Truncated Glucagon-like Peptide-1 and Exendin-4 α-Conotoxin pl14a Peptide Chimeras Maintain Potency and α-Helicity and Reveal Interactions Vital for cAMP Signaling in Vitro*

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Glucagon-like peptide-1 (GLP-1) signaling through the glucagon-like peptide 1 receptor (GLP-1R) is a key regulator of normal glucose metabolism, and exogenous GLP-1R agonist therapy is a promising avenue for the treatment of type 2 diabetes mellitus. To date, the development of therapeutic GLP-1R agonists has focused on producing drugs with an extended serum half-life. This has been achieved by engineering synthetic analogs of GLP-1 or the more stable exogenous GLP-1R agonist exendin-4 (Ex-4). These synthetic peptide hormones share the overall structure of GLP-1 and Ex-4, with a C-terminal helical segment and a flexible N-terminal tail. Although numerous studies have investigated the molecular determinants underpinning GLP-1 and Ex-4 binding and signaling through the GLP-1R, these have primarily focused on the length and composition of the N-terminal tail or on how to modulate the helicity of the full-length peptides. Here, we investigate the effect of C-terminal truncation in GLP-1 and Ex-4 on the cAMP pathway. To ensure helical C-terminal regions in the truncated peptides, we produced a series of chimeric peptides combining the N-terminal portion of GLP-1 or Ex-4 and the C-terminal segment of the helix-promoting peptide α-conotoxin pl14a. The helicity and structures of the chimeric peptides were confirmed using circular dichroism and NMR, respectively. We found no direct correlation between the fractional helicity and potency in signaling via the cAMP pathway. Rather, the most important feature for efficient receptor binding and signaling was the C-terminal helical segment (residues 22–27) directing the binding of Phe22 into a hydrophobic pocket on the GLP-1R.

Secretion of insulin is considerably higher in response to oral administration of glucose compared with intravenous delivery (1). This phenomenon, known as the “incretin effect,” is primarily mediated by two peptide hormones called incretins: glucagon like peptide 1 (GLP-1)5 (2) and glucose-dependent insulinoptropic polypeptide (3). The incretin effect is often greatly reduced in patients suffering from type 2 diabetes mellitus (4), and a possible therapeutic approach for this condition is incretin supplement therapy. Because type 2 diabetes mellitus sufferers continue to respond to GLP-1, but not to glucose-dependent insulinoptropic polypeptide (5), recent therapeutic supplement strategies have focused on GLP-1 and the development of synthetic analogs (6).

GLP-1 exerts its physiological activity in the 0.1–1 nM range (7) by signaling through the glucagon-like peptide-1 receptor (GLP-1R), a secretin/family B of G protein-coupled receptors (GPCR) (8). Primary GLP-1R signaling occurs via binding to Gα subunits that activate the intracellular cAMP pathway. Additional signaling also occurs via both β-arrestin and mobilization of intracellular calcium. Indeed, the level of variability in response reflects the diverse physiological functions of GLP-1R in different tissues (9). As with other members of GPCR receptor family, the GLP-1R has a large extracellular N-terminal domain (NTD) made up of one α-helix and two antiparallel β-sheets (10) and is expected to have a seven-transmembrane subdomain like other class B GPCRs (11). The crystal structure of the extracellular NTD of the GLP-1R in complex with GLP-1 revealed that the ligand binds to the NTD of the receptor through a C-terminal helix, whereas the N-terminal portion of the peptide is unstructured (Fig. 1A) (12). Binding and signaling through the GLP-1R is proposed to occur through a two-do-

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5 The abbreviations used are: GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GPCR, G protein-coupled receptor; Ex-4, exendin-4; NTD, N-terminal domain; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect (NOE) spectroscopy; ECOSEY, exclusive correlation spectroscopy; HSQC, heteronuclear single quantum correlation; r.m.s.d., root mean square deviation.
main model; the helical C terminus of the ligand first binds the NTD of the receptor, dictating binding affinity and specificity (Fig. 1A), before the ligand N terminus interacts with the seven-transmembrane bundle domain core of the receptor to affect signaling potency and specificity (13). The mechanism by which the N terminus of GLP-1R interacts with the binding pocket of the seven-transmembrane bundle domain and how this initiates receptor activation is presently unknown.

An exogenous GLP-1R agonist with a similar pharmacological profile to GLP-1, named exendin-4 (Ex-4; Fig. 1B), has been isolated from the saliva of the Gila monster (Heloderma suspectum) (14). Ex-4 and GLP-1 share 50% sequence identity, with Ex-4 being a slightly more potent agonist (15). A comparison of the crystal structures of the NTD of the GLP-1R in complex with either GLP-1 or Ex-4 revealed that they bind at the same site of the extracellular domain (10, 12), although comprehensive structure and function studies have revealed mechanistic differences in binding and signaling (16). These studies are primarily based upon binding affinity and cAMP signaling and include Ala scans, N-terminal truncations, and chimeras between GLP-1 and Ex-4 (15). The effect of C-terminal truncation of GLP-1 and Ex-4 has been much less extensively investigated. Removing the last two C-terminal residues of GLP-1 resulted in 40% reduction in insulin release from perfused rat pancreas at 100 pM (7) and a 10-fold reduction in binding affinity for the GLP-1R (17). Consistent with a role for the C terminus of GLP-1 in GLP-1R binding and signaling, replacing the C-terminal sequence VKGR of GLP-1 with the corresponding MNT of glucagon caused a 475-fold reduction in affinity (18), and removal of the last 10 C-terminal residues resulted in no binding or cAMP signaling at 10 μM (19).

It has been suggested that the helical structures of class B GPCR ligands, including GLP-1 and Ex-4, are important for receptor binding and signaling (20), and it may be that C-terminal truncations or mutations result in reduced helicity and thereby signaling efficacy. There are a number of chemical methods available for the stabilization of truncated helices, including lactam or disulfide bridges, hydrocarbon staples, and hydrogen bond surrogate approaches (21, 22). Lactam bridges have been used extensively to test the extent to which helicity in...
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GLP-1 affects receptor binding and signaling (23–26); however, to date there has been no investigation into the effect of C-terminal truncation of GLP-1 or Ex-4 on peptide helicity nor has there been research into the effect of inducing helicity in truncated analogs on receptor binding and signaling. Another approach to stabilizing helices is grafting of these secondary structural motifs into highly constrained helical peptide scaffolds (27). One such class of scaffolds is the disulfide-rich conotoxins isolated from marine cone snail venoms that naturally target nicotinic acetylcholine receptors. We recently used an α-conotoxin (pc16a) to engineer a potent GLP-1R agonist by combining the conotoxin disulfide bond connectivity with features of a previously published 11-amino acid peptidomimetic GLP-1R agonist (28). Another α-conotoxin pl14a (Fig. 1C), first isolated from the cone snail Conus planorbis (29), shares several properties with GLP-1 and Ex-4 as both classes of peptides have a flexible N termini followed by an α-helix and both target membrane integrated receptors. However, in contrast to the incretins, the α-helix in pl14a is highly constrained via two disulfide bonds (29), making the secondary structure less dependent on the amino acid sequence. These properties make pl14a an ideal scaffold for grafting peptides that have C-terminal helices and flexible N termini.

In this study we produced a series of C-terminally truncated GLP-1 and Ex-4 peptides both in their linear form and with the sequences grafted into the N-terminal flexible and central helical portions of the α-conotoxin pl14a. These peptides were structurally characterized using circular dichroism and NMR, and their ability to elicit cAMP signaling in vitro was evaluated. We found that although the pl14a scaffold could induce increased helicity in GLP-1 and Ex-4 sequences, signaling potency strongly correlated with the peptide length of the grafted peptide rather than overall helicity. Furthermore, the strongest determinant of GLP-1R agonist signaling through the cAMP pathway was found to be the presence or absence of Phe22. These findings provide new insights into the mechanism of GLP-1R activation and may guide future development of minimized and disulfide-constrained GLP-1 or Ex-4 analogs for the treatment of type 2 diabetes mellitus.

Experimental Procedures

Peptide Synthesis, Purification, and Mass Determination—Peptides were assembled on either rink-amide (0.59 mmol/g; Chem-Impex) or 2-chlorotrityl resins (0.80 mmol/g; Chem-Impex) at a 0.25-mmol scale using Fmoc (N-(9-fluorenylmethoxycarbonyl) solid-phase peptide synthesis on a Symphony Peptide synthesizer. Reagents and methods of peptide synthesis, purification, and mass spectroscopy were as previously described (30).

Circular Dichroism—Peptides were solubilized in either water or 10–20% acetonitrile (pH 3–4) at concentrations between 50 and 100 μM. Far ultraviolet spectra were recorded at room temperature using a Jasco J-810 spectropolarimeter and a 1-mm path length. Spectral data were collected from 3 scans from 260 to 190 nm with a scan speed of 100 nm min−1 and 0.5-nm wavelength steps. Solvent signal was subtracted before smoothing of the data (31) using the JASCO Spectra Manager software. Millidegree values were converted to mean residue ellipticity with units of degree-cm²-dmol⁻¹. Fractional helicity was calculated from the mean molar ellipticity at 220 nm, as previously described (32), assuming that the ellipticity of a completely helical peptide of infinite length is −37,000 degrees-cm²-dmol⁻¹.

CHO cAMP Accumulation Assay—CHO cells stably transfected with hGLP-1R were grown at 37 °C, 95% O₂, 5% CO₂ in 75-cm flasks containing DMEM/F-12 (1:1) medium with added 1% GlutaMAX™ (Gibco®), 1% PenStrep, and 1% Geneticin® (Gibco®) and grown until 90% confluent. Cells were then washed with PBS, lifted (cell dissolution solution; Sigma), counted, and used for cAMP accumulation assays and/or passaged (1:10). Following the manufacturer’s instructions for the LANCE® Ultra cAMP assay (PerkinElmer Life Sciences), cells transfected with hGLP-1R were centrifuged (1500 rpm, 5 min), resuspended in cAMP assay buffer (Hanks’ balanced salt solution, 5.56 mM glucose, 0.1% BSA, 0.5 mM isobutylmethylxanthine, 5 mM HEPS), and seeded at 1000 cells per well in a ProxiPlate-384 Plus plate (PerkinElmer Life Sciences). Cells were treated with compounds diluted in assay buffer over a range of concentrations (10 μM to 100 μM) and incubated for 30 min. Cell lysis buffers (Tracer (1:50) and Ulight (1:150)) were added to each well and incubated at room temperature for 2 h before reading the plates on a PHERAtstar FS (BMG Labtech). Raw signals from three technical replicates were normalized as a percentage of GLP-1 maximum before determining EC₅₀ values using GraphPad Prism 6 from three independent experiments.

NMR Spectroscopy—NMR experiments were carried out as described by Conibear et al. (33). Briefly, Ex-4[1–16]/pl14a and Ex-4[1–27]/pl14a were dissolved in 90% H₂O/10% D₂O or 99.96% D₂O (Cambridge Isotope Laboratories) at a concentration of 0.2 mM and pH 3.6 (uncorrected for isotope effects). Spectra were recorded on a Bruker Avance-600 equipped with a cryoprobe at 280 K (Ex-4[1–16]/pl14a) and 298 K (Ex-4[1–27]/pl14a). NMR experiments included TOCSY using a MLEV-17 spin lock sequence with a 80-ms mixing time, NOESY, ECOSY, and HMQC, and a 1 DANESO. Spectra were recorded with 4096 data points in the F₂ dimension and 512 increments in the F₁ dimension. The spectra were referenced to water at 4.97 ppm at 280 K and 4.76 ppm at 298 K. No 3JNH-Hα coupling constants could be measured from the one-dimensional spectra due to overlap of the peaks. 3JHα-Hα coupling constants for Ex-4[1–16]/pl14a were measured from the ECOSY spectrum, and together with intensities of intra-residual NOEs, these were used for stereo-specific assignments. Temperature coefficients were derived from a series of TOCSY experiments run at 280–310 K for Ex-4[1–16]/pl14a.

A 200-ms NOESY spectrum run at 280 K and 298 K for Ex-4[1–16]/pl14a and Ex-4[1–27]/pl14a, respectively, was used for assignment, integration, and measurement of Hα chemical shifts. A complete list of interproton distances was generated from chemical shifts and NOE intensities using the AUTO function in CYANA 3.0 (34). Several rounds of AUTO were run to ensure correct assignments of the peaks for both peptides. When >90% of all the picked peaks (463/483 for Ex-4[1–16]/pl14a and 651/731 for Ex-4[1–27]/pl14a) were appropriately assigned, the chemical shifts and distance restraints list gener-
ated by the AUTO function in CYANA was used for further structure calculations. Constraints for the $\phi$ and the $\psi$ backbone dihedral angles were generated using TALOS-N (35) from Hα, Ca, Cβ, HN, and N chemical shifts derived from $^{13}$C HSQC and $^{15}$N HSQC. In total, 11 $\phi$, 13 $\psi$ backbone dihedral angles, and 5 $\chi_1$ side-chain dihedral angles for Ex-4[1–16]/pl14a, and 19 $\phi$ and 19 $\psi$ backbone dihedral angles were included in the structure calculation for Ex-4[1–27]/pl14a. Disulfide connectivity of Cys$\gamma_1$-Cys$\gamma_{11}$ and Cys$\gamma_7$-Cys$\gamma_{14}$ (Cys$\gamma_7$-Cys$\gamma_{22}$ and Cys$\gamma_1$-Cys$\gamma_{24}$ for Ex-4[1–16]/pl14a Cys$\gamma_7$-Cys$\gamma_9$ and Cys$\gamma_1$-Cys$\gamma_{25}$ for Ex-4[1–16]/pl14a) previously reported for pl14A (29) were included as restraints. For Ex-4[1–16]/pl14a, amide protons for residues 12–17 and 25 shifted $< -4.6$ ppb/K, were considered to be shielded, and were included as hydrogen bond restraints.

Due to extensive flexibility observed in the N terminus of Ex-4[1–27]/pl14a, only the structure of Ex-4[1–16]/pl14a was refined using protocols from the RECOORD database (36) to calculate an ensemble of 50 structures within the CNS software (37) using the force-field distributed with Haddock 2.0 (38); the 50 structures generated were then further refined in a water shell, as previously described (33). A set of 20 structures with the lowest energy and no NOE violations $>0.2$ Å and few dihedral violations $>3^\circ$ was selected for MolProbity analysis (39).

### Molecular Modeling—The lowest energy NMR structures of Ex-4[1–27]/pl14a and Ex-4[1–16]/pl14a were aligned with Ex-4 bound to the NTD of the GLP-1R structure (PDB 3C5T). For Ex-4[1–27]/pl14a, the coordinates of residues 21–39 were based on the NMR constraints, although residues 9–20 were taken from the crystal structure. For Ex-4[1–27]/pl14a and Ex-4[1–30], the last eight N-terminal residues originated from an Ex-4 solution structure (PDB 1JRJ). Systems were solvated with TIP3P water and neutralized by Na$^+$/Cl$^-$ counterions using VMD1.9.1. This generated systems of 22,000–26,000 atoms, including 19,000–23,000 water molecules. Each protein complex was equilibrated using a stepwise relaxation procedure over 2.5 ns before production runs of 5 ns were carried out for each system using NAMD 2.9 CUDA and CHARMM27 force-field parameters, as previously described (30). Coordinates were saved every 500 simulation steps, producing the 5000 frames per simulation trajectories used for analysis in VMD. Interaction energies were calculated using the NAMD Energy plugin in VMD.

### Results

**Design of Truncated GLP-1 and Ex-4 Conotoxin Chimeras**—Although previous work suggested that the C-terminal segment of GLP-1 is important for efficient cAMP signaling via the GLP-1R (7, 17–19), the underlying mechanism(s) of action is poorly understood and may be a result of loss of either C-terminal helical structure, binding interactions, or both. To investigate the role of the C-terminal helical segment in GLP-1R signaling, a series of C terminally truncated GLP-1 and Ex-4 peptides was produced. These were either linear or grafted into the bicyclic, helical $\alpha$-conotoxin pl14a to stabilize the C-terminal helix (Fig. 1, B–E). Binding of the C-terminal helix of both GLP-1 and Ex-4 occurs in a groove in the GLP-1R NTD (Fig. 1A). To prevent steric hindrance upon binding, all possible chimeras for residues 13–30 of GLP-1 and Ex-4 were modeled (data not shown), and variants with minimal steric clashes were selected for synthesis (Fig. 1B).

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### Table 1

| Energies (kcal/mol) | Overall | Bonds | Angles | Improper | van der Waals | NOE | cDiH | Dihedral | Electrostatic |
|---------------------|---------|-------|--------|----------|---------------|-----|------|---------|---------------|
|                     | -894.3 ± 55.4 | 10.6 ± 1.3 | 30.0 ± 5.2 | 14.7 ± 2.7 | -82.0 ± 5.6 | 0.1 ± 0.03 | 0.4 ± 0.3 | 121.7 ± 1.9 | -989.9 ± 55.2 |

**MolProbity statistics**

|                  | Clashes ($>0.4$ Å/1000 atoms) | Poor rotamers | Ramachandran outliers (%) | Ramachandran favored (%) | MolProbity score | MolProbity score percentile |
|------------------|-------------------------------|---------------|--------------------------|-------------------------|-----------------|--------------------------|
|                  | 8.25 ± 2.2                    | 0.05 ± 0.2    | 0.6 ± 1.4                | 85.0 ± 5.6              | 2.1 ± 0.2       | 70 ± 9.5                 |

**Atomic r.m.s.d. (Å)**

|                    | Mean global backbone (9–16) | Mean global heavy (9–16) | Mean global backbone (9–28) | Mean global heavy (9–28) |
|--------------------|-----------------------------|--------------------------|----------------------------|--------------------------|
|                   | 0.25 ± 0.1                  | 1.30 ± 0.3               | 1.79 ± 0.2                 | 2.09 ± 0.5               |

**Distance restraints**

|                      | Intraresidue (i−j = 0) | Sequential (i−j−i−j−1) | Medium range (i−j−i < 5) | Long range (i−j−i−j−i−j−5) | Hydrogen bonds | Total |
|----------------------|------------------------|-------------------------|--------------------------|-----------------------------|---------------|-------|
|                     | 116                    | 154                     | 72                       | 11                          | 14            | 347   |

**Dihedral angle restraints**

|                   | $\phi$ | $\chi_1$ | Total |
|-------------------|--------|----------|-------|
|                   | 11     | 14       | 29    |

**Violations from experimental restraints**

|                      | NOE violations exceeding $0.3$ Å | Dihedral violations exceeding $3^\circ$ |
|----------------------|--------------------------------|---------------------------------------|
|                      | 0                               | 0                                     |
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There was a >500-fold reduction in cAMP potency compared with Ex-4[1–30], whereas for the linear Ex-4[1–27] F22A, Ex-4[1–24] F22A, and chimera Ex-4[1–27]/pl14a F22A, the reductions were 1700-, 45-, and 190-fold, respectively. Interestingly, Ex-4[1–16]/pl14a, which has a Tyr residue at position 22 (from the conotoxin scaffold), showed much more potent cAMP activity than other short chimeras lacking an aromatic residue at this position (Ex-4[1–19]/pl14a and Ex-4[1–20]/pl14a). Consistent with this, substituting Tyr22 with Ala22 in Ex-4[1–16]/pl14a resulted in a peptide (Ex-4[1–16]/pl14a Y22A) with a 30-fold lower cAMP activity.

Increased Helicity Does Not Correlate with Higher Potency—To evaluate the effect of grafting the truncated GLP-1 and Ex-4 peptides into the helical conotoxin pl14a on overall helicity, circular dichroism was measured, and helicity was estimated for all peptides (Table 2; Fig. 2, A–E). The fraction helicity of GLP-1 and Ex-4 corresponded well with those determined previously in aqueous buffer at pH 3.5 (40). Overall, incorporation of the truncated GLP-1 and Ex-4 into the helical pl14a resulted in increased helicity. A comparison of the potency of cAMP signaling for the various GLP-1 and Ex-4 variants did not show any correlation with fraction helicity (Table 2; Fig. 2F), which is in agreement with previous reports for lactam-bridged GLP-1 analogs (23, 24).

NMR Spectroscopy—Comparison of the C-terminal section of Ex-4[1–16]/pl14a (PDB 2NAW) and Ex-4[1–27]/pl14a (PDB 2NAW) with the native pl14a showed an r.m.s.d. of 0.32 Å and 0.40 Å across the heavy backbone atoms of the α-helix (Ex-4[1–16]/pl14a residues 9–16, Ex-4[1–27]/pl14a residues 20–27, and pl14a residues 6–13), suggesting that conotoxin pl14a can...
be used as a stabilizing scaffold and replace the Trp-cage present in native exendin-4. A comparison of the Hα chemical shifts to random coil values (41) showed stretches of negative secondary shifts, which in agreement with the literature, indicate an α-helix secondary structure from residues 8–16 in Ex-4[1–16]/pl14a and 8–28 in Ex-4[1–27]/pl14a (Fig. 3, A and B; Table 3) (42). This also corresponds well with circular dichroism data acquired for these two peptides, which suggests the presence of a large degree of helicity (Fig. 2). This helicity was observed in the three-dimensional structure of Ex-4[1–16]/pl14a, but not Ex-4[1–27]/pl14a, due to the lack of sufficient NOEs in the region despite the presence of several i–i+4 NOEs.

**Molecular Modeling**—Molecular dynamics was used to investigate how truncated Ex-4 variants might interact with the NTD of the receptor. In the case of Ex-4[1–27]/pl14a, where incomplete NMR constraints were available, solution and crystallography structures were used to create potential models of the bound peptides (see "Experimental Procedures"). Simulations of Ex-4[1–27] and Ex-4[1–27] F22A bound to the NTD of GLP-1 resulted in average simulation structures that closely aligned with the crystal structures of Ex-4/GLP-1R with 0.72 and 0.74 Å Cα r.m.s.d., respectively (Fig. 4A). Despite the lack of structural change between Ex-4[1–30] and Ex-4[1–30] F22A, a reduction in the interaction energy between the NTD of GLP-1R was observed for the F22A mutant (Table 3). The average simulation structure of the chimeric peptide Ex-4[1–27]/pl14a also closely aligned with the crystal structure (Cα r.m.s.d. 0.75 Å; Fig. 4, A and B), and the corresponding F22A mutation in Ex-4[1–27]/pl14a F22A was associated with a similar reduction in interaction energy as Ex-4[1–30] F22A, although Ex-4[1–27] F22A adopted a slight change in the spatial extension of the N-terminal end of the Ex-4 helix, resulting in a somewhat higher degree of deviation compared with the crystal structure (Cα r.m.s.d. 0.97 Å). These
reductions in interaction energies in silico for F22A mutants follow the trend seen in the cAMP assays but were not significant or absolutely proportional to the changes in EC₅₀ values. Attempts were made to model the binding between the NTD of GLP-1 and Ex-4[1–16]/pl14a, but the overlapping binding interface was determined too small to provide a realistic simulation.

**Discussion**

In this study we produced and characterized a series of C-terminally truncated GLP-1 and Ex-4 peptides, both in their linear forms and as α-conotoxin pl14a chimeric peptides. We found that residues 28–30 of GLP-1 and residues 28–39 of Ex-4 were not essential for either peptide helicity or GLP-1R signaling via the cAMP pathway in vitro. Further C-terminal truncations were associated with loss of α-helicity and GLP-1R signaling potency for both peptide hormones. Incorporating C-terminally truncated peptides into the α-conotoxin scaffold often increased helicity but did not similarly improve signaling potency. The most prominent determinant of GLP-1R signaling in vitro was found to be Phe²² in either peptide hormone. These findings might help to guide the future design of minimized and/or constrained GLP-1 or Ex-4 peptide analogs suitable for the treatment of type 2 diabetes mellitus.

The results presented in this study have clarified the relative contribution of the C-terminal helical segments of GLP-1 and Ex-4 to GLP-1R binding and signaling. Although there are a few previous studies involving C-terminal truncations of GLP-1 using different methods (7, 17, 19), ours is the first to systematically explore the effects of C-terminal truncation of GLP-1 and Ex-4. We found that C-terminal removal of three residues from GLP-1 and 12 residues from Ex-4 had only small effects upon cAMP signaling for both linear and chimeric peptides (Fig. 5A). Conversely, removal of three more residues (residues 25–27) from the C terminus of either peptide resulted in a potency loss equal to at least three orders of magnitude. A similar dominating effect was seen for Phe²² in both GLP-1 and Ex-4, where peptides that included Phe²² generally showed more potent cAMP signaling. The positive effect of Phe²² was particularly strong for Ex-4, and a series of F22A mutant peptides confirmed the importance of Phe²² for cAMP signaling (Fig. 5B). These findings are in line with previous Ala scans of GLP-1, which also found that the F22A substitution caused the largest loss in binding affinity (1300-fold) and cAMP signaling (1000-fold) out of all Ala mutants (43). Combined, these findings suggest that the central portion of both GLP-1 and Ex-4 acts as a spacer to position a C-terminal helical segment (residues 22–27) to correctly align Phe²² with a vital binding pocket on the receptor (Fig. 5C). However, future studies need to explore to what extent these structural determinants modulating cAMP signaling translates to insulin secretion.

**FIGURE 3. Secondary Hα chemical shifts and solution structures of selected Ex-4/pl14a chimeras.** A, Ex-4[1–16]/pl14a (PDB 2NAV) secondary Hα chemical shifts (left) and ribbon plots of solution structures (right) with α-helices shown in blue, and disulfide bonds are in yellow. B, Ex-4[1–27]/pl14a (PDB 2NAW) secondary Hα chemical shifts (left) and solution structures (right). Residues with secondary Hα chemical shifts indicating helical structure are indicated with gray helices above the residue numbering.

**TABLE 3**

Calculated interaction energies for the GLP-1 NTD and Ex-4 variants

| Peptide                      | Interaction energy (kcal/mol ± S.D.) |
|------------------------------|-------------------------------------|
| Ex-1[1–30]                   | −258.0 ± 38.4                       |
| Ex-1[1–30] F22A              | −215.7 ± 30.6                       |
| Ex-4[1–27]/pl14a             | −194.0 ± 32.8                       |
| Ex-4[1–27]/pl14a F22A        | −158.3 ± 19.2                       |
FIGURE 4. Molecular modeling of the GLP-1R NTD and Ex-4/Ex-4 pl14a chimeras. A, overlay of the average molecular dynamics simulation secondary structures of Ex-4[1–30] (light blue), Ex-4[1–30] F22A (cyan) and a previous crystal structure (PDB 3C5T; dark blue). Phe22 is labeled and shown in stick model. B, overlay of the average molecular dynamics simulation secondary structures of Ex-4[1–27] pl14a (light blue) and PDB 3C5T (dark blue). The area delineated by the gray dotted rectangle is shown in greater detail for the overlay of Ex-4[1–27] pl14a and PDB 3C5T (C) as well as for Ex-4[1–27] pl14a (D) and PDB 3C5T (E) alone, where the ligand’s secondary structure (green) and stick models (green, carbon; blue, nitrogen; red, oxygen; yellow, sulfur) are shown bound to the electrostatic surface of the GLP-1R NTD (blue, positive; red, negative; white, neutral).

FIGURE 5. Molecular determinants of cAMP signaling. A, correlation between the number of residues of GLP-1 or Ex-4 grafted into conotoxin pl14a and the resulting cAMP signaling Log EC50 values. B, alignment of various Ex-4 (blue) and conotoxin pl14a (black) chimeras and their EC50 values with residue 22 are bound by a black box. Mutated and Cys residues are highlighted in red and yellow, respectively. C, ribbon plot of a crystal structure (PDB 3C5T) of Ex-4 (green) bound to the NTD of the GLP-1R (blue), showing residues interacting in the hydrophobic patch as stick models.
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A recent study involving GLP-1R mutants concluded that a complementary "hydrophobic patch" on GLP-1/Ex-4 and the receptor is needed for correct positioning of the N terminus for GLP-1R activation, a feature that may extend to all class B GPCRs (44). This conclusion was based on GLP-1R mutagenesis of L32A (44), on studies showing the importance of Phe22/Ile23 in GLP-1 for receptor activation (43, 45) and on the presence of a similar hydrophobic patch on glucagon (needed for efficient binding to the glucagon extracellular domain) (46). Although Phe22 only forms part of this hydrophobic patch, we have shown that mutating Phe22 has an impact that is orders of magnitude greater than other hydrophobic residues in this patch.

These findings in conjunction with previous studies provide novel insight into the mechanistic foundations for GLP-1R receptor activation. Considering the size and nature of the binding interface between GLP-1/Ex-4 and the GLP-1R, the mutation of a single residue alone is unlikely to explain such a large change in signaling efficacy; rather, it suggests a mechanistic cause. It may well be that the correct spatial orientation of Phe22 to the pocket in the receptor triggers conformational change in the receptor that affects the relative orientation of the N-terminal and transmembrane domains. This may also explain why much smaller 11-residue peptides, which share the N-terminal portion of GLP-1/Ex-4 but have various biphenyl and/or phenylalanine derivatives at the C-terminal end, achieve similar activation profiles to that of GLP-1 and Ex-4 (28, 47, 48). It is possible that, for these compounds, the biphenyl derivatives bind to the same pocket as Phe22 in GLP-1/Ex-4 and trigger a conformational change in the receptor that allows the N terminus of these shorter peptides to interact with the core of the receptor. If so, one may envision future GLP-1R ligands that consist of a C-terminal hydrophobic anchor segment, a central spacer segment of correct length, and an N-terminal activation segment that binds to the core of the receptor.

Further studies need to be undertaken to fully clarify the role of the hydrophobic patch in receptor activation and signaling. For example, further GLP-1R mutagenesis studies of all residues involved in this hydrophobic interaction may shed further light on the extent of the patch and the relative contribution of various receptor residues. In particular, comparing the effect of GLP-1R mutation on binding affinity and signaling using both full-length peptide agonists and the 11-residue peptides should give a good indication of whether they capitalize on the same interactions. Cross-linking experiments between the 11-residue peptides and the GLP-1R may also be used to further pinpoint the binding area of the biphenyl derivatives.

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