]. The central region of Exendin-4 (residues 10–30) only shares eight identical residues with GLP-1. However, NMR analysis of Exendin-4 has shown this region to be largely helical [18] and it is interesting to observe that the eight residues shared by GLP-1 and Exendin-4 in this region all lie on the same face of an ideal α-helix (Fig. 1B), suggesting that it is this face of the helix that makes the critical contact with the receptor. While Exendin-4 can be truncated without loss of receptor affinity, GLP-1 itself is highly sensitive to N-terminal cleavage [13,14].

The receptor for GLP-1 is a 'family B' G protein-coupled

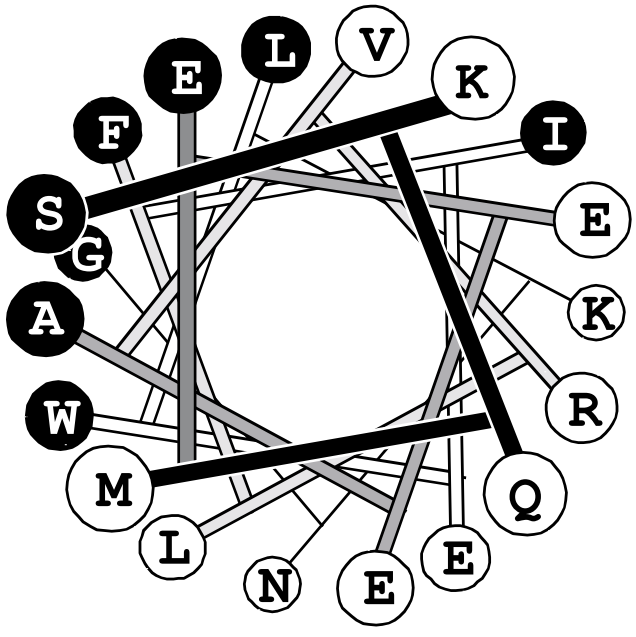

Fig. 1. A: The aligned sequences of various GLP-1 receptor peptides used in this analysis. B: A helical wheel showing the sequence of Exendin-4 from residues Ser11 to Gly29. Residues identical to those in GLP-1 are shown in white lettering on a black background while those unique to Exendin-4 are shown in black lettering.

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Abbreviations: GLP-1, glucagon-like peptide-1(7–36)amide; DMEM, Dulbecco's modified Eagle's medium; GPCR, G protein-coupled receptor

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receptor (GPCR) [19]. GLP-1 binds predominantly to the N-terminal domain of its receptor [20–22] although other regions of the receptor are also involved (e.g. [21,23]). The aim of this work is to determine the role of the negatively charged residue Asp198, located near the extracellular boundary of transmembrane helix 2, in agonist affinity and activation. Asp198 is conserved in many family B GPCR families and has previously been shown to play a role in ligand binding and receptor activation [21,24–26] although the precise nature of this residue's role in agonist and antagonist binding has not been elucidated.

2. Materials and methods

2.1. Mutagenesis

Asp198 was mutated to alanine or asparagine using the QuikChange® Site-Directed Mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The template vector was pcDNA3 (Invitrogen) containing the cDNA sequence encoding the rat GLP-1 receptor cDNA [19].

2.2. Cell culture and transfection

HEK-293 cells were subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were stably transfected using the SuperFect® Transfection Reagent (Qiagen, Crawley, UK.) and transfected clones were selected by addition of 800 μg ml^{-1} G418 antibiotic.

2.3. Membrane preparations

HEK-293 cells cultured to confluence on five ~85-cm^2 Petri dishes (pre-coated with poly-lysine) were washed with phosphate-buffered saline (PBS). Lysis was achieved by addition of 15 ml ice-cold sterile water and incubation on ice for 5 min. Cells were thoroughly washed with ice-cold PBS and scraped from the plates. Membranes were pelleted by centrifugation and resuspended in 1 ml binding buffer (BB: 2 mM HEPES pH 7.4, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mg l^{-1} bacitracin) and homogenised through a 23G needle. Aliquots were snap-frozen in liquid N_2 and stored at −80°C. Total protein content was estimated using the bicinchoninic acid protein assay.

2.4. Radioligand binding assays

Specific binding of 50 pM [^{125}I]GLP-1 or 50 pM [^{125}I]Exendin(9–39) to membrane preparations was calculated in the absence and presence of a range of concentrations of unlabelled GLP-1, GLP-1(15–36)amide, Exendin-4 or Exendin(9–39). Components were mixed in a total volume of 300 μl in BB and incubated for 1 h at room temperature. Unbound ligand was removed by washing with cold PBS through Whatman GF/G fibre-glass paper pre-soaked in 5% non-fat powdered milk. Filters were cut, allowed to dry and radioactivity was counted using a gamma counter.

2.5. cAMP accumulation assays

Transfected cells were seeded into six-well plates and grown to confluence. On the day of the assay, cells were incubated in DMEM with 2 μCi ml^{-1} [^{3}H]adenine for 2 h at 37°C. After several washes with pre-warmed (37°C) PBS, cells were stimulated for 12 min with various concentrations of pre-warmed (37°C) agonist made up in DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine. The supernatant was aspirated and the cells lysed using 5% trichloroacetic acid containing 2.5 nCi ml^{-1} [^{14}C]cAMP. The intracellular [^{3}H]cAMP produced was purified (alongside the [^{14}C]cAMP internal column standard) by the sequential use of Dowex and alumina columns and counted using a liquid scintillation counter calibrated for dual isotope counting [27].

2.6. Data analysis

IC_{50} and EC_{50} values were calculated using non-linear regression with the aid of the GraphPad PRISM® Version 3.0 software (San Diego, CA, USA). Expression levels were calculated by the equation $B_{max} = B_0 \cdot IC_{50}[L^*]^{-1}$ [28], where B_0 is the specific binding of [^{125}I]-Exendin(9–39) and [L*] is the free concentration of [^{125}I]Exendin-(9–39).

3. Results and discussion

Substitution of Asp198 for Asn had no significant effect upon the affinity for GLP-1, demonstrating that the negative charge at Asp198 is not critical for affinity. Conversely, the Asp198-Ala mutant receptor did not display detectable GLP-1 binding (Table 1, Fig. 2A). The absence of detectable [^{125}I]GLP-1 binding at Asp198-Ala could be due to incorrect trafficking or folding of the mutant protein. However, since the receptor maintained high affinity for the antagonist Exendin(9–39) (Table 1, Fig. 2B), it is clearly expressed in the membrane as a folded protein. Alternatively, the absence of GLP-1 binding at the Asp198-Ala mutant receptor could be a result of a reduction in agonist affinity, which easily becomes undetectable due to the low concentration (50 pM) of [^{125}I]GLP-1 tracer used in the binding experiments.

Since the Asp198-Ala mutant maintained high affinity for the antagonist Exendin(9–39), this peptide was used as the radiolabelled tracer in the subsequent competition binding assays. From such experiments (Fig. 3A, Table 2) it was observed that the Asp198-Ala mutation resulted in a >60-fold reduction in GLP-1 affinity which also manifested itself as a similar increase in the EC_{50} value for the activation of the cAMP pathway in intact HEK-293 cells expressing the wild type or mutant receptors (Fig. 3B, Table 3).

Exendin(9–39) can be viewed as a truncated analogue of GLP-1, and therefore the reduction in GLP-1 affinity, coupled with the maintenance of Exendin(9–39) affinity, suggested the possibility that Asp198 was involved in forming the structure of the binding site for the N-terminal region of the natural agonist. GLP-1 requires its N-terminus for maintaining high affinity, in addition to it being critical for efficacy [13,14]. Hence disruption of the binding site on the receptor for this region of the peptide would be expected to reduce GLP-1 affinity. The agonist Exendin-4 is not dependent upon its N-terminus for maintaining receptor affinity, since it retains high affinity with the truncation of up to eight residues [14–17] and hence disruption of the binding site for this region of Exendin-4 should have little effect upon its affinity. Indeed this was the case since the Asp198-Ala receptor displayed a <8-fold reduction in affinity (Table 2, Fig. 4) which manifested itself in a similar increase in the EC_{50} value for the activation of the cAMP pathway (Fig. 4B, Table 3).

To further demonstrate the role of Asp198 in forming the

Table 1
IC_{50} values (nM) from homologous competition binding assays

	Wild type	Asp198-Ala	Asp198-Asn
[^{125}I]GLP-1/GLP-1	4.4 ± 1.3 (5)	Not detectable	3.2 ± 0.7 (3)
[^{125}I]Exendin(9–39)/Exendin(9–39)	7.8 ± 1.8 (7)	36.0 ± 7.8 (3)	32.5 ± 19.1 (3)
B_{max} (pmol receptor mg^{-1} protein)	67.7 ± 12.1 (7)	55.6 ± 7.2 (3)	208.8 ± 22.0 (3)

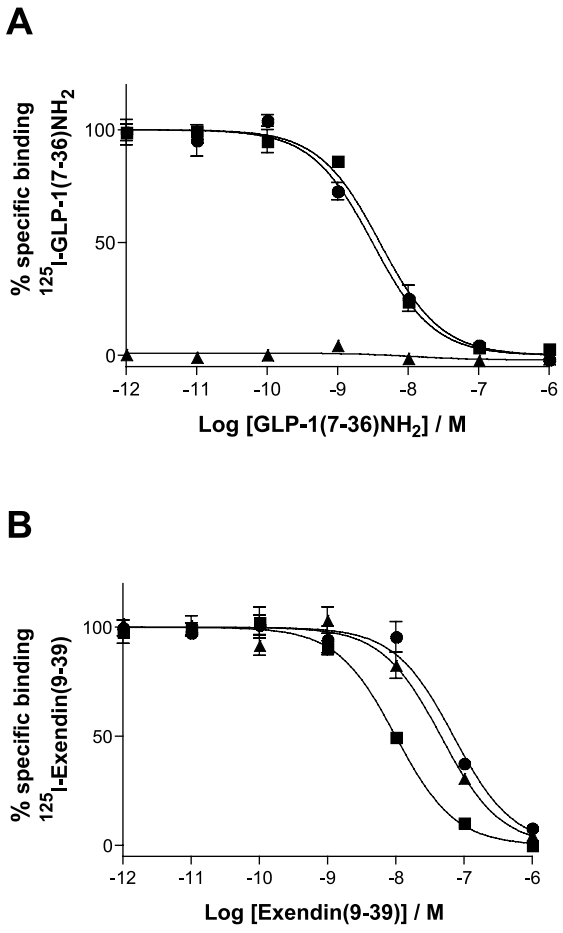

Fig. 2. Homologous competition binding curves for wild type (squares), Asp198-Ala (triangles) and Asp198-Asn (circles) receptors for GLP-1 (A) and Exendin(9–39) (B) (see Tables 1 and 2 for values). All curves represent one of at least three independent experiments for which each point represents the mean of triplicate values with S.E.M. displayed as error bars. Counts were normalised to the maximal specific binding within each data set except for the GLP-1 binding to Asp198-Ala (A) which was normalised to the maximal binding in the wild type data set.

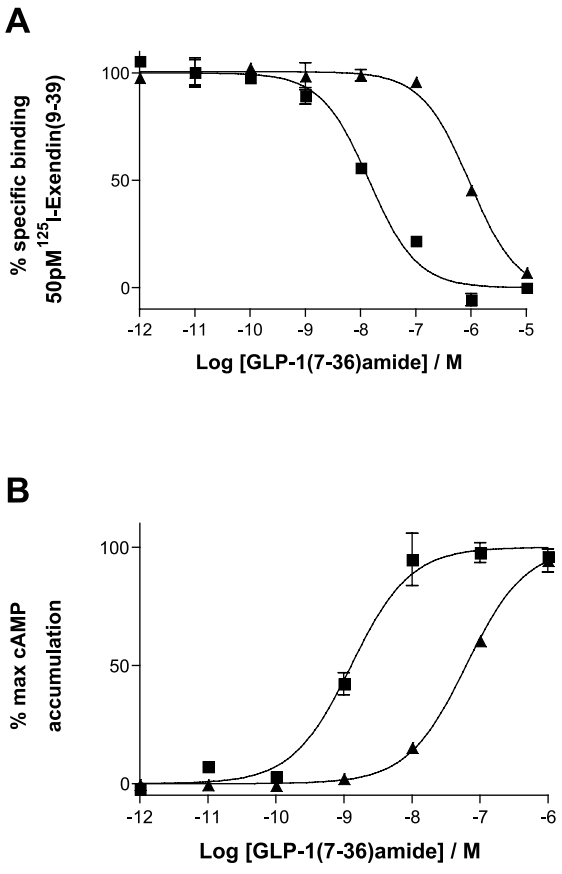

Fig. 3. A: Non-homologous competition binding curve using radiolabelled antagonist and unlabelled GLP-1. B: cAMP accumulation curves for GLP-1 (squares = wild type; triangles = Asp198-Ala). Curves represent one of at least three independent experiments (see Tables 1 and 2). Each point represents the mean of triplicates with S.E.M. displayed as error bars.

binding site for the N-terminal region of GLP-1, the affinity of GLP-1(15–36)amide was analysed at the wild type and Asp198-Ala receptors. If the mutation specifically affects the binding of the N-terminal region of GLP-1, the mutant would be expected to have similar affinity to the wild type receptor for a peptide lacking the N-terminal region. As expected, the N-terminally truncated analogue GLP-1(15–36)amide (Fig. 1A) had a reduced affinity (>35-fold) compared with GLP-1 for the wild type receptor while in contrast, the Asp198-Ala receptor displayed only a 2.1-fold reduction in affinity for GLP-1(15–36)amide. Hence, the >60-fold reduction in GLP-1 affinity observed by mutating Asp198 to Ala was reduced to a <4-fold selectivity for GLP-1(15–36)amide (Table 3). Fig. 5 shows how the mutation of Asp198 to Ala causes the selective reduction of GLP-1 affinity relative to the two N-terminally truncated peptide analogues.

Asp198 is conserved in several family B GPCRs. In the $VPAC_1$ receptor, mutation of the equivalent Asp to Ala resulted in undetectable [^{125}I]VIP binding and a markedly increased EC_{50} for cAMP production [24]. In the secretin receptor [25,26], the substitution of the equivalent Asp by Ala resulted in undetectable receptor expression, while substitution by Asn resulted in a 25-fold increase in EC_{50} for adenylate cyclase activity. Xiao et al. [21] demonstrated that mutation of Asp198 to Ala resulted in a 10-fold reduction in GLP-1 affinity in whole COS-7 cells transiently expressing the GLP-1 receptor.

The data presented here suggest that the structure of the binding site for the N-terminal region of the hormone is dependent upon Asp198. However, this does not necessitate a direct contact between Asp198 and the N-terminus of the

Table 2
IC_{50} values (nM) using radiolabelled antagonist [^{125}I]Exendin(9–39)

	Wild type	Asp198-Ala	Fold change
GLP-1	7.9 ± 3.2 (3)	498.5 ± 174.9 (3)	63.1
Exendin-4	1.8 ± 0.4 (3)	13.9 ± 2.4 (3)	7.7
GLP-1(15–36)amide	287.3 ± 110.3 (3)	1034.0 ± 118.9 (3)	3.6

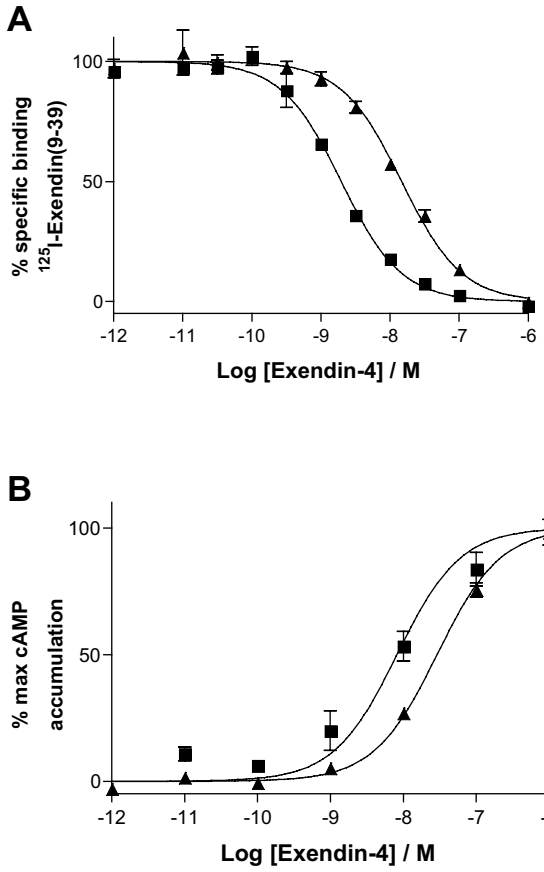

Fig. 4. A: Non-homologous competition binding curve using radiolabelled antagonist and the unlabelled agonist Exendin-4. B: cAMP accumulation curves for Exendin-4 (squares = wild type; triangles = Asp198-Ala). Curves represent one of at least three independent experiments (see Tables 1 and 2). Each point represents the mean of triplicates with S.E.M. displayed as error bars.

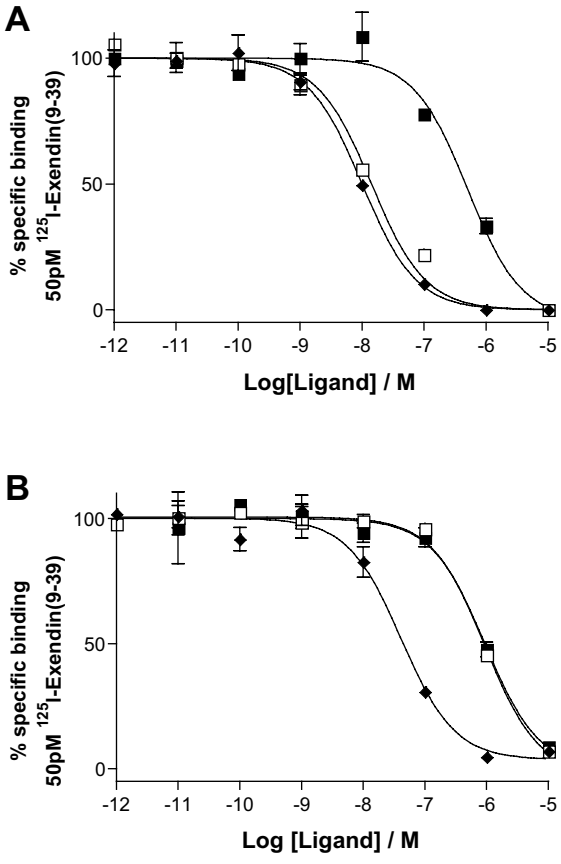

Fig. 5. Non-homologous competition binding curves for wild type (A) and Asp198-Ala (B) using GLP-1 (empty squares), GLP-1(15–36)amide (filled squares) and Exendin(9–39) (diamonds). Curves represent one of at least three independent experiments (see Tables 1 and 2). Each point represents the mean of triplicates with S.E.M. displayed as error bars.

hormone. Rather, the local structure of this region of the hormone-binding site, located close to the boundary of transmembrane helix 2 and the first extracellular loop, likely requires a hydrogen bond from the side chain of Asp198, which can be maintained by substitution with Asn but not Ala. The absence of this hydrogen bond does not completely destroy the ability of the receptor to form its active state and activate its G protein. Rather, it appears to reduce agonist affinity so that receptor activation and the subsequent intracellular signalling events are observed at higher agonist concentrations compared to the wild type receptor.

Although the N-terminal domain of the GLP-1 receptor has been demonstrated to form a direct hormone–receptor interaction [20–22], the first extracellular loop has also been implicated in agonist binding (e.g. [21]). The data presented here further demonstrate that this region is necessary for the formation of the binding site of the GLP-1 hormone. In addition, we have shown that a critical determinant for maintaining this region of the binding site is the hydrogen bonding capability, rather than the negative charge, of Asp198. Moreover, we have identified that this region on the receptor is responsible for forming the binding site of the N-terminal region of the hormone.

Table 3
EC_{50} values (nM) for the stimulation of cAMP

	Wild type	Asp198-Ala
GLP-1	1.7 ± 0.6 (5)	74.6 ± 25.1 (4)
Exendin-4	2.7 ± 0.6 (5)	28.0 ± 5.4 (4)

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