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A β-Peptide Agonist of the GLP-1 Receptor, a Class B GPCR

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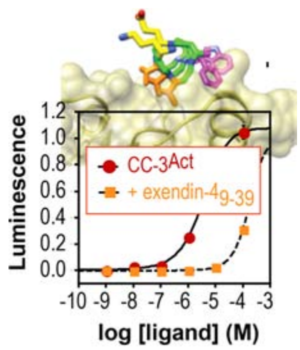
ABSTRACT



Previous work has shown that certain β^3-peptides can effectively mimic the side chain display of an α-helix and inhibit interactions between proteins, both *in vitro* and in cultured cells. Here we describe a β^3-peptide analog of GLP-1, CC-3^{Act}, that interacts with the GLP-1R extracellular domain (nGLP-1R) *in vitro* in a manner that competes with exendin-4 and induces GLP-1R-dependent cAMP signaling in cultured CHO-K1 cells expressing GLP-1R.

The primary role of pancreatic β cells is to synthesize and secrete insulin. This function is modulated in part by a set of heterotrimeric G proteins that are the immediate downstream targets of diverse G protein-coupled receptors (GPCRs). A number of GPCRs expressed by pancreatic β cells regulate insulin secretion. One, the receptor for the glucagon-like peptide-1 (GLP-1R), binds to and is activated by glucagon-like peptide-1 (GLP-1),[1,2] a 30-aa member of the incretin hormone family. In the presence of glucose, activated GLP-1R signals through the associated G protein G_S to activate the adenylyl cyclase pathway and stimulate insulin secretion.[3] Indeed, GLP-1R is a validated target for the treatment of type 2 diabetes mellitus.[3,4] Exendin-4, a 39-aa GLP-1 ortholog[5] possessing improved serum stability, was approved for use in 2005. Additional GLP-1 mimetics with improved pharmacodynamics are in development,[6,7] and efforts have begun to identify

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low molecular weight compounds that may allow oral administration.[8−15] Furthermore, new bifunctional peptides that activate GLP-1R and other molecular targets are being explored in preclinical models for enhanced antidiabetic pharmacology.[16,17]

Previous work has shown that certain β^3-peptides can effectively mimic the side chain display of an α-helix and inhibit interactions between proteins, both *in vitro*[18−20] and in cultured cells.[21−23] Oligomers containing mixtures of both α- and β-amino acids have also shown success.[24] Here we describe a potential β^3-peptide analog of GLP-1, CC-3^{Act}, that interacts with the GLP-1R extracellular domain (nGLP-1R) *in vitro* in a manner that competes with exendin-4 and induces GLP-1R-dependent cAMP signaling in cultured CHO-K1 cells expressing GLP-1R.

Our design of CC-3^{Act} began with the sequence of exendin-4. This sequence can be divided into two regions: a 22 residue C-terminal region that associates as an α-helix (shown in purple in Figure 1) with nGLP-1R (shown in gray) and functions in isolation as a receptor antagonist (K_d = 5 nM[5]) and a 9 residue N-terminal region of

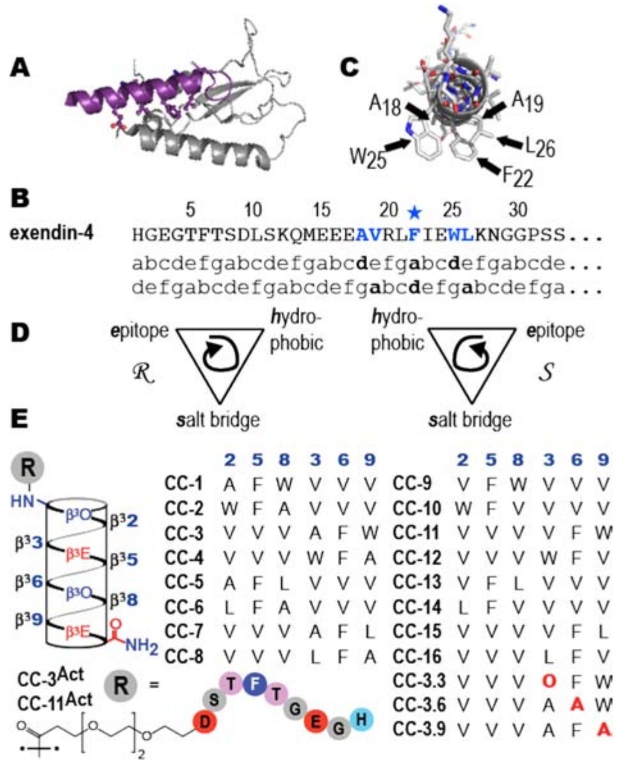

Figure 1. (A) View of exendin-4 (purple) in complex with nGLP-1R (gray) (PDB 3C5T) illustrating relative orientation of the incretin α-helix and the Sushi fold of the receptor extracellular domain. (B) Sequence of exendin-4 aligned with two heptad repeats. (C) View of GLP-1 looking down the helix axis to illustrate side chain arrangement at the ligand−receptor interaction face. (D) Cartoon illustrating the stereochemical relationships between the three 14-helix faces. (E) Helical net and sequences of β-peptides evaluated herein.

undefined structure that is required for receptor activation.[5,25,26] A large number of side chains within the C-terminal region contribute to exendin-4/GLP-1R binding and/or receptor activation. These include E_{15}, F_{22}, I_{23}, and L_{26}; modest contributions are also made by L_{14}, K_{20}, A_{24}, W_{25}, V_{27}, and K_{28}. Of these side chains, five—A_{18}, W_{25}, F_{22}, L_{26}, and A/V_{19}—are conserved between exendin-4 and GLP-1 and localize on the bound α-helix at the ligand−receptor interface (Figure 1C).[27]

Our design was further refined by three principles uncovered during previous efforts to develop β^3-peptide mimics of less complex α-helical segments.[18,20−23,28,29] These efforts revealed that homo-oligomeric β^3-peptides presenting three interacting residues on one 14-helix face

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often perform as designed, binding their targets with affinities < 1 μM, whereas those presenting four interacting residues usually do not. These efforts also revealed that the axial orientation of the three interacting residues (N to C vs C to N) and their stereochemical relationship (Figure 1D) could modulate K_d by more than 100-fold. The side chain that contributes most significantly to exendin-4•GLP-1R interaction is F_{22}.[30] Thus, we began by identifying a collection of side chain triads containing F_{22} and other components of the bound α-helix interface that could be displayed on each of two 14-helix faces and in either the N to C or C to N direction. For example, were F_{22} to occupy position *d* of a heptad repeat, the *ada* side chain triad would include V_{19}, F_{22}, and L_{26}; were it to occupy position *a*, the *dad* triad would be A_{18}, F_{22}, and W_{25}. Each of these three residue epitopes can be presented in two axial directions and on two 14-helix faces to generate a collection of 8 β^3-peptides. β^3-Peptides presenting VFW and AFL epitopes (formally *adg* and *gda*) were also prepared to generate a collection of 16 molecules (Figure 1E).

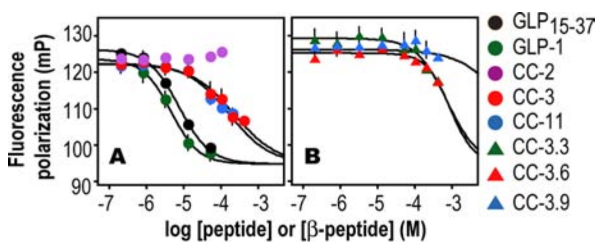

Figure 2. Fluorescence polarization (FP) competition analysis of interactions between nGLP-1R and either peptides (GLP-1 and GLP1_{15-37}) or β^3-peptides. Plots illustrate the change in the polarization of 5 nM exendin-4^{Flu} as a function of the concentration of the ligand indicated; [nGLP-1R] = 125 nM.

First we used an *in vitro* fluorescence polarization (FP) competition assay to compare the relative affinities of these 16 β^3-peptides for recombinant nGLP-1R (Figure S1). Each β^3-peptide was incubated at a concentration of 10 or 50 μM with 5 nM exendin-4^{Flu} in the presence of 125 nM nGLP-1R, and the polarization of the mixture was monitored at equilibrium. Of the 16 β^3-peptides evaluated, 14 had no effect on the observed polarization, even at 50 μM concentration, indicating that they had little or no effect on the fraction of exendin-4^{Flu} bound to the nGLP-1R under these conditions. β^3-Peptides CC-3 and CC-11, however, both significantly reduced the observed polarization values (22% and 33% relative to exendin-4), suggesting that they compete with exendin-4^{Flu} for the nGLP-1R binding pocket. Incubation of 5 nM exendin-4^{Flu} and 125 nM nGLP-1R with between 10 nM and 500 μM CC-3 or CC-11 led to a concentration-dependent decrease in the fraction of exendin-4^{Flu} bound (Figure 2A); subsequent data analysis suggested IC_{50} (and K_i) values of 228 ± 35 μM (115 ± 18) and 116 ± 31 (84 ± 16) μM for CC-3 and CC-11, respectively. In comparison, the α-peptide antagonist GLP-1_{15-37} was only 15−20 times more potent than CC-11 in this assay, competing with exendin-4^{Flu} with IC_{50} and K_i values of 7.53 ± 0.54 and 3.75 ± 0.23 μM, respectively. Differences between exendin-4 and GLP-1 binding to the GLP-1R ectodomain *in vitro* are well described.[31] Even the modest affinity of GLP-1 for nGLP-1R is sufficient for subnanomolar potency in the context of the full-length receptor and peptide.

Three lines of evidence suggest that CC-3 and CC-11 mimic the α-helical regions of exendin-4 and GLP-1 in their interactions with nGLP-1R. First, substitution of each component of the 'AFW/VFW' epitope for alanine (CC-3.6, CC-3.9) or ornithine (CC-3.3) led to β^3-peptides that compete poorly with exendin-4^{Flu} for binding to nGLP-1R (Figure 2B). Second, CC-3 and CC-11 differ by only two methyl groups, presenting either an AFW (CC-3) or VFW (CC-11) triad in the N-to-C orientation on the same β^3-peptide face. Six other collection members carry one of these two side chain triads (CC-1, 2, and 4; CC-9, 10, 12), but differ from CC-3 and CC-11 in the relative orientation of the three side chains (N-to-C or C-to-N) or the stereochemical relationship of the epitope, hydrophobic, and salt bridge faces. Yet, only CC-3 and CC-11 competed effectively with exendin-4^{Flu} for binding to nGLP-1R (Figure 2B).

Finally, *post hoc* modeling experiments provide a structural rationale for the observed differences in affinity among β^3-peptides possessing alternate arrangements of the same binding epitope. We used the program *pepz*[32] to construct models for β^3-peptides CC-1−4 and CC-9−12 in an ideal 3_{14}-helix conformation.[33] After sampling of the epitope side chains, the Cβ, Cγ, Cδ, Cε1, and Nζ1 atoms of the epitope residues were used to align each β^3-peptide to the corresponding side chain atoms (Cβ, Cγ, Cδ, Cε1, and Nζ1) of exendin-4 in the exendin-4•nGLP-1R complex structure (PDBID 3c5t). The RMSD of these alignments varied between 1.1 and 1.4 Å (shown in Figure S2). While these models are too coarse to reveal detailed interactions or the precise placement of each side chain in the binding pocket, they do identify the relative orientation of side chains presented on each 3_{14} helix face. In the case of CC-3 and CC-11, alignment of the AFW/VFW epitopes into the binding pocket orients the β^3-peptide hydrophobic face, which contains three valine side chains, toward a hydrophobic groove on the GLP-1R surface, and orients the salt-bridge face, which contains acidic and basic side chains, toward solvent. Reversing the C-to-N direction of the AFW/VFW epitope as in CC-1 and CC-9, or reversing the relative orientation of the salt-bridge and valine faces with respect to the epitope face as in CC-4 and CC-12, directs the salt-bridge face toward the hydrophobic groove on the nGLP-1R surface and points the hydrophobic

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valine side chains toward solvent. These two side chain arrangements are likely to be energetically unfavorable and destabilize the interaction of these β^3-peptides with nGLP-1R. Interestingly, reversal of *both* the face ordering and the N-to-C directionality (as in CC-2 and CC-10) leads to side-chain placement similar to that of CC-3 and CC-11. In this case, the reduced 3_{14} helicity of CC-2 and CC-10 may be the primary cause for their reduced binding (Figure S3).

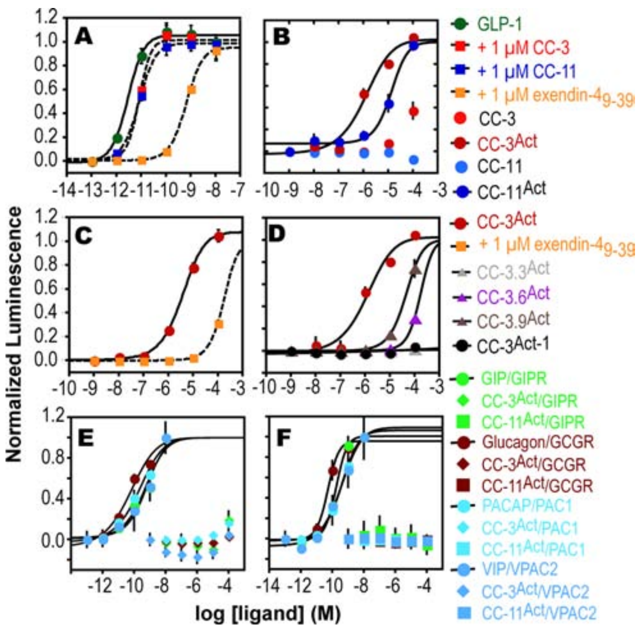

Figure 3. Effect of β^3-peptides and ligands on cAMP production in cells expressing class B1 GPCRs. (A–D) Effect of various β^3-peptides and exendin-4 on cAMP accumulation in GLP-1R+ CHO-K1 cells. (E,F) cAMP accumulation in GIPR+, PAC1+, GCCR+, and VPAC+CHO-K1 cells upon treatment with the cognate ligand, CC-3^{Act} or CC-11^{Act}.

Next, CC-3 and CC-11 were converted into the potential β^3-hormones CC-3^{Act} and CC-11^{Act} by extending their sequence at the N-terminus with a 13-atom polyethylene glycol (PEG) chain followed by the α-peptide NH_2-HGEGTFTSD, which corresponds to the nine N-terminal residues of exendin-4 and are critical for GLP-1R activation[30] (Figure 1). Procedures to minimize aspartimide formation[34] were employed and validated,[35,36] and the products were purified using pristine HPLC columns to avoid cross-contamination with GLP-1 itself. Ligand-dependent GPCR activation was monitored in GLP-1R+ CHO-K1 cells using a luciferase reporter gene under the control of a cAMP response element promoter.[30] As expected, GLP-1 was a potent GLP-1R agonist (EC_{50} = 2.86 ± 0.70 pM) and was inhibited by 1 μM exendin-4_{9-39}, CC-3 or CC-11, in accord with the relative *in vitro* affinities of these ligands (Figure 3A). Both CC-3^{Act} and CC-11^{Act} activated GLP-1R in CHO-K1 cells (EC_{50} = 1.2 ± 0.74 and 13.2 ± 2.5 μM respectively (Figure 3B). Activation of GLP-1R by CC-3^{Act} was reduced 50-fold by 1 μM exendin-4_{9-39} (Figure 3C), suggesting that the observed increase in cAMP resulted from a direct interaction of CC-3^{Act} with the GLP-1R ligand-binding domain. In addition, loss of any of the three side chains that contributed to receptor affinity *in vitro* (Figures 1 and 2) decreased potency in the cell based assay (Figure 3D). Finally, an analog of CC-3^{Act} lacking a PEG linker (CC-$3^{Act\text{-}1}$) was inactive (Figure 3D), consistent with the need for a discrete structural relationship between the C- and N-terminal domains.

GLP-1R is one of several homologous class B1 GPCRs expressed in the pancreas. Other family members include the receptors activated by peptides known as GIP, glucagon, VIP, and PACAP.[37−39] To investigate the selectivity of CC-3^{Act} as a GLP-1R agonist, we examined its effect on the activation of these four receptors in CHO-K1 cells. Although each of these receptors were activated potently by their cognate ligands (Figure 3E), none were activated by CC-3^{Act} (or CC-11^{Act}), even at high concentration (Figure 3F).

Although the results reported herein suggest that CC-3^{Act} activates GLP-1R through interactions that mimic those of GLP-1 and exendin-4, its potency was modest, a full 6 orders of magnitude lower than GLP-1. Even mM concentrations of CC-3^{Act} did not inhibit forskolin-dependent activation of the adenylate cyclase pathway (Figure S4), ruling out the possibility that the lower-than-expected potency of CC-3^{Act} was due to unexpected interference with a later step in the activation pathway. We note, however, that the high *in vitro* potencies of GLP-1 and orthologs are counterbalanced by the rapid elimination and short half-lives of the peptides *in vivo*. β^3-Peptides are not subject to the same degradation processes, and all available studies indicate dramatically enhanced stability *in vivo*.[40−43] Further optimization of these sequences is ongoing in our laboratory.

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Supporting Information Available. Peptide synthesis and characterization, assay procedures, and supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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