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A model for receptor–peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors

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1 The receptor for glucagon-like peptide-1 (GLP-1) can be activated by both its physiological hormone and a peptide discovered in the venom of the Gila Monster, exendin-4, which shows promise as an antidiabetic agent.

2 Exendin-4 displays receptor-binding properties not observed for GLP-1. Firstly, exendin-4 can be truncated by up to eight residues at its N-terminus without a significant loss of affinity. Secondly, exendin-4 maintains high affinity for the isolated N-terminal domain of the receptor, suggesting that exendin-4 makes additional contacts with this domain of the receptor, which nullify the requirement for ligand–receptor interactions involving the extracellular loops and/or transmembrane helices of the receptor's core domain.

3 In order to further understand the nature of the receptor–peptide interaction, a variety of full length and truncated peptide analogues were used to quantify the contribution of each distinct region of exendin-4 and GLP-1 to receptor affinity.

4 Our data show that, for both exendin-4 and GLP-1, the primary interaction is between the putative helical region of the peptide and the extracellular N-terminal domain of the receptor.

5 However, we demonstrate that the contribution to receptor affinity provided by the N-terminal segment of GLP-1 is greater than that of exendin-4, while the C-terminal nine residue extension of exendin-4, absent in GLP-1, forms a compensatory interaction with the N-terminal domain of the receptor.

6 We describe a peptide–receptor binding model to account for these data.
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Keywords: Receptor; incretin; GLP-1; exendin; pancreas; Trp-Cage; diabetes

Abbreviations: DPP IV, dipeptidyl peptidase IV; EX-4, exendin-4; GLP-1, glucagon-like peptide-1[7–36]amide; GLP-1R, GLP-1 receptor; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; NT, N-terminal receptor domain; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; TM, transmembrane helix

Introduction

Glucagon-like peptide-1(7–36)amide (termed GLP-1 throughout this paper) is a 30-residue peptide hormone derived from the post-translational modification of proglucagon in intestinal L cells (Kieffer & Habener, 1999). It binds to a 'Family B' G protein-coupled receptor (GPCR), a subgroup of peptide receptors characterised by a related N-terminal domain of 100–150 amino acids and a transmembrane (TM) core domain (Segre & Goldring, 1993). The extracellular N-terminal domain contains three disulphide bonds and is critical for peptide binding, while the core domain contains the seven TM segments and connecting loop regions typical of all GPCRs.

Since the predominant physiological role of GLP-1 is in maintaining blood sugar levels *via* a glucose-dependent mechanism, the GLP-1 receptor (GLP-1R) is a potential target for glucose-dependent therapeutic agents designed to treat hyperglycaemia resulting from type II diabetes (Gutniak *et al*., 1992; Moller, 2001). Unfortunately, the half-life of GLP-1 itself is very short due to dipeptidyl peptidase IV (DPP IV) cleavage of the first two N-terminal residues (Kieffer *et al*., 1995), and therefore the discovery of more physiologically stable GLP-1 receptor agonists is necessary. One such protease-resistant GLP-1R agonist is the 39 amino-acid peptide exendin-4 (EX-4; Göke *et al*., 1993), discovered in the venom of the Gila monster *Heloderma suspectum*, which shows promise as a therapeutic agent for the treatment of type II diabetes due to its longer half-life *in vivo* (e.g. Young *et al*., 1999; Taylor *et al*., 2002).

EX-4 shares approximately 50% sequence identity with GLP-1 itself, and is a potent GLP-1R agonist (Göke *et al*., 1993; Thorens *et al*., 1993). Its agonist properties can be attributed to the high level of sequence identity shared with GLP-1 in the N-terminal region, where eight of the first nine residues are conserved (Figure 1a). However, the absence of total conservation in this N-terminal region, due to the single substitution of Ala-2 in GLP-1 by Gly in EX-4, is responsible for an increased DPP IV resistance of several orders of

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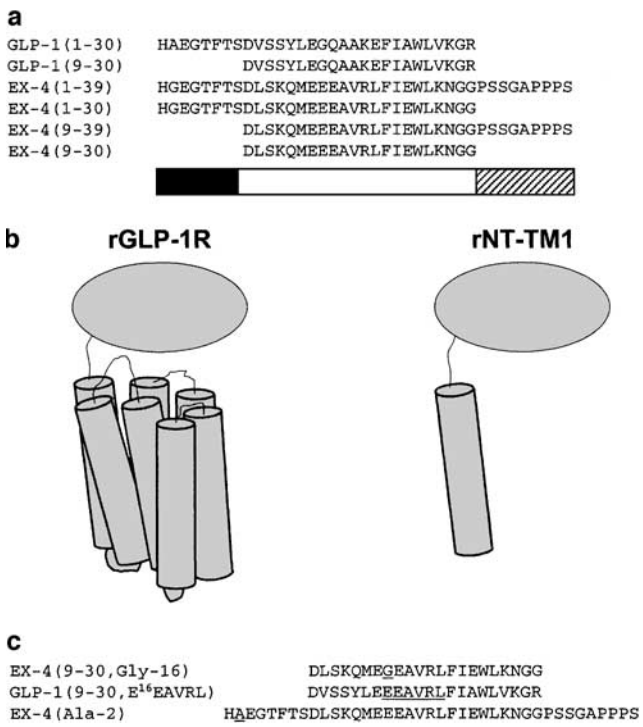

Figure 1 Peptides and receptor constructs used in this work. (a) An alignment of the full-length and truncated peptides used in this study. In order to facilitate direct comparison with EX-4, the numbering of GLP-1 has been modified from the conventional system, so that its first residue is His-1. The three distinct regions of the peptides are highlighted by the bar below the sequences, such that the central helix is shown as white, the N-terminal region as black and the C-terminal region of EX-4 as hatched. This pictorial shorthand system will be used in Figures 2–5. All the peptides were C-terminally amidated. (b) Pictorial representation of the full-length (rGLP-1) and truncated (rNT-TM1) receptors used in this study. The extracellular N-terminal domain is shown by a shaded oval, while the TM helices are depicted as cylinders. (c) An alignment of three modified peptides based upon GLP-1 and EX-4. The modifications to the sequence are underlined. All the three peptides were C-terminally amidated.

magnitude for EX-4 (Thum *et al*., 2002). Interestingly, while the affinity of GLP-1 is highly sensitive to N-terminal cleavage, EX-4 can be truncated by up to eight residues at its N-terminus without major loss of affinity: in this paper, we term this property 'N-independent affinity'. Truncation is not completely without consequence since the first two amino acids of EX-4 are essential for the efficacy of exendin peptides and, once removed, the truncated exendin peptides function as potent antagonists (Göke *et al*., 1993; Thorens *et al*., 1993; Montrose-Rafizadeh *et al*., 1997; Serre *et al*., 1998).

While the N-termini of GLP-1 and EX-4 are highly conserved, EX-4 contains an additional nine amino acids at its C-terminus, which have been shown by NMR analysis to form a compact folding unit called a 'Trp-Cage' (Neidigh *et al*., 2001). Interestingly, this Trp-Cage motif can be further stabilised in mutated and truncated analogues of EX-4, such that it forms the smallest example to date of a cooperatively folded tertiary structure (Neidigh *et al*., 2002). The NMR analysis also reveals that the central region of EX-4 is α-helical, while the N-terminus is significantly more frayed (Neidigh *et al*., 2001). In addition, we have demonstrated that although the central region of EX-4 (comprising residues 10–30) only shares eight identical residues with GLP-1, they all lie on the same face of an ideal α-helix, suggesting that this conserved face of the helix contacts the binding pocket on the receptor (López de Maturana & Donnelly, 2002).

Hence, the structure of EX-4 can be considered to consist of three distinct regions: the N-terminal region comprising residues 1–8, the central helical region comprising residues 9–30, and the C-terminal region comprising residues 31–39, which may form a Trp-Cage motif in conjunction with part of the central helix. GLP-1 shares two of these regions with its longer reptilian counterpart: the N-terminal region which is similar to that of EX-4, differing only at position 2, and the central helical region, which has been postulated to be less helical than that of EX-4 due to the substitution of Glu-16 for Gly in GLP-1 (Neidigh *et al*., 2001). The N-independent affinity of EX-4 is interesting, and suggests that the central helical and/or C-terminal putative Trp-Cage of EX-4 can form additional stabilising contacts with the receptor, which are absent from GLP-1 (López de Maturana *et al*., 2003).

The aim of the work described in this paper was to define the relative contributions of the distinct regions of EX-4 and GLP-1 to GLP-1R affinity, by analysing the full-length peptides and various truncated analogues missing one or more of these regions. In this way, we aimed to identify the region of EX-4 responsible for its N-independent affinity. We also set out to identify the region that enables EX-4, but not GLP-1, to bind with high affinity to the isolated N-terminal domain of the receptor. In addition, we aimed to define the receptor domain, the N-terminal domain or the 7TM core domain, responsible for binding each of the three distinct regions of the peptides.

Methods

Materials

EX-4 and EX-4(9–39) were from Bachem (Saffron Walden, U.K.), while GLP-1 and all other truncated peptide ligands were custom synthesised by Genosphere Biotechnologies (Paris, France). ^{125}I-EX-4(9–39), labelled *via* Bolton–Hunter reagent at Lys-12, was purchased from NEN-Perkin-Elmer (Boston, MA, U.S.A.). [^{3}H]adenine and [^{14}C]cyclic adenosine monophosphate (cAMP) were obtained from Amersham. Dowex 50W-X4 and alumina were obtained from Bio-Rad. Cell culture reagents were obtained from Gibco-Invitrogen and Sigma-Aldrich (Poole, U.K.). General chemicals were from BDH-Merck (Poole, U.K.) and Sigma-Aldrich.

Methods

Constructs The pcDNA3 vector containing the full-length rat GLP-1R cDNA (López de Maturana & Donnelly, 2002), originally provided in pcDNA1 by Dr B. Thorens (Thorens, 1992), was used to express the full-length receptor rGLP-1R. As described previously (López de Maturana *et al*., 2003), the cDNA encoding amino acids Met^{1}–Leu^{171} (N-terminal domain, including the putative signal sequence, and first TM α-helix) of the rat GLP-1 receptor was synthesised by PCR, using the pcDNA3 vector containing the full-length rat GLP-1R cDNA as a template.

Isolation of cell lines HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg/ml streptomycin. Cells were transfected with pcDNA3 containing the cDNA encoding rGLP-1R or rNT-TM1, using the SuperFect® Transfection Reagent (Qiagen Ltd, Crawley, U.K.), and stable clones were selected with the G418 antibiotic as follows.

Cells were seeded into a 25 cm^2 flask containing 10 ml of media, and transfected when they reached 50–80% confluence. SuperFect (20 μl) was mixed with a DNA solution consisting of 5 μg plasmid DNA in 150 μl DMEM. The DNA was incubated with the reagent for 10 min at room temperature. The media (1 ml) was then added and mixed gently. The cells were washed once with sterile PBS before the transfection mixture was added, and incubated for 3 h at 37°C. The cells were then washed three times with PBS before the addition of fresh media. After 3 days, the supernatant was removed, and the cells were washed with PBS before fresh media was added. Selection of transfected cells was achieved by addition of 800 μg ml^{-1} geneticin (G418 antibiotic). The media, containing antibiotic, was replaced every 3 days until individual colonies were clearly visible. Individual colonies were isolated using cloning rings and further cultured.

HEK-293 cell membranes: preparation and binding HEK-293 cells cultured to confluence on five 160-cm^2 Petri dishes (precoated with poly-D-lysine) were washed with PBS, followed by the addition of 15 ml of ice-cold sterile double-distilled water to induce cell lysis. Following 5 min incubation on ice, the ruptured cells were thoroughly washed with ice-cold PBS before being scraped from the plates and pelleted by centrifugation in a bench-top centrifuge (13,000 × *g* for 30 min). The crude membrane pellet was resuspended in 1 ml membrane-binding solution (MBS; 25 mM HEPES pH 7.4, 2.5 mM2, 1 mM $MgCl_2$, 50 mg l^{-1} bacitracin), and forced through a 23 G needle. Aliquots (0.1 ml) were snap-frozen in N_2(l) and stored at −70°C. Total protein content was estimated using a bicinchoninic acid protein assay.

The affinity of ^{125}I-EX-4(9–39) was assumed to approximate that of unlabelled EX-4(9–39), since the addition of a large γ-L-glutamoyl(Nα-hexadecanoyl) at position 12 had no adverse effect upon the affinity of a related GLP-1R ligand (Knudsen *et al*., 2000). Hence, a concentration of 50 pM was considered to be within the appropriate range for competition-binding studies, since it is >100-fold below the estimated affinity constant but sufficiently high to avoid significant depletion of radioligand during the assay (e.g. Motulsky & Christopoulos, 2003). Membranes slowly thawed on ice were diluted appropriately in MBS, and 75 μl was then mixed with 75 μl of 0.2 nM ^{125}I-EX-4(9–39) (50 pM final concentration), 75 μl of MBS and 75 μl of unlabelled peptides, at a final concentration ranging from 1×10^{-5} to 1×10^{-12} M, diluted in MBS. Reactions were incubated for 1 h at 23°C. The unbound ligand was washed by rapid vacuum filtration (Brandel cell harvester; Gaithersburg, MD, U.S.A.) through glass-fibre paper (Whatman GF/C grade filter paper) presoaked in 5% w v^{-1} nonfat powdered milk. Filters were washed three times with 5 ml of ice-cold PBS, and filter-bound radioactivity was measured in a gamma counter (RiaStar 5405 counter; Packard, Pangbourne, U.K.).

cAMP accumulation assay The assay was adapted from the protocol of Salomon *et al*. (1974), which is based on a sequential chromatography technique using a Dowex 50W-X8 cation-exchange resin and alumina. The Dowex columns were equilibrated and regenerated by washing gradually with 4 ml of 1 M HCl and 8 ml of deionised water. The alumina columns were equilibrated by washing sequentially with 4 ml of 1 M imidazole-HCl (pH 7.4) and 8 ml of 0.1 M imidazole-HCl (pH 7.4). Cells were seeded into six-well plates (containing 2 ml CM10) and grown to 80–90% confluence (2–3 days). On the day of the assay, the old media was removed and 2 ml fresh media containing 2 μCi ml^{-1} of [^{3}H]adenine was added. The plates were incubated at 37°C for 2 h. The cells were then washed three times with PBS (prewarmed to 37°C) and incubated for 12 min with various concentrations of agonist made up in DMEM, including 5×10^{-4} M IBMX. The supernatant was aspirated and 1.5 ml of ice-cold 5% TCA, containing 2.5 nCi ml^{-1} [^{14}C]cAMP, was added. The TCA extract (1 ml) was then gently applied to the Dowex columns. Following the addition of 2 ml of 0.1 M HCl and then 3 ml of deionised water (not collected), the [^{3}H]cAMP was eluted directly onto the alumina columns with 6 ml of deionised H_2O. Following the elution from the alumina columns of 0.5 ml, a further 3 ml of 0.1 M imidazole-HCl was used to elute the [^{3}H]cAMP directly into scintillation vials containing 12 ml of scintillation fluid. Finally, both [^{3}H] and [^{14}C] counts were counted using a liquid scintillation counter preprogrammed with the appropriate quench curves for dual isotope counting. The [^{14}C] counts acted as a control to quantify column efficiency, to which the equivalent [^{3}H] counts from the same column were normalised.

Data analysis Binding curves in the figures represent one of at least three independent experiments for which each point is the mean of triplicate values, with s.e.m. displayed as error bars. Counts were normalised to the maximal specific binding within each dataset. IC_{50} values were calculated with a single-site-binding model fitted using nonlinear regression, with the aid of the GraphPad PRISM 3.0 software (San Diego, CA, U.S.A.). EC_{50} values were calculated in an analogous manner following fitting with a sigmoidal concentration–response curve. Values in the tables represent the mean, with s.e.m. calculated from the pIC_{50} values ($-\log IC_{50}$) and pEC_{50} values ($-\log EC_{50}$) from at least three independent experiments. The significance of the differences in the pIC_{50} values of the peptides compared in Figures 2–4 were analysed from the mean values of the experiments (Table 1), using a two-tailed unpaired Student's *t*-test.

Results & discussion

The binding and activation properties of EX-4, GLP-1 and their truncated analogues (detailed in Figure 1a) are shown in Table 1 at both the wild-type and truncated receptors (see Figure 1b for a schematic diagram of the two receptors used in this analysis). Examples of individual experiments are displayed in Figures 2–5, and are discussed below. Table 2 shows the binding properties of three further peptides containing elements of both GLP-1 and EX-4 (see Figure 1c).

In order to simplify the comparison of GLP-1 and EX-4, the residue-numbering scheme of EX-4 was applied to GLP-1 such that the first residue of GLP-1, His-7, was renamed as His-1, Ala-8 of GLP-1 was renamed Ala-2, etc. To clarify direct

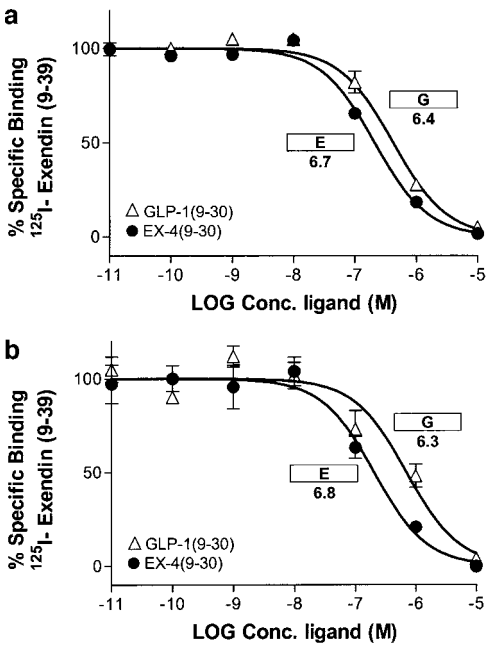

Figure 2 Binding of the helical regions of EX-4 and GLP-1 to rGLP-1R and rNT-TM1. ^{125}I-exendin-4(9–39) competition-binding assays for (a) rGLP-1R and (b) rNT-TM1 with EX-4(9–30) and GLP-1(9–30). The figures are representative of one of at least three independent experiments to assess the affinity of the central helical region of EX-4 and GLP-1. pIC_{50} values from Table 1 are given next to the symbol of each peptide.

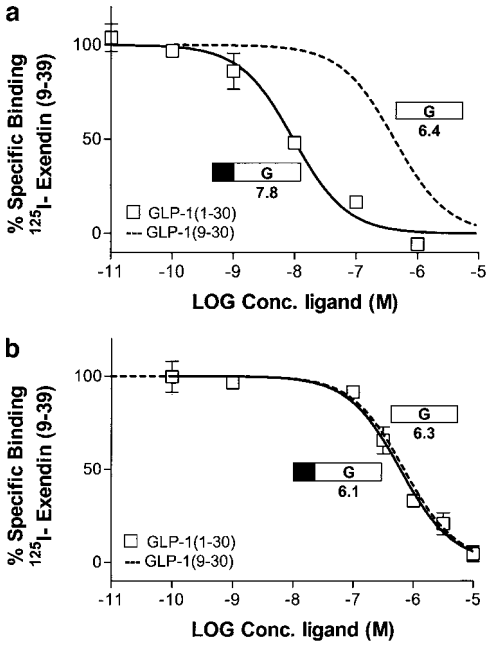

Figure 3 Binding of N-terminal region of GLP-1 to rGLP-1R, and rNT-TM1. ^{125}I-exendin-4(9–39) competition-binding assays for (a) rGLP-1R and (b) rNT-TM1 with GLP-1(1–30). The figures are representative of one of at least three independent experiments to assess the affinity of the N-terminal region of GLP-1 by comparing GLP-1(1–30) with GLP-1(9–30). The dotted lines represent the GLP-1(9–30) curves from Figure 2. pIC_{50} values from Table 1 are given next to the symbol of each peptide. The figure highlights the large improvement in affinity at rGLP-1R, resulting from the addition of the N-terminal region of GLP-1 in (a) (significantly different, $P<0.0001$). The difference in pIC_{50} at rNT-TM1 (b) is not significant ($P<0.05$).

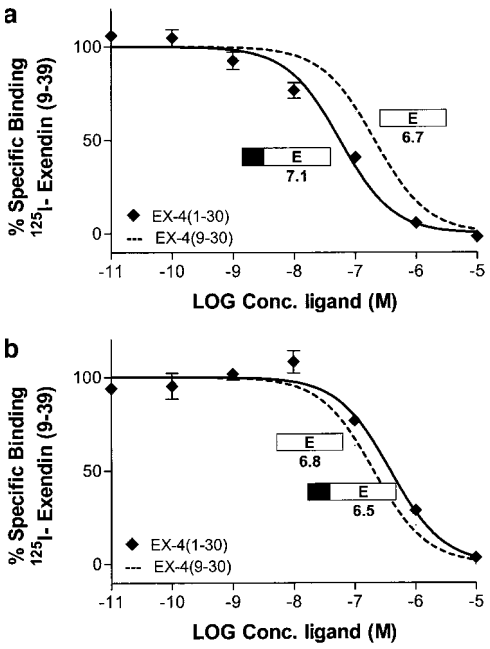

Figure 4 Binding of the N-terminal region of EX-4 to rGLP-1R and rNT-TM1. ^{125}I-exendin-4(9–39) competition-binding assays for (a) rGLP-1R and (b) rNT-TM1 with EX-4(1–30). The figures are representative of one of at least three independent experiments to assess the affinity of the N-terminal region of EX-4 by comparing EX-4(1–30) with EX-4(9–30). The dotted lines represent the EX-4(9–30) curves from Figure 2. pIC_{50} values from Table 1 are given next to the symbol of each peptide. Panel (a) highlights the small improvement in affinity resulting from the addition of the N-terminal region of EX-4 (significantly different, $P<0.01$). This small enhancement of affinity is of the same magnitude and significance to that observed when comparing EX-4(1–39) with EX-4(9–39) (see Table 1).

Table 1 Binding and activation properties of GLP-1, EX-4 and their truncated analogues

	rGLP-1R			*rNT-TM1*
	pIC_{50}	pEC_{50}	E_{max}	pIC_{50}
GLP-1(1–30)	7.8±0.10	8.1±0.01	100	6.1±0.11
GLP-1(9–30)	6.4±0.03	ND	ND	6.3±0.08
EX-4(1–39)	8.5±0.12	8.5±0.13	104±12	7.9±0.13
EX-4(9–39)	8.1±0.07	ND	ND	7.9±0.11
EX-4(1–30)	7.1±0.11	7.5±0.12	75±5	6.5±0.11
EX-4(9–30)	6.7±0.06	ND	ND	6.8±0.13

The means±s.e. are shown for three independent experiments. ND refers to peptides which, at 1 μM, displayed no detectable elevation of cAMP levels above the basal. E_{max} values are maximum elevations of cAMP levels, as a percentage of that produced by GLP-1(1–30). pIC_{50} refers to $-\log IC_{50}/M$. pEC_{50} refers to $-\log EC_{50}/M$. B_{max} values estimated from the EX-4(9–39) competition data suggest expression levels of 13.5±2.2 pmol mg^{-1} for rGLP-1R and 2.4±0.4 pmol mg^{-1} for rNT-TM1.

comparisons of full-length and truncated peptides, the full-length peptides EX-4 and GLP-1 will sometimes be referred to as EX-4(1–39) and GLP-1(1–30). In addition, the schematic representation shown below the sequences in Figure 1a will be used in Figures 2–5 to highlight the peptides used, and to simplify the comparison of binding data in these Figures.

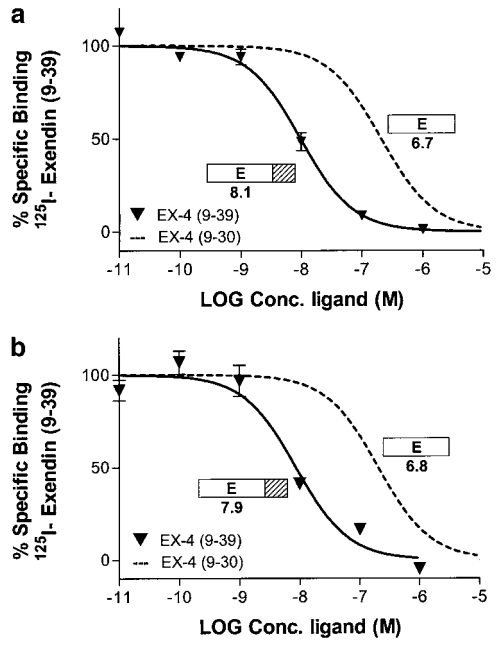

Figure 5 Binding of the C-terminal region of EX-4 to rGLP-1R and rNT-TM1. ^{125}I-exendin-4(9–39) competition-binding assays for (a) rGLP-1R and (b) rNT-TM1 with EX-4(9–39). The figures are representative of one of at least three independent experiments to assess the affinity of the C-terminal region of EX-4 by comparing EX-4(9–39) with EX-4(9–30). The dotted lines represent the EX-4(9–30) curves from Figure 2. pIC_{50} values from Table 1 are given next to the symbol of each peptide. The figure highlights the large improvement in affinity at both rGLP-1R and rNT-TM1 (significantly different, $P<0.0001$ and $P<0.0005$, respectively) resulting from the addition of the C-terminal region of EX-**4.** The enhancement of affinity is of a similar magnitude to that observed when comparing EX-4(1–39) with EX-4(1–30) (significantly different for both receptors, $P<0.0001$; see Table 1).

Table 2 Binding properties of modified peptide ligands at rGLP-1R

	pIC_{50}
GLP-1(9–30, E^{16}EAVRL)	6.4±0.20
EX-4(9–30, Gly-16)	6.7±0.13
EX-4(Ala-2)	8.7±0.10

The mean±s.e. is shown for three independent competition-binding assays using ^{125}I-exendin(9–39) tracer and the unlabelled peptides shown in Figure 1c. pIC_{50} refers to $-\log IC_{50}/M$.

Receptor activation

As expected from previous studies (e.g. Göke *et al*., 1993; Montrose-Rafizadeh *et al*., 1997), all peptides containing a complete N-terminal sequence were shown to be agonists, while those lacking the eight N-terminal residues were unable to elevate intracellular cAMP levels at 1 μM (Table 1). This highlights the importance of the N-terminal sequence for receptor activation. However, no activation data are displayed for the truncated receptor, since no elevation of intracellular cAMP levels was observed with 1 μM of either EX-4 or GLP-1. This was presumably due to the absence of the intracellular loops, which disables any possible interaction between the receptor and the G protein.

Putative helical region of the peptide ligands

The binding of the putative helical region of both GLP-1 and EX-4 was analysed using GLP-1(9–30) and EX-4(9–30). Both peptides had measurable binding affinity at rGLP-1R (Figure 2a, Table 1), with the affinity of the EX-4-derived peptide being only slightly higher than its GLP-1-based analogue (difference in pIC_{50} of 0.3, significantly different at $P<0.005$). However, this small difference in pIC_{50} magnitude does not account for the N-independent affinity of EX-4. The two truncated peptides bound to the rNT-TM1 receptor with affinities that were not significantly different ($P<0.1$) to that observed at the full-length receptor (Figure 2, Table 1). Since the removal of all the three extracellular loops and six of the TM helices from the receptor resulted in no noticeable effect on the binding of these peptides, it demonstrates that the putative helical region interacts with the receptor's N-terminal domain rather than its core domain. Again, as with rGLP-1R, the difference in pIC_{50} of 0.4 between EX-4(9–30) and GLP-1(9–30) at NT-TM1 is significant ($P<0.005$), albeit of a small magnitude (Table 1, Figure 2a).

NMR analysis of EX-4 and GLP-1 has indicated that the helix in EX-4 is more regular than that in GLP-1 (Neidigh *et al*., 2001), possibly due to the presence of a helix-stabilising Glu-16 : Arg-20 interaction in EX-4 compared with a helix-destabilising Gly-16 in GLP-1. Since the affinity of EX-4(9–30) at both rGLP-1 and rNT-TM1 was observed to be slightly better than that of GLP-1(9–30), we decided to analyse this further. In order to test whether the small difference in affinity between EX-4(9–30) and GLP-1(9–30) at rGLP-1 and rNT-TM1 was due to the stability of the helix, we tested an analogue of EX-4(9–30) in which Glu-16 was replaced by Gly. However, comparison of the binding data for EX-4(9–30) and EX-4(9–30, Gly-16) (Tables 1 and 2) demonstrates that the disruption of the putative helical structure did not result in a loss of affinity, since pIC_{50} values for EX-4(9–30) and EX-4(9–30, Gly-16) are not significantly different ($P<1$). This suggests that the small difference in affinity of GLP-1(9–30) and EX-4(9–30) is due to some other factor.

N-terminal region of the peptide ligands

In order to estimate the contribution to affinity of the N-terminal region of GLP-1 and EX-4, the affinities of GLP-1(9–30) and EX-4(9–30) were, respectively, compared with GLP-1(1–30) and EX-4(1–30) (e.g. Figures 3 and 4). As can be seen from Table 1, the N-terminal region of GLP-1 improves the affinity of its shorter analogue by a much larger degree than the equivalent region of EX-4 (increase in pIC_{50} of 1.4 for GLP-1, $P<0.0001$; compared with 0.4 for EX-4, $P<0.006$). It is worth noting from Table 1 that the difference in the magnitude of the pIC_{50} values obtained by the comparison of EX-4(9–30) and EX-4(1–30) is equivalent to that observed between their C-terminally extended counterparts EX-4(9–39) and EX-4(1–39). This demonstrates that there are no cooperative effects upon binding affinity, resulting from the interactions of the N- and C-termini of the peptide.

Interestingly, the addition of the N-terminal region of GLP-1 provides no improvement to peptide affinity at the rNT-TM1 receptor (pIC_{50} values for GLP-1(1–30) and GLP-1(9–30) are not significantly different at $P<0.05$; Figure 3b; Table 1). This

further demonstrates that the N-terminal region of the peptide interacts with the receptor's core domain, as suggested previously (Bergwitz *et al*., 1996; Hjorth & Schwartz, 1996; López de Maturana & Donnelly, 2002; López de Maturana *et al*., 2003; Runge *et al*., 2003). The comparable affinities of GLP-1(1–30) and GLP-1(9–30) at rNT-TM1 also demonstrate that the truncation of the peptide has not prevented the helical region of GLP-1(9–30) from forming the same structure as its full-length counterpart.

C-terminal region of EX-4

The contribution provided by the nine-residue extension at the C-terminus of EX-4 can be estimated either by comparing EX-4(1–39) with EX-4(1–30), or else by comparing EX-4(9–39) with EX-4(9–30) (e.g. Figure 5a). As can be seen from Table 1, both these comparisons yield the same difference in pIC_{50} of 1.4 at rGLP-1R (significantly different, $P<0.0001$), demonstrating that this region provides a major enhancement to affinity and can explain the N-independent affinity of EX-4. The improved affinity provided by the C-terminal region is also seen at the truncated rNT-TM1 receptor (Figure 5b), with the difference in pIC_{50} of 1.4 for EX-4(1–39) compared to EX-4(1–30) ($P<0.0001$), and 1.1 for EX-4(9–39) compared to EX-4(9–30) ($P<0.0005$). This demonstrates that the C-terminal region of EX-4 binds to the N-terminal domain of the receptor, and provides an additional interaction that explains how EX-4 is able to maintain high affinity for the isolated N-terminal domain of GLP-1R (López de Maturana *et al*., 2003).

Montrose-Rafizadeh *et al*. (1997) published an interesting study which, among other things, analysed two series of N-terminally truncated chimaeric peptides, all of which contained the intact C-terminal region of EX-4. The peptide series displayed an increasing C-terminal component of EX-4 and a decreasing N-terminal component of GLP-1. The chimaeric peptides with the junction of the GLP-1- and EX-4-derived sequences between either positions 31–32 or 23–24 (chimeras 1 and 4 and 2 and 5 in Montrose-Rafizadeh *et al*., 1997) displayed poor affinity, while those with the junction between 15 and 16 (chimeras 3 and 6 in Montrose-Rafizadeh *et al*., 1997) regained affinity by more than an order of magnitude. One conclusion from this work was that the E^{16}EAVRL region of EX-4 was a principle determinant of the ability of EX-4 to maintain high affinity without its N-terminal residues. To test this, we substituted the E^{16}EAVRL sequence into the equivalent region of GLP-1(9–30), the N-terminally truncated version of GLP-1. As can be seen from Table 2, the affinity of GLP-1(9–30) was not improved by the inclusion of the EX-4-derived sequence (pIC_{50} values not significantly different, $P<1$), suggesting that it is not the critical factor for N-independent affinity.

While, at first sight, the data from the chimaeric peptides of Montrose-Rafizadeh *et al*. (1997) appear to be contradictory with our data, they are in fact entirely compatible once the more recently determined information on the structure of EX-4 is taken into account (Neidigh *et al*., 2001; 2002). As detailed above, NMR data demonstrate that the C-terminal region of EX-4 can fold back onto part of the central helix, to form a Trp-Cage motif. While all the chimaeric peptides in Montrose-Rafizadeh *et al*. (1997) contain the final eight residues of EX-4, it is unlikely that the Trp-Cage can form in chimaeras 1, 2, 4 and 5. In the chimaeric peptides 1 and 4, with the GLP-1 and EX-4 junction between positions 31 and 32, the conformationally critical N^{28}GGP sequence of EX-4, which forms a tight bend in the structure made possible by the positive *phi* torsion angles of the two Gly residues, is absent in the chimaera. In the chimaeric peptides 2 and 5, with the GLP-1 and EX-4 junction between positions 23 and 24, there is a helix destabilising i to $i+3$ charge–charge interaction between Glu-21 from the GLP-1 sequence and Glu-24 of the EX-4 segment. Helix stability in this region is critical for the formation of the Trp-Cage motif (Neidigh *et al*., 2002). Only for the chimaeric peptides 3 and 6, with the GLP-1 and EX-4 junction between positions 15 and 16, is the Trp-Cage structure likely to form. Hence, we propose that the simplest explanation to account for all the data to date is that the Trp-Cage motif in EX-4 is the essential determinant of N-independent affinity, as well as for the ability of EX-4 and its N-terminally truncated analogues to maintain high affinity for the isolated N-terminal domain of the receptor. It is therefore possible that GLP-1 affinity could be enhanced by extending its C-terminal region, so long as careful consideration is given to the structural requirements of Trp-Cage formation.

The enhancement of peptide affinity and activity at GLP-1R by the alteration of the C-terminal end of the ligand has been observed in other studies. For example, Knudsen *et al*. (2000) have shown that the addition of a ω-carboxyundecanoyl or ω-carboxypentadecanoyl group at the C-terminus of a GLP-1 analogue can enhance activity by 10-fold. Hjorth *et al*. (1994) have also demonstrated that the replacement of the final three residues of glucagon (a 29-residue peptide) with the final four residues of GLP-1 results in a 169-fold improvement in affinity at GLP-1R. Furthermore, the >1000-fold selectivity of GLP-1R for GLP-1 over glucagon (Hjorth *et al*., 1994) is reduced to only a 50-fold difference when glucagon is extended by eight residues to give oxyntomodulin (Dakin *et al*., 2001). Conversely, the replacement of the final four residues of GLP-1 with the final three residues of glucagon reduces affinity at GLP-1R by 475-fold (Hjorth *et al*., 1994). Truncation of GLP-1 at its C-terminus also results in a reduction of receptor affinity (Mojsov, 1992). Hence, the interaction of the C-terminal region of the peptide ligand with the receptor is clearly important for affinity, and could be exploited in the design of modified ligands with increased activity. These data are also compatible with our model for enhanced EX-4 affinity *via* its extended C-terminal sequence.

Conclusion and model

The data presented in this work are compatible with models for peptide–receptor binding at other family B GPCRs (e.g. Bergwitz *et al*., 1996; Hjorth & Schwartz, 1996; Runge *et al*., 2003), as well as with the model previously proposed by our group (López de Maturana *et al*., 2003). In this latter model, we defined 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. It was proposed 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 **H** interaction is between the receptor's

N-terminal domain and the helical region of the peptide, in particular the face of the helix composed of the conserved residues Ser-11, Glu-15, Ala-18, Phe-22, Trp-25 and Leu-26 (Figure 6a; López de Maturana & Donnelly, 2002). The **Ex** interaction was defined as one or more interaction(s) unique to EX-4, which enables its N-independent affinity and also its ability to bind with high affinity to the isolated N-terminal domain. However, although our previous data showed that it clearly involved the N-terminal domain of the receptor, the region responsible for the **Ex** interaction was not previously localised on EX-4 (López de Maturana *et al*., 2003).

The latest data now allow us to propose that the **Ex** interaction is formed between the N-terminal domain of the receptor and the C-terminal region of EX-4, probably *via* its putative Trp-Cage motif. In addition, we can now observe that

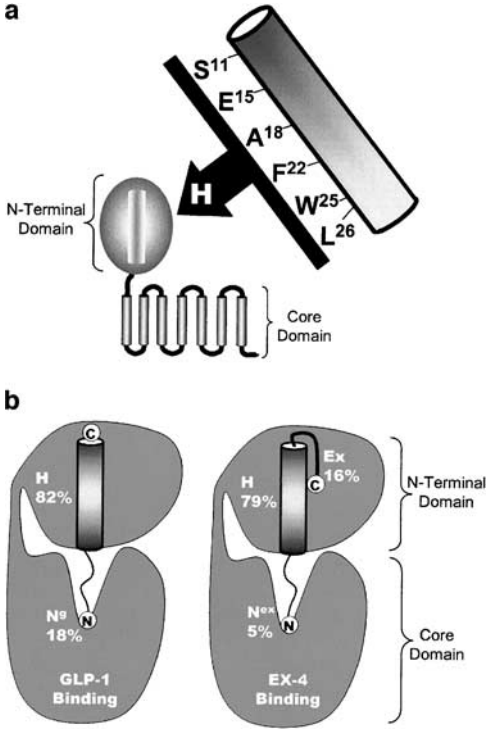

Figure 6 A model for peptide–receptor binding. (a) Schematic model for the **H** interaction between a putative groove on the N-terminal domain of the receptor and the conserved face of the helical region of the peptides. (b) Two schematic diagrams depicting the binding of GLP-1 (left) and EX-4 (right) to GLP-1R. The receptor is shown as consisting of two domains, the 'N-terminal domain' and the 'core domain'. The peptides are displayed between the circled symbols N and C, with their putative helical regions shown as cylinders. GLP-1 binding (left) involves an interaction **H** between its helical region and the receptor's N-terminal domain, which accounts for approximately 82% of the total binding energy of GLP-1 (see Discussion). The remaining binding energy comes from the interaction $\mathbf{N^g}$ between the N-terminal sequence of GLP-1 and the core domain. The interaction between EX-4 and GLP-1R (right) is also predominantly *via* the peptide's helical region and the receptor's N-terminal domain, with this **H** interaction accounting for approximately 79% of the total binding energy of EX-4. However, the $\mathbf{N^{ex}}$ only contributes 5% of the binding energy of the full-length receptor. In addition, EX-4 forms an additional **Ex** interaction *via* its C-terminal region and the N-terminal domain of the receptor. This **Ex** interaction accounts for approximately 16% of the total binding energy of EX-4. The magnitude of the $\mathbf{N^g}$ interaction is equivalent to that of the **Ex** interaction.

the **N** interaction formed by GLP-1 ($\mathbf{N^g}$) is, clearly, of a greater magnitude to that formed by EX-4 ($\mathbf{N^{ex}}$). Since GLP-1 and EX-4 result in equivalent levels of receptor activation, it demonstrates that the component of the **N** interaction essential for receptor activation is maintained for both $\mathbf{N^g}$ and $\mathbf{N^{ex}}$. However, the greater strength of the $\mathbf{N^g}$ interaction suggests that additional peptide–receptor interactions are made with the N-terminal region of the natural hormone. The different interactions are unlikely to be due to the single residue change at position 2, since the substitution of Ala-2 by Gly in GLP-1 has only a minimal effect of affinity (Xiao *et al*., 2001). In order to test this further, we analysed the affinity of the peptide EX-4(Ala-2), which is identical to EX-4 except for the substitution of Gly 2 for Ala. Despite having an identical N-terminal sequence to GLP-1, we observed no improvement in the affinity of EX-4(Ala-2) compared with EX-4 (Tables 1, 2; not significantly different, $P<0.2$). Hence, the absence of a significant affinity-generating component in the N-terminus of EX-4, compared with GLP-1, is likely due to a slightly different orientation of each peptide's N-terminal region in the binding site on the receptor core domain. Rather than being due to differences in the N-terminal sequence, these alternative binding modes likely result from differences in the more distant regions of the binding site for the less-conserved central and C-terminal regions on the receptor's N-terminal domain. Further evidence for a difference between the **N** interactions of GLP-1 and EX-4 comes from the observation that GLP-1(3–30) is a partial agonist, while EX-4(3–39) is an antagonist, despite identical N-terminal sequences (Montrose-Rafizadeh *et al*., 1997).

If we make the assumption that the IC_{50} value approximates the affinity constant K, we can use the binding data at rGLP-1R to approximate the relative contribution of each region of GLP-1 and EX-4 to the total binding energy of the full-length peptide. Using the equation $\Delta G = -RT\ln K$ (where R is the universal gas constant, T is the temperature of the binding assay in degrees Kelvin and K is the affinity constant approximated by IC_{50}), this comparison can be achieved by calculating the values of ΔG for each truncated peptide as a percentage of its full-length counterpart, hence giving the relative contribution to binding of each region. These approximate and relative values are displayed on the schematic representation of our model in Figure 6b, and serve to emphasise that the primary interaction between the receptor and peptide is *via* the interaction of the peptide's helical region and the receptor's N-terminal domain, while the most significant additional interactions are made at opposite ends of each peptide ligand and also with different domains of the receptor.

Therefore, we present a model for peptide binding at GLP-1R, which demonstrates that while the primary receptor interaction with GLP-1 and EX-4 is *via* a similar mechanism, the additional interactions occur *via* opposite ends of each peptide, and also *via* different domains on the receptor. Such differences could be exploited in the search for novel GLP-1R agonists. The model therefore accounts for the N-independent affinity of EX-4, as well as the high affinity of EX-4 for the isolated N-terminal domain of the receptor.

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