

The importance of the nine-amino acid C-terminal sequence of exendin-4 for binding to the GLP-1 receptor and for biological activity

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Abstract

Exendin-4, a 39-amino acid (AA) peptide, is a long-acting agonist at the glucagon-like peptide-1 (GLP-1) receptor. Consequently, it may be preferable to GLP-1 as a long-term treatment for type 2 diabetes mellitus. Exendin-4 (Ex-4), unlike GLP-1, is not degraded by dipeptidyl peptidase IV (DPP IV), is less susceptible to degradation by neutral endopeptidase, and possesses a nine-AA C-terminal sequence absent from GLP-1. Here we examine the importance of these nine AAs for biological activity of Ex-4, a sequence of truncated Ex-4 analogs, and native GLP-1 and GLP-1 analogs to which all or parts of the C-terminal sequence have been added. We found that removing these AAs from Ex-4 to produce Ex (1–30) reduced the affinity for the GLP-1 receptor (GLP-1R) relative to Ex-4 (IC₅₀: Ex-4, 3.22 ± 0.9 nM; Ex (1–30), 32 ± 5.8 nM) but made it comparable to that of GLP-1 (IC₅₀: 44.9 ± 3.2 nM). The addition of this nine-AA sequence to GLP-1 improved the affinity of both GLP-1 and the DPP IV resistant analog GLP-1 8-glycine for the GLP-1 receptor (IC₅₀: GLP-1 Gly⁸ [GG], 220 ± 23 nM; GLP-1 Gly⁸ Ex (31–39), 74 ± 11 nM). Observations of the cAMP response in an insulinoma cell line show a similar trend for biological activity. Published by Elsevier Science B.V.

Keywords: Insulinotropic compounds; Peptide structure/function

1 Introduction

Glucagon-like peptide-1 (GLP-1) is an enteroendocrine hormone that has a capacity, when administered exogenously, to normalize blood glucose levels in type 2 diabetes mellitus [1,2]. It is now also well established that GLP-1 and GLP-1 receptor (GLP-1R) agonists are insulinotropic in rodents causing beta (β)-cell proliferation and increasing β-cell mass [3,4]. GLP-1 is degraded rapidly by dipeptidyl peptidase IV (DPP IV) [5] and by many ectopeptidases [6]. Hence, the search for long-acting GLP-1 analogs led to the identification of exendin-4 (Ex-4), which is an agonist at the GLP-1R. Ex-4 is a 39-amino acid (AA) peptide produced in the salivary gland of the gila monster lizard and has a 53% amino acid homology with GLP-1 [7]. Its N-terminal sequence (His:Gly:Glu) is not recognized by DPP IV, which rapidly cleaves the His:Ala:Glu sequence found on the N-

terminus of GLP-1 [8]. Ex-4 also lacks many of the neutral endopeptidase substrate sites present in GLP-1 [9] and has a nine-AA sequence at the C-terminus absent from GLP-1. In both rodents [10,11] and humans [12,13], Ex-4 is a more potent insulinotrope. Ex-4 binds with greater affinity to the GLP-1R [14] and has a longer half-life than GLP-1 in vivo [15]. Nothing is known of the importance of the nine-AA C-terminal sequence in the (1) binding of the compound to GLP-1R and (2) of the increased potency of Ex-4 relative to GLP-1. Here we study the affinity of a series of C-terminal truncated versions of Ex-4 for the GLP-1R. We also studied the effect of adding these nine C-terminal AAs to GLP-1 and its longer-acting analog GLP-1 8-glycine (GLP-1 Gly⁸) [16–18] that is not a substrate for DPP IV.

2 Materials and methods

2.1. Materials and cell lines

Peptides were synthesized as before [18]. All peptides were of 95% or greater purity. Table 1 shows the sequences

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Table 1

The amino acid sequences of the GLP-1 and exendin-4 analogs studied

GLP-1(7–36)	GLP-1	7 11 16 21 26 31 36
GLP-1(7–36), Exendin (31–39)	GLP-1 ET	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRPSSGAPPPS
Exendin-4	Ex-4	1 5 10 15 20 25 30 35
Exendin (1–36)	Ex (1–36)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGPSSGAPPPS
Exendin (1–35)	Ex (1–35)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGPSSGAP
Exendin (1–33)	Ex (1–33)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGPSSGA
Exendin (1–30)	Ex (1–30)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNGGPSS
Exendin (1–28)	Ex (1–28)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKNNG
Exendin (1–26)	Ex (1–26)	HGEGTFTSDLSKQMEEEEAVRLFIEWLKN
Exendin (1–23)	Ex (1–23)	HGEGTFTSDLSKQMEEEEAVRLFIEWL
Exendin (1–20)	Ex (1–20)	HGEGTFTSDLSKQMEEEEAVRLF
Exendin (1–17)	Ex (1–17)	HGEGTFTSDLSKQMEEEEAVR
Exendin (1–14)	Ex (1–14)	HGEGTFTSDLSKQMEEEE
Exendin (1–11)	Ex (1–11)	HGEGTFTSDLSKQM
		HGEGTFTSDLS
GLP-1Gly ⁸ (7–36)	GG	7 11 16 21 26 31 36
GLP-1Gly ⁸ (7–36), Exendin (31–39)	GG1	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGR
GLP-1Gly ⁸ (7–36), Exendin (31–36)	GG2	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRPSSGAPPPS
GLP-1Gly ⁸ (7–36), Exendin (31–33)	GG3	HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRPSSGAP
		HGEGTFTSDVSSYLEGQAAKEFIAWLVKGRPSS

The underlined amino acids refer to those from the Ex-4 sequence under investigation.

of the GLP-1 and Ex-4 analogs studied. 3-Isobutyl-1-methylxanthine (IBMX) was purchased from Calbiochem (La Jolla, CA). Ex-4 and GLP-1(7–36) amide were obtained from Bachem (Torrance, CA). The cloned rat insulinoma cell line RIN 1046-38 was a gift from Dr. Samuel A. Clark (Bio Hybrid Technologies, Shrewsbury, MA) and was routinely cultured in M199 with Earle's salts (Mediatech, Herndon, VA) supplemented with glucose (11 mM), 50 U/ml penicillin, 50 g/ml streptomycin, and glutamine (2 mM) in a humidified 5% CO₂–95% air incubator at 37 °C. Chinese hamster ovary (CHO) cells were stably transfected with the human GLP-1 receptor (CHO/GLP-1R cells) as previously described [14].

2.2. Insulin secretion in vitro

RIN 1046-38 cells grown on 12-well plates that had reached 50–60% confluency were washed in glucose-free insulin secretion buffer (9), followed by two 30 min preincubation periods in fresh insulin secretion assay buffer (1 ml). The buffer was replaced and the cells were incubated with the peptides and glucose (5 mM) for 1 h at 37 °C. The supernatant was then collected and saved for determination of insulin content by EIA (Crystal Chem, Chicago, IL). Cells were lysed by the addition of hydrochloric acid (300 l, 0.1 M, 20 min, RT) and the protein content quantified using the Bradford method.

2.3. Intracellular cAMP determination

RIN 1046-38 cells were plated 48 h prior to the experiment on 12-well plates and grown to 60–70% confluency.

On the day of the experiment, the peptides and IBMX (1 mM) were added to the medium and incubated for 30 min. The intracellular cAMP was then extracted by incubating the cells in ice-cold perchloric acid (0.6 M, 1 ml, 5 min). After adjusting the pH of the samples to 7.0 using potassium carbonate (5 M, 84 l), sample tubes were vortexed and the precipitate formed was sedimented by centrifugation (5 min, 2000 g, 4 °C). The supernatant

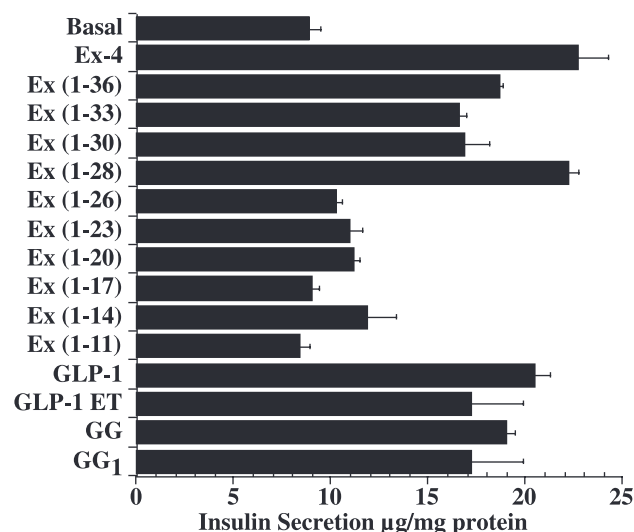


Fig. 1. Static in vitro insulin secretion. RIN 1046-38 cells were incubated with glucose (5 mM) in the absence or presence peptides (10 nM) for 1 h at 37 °C. The amount of insulin secreted into the medium was then determined. Data are the mean S.E.M. of two or three experiments performed in triplicate.

was vacuum-dried and solubilized in 0.05 M Tris (pH 7.5) containing 4 mM EDTA (200 μ l). Sodium carbonate (0.15 M) and zinc sulfate (0.15 M) were added to the samples and were then incubated for 15 min on ice. The resulting salt precipitate was removed by centrifugation (5 min, 2000 g, 4 $^{\circ}$ C). The samples were assayed in duplicate aliquots (50 μ l) using a [^{3} H]cAMP competitive binding assay kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

2.4. Competitive binding of peptides to GLP-1 receptor in intact cells

Binding studies were performed as described by Montrose-Rafizadeh et al. [14]. Briefly, CHO/GLP-1R cells were grown to confluency on 12-well plates and washed with serum-free ham F-12 medium for 2 h before the experiment. After two washes in 0.5 ml binding buffer [14], cells were incubated overnight at 4 $^{\circ}$ C with 0.5 ml buffer containing 2% bovine serum albumin (BSA, RIA grade Sigma), 50 μ M DPP-IV inhibitor (Linco, St. Charles, MO), 400 kallikrein inactivator units (KIU) aprotinin (ICN, Aurora, OH), 10 mM glucose, 1–1000 nM GLP-1 or other peptides and 30,000 cpm [^{125} I]GLP-1 (Phoenix Pharmaceuticals, Belmont, CA). At the end of the incubation, the supernatant was discarded and the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated at room temperature with 0.5 ml of 0.5 M NaOH and 0.1% sodium dodecylsulfate for 10 min. Radioactivity in cell lysates was measured in an ICN Apec-Series γ -counter (ICN Biomedicals, Costa Mesa, CA). Specific binding was determined as total binding minus the radioactivity associated with cells incubated in the presence of a large excess of unlabeled GLP-1 (1 μ M).

2.5. Statistical analysis

All values are shown as the mean $\pm$ S.E.M. The curves for Figs. 1 and 2 were fitted with a four-parameter sigmoid

logistic regression equation using an iterative computer program [19], and the EC₅₀ and IC₅₀ values in Table 2 were calculated from the fitted data.

3 Results

3.1. Insulin secretion in vitro

We initially screened the derivatives for biological activity in terms of the amount of insulin secreted into the medium of RIN 1046-38 cells over a 1-h period. The concentration (10 nM) at which maximum insulin secretion was obtained with GLP-1 and Ex-4 was used to assess the potential of the peptides as insulin secretagogues. At this concentration, there is no statistical significant difference between the insulin secreted in response GLP-1 and Ex-4, with both producing an approximate 2.3- and 2.5-fold increase, respectively, in insulin above basal levels. With the exception of truncations of Ex-4 that are shorter than 28 amino acids, all of the peptides displayed an approximate twofold increase in insulin secretion that was significantly different from basal ($p < 0.05$). The amount of insulin secreted in response to Ex-4 was also significantly higher ($p < 0.05$) than the amount secreted in response to the shortest truncated analog Ex (1–11).

3.2. Competitive binding of GLP-1 and GLP-1 Gly⁸ analogs and truncated Ex-4 analogs to the GLP-1 receptor

The potential of the GLP-1 and Ex-4 analogs to displace [^{125} I]GLP-1 by binding competitively to the human GLP-1R was first examined. CHO/GLP-1R cells were incubated with ^{125} I-labeled GLP-1 in the absence and presence of varying concentrations of the peptides. The IC₅₀ values are shown in Table 2. The addition of the nine-AA C-terminal sequence to the C-terminus of GLP-1 led to a modest but significant ($p = 0.003$, difference in relation to IC₅₀) improvement in binding to GLP-1R as illustrated by the reduction in the half-maximal binding. The addition of this C-terminal sequence to the GLP-1 analog GLP-1 Gly⁸ (GG) decreased the IC₅₀ tenfold ($p = 0.003$) relative to the parent compound. The addition of the 31–36 portion of Ex-4 to GG halved the IC₅₀ relative to the parent compound. However, the addition of the 31–33 sequence of Ex-4 to GG produced a compound with an IC₅₀ comparable to that of native GLP-1 and GG₁. The affinity of Ex-4 for the GLP-1R is highest of all of the compounds (Fig. 1, Table 2, and Ref. [14]). The stepwise truncation of the nine-AA C-terminal sequence resulted in a progressive increase in the half-maximal binding with only Ex (1–36) and Ex (1–35) having an IC₅₀ that is not significantly different from Ex-4. The IC₅₀ of the shorter peptides Ex (1–33) and Ex (1–30) are significantly different from Ex-4 ($p = 0.008$ and $p = 0.0034$), respectively (Fig. 1C).

Table 2

The IC₅₀ and EC₅₀ values derived from the competitive binding in CHO/GLP-1R cells and cAMP assays in RIN 1046-38 cells, respectively

Peptide name	IC ₅₀ (nM)		EC ₅₀ (nM)	
GLP-1	44.9	3.2	0.15	0.01
GG	220	23	1.11	0.2
GG ₁	74	11	0.6	0.3
GG ₂	129	39	ND	
GG ₃	34.5	14.5	ND	
GLP-1 ET	21.2	2.9	0.19	0.03
Ex-4	3.22	0.9	0.08	
Ex (1–36)	8.8	1.4	ND	
Ex (1–35)	7.0	2	ND	
Ex (1–33)	49.0	1.1	ND	
Ex (1–30)	32.0	5.8	0.7	0.1

The concentration that reached 50% of [^{125} I]GLP-1 binding was calculated in three or four separate experiments performed in triplicate.

We also examined compounds Ex (1–28) through to Ex (1–11) and found that the peptides Ex (1–26) onwards do not competitively displace GLP-1 at GLP-1R (data not shown), i.e. the last in the series of C-terminal truncations of Ex-4 to bind to GLP-1R is Ex (1–28).

3.3. *In vitro* intracellular cAMP production

The intracellular cAMP production in an insulinoma cell line in response to the GLP-1 analogs with and without the nine-AA C-terminal sequence was compared with both Ex-4

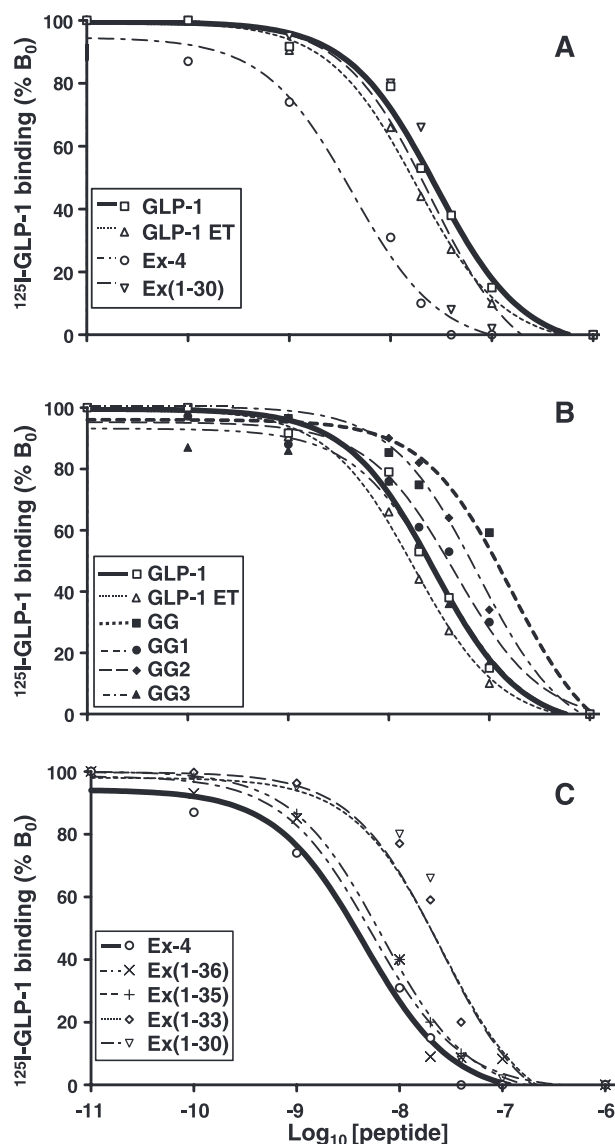


Fig. 2. Displacement of [^{125} I]GLP-1 binding to CHO/GLP-1R cells with the analogs of GLP-1, GLP-1 Gly⁸, and truncated analogs of Ex-4. [^{125} I]GLP-1 binding to intact CHO/GLP-1R cells was competed with various concentrations of the peptides shown. The data are normalized to maximum values obtained in the presence of 10 nM of the respective peptides. The data points represent the mean of three to five experiments performed in triplicate. The IC₅₀ value for each GLP-1 analog is shown in Table 2. B₀, maximum binding in the absence of cold peptide.

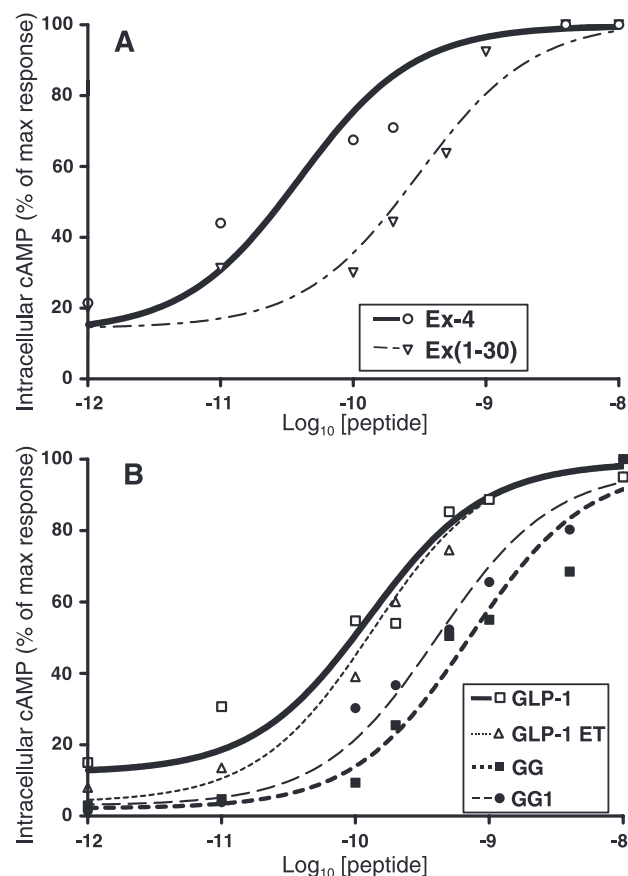


Fig. 3. cAMP dose response curves for selected peptides. Intracellular cAMP levels were measured in CHO/GLP-1R cells after treatment with the indicated concentrations of the peptides for 30 min at 37 °C. Ex-4 is compared with the truncated analog Ex (1–30) (A). GLP-1 and GLP-1 Gly⁸ [GG] are compared with their respective analogs in which the PSSGAPPPS sequence has been added (B). The data are normalized to maximum values obtained in each experiment in the presence of the 10 nM of the individual peptide. The data points represent the mean of three experiments performed in triplicate.

and Ex (1–30). The induction of cAMP in response to varying concentrations of the peptides in the presence of IBMX was measured (Fig. 2) during a 30-min incubation at 37 °C. Table 2 shows the EC₅₀ values of these compounds derived from this data. The trends are similar to those observed with the binding data. Addition of the nine-AA C-terminal sequence to GG results in a twofold improvement in cAMP response (Fig. 3), which is similar to Ex (1–30) as shown in Fig. 2 and Table 2. Addition of the PSSGAPPPS sequence to GLP-1 did not increase the cAMP response. Removing the nine C-terminal AAs from Ex-4 resulted in a dramatic drop in potency from an EC₅₀ of 0.08 nM with Ex-4 to 0.7 nM for Ex (1–30).

4 Discussion

One of the obvious sequence differences between GLP-1 and Ex-4 is the nine-AA sequence, PSSGAPPPS, at the C-

terminal end of Ex-4 that is absent from the GLP-1 molecule. In this study, we investigated the extent to which this contributes to the increased affinity of Ex-4 for the GLP-1 receptor and the increased potency of the compound *in vitro*, relative to GLP-1 itself. We found a progressive reduction in the affinity for the GLP-1 receptor for a series of C-terminal truncated Ex-4 analogs such that Ex (1–30) (the peptide of equivalent length to GLP-1) had a binding curve and cAMP response resembling GLP-1 rather than Ex-4. Similarly, the addition of the PSSGAPPPS sequence to GLP-1 marginally improved the affinity for GLP-1R but did not make it comparable to Ex-4. Previously, we and others have shown that substitution of Gly for Ala in position 8 of GLP-1 reduced the affinity of the compound for the GLP-1 receptor (16,17,18). Here we show that the addition of the PSSGAPPPS sequence to the C-terminal end of the GLP-1 analog compensates for the disruption to the binding affinity caused by the substitution at the N-terminal end. Thus, GLP-1 Gly⁸ Ex (31–39) is almost as effective as GLP-1 in terms of binding affinity and demonstrates an improved cAMP response relative to GLP-1 Gly⁸ itself. In a series of truncations of Ex-4, we found that peptides shorter than Ex (1–28) did not increase insulin secretion in the RIN 1046-38 cells to a significant extent as they do not bind to the GLP-1 receptor.

GLP-1 and GLP-1 Ex (31–39) displayed similar affinities for the GLP-1 receptor and similar cAMP response curves. In all cases, no peptide was as effective as Ex-4 in binding to the GLP-1 receptor, hence the increased potency of this compound in the cAMP assay. In binding to a receptor, a peptide undergoes conformational restriction and thus becomes more ordered with a consequent decrease in entropy. If the molecule is already highly ordered, then the decrease in entropy will be less and association with the receptor more favorable. The solution structure of both GLP-1 and Ex-4 were described recently by Neidigh et al. [20] using NMR structural analysis of the compounds in solution and DPC micelle-bound state. The latter condition is believed to be more representative of the compound just prior to association with the receptor. These authors showed that both GLP-1 and Ex-4 display a significant helicity from residues 7–28 in solution and in the dodecylphosphocholine (DPC) micelle-bound state. The helical structure of Ex-4 is more stable in nature than GLP-1 because the helix disrupting Gly at position 22 in GLP-1 is absent in Ex-4 (the Ex-4 equivalent is a Glu at position 16, Ref. [20]). Moreover, the presence of the Trp-cage fold in which the Trp ring on residue 25 of Ex-4 is enclosed in a hydrophobic cage formed by extensive hydrogen bonding is a stabilizing influence on the helical structure [20]. Thus, the structure of Ex-4 is more restricted in free rotation around the bonds; hence, the reduction in entropy upon going from the free to the receptor bound state will be less for Ex-4 than for GLP-1.

The compounds Ex (1–36) and Ex (1–35) have EC₅₀s of the same order of magnitude as Ex-4, as all the hydrogen

bonding contributing to the Trp-cage fold formation remains intact in these analogs. There is a loss of affinity for the GLP-1 receptor of one order of magnitude upon going from Ex (1–35) to Ex (1–33), and this might be explained by the removal of the hydrogen bonding between Trp²⁵–NH → O=C–Ala³⁵ with the consequent destabilization of the Trp-cage fold feature. Ex (1–30) has a similar affinity for the GLP-1 receptor as GLP-1 itself. However, the addition of the nine-AA sequence does not increase the binding of GLP-1 to its receptor to a significant extent. This is possibly due to the presence of the Gly at position 22 in GLP-1 that allows for greater inherent flexibility than that present in the Ex-4 molecule.

Addition of the PSSGAPPPS sequence to the C-terminal end of GLP-1 Gly⁸ improves the binding affinity of this compound to that of GLP-1 itself. This is also reflected in an increased potency in the cAMP assay.

In summary, we have shown that while the PSSGAPPPS sequence of Ex-4 is not essential for either binding to the GLP-1 receptor or biological activity, the absence of this sequence in Ex (1–30) reduces the affinity of the peptide for the GLP-1 receptor and hence the cAMP response in an insulinoma cell line. The addition of this sequence to GLP-1 and its analogs does not compensate for the helix disrupting influence of the Gly/22 in GLP-1 but does compensate for the loss of affinity for the GLP-1 receptor found with the GLP-1 Gly⁸ analog. Thus, through the knowledge we gained on the functional importance of the PSSGAPPPS C-terminal sequence in Ex-4, we have produced a GLP-1 analog, GLP-1 Gly⁸ Ex (31–39) affinity for the GLP-1 receptor. Thus, the GLP-1 Gly⁸ Ex (31–39) compound has the advantage of the improved metabolic stability of GLP-1 Gly⁸ but an enhanced affinity for the target receptor.

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References

- [1] Nathan DM, Schreiber E, Fogel H, Mojsov S, Habener JF. Insulinotropic action of glucagonlike peptide-I(7–37) in diabetic and non-diabetic subjects. *Diabetes Care* 1992;15:270–6.
- [2] Zander M, Madsbad S, Madsen JL, Holst JJ. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and beta-cell function in type 2 diabetes: a parallel-group study. *Lancet* 2002;359:824–30.
- [3] Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF, et al. Insulinotropic glucagon-like peptide 1 agonists

- stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes* 2000;49:741–8.
- [4] Perfetti R, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 induces cell proliferation and pancreatic-duodenum homeobox-1 expression and increases endocrine cell mass in the pancreas of old, glucose-intolerant rats. *Endocrinology* 2000;141:4600–5.
- [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;136:3585–96.
- [6] Hupe-Sodmann K, Goke R, Goke B, Thole HH, Zimmermann B, Voigt K, et al. Endoproteolysis of glucagon-like peptide (GLP)-1 (7–36) amide by ectopeptidases in RINm5F cells. *Peptides* 1997;18:625–32.
- [7] Eng J, Kleinman WA, Singh L, Singh G, Raufman JP. Isolation and characterization of exendin-4, an exendin-3 analogue, from *Heloderma suspectum* venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. *J Biol Chem* 1992;267:7402–5.
- [8] Hansen L, Deacon CF, Orskov C, Holst JJ. Glucagon-like peptide-1-(7–36)amide is transformed to glucagon-like peptide-1-(9–36)amide by dipeptidyl peptidase IV in the capillaries supplying the L cells of the porcine intestine. *Endocrinology* 1999;140:5356–63.
- [9] Hupe-Sodmann K, McGregor GP, Bridenbaugh R, Goke R, Goke B, Thole H, et al. Characterisation of the processing by human neutral endopeptidase 24.11 of GLP-1(7–36) amide and comparison of the substrate specificity of the enzyme for other glucagon-like peptides. *Regul Pept* 1995;58:149–56.
- [10] Greig NH, Holloway HW, De Ore KA, Jani D, Wang Y, Zhou J, et al. Once daily injection of exendin-4 to diabetic mice achieves long-term beneficial effects on blood glucose concentrations. *Diabetologia* 1999;42:45–50.
- [11] Young AA, Gedulin BR, Bhavsar S, Bodkin N, Jodka C, Hansen B, et al. 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;48:1026–34.
- [12] Edwards CM, Stanley SA, Davis R, Brynes AE, Frost GS, Seal LJ, et al. Exendin-4 reduces fasting and postprandial glucose and decreases energy intake in healthy volunteers. *Am J Physiol Endocrinol Metab* 2001;281:E155–61.
- [13] Egan JM, Clocquet AR, Elahi D. The insulinotropic effect of acute exendin-4 administered to humans: comparison of nondiabetic state to type 2 diabetes. *J Clin Endocrinol Metab* 2002;87:1282–90.
- [14] Montrose-Rafizadeh C, Yang H, Rodgers BD, Beday A, Pritchette LA, Eng J. High potency antagonists of the pancreatic glucagon-like peptide-1 receptor. *J Biol Chem* 1997;272:21201–6.
- [15] Parkes D, Jodka C, Smith P, Nayak S, Rinehart L, Gingerich R, et al. Pharmacokinetic actions of exendin-4 in the rat: comparison with glucagon-like peptide-1. *Drug Dev Res* 2001;53:260–7.
- [16] 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;41:271–8.
- [17] Burcelin R, Dolci W, Thorens B. Long-lasting antidiabetic effect of a dipeptidyl peptidase IV-resistant analog of glucagon-like peptide-1. *Metabolism* 1999;48:252–8.
- [18] Doyle ME, Greig NH, Holloway HW, Betkey JA, Bernier M, Egan JM. Insertion of an N-terminal 6-aminohexanoic acid after the 7 amino acid position of glucagon-like peptide-1 produces a long-acting hypoglycemic agent. *Endocrinology* 2001;142:4462–8.
- [19] Delean A, Munson PJ, Gaurdabasso V, Rodbard D. A User's Guide to ALLFIT. Washington, DC: NIH; 1988.
- [20] 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;40:13188–200.