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Functional association of the N-terminal residues with the central region in glucagon-related peptides

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GLP-1 is an incretin peptide involved in the regulation of glucose metabolism and the glucose-dependent stimulation of insulin secretion. Ex-4 is a paralog of GLP-1 that has comparable GLP-1R potency but extended physiological action. GLP-1 and Ex-4 are helical peptides that share ∼50% sequence homology but differ at several residues, notably the second amino acid which controls susceptibility to DPP-IV cleavage. This single amino acid difference yields divergent receptor potency when studied in the context of the two hormone sequences. Ex-4 uniquely tolerates Gly2 through select amino acid differences in the middle region of the peptide that are absent in GLP-1. We report that substitution of Ex-4 amino acids Glu16, Leu21, and Glu24 to the GLP-1 sequence enabled Gly2 tolerance. The coordination of the N-terminus with these central residues shows an interaction of substantial importance not only to DPP-IV stability but also to receptor activation. Extension of this observation to glucagon-based co-agonist peptides showed different structural requirements for effective communication between the N-terminus and the mid-section of these peptides in achieving high potency agonism at the GLP-1 and GCGRs. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: GLP-1; Ex-4; glucagon; GLP-1R; CGCR; co-agonist; DPP-IV

Introduction

GLP-1 is an incretin hormone that facilitates insulin secretion from pancreatic β-cells to promote glucose homeostasis. GLP-1 is produced by intestinal L-cells and released upon nutrient ingestion to act at a specific GLP-1R in a glucose-dependent manner (the incretin effect) [1]. Treatment of adult-onset diabetics with the GLP-1R agonist Ex-4 has proven clinical benefit in lowering blood glucose and the reduction of body weight [2]. The actions of GLP-1 include the suppression of glucagon, decreasing gastric motility, and increasing β-cell mass: each representing beneficial elements for treatment of Type II diabetes. However, native GLP-1 is cleaved proteolytically and inactivated by DPP-IV within minutes, minimizing its effectiveness as a therapeutic agent [3–6]. The short half-life of native GLP-1 has led to the identification of analogs with more favorable therapeutic profiles [7–9].

Ex-4 has been shown to have a comparable *in vitro* profile of slightly increased potency as native GLP-1 but shows much extended *in vivo* action [10–13], in part because of Ex-4 being appreciably resistant to DPP-IV cleavage. An amino acid difference at position 2 in Ex-4 (Gly), relative to GLP-1 (Ala), has largely been attributed for this difference in the kinetics of degradation (Figure 1), but other residues have also been implicated [11]. Ex-4 does not discriminate between either residue at position 2 [14] whereas GLP-1 loses approximately fourfold potency when substituted with glycine at this position [15–19]. The N-terminal histidine has been shown to be essential for binding with subtle alterations in the structure of this residue making considerable changes in activity [20–23]. Though structure–function studies have focused on position 2 to improve proteolytic stability, the structural mechanism by which Gly2 is tolerated by the GLP-1R in Ex-4 but not GLP-1 has yet to be addressed. We hypothesized that Ex-4 contains unique sequence distal to position 2 that enable substitution, and that the biophysical characterizations of peptide–ligand association with the GLP-1R extracellular domain do not fully provide a molecular basis for these biochemical observations.

GLP-1 and Ex-4 are both peptides with sizable helical content, but GLP-1 is considerably less helical than Ex-4 when studied in aqueous solution [24,25]. NMR studies have shown that GLP-1 has a discontinuous helix around Gly16 whereas Ex-4 adopts a continuous helix [26–28]. The presence of Glu16 in Ex-4 promotes alpha helicity which is further reinforced by the alternating charge network in its backbone (Figure 1). Another distinctive feature of Ex-4 is its C-terminal sequence extension (Cex), which folds back on the C-terminus (Trp25) to form the Trp-cage [27,29]. While Cex has been implicated in protecting Ex-4 *in vivo* [11], the C-terminal extension was not required for receptor recognition by either the agonist or the N-terminally truncated antagonist [24,30]. Deletion of Cex from Ex-4 had minimal effect on helicity, but C-terminal extension of GLP-1 with Cex bestowed helicity intermediate to

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Abbreviations used: *GLP-1, glucagon-like peptide-1; Ex-4, exendin-4; GLP-1R, glucagon-like peptide-1 receptor; GCGR, glucagon receptor; DPP-IV, dipeptidyl peptidase IV; cAMP, cyclic adenosine monophosphate.*

Figure 1. Primary sequences of GLP-1 (red), Ex-4 (blue), glucagon (purple), and hybrid peptides. Positions 2 and 16 are indicated with an asterisk (*), and the C-terminal extension of Ex-4 (Cex) is underlined. Regions of sequence involved in substitution are boxed. Top, conserved residues between GLP-1 and Ex-4 are black and residues unique to GLP-1 and Ex-4 are red and blue respectively. Bottom, residues conserved between GLP-1, Ex-4, and glucagon are shown in black. Shared residues with either GLP-1 or Ex-4 are red or blue respectively, and residues unique to glucagon are purple.

GLP-1 and Ex-4 [30]. Moreover, the addition of Cex to GLP-1 was further shown to partially reduce potency loss upon Gly2 substitution [16]. To identify the specific Ex-4 residues required for full Gly2 tolerance, we studied a series of GLP-1/Ex-4 hybrid peptides that mapped the basis for tolerance to the central to C-terminal region of the hormone. We also prepared glucagon and glucagon-based receptor co-agonists of the GLP-1 and GCGRs to explore the influence of differing peptide backbones on position 2 promiscuity at the structurally related GCGR (Figure 1).

Experimental Procedures

Peptide Synthesis

Peptides were generated by solid-phase peptide synthesis. An *in situ* neutralization protocol for Boc-chemistry was followed as described by Kent and colleagues [31]. Peptide synthesis was performed using 0.2 mmol 4-methylbenzhydrylamine resin (Midwest Biotech, Fishers, IN, USA) on a modified Applied Biosystems 430A synthesizer (Applied Biosystems, Foster City, CA, USA). Amino acids (Midwest Biotech) were side-chain protected with the following groups: Arg(Tos), Asp(OcHex), Asn(Xan), Glu(OcHex), His(BOM), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Trp(CHO), and Tyr(Br-Z). Activation of amino acids (2 mmol) was performed with 0.5 M 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one and DIEA (4 : 1 v/v). Cleavage from the solid support used HF/*p*-cresol (95 : 5 v/v) for 1 h at 0 °C followed by *in vacuo* HF evacuation. Peptides were next precipitated in diethyl ether, collected using a 50 μm Teflon filter funnel, and solubilized in 10% acetic acid. Peptides were then lyophilized and stored until purification.

Peptide Purification

RP-HPLC was employed for purification of peptides. A C_{18} stationary phase (Vydac, Deerfield, IL, USA: 218TP, 250 mm × 2 mm, 10 μm) was utilized with a linear acetonitrile gradient in 0.1% TFA during preparative purifications. Peptide identity and purity were assessed by analytical RP-HPLC and ESI- or MALDI-MS. Analytical RP-HPLC analysis was performed on peak fractions with a C_8 column (Zorbax, Santa Clara, CA, USA: 300SB, 4.6 mm × 50 mm, 3.5 μm) using a linear acetonitrile gradient in 0.1% TFA. All peptides were found to have the correct molecular weight and >90% purity. Lyophilized peptides were stored at 4 °C.

GLP-1R- and GCGR-Mediated cAMP Induction

The ability of peptides to stimulate cAMP induction at the human GLP-1 and GCGRs was examined by a luciferase reporter gene assay. Co-transfection of HEK293 cells with the receptor and a cAMP response element-linked luciferase gene enabled receptor activation measurements. Bioassays were performed by incubating serum deprived cells with serial dilutions of peptides for 5 h at 37 °C and 5% CO_2 in 96-well poly-D-lysine-coated tissue culture plates (BD Biosciences, Wellesley, MA, USA) using Dulbecco-modified Eagle's medium supplemented with 0.25% Bovine Growth Serum (HyClone, Logan, UT, USA) as diluent. Assays were stopped by the addition of an equivalent volume (100 μl) of LuCLite luminescence substrate reagent (Perkin-Elmer, San Jose, CA, USA). Plates were shaken at 600 rpm for 3 min, and luminescence signal was quantified with a MicroBeta-1450 liquid scintillation counter (Perkin-Elmer). Data was plotted using Origin software (OriginLab, Northampton, MA, USA), and the half maximal effective concentration (EC_{50}) was determined by sigmoidal curve fitting.

Circular Dichroism

Peptides were analyzed by CD spectroscopy from 250 to 190 nm using a JASCO J-715 (Easton, MD, USA) spectropolarimeter. Samples were prepared at 10 μM peptide in 10 mM aqueous sodium phosphate buffer (pH 5.9). Nitrogen was streamed over the samples at 25 °C, and data was collected for five scans in a 1 mm path length cell at a scan speed of 100 nm per min and a 0.5 nm wavelength step. Spectra were normalized by subtracting background solvent signal and then Savitsky–Golay smoothing was applied. Millidegree data values were converted to mean residue ellipticity (deg cm^2 $dmol^{-1}$) allowing calculation of percent helicity.

Results

Improved Tolerance for Gly2 Substitution by Glu16

GLP-1 (1–30)a (**1**) is the endogenous ligand of the GLP-1R (EC_{50} = 0.036 nM), but its reptilian counterpart Ex-4 (1–39)a (**2**) (EC_{50} = 0.017 nM) stimulates cAMP induction with twofold increased potency (Figure 2(A) and Table 1) [13]. Ex-4 receptor activation was not dependent on the presence of Cex, as Ex-4 (1–30)a (**3**) proved equally effective [30]. While Cex moderately increased the activity of GLP-1 (**4**), simultaneous substitution of Glu16 and Cex (**5**) yielded a hormone equivalent to Ex-4 (**2**) boosting GLP-1 potency by approximately twofold (Figure 2(A) and Table 1). However, incorporation of Glu16 to GLP-1 (1–30)a (**6**) was sufficient to reach equivalent potency with the comparable Ex-4-based peptide **3** (Figure 2(A)). Our results are consistent with the observation that α helix stabilizing residues improve GLP-1R potency [24,32,33] and set a foundation to determine specific Ex-4 and GLP-1 sequences tolerant to position 2 substitutions.

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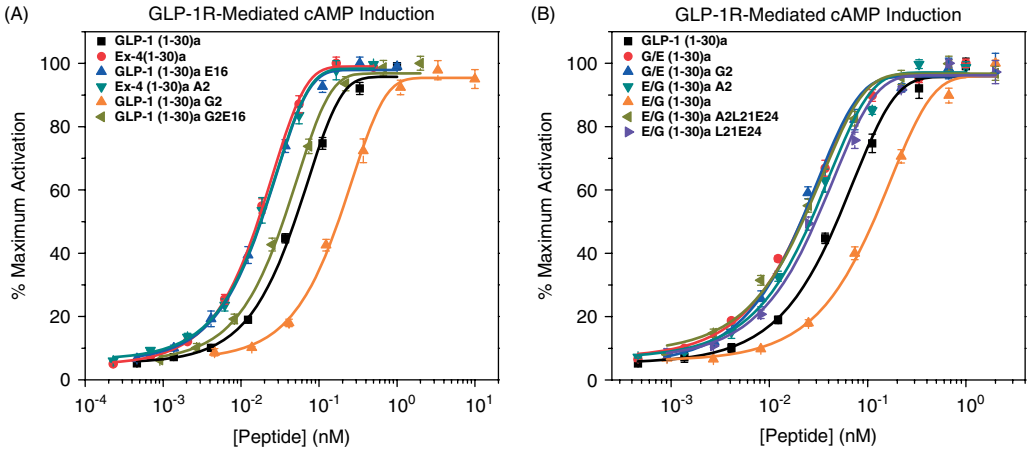

Figure 2. (A) GLP-1R-mediated cAMP induction by GLP-1, Ex-4, and GLP-1 Glu16/Cex analogs. (B) Stimulation of cAMP release by GLP-1/Ex-4 hybrid peptides substituted at position 2.

Table 1. Summary of GLP-1R activation for GLP-1/Ex-4 hybrids. Numbering for GLP-1 (7–36)a corresponds to positions (1–30)a for consistency with Ex-4[a,b]

#	Peptide	GLP-1R, EC_{50} (nM)
1	GLP-1 (1–30)a	0.036 ± 0.007
2	Ex-4 (1–39)a	0.017 ± 0.005
3	Ex-4 (1–30)a	0.019 ± 0.006
4	GLP-1-Cex (1–39)a	0.025 ± 0.007
5	GLP-1-Cex (1–39)a Glu16	0.018 ± 0.005
6	GLP-1 (1–30)a Glu16	0.020 ± 0.007
7	Ex-4 (1–30)a Ala2	0.020 ± 0.004
8	GLP-1 (1–30)a Gly2	0.15 ± 0.03
9	GLP-1 (1–30)a Gly2Glu16	0.039 ± 0.008
10	G/E (1–30)a	0.021 ± 0.004
11	G/E (1–30)a Gly2	0.019 ± 0.005
12	E/G (1–30)a Ala2	0.028 ± 0.007
13	E/G (1–30)a	0.081 ± 0.02
14	E/G (1–30)a Ala2Leu21Glu24	0.022 ± 0.007
15	E/G (1–30)a Leu21Glu24	0.026 ± 0.006
16	GLP-1 (1–30)a Glu16Leu21Glu24	0.018 ± 0.003
17	GLP-1 (1–30)a Gly2Glu16Leu21Glu24	0.020 ± 0.004

[a] Peptides were assayed with an n value ≥ 3 (n, the number of duplicate experiments performed).
[b] Assays were normalized to the EC_{50} value of GLP-1.

The amino acid substitution of Ala2 to Gly2 in GLP-1 confers partial plasma DPP-IV resistance but decreases potency [15–19]. While GLP-1 was reduced in potency by fourfold with Gly2 substitution (**8**) (Figure 2(A) and Table 1), Ex-4 (1–39)a accommodates this residue equally well [14]. To evaluate if specific sequence elements enable Gly2 tolerance in the absence of Cex, we prepared both Ala2 and Gly2 versions of the GLP-1, GLP-1 Glu16, and Ex-4 (1–30)a peptides. Ex-4 (1–30)a, like its C-terminally extended counterpart, did not discriminate between Ala2 (**7**) and Gly2 (**3**) thus eliminating Cex as the source of the differential activity. GLP-1 (1–30)a Glu16 displayed a response that was intermediate to those of the Ex-4 and GLP-1 peptides (**6,9**) (Figure 2(A)). While Glu16 was the minimal change required to improve *in vitro* potency of GLP-1 to that of Ex-4, it proved insufficient to provide full tolerance to Gly2 substitution. Hence, additional elements contribute to the equivalence of Gly and Ala at position 2 within the Ex-4 sequence.

Leu21 and Glu24 Support Gly2 Acceptance

Peptides devoid of Cex were prepared to identify the additional Ex-4 sequence elements that facilitate Gly2 tolerance. Analogs were designed to contain either GLP-1 or Ex-4 at the N- or C-termini with Glu16 as the central pivot point (Figure 1). Hybrid peptides **10** and **11** containing Ex-4 sequence at the C-terminus (G/E) tolerated Ala2 and Gly2 equally well (**10** EC_{50} = 0.021 nM, **11** EC_{50} = 0.019 nM), relative to Ex-4 (**3, 7**) (Figure 2(B) and Table 1). On the contrary, analogs with GLP-1 sequence at the C-terminus, **12** and **13** (E/G), displayed a strong sensitivity to Gly2 substitution, reminiscent of native GLP-1 (Figure 2(B)). Peptide E/G preferred Ala2 to residue Gly2 by threefold (**12** EC_{50} = 0.028 nM, **13** EC_{50} = 0.081 nM) in comparison to GLP-1 Glu16 which exhibited a twofold preference (**6, 9**) (Table 1). The additional sequence elements conferring N-terminal position 2 tolerance appeared to be located within residues 17–28 encouraging further study of C-terminal Ex-4 substitutions.

We reasoned that if the alternating charge network present in the Ex-4 sequence reinforces its structure, then substitution of these residues to GLP-1 might similarly improve Gly2 tolerance. With this as an objective, Leu21 and Glu24 were incorporated into the E/G backbone to stabilize the hormone's secondary structure (Figure 1). Incorporation of Leu21 removes a potential negative charge with GLP-1 Glu21 that could disrupt the alternating charge network. Indeed, E/G (1–30)a Leu21Glu24 did not discriminate between Ala2 (**14**) and Gly2 (**15**) substitution (**14** EC_{50} = 0.022 nM, **15** EC_{50} = 0.026 nM) (Figure 2(B) and Table 1). The GLP-1-based peptide GLP-1 (1–30)a Glu16Leu21Glu24 also did not discriminate between Ala2 (**16**) and Gly2 (**17**) and maintained Ex-4-like potency (**16** EC_{50} = 0.018 nM, **17** EC_{50} = 0.020 nM) (Table 1). Thus, the results provide evidence that the stabilized helix associated with the alternating charge network of Ex-4 is serving a functional role in receptor activation.

GLP-1R Activity in Glucagon-Based Hormones

To expand our investigation to the GCGR, we synthesized glucagon-based hormones modified at position 2 with Ala, Gly, Aib,

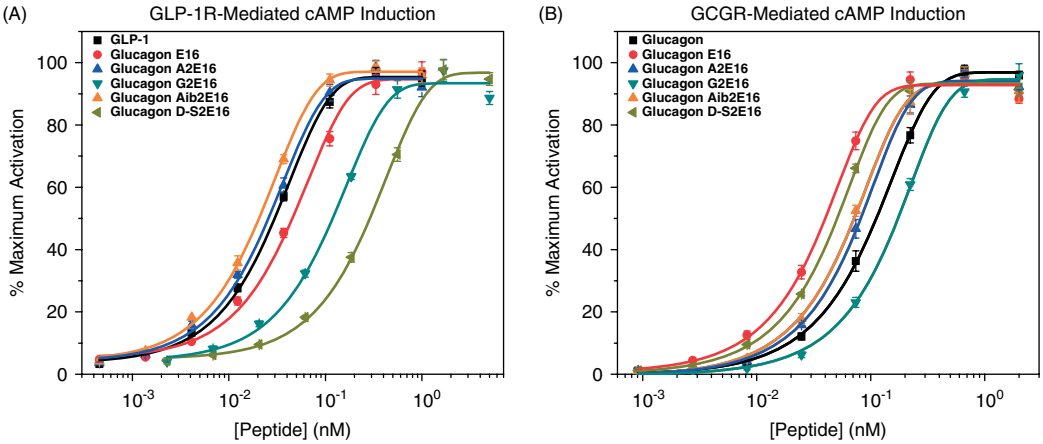

Figure 3. cAMP induction by glucagon (1–29)a Glu16 analogs at the GLP-1 (A) and glucagon (B) receptors.

Table 2. Summary of agonism at the GLP-1 and GCGRs for glucagon-based analogs[a,b]

#	Peptide	GLP-1R, EC_{50} (nM)	GCGR, EC_{50} (nM)
1	GLP-1 (1–30)a	0.036 ± 0.007	n/a
18	Glucagon (1–29)a	1.8 ± 0.4	0.068 ± 0.02
19	Glucagon (1–29)a Glu16	0.041 ± 0.006	0.022 ± 0.006
20	Glucagon (1–29)a Ala2Glu16	0.026 ± 0.005	0.041 ± 0.005
21	Glucagon (1–29)a Gly2Glu16	0.13 ± 0.01	0.084 ± 0.008
22	Glucagon (1–29)a Aib2Glu16	0.024 ± 0.004	0.036 ± 0.005
23	Glucagon (1–29)a D-Ser2Glu16	0.20 ± 0.04	0.028 ± 0.004
24	Chimera 2X (1–29)a	0.027 ± 0.007	0.056 ± 0.01
25	Chimera 2X (1–29)a Ala2	0.026 ± 0.006	0.68 ± 0.2
26	Chimera 2X (1–29)a Gly2	0.041 ± 0.01	1.5 ± 0.4
27	Chimera 2X (1–29)a Aib2	0.019 ± 0.005	0.62 ± 0.1
28	Chimera 2X (1–29)a D-Ser2	0.050 ± 0.02	0.061 ± 0.02
29	Chimera 2 (1–29)a	0.030 ± 0.006	0.050 ± 0.01
30	Chimera 2 (1–29)a Ala2	0.027 ± 0.005	0.088 ± 0.02
31	Chimera 2 (1–29)a Gly2	0.043 ± 0.007	0.20 ± 0.03
32	Chimera 2 (1–29)a Aib2	0.022 ± 0.004	0.70 ± 0.1
33	Chimera 2 (1–29)a D-Ser2	0.069 ± 0.01	0.059 ± 0.02

[a] Peptides were assayed with an n value ≥ 3 (n, the number of duplicate experiments performed).
[b] Assays were normalized to the GLP-1 or glucagon EC_{50} value at their respective receptors.

or D-Ser. We included Aib and D-Ser substitutions in this series as the altered chirality of these amino acids could serve as additional probes into the function of N-terminally modified hormones. However, glucagon (1–29)a (**18**) does not activate the GLP-1R with similar potency as it does the GCGR (GLP-1R EC_{50} = 1.8 nM, GCGR EC_{50} = 0.068 nM) (Figure 3 and Table 2). We have previously reported the balanced co-agonism of glucagon (1–29)a Glu16 (**19**) (Figure 1) [33]. Substitution to Glu16 in glucagon provided a nearly 50-fold increase in GLP-1R potency, without disrupting GCGR activity (GLP-1R EC_{50} = 0.041 nM, GCGR EC_{50} = 0.022 nM). This provided a suitable glucagon-based sequence to explore the effect of position 2 structural substitution on activity at both receptors.

We prepared position 2 analogs of glucagon (1–29)a Glu16 (**19**–**23**) and for comparative purposes additional studies were performed in a second and third set of glucagon-based co-agonists. These two sets were distinguished by the substitution of the C-terminal residues of glucagon (17–24) by either the corresponding sequence in Ex-4 or GLP-1. The Ex-4-based co-agonist set was termed Chimera 2X (**24**–**28**) and the GLP-1-based co-agonist set Chimera 2 (**29**–**33**) (Figure 1). The comparison of Ala2 (**20**) and Gly2 (**21**) analogs of glucagon (1–29)a Glu16 revealed a differential activity at the GLP-1R similar to that which we previously observed with GLP-1-based peptides, with Ala2 being fivefold more potent (Figure 3(A) and Table 2). Once again consistent with our prior observations, the substitution of Ex-4 amino acids 17–24 significantly eliminated this difference at the GLP-1R (**25** vs **26**) (Figure 4(A)). The position 2 analogs for these two sets of peptides (**24**–**33**) varied by no more than threefold potency difference at the GLP-1R. Furthermore, there was alignment in potency across Chimera 2X and Chimera 2 peptides with Aib2 (**27,32**) proving most favorable and D-Ser (**28,33**) least (Figures 4(A) and 5(A)). While the dynamic range observed for potency at the GLP-1R was increased in the glucagon-based set of peptides (**19**–**23**), Aib2 (**22**) still proved most potent and D-Ser (**23**) least active (Figure 3(A)).

GCGR Activity in Glucagon-Based Hormones

A comparable analysis of these same peptide analogs (**19**–**33**) at the GCGR proved highly informative and with notable differences in potency observed between Ex-4 (**24**–**28**) and GLP-1 (**29**–**33**) sequence substitutions. The glucagon-based set with only the single non-native Glu16 (**19**–**23**) showed similar GCGR potency in a range of 0.022–0.041 nM, with only one exception (Figure 3(B) and Table 2). The Gly2 analog (**21**) exhibited a lower potency of 0.084 nM. The Chimera 2X sequence, which had proven effective in minimizing position 2 differences in potency at the GLP-1R, showed a range of potencies that differed by nearly 30-fold (**24**–**28**) (Figure 4(B) and Table 2). Ser2 (**24**) and D-Ser2 (**28**) peptides were nearly as effective as the most potent glucagon (1–29)a Glu16 based peptides. The Ala (**25**) and Aib2 (**27**) peptides were reduced in potency approximately tenfold illustrating the importance of the serine hydroxyl group. This structural preference was not apparent in the set of peptide analogs with more native glucagon sequence (**19**–**23**) (Figure 3(B)). Finally, the Gly2 Chimera 2X analog (**26**) proved less than 10% the potency of the Gly2 glucagon-based analog (**21**) (Figure 4(B) and Table 2). This further illustrates the

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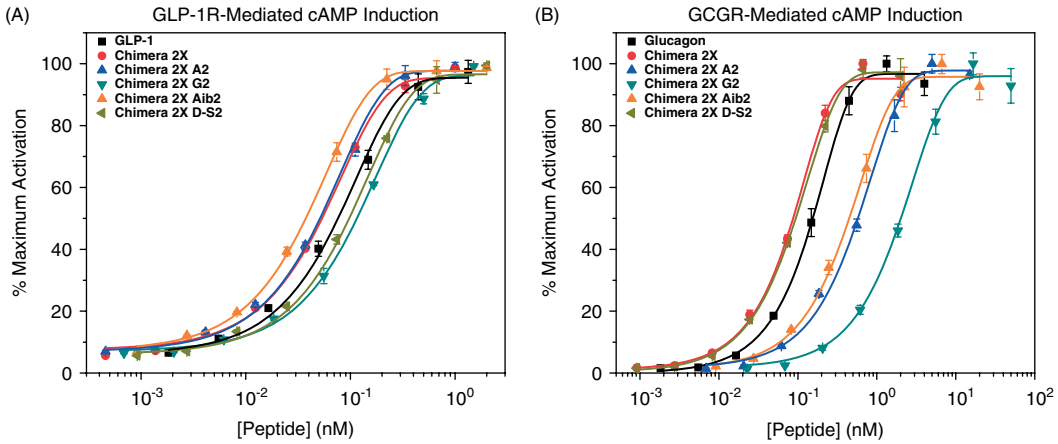

Figure 4. Stimulation of cAMP production by glucagon/Ex-4 hybrid peptides at the GLP-1 (A) and glucagon (B) receptors.

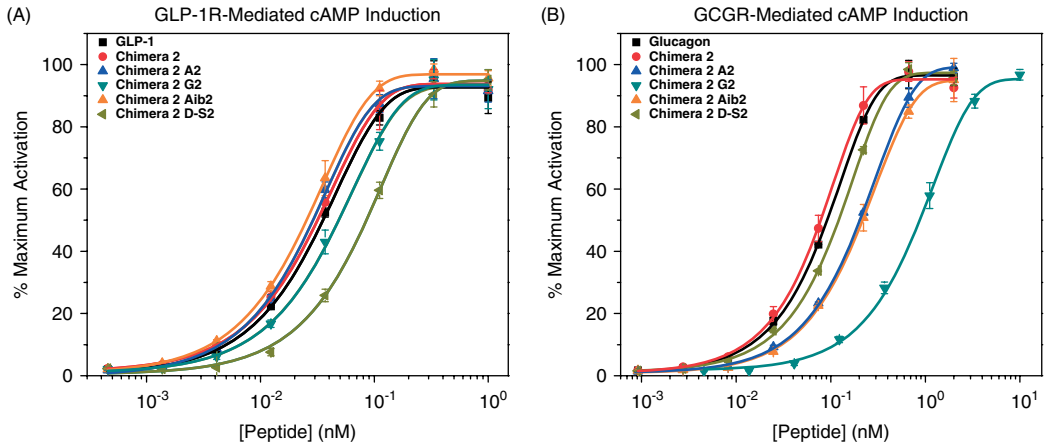

Figure 5. Stimulation of cAMP synthesis by glucagon/GLP-1 hybrid peptides at the GLP-1 (A) and glucagon (B) receptors.

importance of an optimal peptide backbone in minimizing the destructive effect of a less optimal amino acid at position 2.

The Chimera 2 peptide series (**29**–**33**) exhibited GCGR potency intermediate to the observations with the two other structurally related peptide sets (**19**–**23** and **24**–**28**) (Figure 5(B)). Serine (**29**) and D-Serine (**33**) proved most effective, but Ala2 (**30**) was only slightly less potent. The Aib2 peptide (**32**) was equally reduced in potency when compared to the same substitution in the Chimera 2X peptide (**27**), while Gly2 (**31**) was nearly eightfold the potency of the same substitution in the peptide of enhanced Ex-4 sequence (**26**) and only one-fourth the potency of the most potent peptide in the Chimera 2 series (Table 2). The data are striking in showing the relative importance of the second amino acid being highly dependent on the specific peptide backbone and the receptor used in analysis. Three homologous peptide sets differing only in relative sequence at a point linearly removed from position two by more than half the total length of the peptide, and without any direct structural contact in three dimensional space, exhibited a spectrum of biological activities.

Discussion

The results of this study indicate that residues within the central region of glucagon-related peptides stabilize the α helix, and this stabilization is sensed structurally at the N-terminus of the molecule to optimize interactions with its cognate receptor. Recent crystal structures have revealed distinctive features of GLP-1 and Ex-4 (9–39)a ligand binding to the GLP-1R extracellular domain [34,35], but they do not provide details into the molecular interactions between the peptide N-terminus with the core domain of the receptor. Extensive structure–function studies on the N-terminus of GLP-1 have been conducted to engineer DPP-IV protease resistance, however, many of these substitutions result in poor activity [9]. Ex-4 potency at the GLP-1R was modestly improved *in vitro* relative to GLP-1 [EC_{50}, GLP-1 (**1**) 0.036 nM; Ex-4 (**2**) 0.017 nM] [13], but Ex-4 did not discriminate between Ala and Gly at the second position (**3,7**) (Figure 2(A) and Table 1). Substitution of Ala2 with Gly2 has been shown to increase resistance to DPP-IV degradation [14–19] although GLP-1 potency was reduced by fourfold with this change (**1,8**). The reduced α helicity of GLP-1 relative to Ex-4 has been attributed to Gly16 which introduces a discontinuous helix whereas Glu16 in Ex-4 stabilizes the backbone (Figure 6 and Table 3) [25,27]. However, substitution of Glu16 to GLP-1 [24], much like Cex [16,30], only partially restored GLP-1R activity and alone was not sufficient to explain how Ex-4 tolerates Gly2 (Figures 2(A) and 6).

The additional elements in Ex-4 that enable promiscuity at the second position were identified through a series of GLP-1/Ex-4 hybrid ligands, G/E (**10,11**) and E/G (**12,13**) (Figures 1 and 2(B)). An approximate threefold sensitivity to Gly2 relative

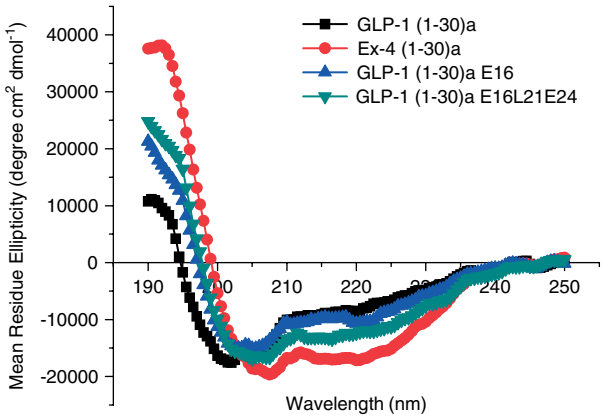

Figure 6. CD of GLP-1, Ex-4, and hybrid (1–30)a peptides (10 μM) in aqueous phosphate buffer (pH 5.9).

Table 3. CD of GLP-1, Ex-4, and, GLP-1/Ex-4 hybrids[a]

#	Peptide	Percent Helicity (%)
1	GLP-1 (1–30)a	22
3	Ex-4 (1–30)a	49
6	GLP-1 (1–30)a Glu16	30
7	Ex-4 (1–30)a Ala2	49
8	GLP-1 (1–30)a Gly2	21
9	GLP-1 (1–30)a Gly2Glu16	28
16	GLP-1 (1–30)a Glu16Leu21Glu24	35
17	GLP-1 (1–30)a Gly2Glu16Leu21Glu24	36

[a] Percent helicity was calculated from mean residue ellipticity values at 222 nM.

to Ala2 remained for the E/G hybrids, but the same change was well tolerated when the hybrid peptide was substituted with Ex-4 sequence at the C-terminus (Table 1). Extension of the charge network in the E/G hybrid and GLP-1 Glu16 backbones through Leu21, Glu24 substitution proved equally tolerant to Gly2 substitution (**14**–**17**) (Figure 2(B)). The increased helical propensity of GLP-1 Glu16 and GLP-1 Glu16Leu21Glu24 relative to the native hormone suggests that position 2 promiscuity may be facilitated by communication of the enhanced central secondary structure with the N-terminus of the hormone (Figure 6 and Table 3). It was previously shown that α,α-dialkylamino acid substitution or backbone lactamization within this region simultaneously increases helicity, potency, and serum stability [32,36]. The influence of the C-terminal hydrophobic amino acids in supporting α helicity of GLP-1 has also been shown through alanine substitution of Phe22 and Ile23 [21], residues flanked by Leu21 and Glu24. Intriguingly, Leu21 and Glu24 bind in close proximity to extracellular domain residues Leu32* and Glu127* respectively, which have been shown to contribute differentially to Ex-4 activity [35]. Additionally, hydrophilic interactions between the central amino acids of the hormone and extracellular domain residue Glu128* have also been shown to be determinants of GLP-1R receptor binding and selectivity between the homologous GLP-1 and GCGRs [37].

We extended this structure–function analysis to the structurally related GCGR with a series of GLP-1/GCGR co-agonists based on the glucagon (1–29)a Glu16 (**19**–**23**), Chimera 2X (**24**–**28**), and Chimera 2 (**29**–**33**) backbones. These co-agonist analogs were varied at positions 17–24 and provided an opportunity to simultaneously explore the effect of position 2 substitution at the two receptors (Figure 1). The glucagon-based hybrid peptides **19, 24**, and **29** were similarly effective as GLP-1R and GCGR agonists with only a slight preference for the native glucagon 17–24 sequence, consistent with previous results [33] (Figures 3–5). At the GLP-1R, these sequences responded similarly and proved moderately tolerant to Gly2 or D-Ser2 substitution. However, the GCGR has proven to be highly sensitive to substitution within the N-terminal portion of the hormone with positions two and three being more restrictive than for GLP-1 [38–40]. The dispersed activity of the co-agonist analogs at the GCGR when the second amino acid position was varied reflects the enhanced sensitivity of this receptor relative to the GLP-1R (Table 2). Glucagon (1–29)a Glu16 was more tolerant to changes in the second position than was Chimera 2 and differentiation was even more evident for Chimera 2X. A similar destructive effect on GCGR potency was reported when using the central Ex-4 sequence relative to the GLP-1 sequence in an attempt to optimize peptides with mixed GLP-1R agonist/GCGR antagonist activities [41].

Notably, Chimera 2X and Chimera 2 peptides substituted with Ala, Gly, or Aib at position two were especially reduced in potency at the GCGR relative to the respective serine analogs of either stereochemistry (Table 2). Given the importance of the central peptide region in generating a balanced co-agonist peptide [33,37], it is probable that substitution of GLP-1 or Ex-4 sequence within this region causes unfavorable interactions with the GCGR when the second amino acid is not a hydroxylated residue. Mutational studies of the GCGR [39] have identified interactions between glucagon Ser2/TM7 and Gln3/TM2 that serve as a secondary selectivity filter and constitute a likely cooperative site in receptor activation [42]. Disruption of such contacts between the N-terminus of the peptide with the receptor core domain through altered binding to the receptor's extracellular domain could be the basis of the enhanced GCGR sensitivity to non-cognate substitutions made within the 17–24 sequence. The α helical nature of the molecule imparts the ability to communicate structural changes throughout the peptide. However, specific contacts with the receptor provide interactions needed for proper orientation of His1 with TM2 in the receptor-bound conformation [43–45].

In conclusion, our observations compliment the recently proposed mechanistic model for Family B G protein-coupled receptor induction where ligand binding is associated with concomitant α helix formation and supported through specific receptor contacts [46]. A similar functional relationship has been reported for parathyroid hormone binding advocating that such higher order features of ligand binding are common to this homologous receptor class [47,48]. It appears clear that the simplistic lock and key model for activation is insufficient to explain the complexity of signal transduction. Physiologically, structural interactions among ligands and receptors must be compatible with other demands imposed by biosynthesis, storage, secretion, and degradation of the specific hormone. Consequently, these higher order structural communications constitute a vital source of affinity and specificity in hormone action. The relative tolerance of the native glucagon sequence to changes at position 2 compared to non-native agonists of comparable potency stands as a reminder of the unexplained wisdom in

Journal of PeptideScience

selection against the destructive effect of potential indiscriminate mutations.

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References

1 Vilsboll T, Holst JJ. Incretins, insulin secretion and Type 2 diabetes mellitus. *Diabetologia* 2004; **47**(3): 357–366.
2 Drucker DJ. The biology of incretin hormones. *Cell Metab.* 2006; **3**(3): 153–165.
3 Oshima I, Hirota M, Ohboshi C, Shima K. Comparison of half-disappearance times, distribution volumes and metabolic clearance rates of exogenous glucagon-like peptide 1 and glucagon in rats. *Regul. Pept.* 1988; **21**(1–2): 85–93.
4 Mentlein R, Gallwitz B, Schmidt WE. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur. J. Biochem.* 1993; **214**(3): 829–835.
5 Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology* 1995; **136**(8): 3585–3596.
6 Hassan M, Eskilsson A, Nilsson C, Jonsson C, Jacobsson H, Refai E, Larsson S, Efendic S. In vivo dynamic distribution of 131I-glucagon-like peptide-1 (7–36) amide in the rat studied by gamma camera. *Nucl. Med. Biol.* 1999; **26**(4): 413–420.
7 Holst JJ, Orskov C. The incretin approach for diabetes treatment: modulation of islet hormone release by GLP-1 agonism. *Diabetes* 2004; **53**(Suppl. 3): S197–204.
8 Kruger DF, Bode B, Spollett GR. Understanding GLP-1 analogs and enhancing patients success. *Diab. Educ.* 2010; **36**(Suppl. 3): 44S–72S; quiz 73S–74S.
9 Hui H, Zhao X, Perfetti R. Structure and function studies of glucagon-like peptide-1 (GLP-1): the designing of a novel pharmacological agent for the treatment of diabetes. *Diab. Metab. Res. Rev.* 2005; **21**(4): 313–331.
10 Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, Garant MJ, Egan JM. Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia* 1999; **42**(1): 45–50.
11 Skovgaard M, Kodra JT, Gram DX, Knudsen SM, Madsen D, Liberles DA. Using evolutionary information and ancestral sequences to understand the sequence-function relationship in GLP-1 agonists. *J. Mol. Biol.* 2006; **363**(5): 977–988.
12 Young AA, Gedulin BR, Bhavsar S, Bodkin N, Jodka C, Hansen B, Denaro M. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (Macaca mulatta). *Diabetes* 1999; **48**(5): 1026–1034.
13 Goke R, Fehmann HC, Linn T, Schmidt H, Krause M, Eng J, Goke B. Exendin-4 is a high potency agonist and truncated exendin-(9–39)-amide an antagonist at the glucagon-like peptide 1-(7–36)-amide receptor of insulin-secreting beta-cells. *J. Biol. Chem.* 1993; **268**(26): 19650–19655.
14 Al-Sabah S, Donnelly D. A model for receptor-peptide binding at the glucagon-like peptide-1 (GLP-1) receptor through the analysis of truncated ligands and receptors. *Br. J. Pharmacol.* 2003; **140**(2): 339–346.
15 Burcelin R, Dolci W, Thorens B. Long-lasting antidiabetic effect of a dipeptidyl peptidase IV-resistant analog of glucagon-like peptide-1. *Metabolism* 1999; **48**(2): 252–258.
16 Doyle ME, Theodorakis MJ, Holloway HW, Bernier M, Greig NH, Egan JM. The importance of the nine-amino acid C-terminal sequence of exendin-4 for binding to the GLP-1 receptor and for biological activity. *Regul. Pept.* 2003; **114**(2–3): 153–158.
17 Deacon CF, Knudsen LB, Madsen K, Wiberg FC, Jacobsen O, Holst JJ. Dipeptidyl peptidase IV resistant analogues of glucagon-like peptide-1 which have extended metabolic stability and improved biological activity. *Diabetologia* 1998; **41**(3): 271–278.
18 Siegel EG, Gallwitz B, Scharf G, Mentlein R, Morys-Wortmann C, Folsch UR, Schrezenmeir J, Drescher K, Schmidt WE. Biological activity of GLP-1-analogues with N-terminal modifications. *Regul. Pept.* 1999; **79**(2–3): 93–102.
19 Xiao Q, Giguere J, Parisien M, Jeng W, St-Pierre SA, Brubaker PL, Wheeler MB. Biological activities of glucagon-like peptide-1 analogues in vitro and in vivo. *Biochemistry* 2001; **40**(9): 2860–2869.
20 Sarrauste de Menthiere C, Chavanieu A, Grassy G, Dalle S, Salazar G, Kervran A, Pfeiffer B, Renard P, Delagrange P, Manechez D, Bakes D, Ktorza A, Calas B. Structural requirements of the N-terminal region of GLP-1-(7–37)-NH2 for receptor interaction and cAMP production. *Eur. J. Med. Chem.* 2004; **39**(6): 473–480.
21 Adelhorst K, Hedegaard BB, Knudsen LB, Kirk O. Structure-activity studies of glucagon-like peptide-1. *J. Biol. Chem.* 1994; **269**(9): 6275–6278.
22 Hareter A, Hoffmann E, Bode HP, Goke B, Goke R. The positive charge of the imidazole side chain of histidine7 is crucial for GLP-1 action. *Endocrinol. J.* 1997; **44**(5): 701–705.
23 Gallwitz B, Witt M, Paetzold G, Morys-Wortmann C, Zimmermann B, Eckart K, Folsch UR, Schmidt WE. Structure/activity characterization of glucagon-like peptide-1. *Eur. J. Biochem.* 1994; **225**(3): 1151–1156.
24 Patterson JT, Ottaway N, Gelfanov VM, Smiley DL, Perez-Tilve D, Pfluger PT, Tschop MH, Dimarchi RD. A novel human-based receptor antagonist of sustained action reveals body weight control by endogenous GLP-1. *ACS Chem. Biol.* 2011; **6**(2): 135–145.
25 Andersen NH, Brodsky Y, Neidigh JW, Prickett KS. Medium-dependence of the secondary structure of exendin-4 and glucagon-like-peptide-1. *Bioorg. Med. Chem.* 2002; **10**(1): 79–85.
26 Thornton K, Gorenstein DG. Structure of glucagon-like peptide (7–36) amide in a dodecylphosphocholine micelle as determined by 2D NMR. *Biochemistry* 1994; **33**(12): 3532–3539.
27 Neidigh JW, Fesinmeyer RM, Prickett KS, Andersen NH. Exendin-4 and glucagon-like-peptide-1: NMR structural comparisons in the solution and micelle-associated states. *Biochemistry* 2001; **40**(44): 13188–13200.
28 Chang X, Keller D, O'Donoghue SI, Led JJ. NMR studies of the aggregation of glucagon-like peptide-1: formation of a symmetric helical dimer. *FEBS Lett.* 2002; **515**(1–3): 165–170.
29 Neidigh JW, Andersen NH. Peptide conformational changes induced by tryptophan-phosphocholine interactions in a micelle. *Biopolymers* 2002; **65**(5): 354–361.
30 Runge S, Schimmer S, Oschmann J, Schiodt CB, Knudsen SM, Jeppesen CB, Madsen K, Lau J, Thogersen H, Rudolph R. Differential structural properties of GLP-1 and exendin-4 determine their relative affinity for the GLP-1 receptor N-terminal extracellular domain. *Biochemistry* 2007; **46**(19): 5830–5840.
31 Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* 1992; **40**(3–4): 180–193.
32 Miranda LP, Winters KA, Gegg CV, Patel A, Aral J, Long J, Zhang J, Diamond S, Guido M, Stanislaus S, Ma M, Li H, Rose MJ, Poppe L, Veniant MM. Design and synthesis of conformationally constrained glucagon-like peptide-1 derivatives with increased plasma stability and prolonged in vivo activity. *J. Med. Chem.* 2008; **51**(9): 2758–2765.
33 Day JW, Ottaway N, Patterson JT, Gelfanov V, Smiley D, Gidda J, Findeisen H, Bruemmer D, Drucker DJ, Chaudhary N, Holland J, Hembree J, Abplanalp W, Grant E, Ruehl J, Wilson H, Kirchner H, Lockie SH, Hofmann S, Woods SC, Nogueiras R, Pfluger PT, Perez-Tilve D, DiMarchi R, Tschop MH. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat. Chem. Biol.* 2009; **5**(10): 749–757.
34 Runge S, Thogersen H, Madsen K, Lau J, Rudolph R. Crystal structure of the ligand-bound glucagon-like peptide-1 receptor extracellular domain. *J. Biol. Chem.* 2008; **283**(17): 11340–11347.
35 Underwood CR, Garibay P, Knudsen LB, Hastrup S, Peters GH, Rudolph R, Reedtz-Runge S. Crystal structure of glucagon-like peptide-1 in complex with the extracellular domain of the glucagon-like peptide-1 receptor. *J. Biol. Chem.* 2010; **285**(1): 723–730.
36 Murage EN, Gao G, Bisello A, Ahn JM. Development of potent glucagon-like peptide-1 agonists with high enzyme stability via

introduction of multiple lactam bridges. *J. Med. Chem.* 2010; **53**(17): 6412–6420.
37 Day JW, Li P, Patterson JT, Chabenne J, Chabenne MD, Gelfanov VM, Dimarchi RD. Charge inversion at position 68 of the glucagon and glucagon-like peptide-1 receptors supports selectivity in hormone action. *J. Pept. Sci.* 2011; **17**(3): 218–225.
38 Hjorth SA, Adelhorst K, Pedersen BB, Kirk O, Schwartz TW. Glucagon and glucagon-like peptide 1: selective receptor recognition via distinct peptide epitopes. *J. Biol. Chem.* 1994; **269**(48): 30121–30124.
39 Runge S, Gram C, Brauner-Osborne H, Madsen K, Knudsen LB, Wulff BS. Three distinct epitopes on the extracellular face of the glucagon receptor determine specificity for the glucagon amino terminus. *J. Biol. Chem.* 2003; **278**(30): 28005–28010.
40 Runge S, Wulff BS, Madsen K, Brauner-Osborne H, Knudsen LB. Different domains of the glucagon and glucagon-like peptide-1 receptors provide the critical determinants of ligand selectivity. *Br. J. Pharmacol.* 2003; **138**(5): 787–794.
41 Pan CQ, Buxton JM, Yung SL, Tom I, Yang L, Chen H, MacDougall M, Bell A, Claus TH, Clairmont KB, Whelan JP. Design of a long acting peptide functioning as both a glucagon-like peptide-1 receptor agonist and a glucagon receptor antagonist. *J. Biol. Chem.* 2006; **281**(18): 12506–12515.
42 Gardella TJ, Luck MD, Fan MH, Lee C. Transmembrane residues of the parathyroid hormone (PTH)/PTH-related peptide receptor that specifically affect binding and signaling by agonist ligands. *J. Biol. Chem.* 1996; **271**(22): 12820–12825.
43 Lopez de Maturana R, Donnelly D. The glucagon-like peptide-1 receptor binding site for the N-terminus of GLP-1 requires polarity at Asp198 rather than negative charge. *FEBS Lett.* 2002; **530**(1–3): 244–248.
44 Lin F, Wang R. Molecular modeling of the three-dimensional structure of GLP-1R and its interactions with several agonists. *J. Mol. Model* 2009; **15**(1): 53–65.
45 Unson CG. Molecular determinants of glucagon receptor signaling. *Biopolymers* 2002; **66**(4): 218–235.
46 Parthier C, Reedtz-Runge S, Rudolph R, Stubbs MT. Passing the baton in class B GPCRs: peptide hormone activation via helix induction? *Trends Biochem. Sci.* 2009; **34**(6): 303–310.
47 Wittelsberger A, Corich M, Thomas BE, Lee BK, Barazza A, Czodrowski P, Mierke DF, Chorev M, Rosenblatt M. The mid-region of parathyroid hormone (1–34) serves as a functional docking domain in receptor activation. *Biochemistry* 2006; **45**(7): 2027–2034.
48 Shimizu M, Shimizu N, Tsang JC, Petroni BD, Khatri A, Potts JT Jr, Gardella TJ. Residue 19 of the parathyroid hormone (PTH) modulates ligand interaction with the juxtamembrane region of the PTH-1 receptor. *Biochemistry* 2002; **41**(44): 13224–13233.

