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The *Xenopus* proglucagon gene encodes novel GLP-1-like peptides with insulinotropic properties

(diabetes/insulinotropic peptides/GLP-1 receptor)

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ABSTRACT **The proglucagon gene encodes several hormones that have key roles in the regulation of metabolism. In particular, glucagon-like peptide (GLP-1), a potent stimulus of insulin secretion, is being developed as a therapy for the treatment of non-insulin-dependent diabetes mellitus. To define structural moieties of the molecule that convey its insulinotropic activity, we have cloned and characterized the proglucagon gene from the amphibian, *Xenopus laevis*. Unexpectedly, these cDNAs were found to encode three unique glucagon-like-1 peptides, termed xenGLP-1A, xenGLP-1B, and xenGLP-1C in addition to the typical proglucagon-derived hormones glucagon and GLP-2. xenGLP-1A, -1B, and -1C were synthesized and tested for their ability to bind and activate the human GLP-1 receptor (hGLP-1R), and to stimulate insulin release from rat pancreas. All three *Xenopus* GLP-1-like peptides bind effectively to the hGLP-1R and stimulate cAMP production. Surprisingly, xenGLP-1B(1–30) demonstrated higher affinity for the hGLP-1R than hGLP-1 (IC_{50} of 1.1 ± 0.4 nM vs. 4.4 ± 1.0 nM, respectively, $P < 0.02$) and was equipotent to hGLP-1 in stimulating cAMP production (EC_{50} of 0.17 ± 0.02 nM vs. 0.6 ± 0.2 nM, respectively, $P > 0.05$). Further studies demonstrated that hGLP-1, xenGLP-1A, -1B, and -1C stimulate comparable insulin release from the pancreas. These results demonstrate that despite an average of nine amino acid differences between the predicted *Xenopus* GLPs and hGLP-1, all act as hGLP-1R agonists.**

Differential processing of the proglucagon precursor in the mammalian pancreas and intestine gives rise to distinct regulatory peptides with diverse functions in the regulation of carbohydrate, amino acid, and lipid metabolism (1). One peptide secreted from the intestinal L-cell, glucagon-like peptide-1 (GLP-1) (7–36) amide, is a potent stimulus of insulin secretion (2). The recent observation that mice expressing a null mutation in the GLP-1 receptor (GLP-1R) are diabetic clearly establishes a major role for GLP-1 in mammalian carbohydrate metabolism (3). The potent insulinotropic activity of GLP-1 has given rise to the possibility that this peptide or agonists of its receptor may be used as a therapeutic agent for the treatment of non-insulin-dependent diabetes mellitus (4, 5). When GLP-1 is administered to patients with non-insulin-dependent diabetes mellitus, postprandial insulin levels are elevated and the meal-induced requirement for exogenous insulin is reduced, further supporting its therapeutic potential (6, 7). The therapeutic potential of this peptide has led to an increased interest in elucidating the structural determinants that define its insulinotropic activity, information that will facilitate the development of more effective GLP-1 analogs.

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Although the structure and the function of glucagon is highly conserved across all species studied thus far (1), those of GLP-1 appear to be more variable. In all mammals studied, GLP-1 acts at the level of the pancreatic β-cell to potentiate insulin secretion. However, in fish, GLP-1 acts directly upon the liver with glucagon-like activity (8). In amphibians (e.g., frogs such as *Xenopus*) the actions of GLP-1 are more consistent with those seen in mammals (8). Thus, amphibians appear to represent one of the more evolutionarily distant species from mammals that use GLP-1 as an insulinotropic hormone. For this reason, we sought to determine the structure of *Xenopus* proglucagon and the sequence of GLP-1. During the initial characterization of *Xenopus* pancreatic proglucagon cDNAs, it became apparent that the predicted proglucagon structure differed significantly from all other known proglucagons, in that the sequence contained three GLP-1-like peptides we term xenGLP-1A, xenGLP-1B, and xenGLP-1C. These observations suggest that xenGLP-1A, -1B, and -1C may have GLP-1-like activities. Results presented here demonstrate that these peptides efficiently bind and activate the human GLP-1R (hGLP-1R) and possess potent insulinotropic activity.

MATERIALS AND METHODS

Materials. Oligonucleotides for PCR were obtained from ACGT (Toronto). Radioisotopes were purchased from Amersham. pCRII cloning vector was from Invitrogen and the λZAPII XR cDNA synthesis kit was from Stratagene. The T7 DNA polymerase sequencing kit was from Pharmacia. Peptides xenGLP-1A(1–32) (HAEGTFTSDV TQQLDEKAAK EFIDWLINGG PS), xenGLP-1A(1–37) (HAEGTFTSDV TQQLDEKAAK EFIDWLINGG PSKEIIS), xenGLP-1B(1–30) (HAEGTYTNDV TEYLEEKAAK EFIEWLIKGK), xenGLP-1B(1–32) (HAEGTYTNDV TEYLEEKAAK EFIEWLIKGK PK), and xenGLP-1C(1–30) (HAEGTFTNDM TNYLEEKAAK EFVGWLINGR) were commercially synthesized and purified (>80% pure) by the Sheldon Biotechnology Centre (Montreal).

***Xenopus* RNA.** For the isolation of RNA, *Xenopus laevis* tissues, pancreas, stomach, intestine, and liver was generously provided by R. Elinson, Department of Zoology, and P. Backx, Department of Physiology, University of Toronto. RNA was isolated from frozen tissue using RNAsol (Canadian Life Technologies, Burlington, ON, Canada). mRNA was purified

Abbreviations: GLP-1 and -2, glucagon-like peptides 1 and 2; xenGLP-1A, xenGLP-1B, and xenGLP-1C, *Xenopus* glucagon-like peptides 1A, 1B, and 1C; hGLP, human GLP; GLP-1R and hGLP-1R, GLP-1 and hGLP-1 receptor; CHO, Chinese hamster ovary.

The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF004432 and AF004433).

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using poly(A)tract magnetic beads (Promega). RNA from the various *Xenopus* tissues were separated in formaldehyde–agarose gels as described (9). Proglucagon mRNA was detected by hybridization with a portion of the proglucagon cDNA at low stringency as described (10). First strand cDNA used to isolate initial proglucagon cDNAs and examine alternative splicing was synthesized with a kit from Canadian Life Technologies.

Isolation of Proglucagon cDNAs. Degenerate primers were synthesized to the conserved N-terminal sequences of vertebrate glucagon (HSQGTF, 5′-GGGAATTCAYTCNCARG-GNACNTT-3′), GLP-1 (HAEGTF, 5′-GAGGATCCTGT-NCCRTCNGCRTG-3′), and GLP-2 (HADGSF, 5′-GAG-GATCCAARCTNCCRTCNGCRTG-3′). The glucagon primer was sense primer, whereas those to GLP-1 and GLP-2 were antisense. Reverse transcription–PCR was as previously described (11), with products cloned into pCRII. *Xenopus* pancreatic cDNA library (composed of $\approx 3 \times 10^6$ independent clones) was constructed in λZAPII XR. Approximately 2.5×10^5 clones of the amplified library were screened by standard methods (9) with the glucagon–GLP-2 PCR product. Potential positive clones were purified through additional screens and excised *in vivo*. PCR and cDNA clones were sequenced with primers flanking the insert and unique internal primers.

Iodination of GLP-1 and Binding Assays. Synthetic human (h) tGLP-1(7–36) amide (Bachem) was used in all binding studies. Radioiodination and purification of [^{125}I]tGLP-1(7–36) amide was performed as described (12, 13) and had a specific activity of approximately 125–250 μCi/μg (1 Ci = 37 GBq). Chinese hamster ovary (CHO)-K1 cells stably expressing the hGLP-1R ($B_{max} = 2.3 \times 10^4 \pm 0.4 \times 10^4$ receptors per cell) were generated as described (2). Binding characteristics of the *Xenopus* peptides with the hGLP-1R were assessed as previously described (14, 15). Briefly, the cells were washed twice with PBS and recovered from plates with 2 mM EDTA in PBS. Cells ($\approx 1 \times 10^6$ cells per tube) were incubated in binding buffer (DMEM containing 0.4% glucose/1% BSA, pH 7.4) with radiolabeled tracer [^{125}I]tGLP1(7–36) amide ($\approx$30,000 cpm) and each of the unlabeled peptides at concentrations of 10^{-12} to 10^{-6} M, in a final volume of 200 μl. Cell suspensions were centrifuged at 12,000 × *g* and the cell-associated radioactivity was counted (Cobra II, Canberra Packard). All peptides were prepared on the day of the assay from concentrated stocks.

cAMP Assays. The *Xenopus* peptides were tested for their ability to activate the hGLP-1 R and stimulate the production of cAMP, the second messenger, as previously described (16). CHO hGLP-1 R cells ($\approx$100,000 per well) were passaged into 24-well plates and allowed to culture for an additional 48 hr. The cells were then washed with PBS followed by a 30-min preincubation at 37°C in assay buffer (DMEM containing 0.4% glucose/1% BSA, pH 7.4). This was followed by a 30-min stimulation period with 3-isobutyl-1-methylxanthine (1 mM) and varying concentrations (10^{-12} to 10^{-6} M) of each of the peptides in a final volume of 500 μl. cAMP was extracted with 80% ethanol. cAMP production was measured by RIA (Biomedical Technologies, Stoughton, MA) as described (16). All peptides were prepared on the day of the assay from concentrated stocks.

Perfused Pancreas. Studies were carried out as described (17). Briefly, overnight fasted rats were anaesthetized (60 mg/kg pentobarbital) and the pancreas and associated duodenum isolated. Perfusate was a modified Krebs–Ringer buffer containing 3% dextran (Sigma) and 0.2% BSA, gassed with 95% O_2/5% CO_2 to achieve a pH of 7.4. The peptides were delivered as a linear gradient of 0–1 nM over a 50 min period in the presence of 16 mM glucose. The experimental procedure and insulin RIA details are as described (17). Results are expressed as the mean integrated insulin response (nmol) over 50 min ± SEM. All animal experimentation was carried out in accordance with guidelines approved by the University of British Columbia Animal Care Committee.

Statistics. All values are expressed as the mean ± SEM of at least three independent observations. Competitive binding-displacement curves and cAMP dose-response curves were generated using PRISM (GraphPad, San Diego). Statistical analysis was performed using ANOVA followed by the Tukey Kramer test (INSTAT software), comparing each *Xenopus* peptide to values obtained for hGLP-1. *P* values greater than 0.05 were considered not significant.

RESULTS

Isolation of *Xenopus* Proglucagon cDNAs. Initial PCR amplifications of pancreatic and intestinal cDNA with the degenerate glucagon and GLP-1 primers yielded a DNA product of about 300 bp. This length for the proglucagon product is larger than the predicted size of 150–200 bp estimated to contain glucagon, intervening peptide 1, and the N terminal of GLP-1 based on the sizes of mammalian, bird, and fish proglucagons. The amplified products were cloned and two similar sequences were identified (data not shown; *Xenopus* was expected to contain two sequences because it is a tetraploid species). Both sequences were found to contain an additional GLP-1-like peptide between glucagon and the binding site of the GLP-1 primer. A possible explanation for this observation was that the GLP-1 primer was instead priming from the sequence encoding the N terminal of GLP-2, and that the internal glucagon-like sequence was GLP-1. To test this possibility, intestinal cDNA was amplified with the glucagon and GLP-2 primers. The observed product ($\approx$500 bp) was larger than the glucagon– GLP-1 product ($\approx$300 bp), and when characterized, two types of related sequences were found and each encoded three GLP-1-like sequences; thus, the *Xenopus* proglucagon gene potentially encodes two novel glucagon-like peptides. Northern blot analysis of proglucagon expression in pancreas, intestine, stomach, and liver demonstrated that two different-sized mRNAs existed. mRNAs of approximately 1,600 and 1,400 nt were observed in the pancreas, whereas only the larger was observed in the intestine and smaller in the stomach (data not shown).

To further characterize the proglucagon sequences we constructed and screened a *Xenopus* pancreatic cDNA library. A total of 11 pancreatic proglucagon cDNA clones were isolated and characterized (Fig. 1). The clones were found to contain two distinct, but very similar, proglucagon sequences, as expected for a tetraploid species. The proglucagon I sequence was represented by nine clones, whereas the proglucagon II sequence was encoded by two clones. In addition, some of the clones encoding the proglucagon I sequence contained a 141 base deletion, which appears to be the result of alternative splicing (Fig. 1). To determine if the difference in size of the intestinal and stomach proglucagon mRNAs had the same basis as the pancreatic difference, reverse transcription–PCR was conducted with primers flanking the internal deletion. As expected, two sizes of fragments were observed in the pancreas, whereas only one was found in each of the intestine and stomach (data not shown). The intestinal mRNA was approximately 1,600 bases and contains the 141 base insert. The smaller stomach mRNA does not contain the 141 base insert. Sequence analysis of the reverse transcription–PCR products demonstrated that like the pancreas, both proglucagon I and proglucagon II are expressed in stomach and intestine and, thus, both genes are alternatively spliced.

Predicted Proglucagon-Derived Peptides. The longest open reading frame of proglucagon I was found to encode predicted peptides of 266 amino acids (Fig. 1). The first 20 amino acids are predicted to encode a signal peptide, similar to other proglucagons (Fig. 1). Unexpectedly, we found that the open reading frame encoded five glucagon-like sequences, rather

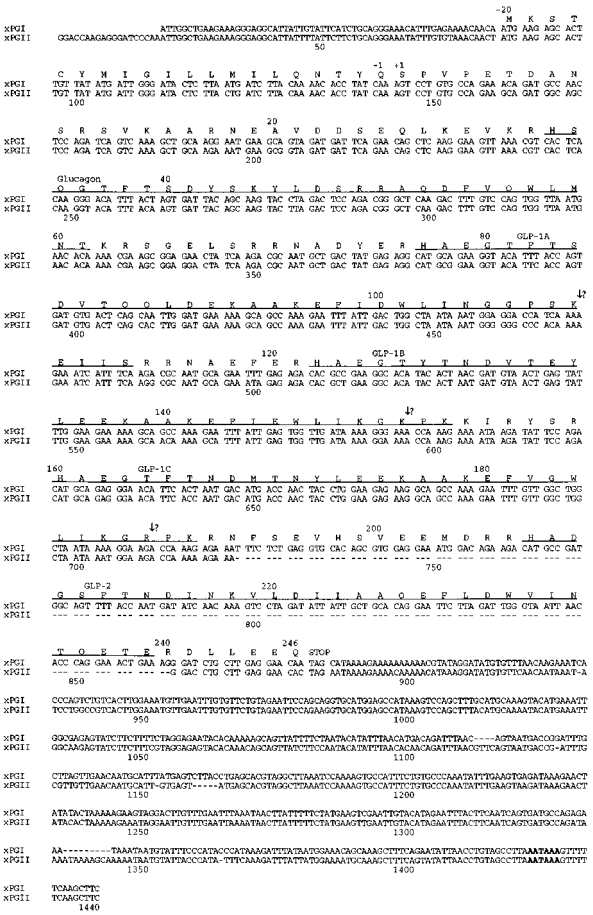

FIG. 1. Nucleotide sequence of *Xenopus* proglucagon cDNAs I and II. The predicted amino acid sequence of *Xenopus* proglucagon I is shown above the cDNA sequences. The DNA sequence is numbered from the 5′ end of the longest cDNA, whereas the amino acid sequence is numbered from the predicted N terminus of proglucagon. Predicted glucagon and glucagon-like peptides are underlined. Potential proteolytic processing sites that yield C-terminally truncated GLP-1-like peptides are indicated by an arrow with a question mark (↓?). A polyadenlyation signal (AATAAA) at bases 1422–1427 is indicated in boldface type. Dashes are gaps introduced to yield maximal alignment. *Xenopus* proglucagon II cDNAs do not encode GLP-2, and some proglucagon I cDNAs had a homologous deletion, removing the GLP-2 sequence, due to alternative splicing.

than the maximum of three found in mammals, birds, and fish (11, 18–22). Identity of the different glucagon-like sequences was made with comparison to those from other amphibians (bullfrog and *Amphiuma*) and mammals (Fig. 2, Table 1); three of the glucagon-like peptides are most similar to GLP-1 and

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Xenopus Glucagon        HSQGTFTSDY SKYLDSRRAQ DFVQWLMNT
Bullfrog Glucagon       .......... .......... .......S.
Amphiuma Glucagon       .......... .....N.... ..I....S.
Human Glucagon          .......... .......... .........

Xenopus GLP-1A(1-37)    HAEGTFTSDV TQQLDEKAAK EFIDWLINGG PSKEIIS
Xenopus GLP-1B(1-32)    .....Y.N.. .EY.E..... ...E...K.K .K-----
Xenopus GLP-1C(1-32)    .......NDM .NY.E..... ..VG...K.R .K-----
Bullfrog GLP-1          ..D......M SSY.E..... ..V....K.R .K-----
Amphiuma GLP-1          ..D..L...I SSF.EKQ.T. ...A..VS.R GRRQ---
Human GLP-1             .......... SSY.EGQ... ...A..VK.R -------

Xenopus GLP-2           HADGSFTNDI NKVLDIIAAQ EFLDWVINTQ ETE
Bullfrog GLP-2          .......S.F ..A...K... .....I...P VK.
Amphiuma GLP-2          .......S.. .....T...K ...N.L.S.K V..
Human GLP-2             ......SDEM .TI..NL..R D.IN.L.Q.K I.D
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FIG. 2. Comparison of amphibian and human glucagon-like peptide amino acid sequences. The predicted amino acid sequences of *Xenopus* glucagon, GLP-1A(1–37) and GLP-2, are shown in single letter code, with differences in the human, bullfrog, *Amphiuma*, and *Xenopus* GLP-1B(1–32), and *Xenopus* GLP-1C(1–32). Identities are shown as dots.

Table 1. Number of amino acid differences between glucagon-like peptides

	Xenopus glucagon-like sequences				
	Glucagon	GLP-1A	GLP-1B	GLP-1C	GLP-2
Xenopus glucagon	0	15	17	16	15
Bullfrog glucagon	1	16	17	16	16
Amphiuma glucagon	3	15	17	16	15
Human glucagon	0	15	17	16	15
Xenopus GLP-1A	15	0	10	11	15
Xenopus GLP-1B	17	10	0	6	18
Xenopus GLP-1C	16	11	6	0	17
Bullfrog GLP-1	14	10	9	5	16
Amphiuma GLP-1	18	16	16	16	20
Human GLP-1	15	9	8	9	17
Xenopus GLP-2	15	15	18	17	0
Bullfrog GLP-2	14	15	18	17	8
Amphiuma GLP-2	15	16	17	17	8
Human GLP-2	18	16	19	19	17

are identified as xenGLP-1A, xenGLP-1B, and xenGLP-1C. The in-frame 141 base deletion found in some proglucagon I and both proglucagon II cDNAs resulted in a shorter open reading frame of 219 amino acids. The 141 base insert encodes GLP-2 (Fig. 1). The N-terminal and C-terminal ends of glucagon and glucagon-like peptides were based on previously published peptide sequences and the locations of potential prohormone processing sites. Prohormone convertases cleave at paired basic amino acids, and sometimes at single basic amino acid sites [e.g., to yield tGLP-1(7–37)] within the prohormone sequence (13, 23). The exposed C-terminal basic amino acid residues are then usually removed by carboxypeptidase E (24). Glucagon is predicted to be encoded by amino acids 33–61 of proglucagon and is flanked by two basic amino acids (KR). *Xenopus* glucagon is identical to human glucagon but differs from bullfrog glucagon by one amino acid (Table 1). A sequence similar to bullfrog and *Amphiuma* GLP-2 is encoded by amino acids 207–239 of proglucagon (Figs. 1 and 2). Paired basic residues N terminal to GLP-2 may serve as a processing signal; whereas, unlike mammalian and bird GLP-2s, only a single basic residue followed amino acid 239. Between glucagon and GLP-2, three GLP-1-like peptide sequences were found, between proglucagon residues 77–113, 122–153, and 160–191 (Fig. 1). As is found for truncated hGLP-1, the N terminal of each of the *Xenopus* GLP-1-like peptides is preceded by a single arginine residue. In fact, xenGLP-1A and xenGLP-1B share with hGLP-1 a potential N-terminal extension of six amino acids, although they do not posses an N-terminal histidine, which is preceded by paired basic amino acids (see Fig. 1). Paired basic residues at the C-terminal ends of the GLP-1-like peptides would yield sequences of 37 [xenGLP-1A(1–37)] or 32 [xenGLP-1B(1–32) and xenGLP-1C(1–30)] amino acids in length, longer than human tGLP-1 (30 amino acids) (Table 1). xenGLP-1B(1–32) and xenGLP-1C(1–30) may have a C-terminal lysine since carboxypeptidase E may not remove basic residues preceded by proline (25). Potential processing at single basic residues 109, 151, and 189 would yield xenGLP-1A(1–32), xenGLP-1B(1–30), and xenGLP-1C(1–30), respectively, peptides more similar in length to hGLP-1.

Binding Properties of xenGLP-1A, xenGLP-1B, and xenGLP-1C. To examine the interaction of the *Xenopus* GLP-1-like peptides with the hGLP-1R, competitive binding-displacement assays were performed on CHO-K1 cells stably expressing the recombinant hGLP-1R (13). A total of five peptides were chosen for study—xenGLP-1A(1–37), xenGLP-1A(1–32), xenGLP-1B(1–32), xenGLP-1B(1–30), and xenGLP-1C(1–30) representing both full-length and C-terminally truncated peptides. Human GLP-1(7–36)NH_2 represents the

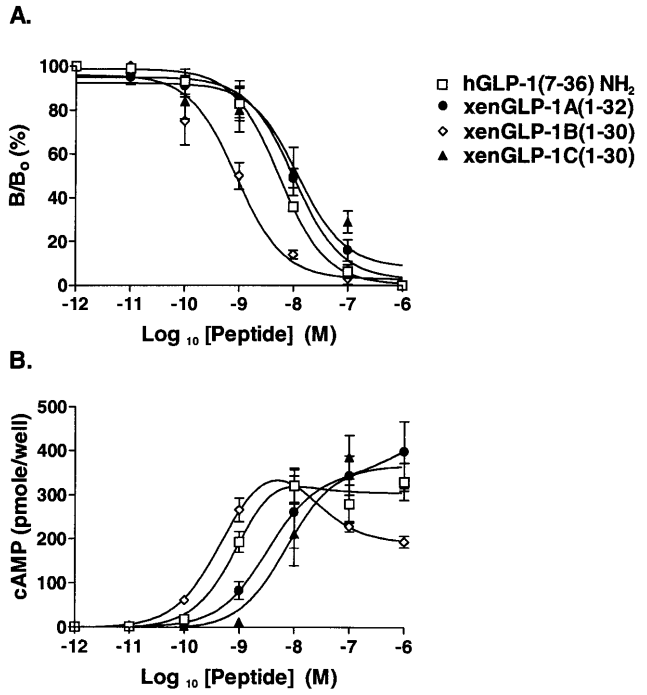

FIG. 3. (*A*) Displacement of [^{125}I]-htGLP-1 from CHO cells stably expressing the hGLP-1R, comparing hGLP-1 with the *Xenopus* peptides xenGLP-1A(1–32), xenGLP-1B(1–30), and xenGLP-1C(1–30). All curves were fit to a one-site model and are representative of $n \geq 3$ independent experiments. (*B*) Stimulation of cAMP accumulation in CHO cells expressing hGLP-1R in response to hGLP-1, xenGLP-1A, -1B, or -1C ($n \geq 3$ for each peptide). Data were fit to fixed-slope dose-response curves. The IC_{50}s and EC_{50}s are summarized in Table 2.

control to allow comparisons between each of the *Xenopus* peptides and the human peptide. Binding-displacement curves for the truncated peptides, xenGLP-1A(1–32), xenGLP-1B(1–30), and xenGLP-1C(1–30), are shown in Fig. 3*A*. A one-site competition model was used to draw the curves that best described the data. Specific binding is expressed as a percentage of binding at 10^{-12} M of peptide (B_o, zero binding). The curves represent three assays performed independently, each time at least in duplicate. As shown in Fig. 3*A*, xenGLP-1A(1–32), xenGLP-1B(1–30), and xenGLP-1C(1–30) were capable of efficiently displacing labeled tGLP-1 from the hGLP-1R, thus indicating that they are binding to the hGLP-1R. The IC_{50} values for each peptide is in the nanomolar range (Table 2). Full-length xenGLP-1A(1–37) and xenGLP-1B(1–32) gave similar results to the corresponding truncated peptides (Table 2). In contrast, the structurally related peptide, glucagon, achieved less than 50% displacement at 10 μM with the hGLP-R (13). xenGLP-1B(1–30) was found to achieve an IC_{50} at significantly lower peptide concentrations than hGLP-1, xenGLP-1A(1–32), or GLP-1B(1–30) ($P < 0.02$ vs. hGLP-1), indicating more affinity for the hGLP-1R. xenGLP-1C(1–30) demonstrated the highest IC_{50}, 43.4 nM ± 10.5 nM vs. 4.4 nM ± 1.0 nM for hGLP-1C, and thus demonstrated the lowest affinity for the hGLP-1R of all the peptides tested. These data suggest that all three *Xenopus* peptides may efficiently activate the hGLP-1R and possess insulinotropic activity.

Effects of Peptides on Second Messenger Activation. Studies have shown that the GLP-1R can couple to at least two G-protein-coupled signaling pathways, adenylyl cyclase and phospolipase C (26). However, studies with the endogenous receptor expressed in CHO cells suggest that cAMP is the main second messenger and along with free cytosolic calcium, triggers insulin secretion (27, 28). Hence, a measure of cAMP response is a good indication of both GLP-1R activation and insulinotropic ability of the peptides. To determine the level of activation of the GLP-1R and the adenylyl cyclase system, cAMP assays were performed on CHO-hGLP-1R cells. Fixed-slope dose-response curves best describe the data obtained for the cAMP responses. Responses for the three truncated peptides, xenGLP-1A(1–32), xenGLP-1C(1–30), and xenGLP-1B(1–30) are shown in Fig. 3*B*. The EC_{50}s for cAMP accumulation for all five peptides are summarized in Table 2. These data demonstrate a reasonable correlation between the IC_{50} values and the half-maximal cAMP responses for each of the peptides, with xenGLP-1B and hGLP-1 demonstrating similar potency. In addition, xenGLP-1B(1–30) produced a significantly lower cAMP response at a 1 μM concentration of peptide compared with the other peptides ($P < 0.05$; Fig. 3*B*), strongly suggesting that it may be desensitizing the receptor at this supramaximal dose. xenGLP-1C(1–30) produced the least response of the peptides tested with EC_{50} of 18.8 nM ± 0.4 nM vs. 0.6 nM ± 0.2 nM for hGLP-1. A similar trend is seen with the binding data. Because it is believed that cAMP is the second messenger that couples GLP-1 to insulin secretion, these results suggest that all xenGLP peptides should possess insulinotropic activity.

Effects of Peptides on Insulin Secretion. To compare the insulinotropic potency of the xenGLPs to hGLP-1, these peptides were presented to the rat pancreas as a gradient of 0–1 nM in the presence of a strong hyperglycemic stimulus (16 mM glucose) as illustrated in Fig. 4 and summarized in Table 2. The three *Xenopus* peptides were insulinotropic, producing an 8- to 10-fold greater increase in insulin secretion than glucose alone. Although the insulin response to xenGLP-1B(1–30) (1,300 nM/50 min ± 200 nM/50 min) appeared greater than that of all the other peptides including hGLP-1 (1,050 nM/50 min ± 170 nM/50 min), the differences among the peptides in the integrated insulin responses were not significant. Thus, the comparative pattern of insulin release appears to reflect receptor binding and cAMP stimulation in CHO cells expressing hGLP-1R, with xenGLP-1B(1–30) and hGLP-1 exhibiting greater potency than xenGLP-1A(1–32) or xenGLP-1C(1–30).

DISCUSSION

In mammals, proglucagon is expressed in the pancreas, intestine, and brainstem, with an identical mRNA transcribed in

Table 2. Summary of binding data, cAMP responses, and insulin responses for *Xenopus* GLPs

Peptides	hGLP-1 (7-36)NH_2	xenGLP-1A (1-32)	xenGLP-1A (1-37)	xenGLP-1B (1-30)	xenGLP-1B (1-32)	xenGLP-1C (1-30)
IC_{50} (nM)	4.4 ± 1.0	17.5 ± 4.7*	12.7 ± 4.3	1.1 ± 0.4†	2.9 ± 0.7	43.4 ± 10.5‡
EC_{50} cAMP (nM)	0.6 ± 0.2	8.6 ± 3.5§	2.0 ± 0.9	0.17 ± 0.02	1.1 ± 0.3	18.8 ± 0.4¶
Insulin (nM/50 min)	1,050 ± 170	850 ± 105	ND	1,300 ± 200	ND	950 ± 150

Data are presented as the mean ± SEM of at least three independent observations. ND, not done.
*$P < 0.02$ comparing hGLP-1 with xenGLP-1A(1–32).
†$P < 0.02$ to xenGLP-1B(1–30).
‡$P < 0.004$ to xenGLP-1C(1–30).
§$P < 0.003$ comparing hGLP-1 with xenGLP-1A(1–32).
¶$P < 0.0001$ to xenGLP-1C(1–30).

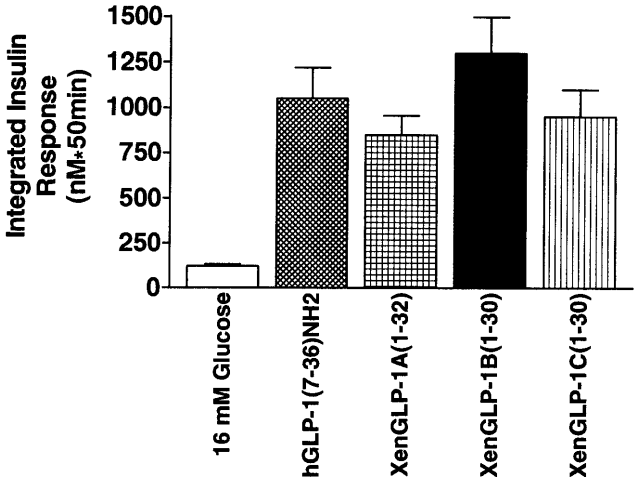

FIG. 4. Integrated insulin responses from the perfused rat pancreas to 16 mM glucose plus a gradient of hGLP-1 or the xenGLPs. The column on the left represents the insulin response to glucose alone ($n \geq 3$ for each peptide).

each of the tissues (29, 30). In contrast, tissue-specific splicing yields distinct mRNAs, and distinct proglucagon polypeptides, in the pancreas and intestine of birds and fish (11). In birds and fish, pancreatic proglucagon does not encode GLP-2 (18–20), whereas in mammals it does (21, 22), suggesting that alternative splicing was lost on the mammalian lineage. Therefore, we would expect that alternative splicing would also occur in amphibians. The reported isolation of GLP-2 from pancreatic extracts of two amphibians (bullfrog and *Amphiuma*) suggested that a mammalian type pattern of proglucagon expression existed in amphibians, i.e., no alternative splicing. Our *Xenopus* cDNAs show that indeed GLP-2 is encoded by proglucagon cDNAs in the pancreas, but that it is also alternatively spliced such that only some of the mRNAs yield GLP-2.

Alternative splicing of *Xenopus* proglucagon differs from that observed in chicken and trout in at least two important aspects: (*i*) mechanism of splicing and (*ii*) tissue-specific distribution. In trout and chicken alternative splicing yields proglucagon mRNAs with different 3′ ends (11). This difference is attributed to the failure of the proglucagon mRNA to splice after the exon encoding GLP-1. In *Xenopus*, the alternatively spliced mRNAs have identical 3′ ends and differ only in the presence or absence of an exon encoding GLP-2 (Fig. 1). In mammals, no tissue-specific alternative splicing is observed (29, 30). *Xenopus* also differs in the tissue-specific distribution of the alternatively spliced mRNAs. GLP-2 encoding mRNAs were found in the intestine but not the pancreas of trout and chicken (11). Like birds and fish, *Xenopus* intestinal proglucagon mRNAs contain GLP-2, and stomach mRNAs did not. In contrast, both mRNAs were found in the pancreas in similar amounts (data not shown).

During the initial characterization of the *Xenopus* proglucagon cDNAs it was discovered that these mRNAs could potentially encode three GLP-1-like peptides (xenGLP-1A, xenGLP-1B, and xenGLP-1C, Figs. 1 and 2). All three xenGLP-1-like sequences were found to be most similar to mammalian GLP-1s (Fig. 2 and Table 1). xenGLP-1C was most similar to a GLP-1 that was identified in bullfrog (31). The three *Xenopus* GLP-1-like sequences are equally similar to the GLP-1 sequence from a divergent amphibian lineage, the *Amphiuma* (32). The GLP-1-like sequence of these peptides raised the possibility that *Xenopus* GLP-1s may possess incretin-like properties. Thus, xenGLP-1A, -1B, and -1C were tested for their ability to (*i*) bind to and activate the recombinant human GLP-1R and (*ii*) release insulin from perfused rat pancreas. The three peptides (Table 2) were found to be similar to hGLP-1 with respect to these parameters. However, xenGLP-1B was superior to all the peptides tested with respect to binding, displaying a greater affinity for the receptor than hGLP-1. xenGLP-1B was at least as potent as hGLP-1 in stimulating cAMP production and insulin in the perfused pancreas model. Thus, despite an average of nine amino acid substitutions between the *Xenopus* peptides and the hGLP-1 sequences, incretin activity has been conserved. Previous studies with alanine substituted analogs of mammalian GLP-1 identified residues 1, 4, 6, 7, 9, 22, and 23 as being sensitive to substitution with alanine (33, 34). The N-terminal amino acids were important for receptor interactions, whereas substitution of the C-terminal residues resulted in conformational changes such that the peptide could not be recognized by the receptor (33, 35). Substitution of the remaining positions with alanine, or if alanine in GLP-1 replacement with the homologous residue in glucagon, did not significantly alter GLP-1 function as measured by receptor binding (33, 34).

The *Xenopus* peptides differ from the hGLP-1 sequence at positions 6, 8, 10, 11, 12, 13, 15, 16, 17, 23, 24, 27, and 28 (Fig. 2). Only one residue, position 23, had previously been identified as crucial for GLP-1 function. Replacement of valine-23 with alanine resulted in a peptide that could not be recognized by the GLP-1R (33–35). The *Xenopus* peptides all have isoleucine instead of the very similar amino acid valine at position 23 (Fig. 2). Thus, a conservative substitution apparently did not alter the conformation of GLP-1-like peptides, whereas the slightly more radical valine to alanine does. Our results also show that the substitution of side chains at many positions in GLP-1 can occur without dramatically altering the activity of GLP-1; similar results have been seen with fish GLPs (36). Many of the observed changes between the mammalian and *Xenopus* peptides are conservative (e.g., serine-11 to threonine), but several are not (e.g., tyrosine-13 to glutamine) (Fig. 2). This complements the previous work that showed that the elimination of these side chains had little or no biological effect (33, 34). If xenGLP-1B is truly better at binding and activating the hGLP-1R, this may suggest that one of the unique replacements at positions 6 (F → Y), 12 (S → E), or 30 (R → K) may be responsible. Since xenGLP-1A and xenGLP-1C may be less effective than hGLP-1, this may suggest that changes at position 12 (S → Q or N) may result in lower biological activity. These results may suggest that the charge of the amino acid at position 12 may have some role in hormone–receptor interactions.

Because GLP-1 has been suggested as a potential therapeutic agent for the treatment of diabetes (6, 7), and as a central satiety factor (37) there is great interest in designing more efficient GLP-1R agonists. Our results have revealed that the *Xenopus* proglucagon gene encodes at least three potential insulinotropic peptides that may act as effectively (or possibly even more effectively in the case of xenGLP-1B) than hGLP-1 on hGLP-1R. Since the three *Xenopus* peptides have differing amino acid sequences, receptor binding, and activation properties, it raises the possibility that they have unique functions, possibly acting through unique (but related) receptors. Further examination of the *Xenopus* GLP-1-like peptides and perhaps their unique receptors may identify other biologically important and beneficial properties of these peptides, and should help facilitate the development of more effective GLP-1 analogs for therapeutic application.

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