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Insulin and Proglucagon-Derived Peptides from the Horned Frog, *Ceratophrys ornata* (Anura:Leptodactylidae)

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Insulin and peptides derived from the processing of proglucagon have been isolated from an extract of the pancreas of the South American horned frog, *Ceratophrys ornata* (Leptodactylidae). *Ceratophrys* insulin is identical to the insulin previously isolated from the toad, *Bufo marinus* (Bufonidae). *Ceratophrys* glucagon was isolated in two molecular forms with 29- and 36-amino acid residues in approximately equal amounts. Glucagon-29 is identical to glucagon from *B. marinus* and from the bullfrog, *Rana catesbeiana* (Ranidae) and contains only 1 amino acid substitution (Thr^{29} → Ser) compared with glucagon from *Xenopus laevis* (Pipidae). Glucagon-36 comprises glucagon-29 extended from its C-terminus by Lys-Arg-Ser-Gly-Gly-Met-Ser. This extension is structurally dissimilar to the C-terminal octapeptide of mammalian oxyntomodulin and resembles more closely that found in C-terminally extended glucagons isolated from fish pancreata. *Ceratophrys* glucagon-like peptide-1 (GLP-1) (His-Ala-Asp-Gly-Thr-Tyr-Gln-Asn-Asp-Val^{10}-Gln-Gln-Phe-Leu-Glu- Glu-Lys-Ala-Ala-Lys^{20}-Glu-Phe-Ile-Asp-Trp-Leu-Ile-Lys-Gly- Lys^{30}-Pro-Lys-Lys-Gln-Arg-Leu-Ser) contains 3 amino acid substitutions compared with the corresponding peptide from *B. marinus*, 8 substitutions compared with GLP-1 from *R. catesbeiana*, and between 4 and 11 substitutions compared with the three GLP-1 peptides identified in *X. laevis* proglucagon. GLP-2 was not identified in the extract of *Ceratophrys* pancreas. The data indicate that, despite its importance in the regulation of glucose metabolism, the primary structure of GLP-1 has been very poorly conserved during evolution, even among a single order such as the Anura.

Although the amino acid sequence of insulin is known for more than 100 species of vertebrates, our knowledge of the primary structures of insulins from amphibia is very limited. Among the Anurans, insulin has been characterized for the cane toad *Bufo marinus* (Bufonidae) (Conlon *et al.*, 1998a), three species of the family Ranidae: the American bullfrog *Rana catesbeiana* (Conlon *et al.*, 1998b), the European marsh frog *Rana ridibunda* (Conlon *et al.*, 1998b), the North American wood frog *Rana sylvatica* (Conlon *et al.*, 1998b), and two molecular forms from the South African clawed toad *Xenopus laevis* (Pipidae) (Shuldiner *et al.*, 1989). Similarly, the structure of insulin has been determined for only two species of salamander, the three-toed amphiuma *Amphiuma tridactylum* (Caudata: Amphiumidae) (Conlon *et al.*, 1996) and the lesser siren *Siren intermedia* Le Conte (Caudata: Sirenidae) (Conlon *et al.*, 1997), and for the caecilian *Typhlonectes natans* (Gymnophiona:Typhlonectidae) (Conlon *et al.*, 1995).

The biosynthesis of proglucagon-derived peptides in amphibia is less well understood than in mammals.

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In the pig pancreas, for example, proglucagon is processed predominantly to glucagon and an unprocessed peptide containing both the GLP-1 and the GLP-2 sequences, whereas in the pig intestine the prohormone is processed primarily to proglucagon (1–69), GLP-(7–37) and/or GLP-1(7–36)amide, and GLP-2 (Orskov *et al.*, 1986). Peptides whose amino acid sequences indicated structural similarity with mammalian GLP-1(7–37) and GLP-2 were isolated, together with glucagon, from extracts of the pancreata of the bullfrog *R. catesbeiana* (Pollock *et al.*, 1988a) and the amphiuma *A. tridactylum* (Cavanaugh *et al.*, 1996) and it has been shown that a single cloned cDNA encoding preproglucagon from the clawed toad *Xenopus laevis* contains the sequence of three peptides with structural similarity to GLP-1(7–37) in addition to sequences corresponding to mammalian glucagon and GLP-2 (Irwin *et al.*, 1997). In contrast, two proglucagon-derived peptides with 32- and 37-amino acid residues displaying structural similarity to human GLP-1(7–37) were isolated together with glucagon from *B. marinus* pancreas but a peptide corresponding to GLP-2 was not identified (Conlon *et al.*, 1998a).

The Argentinean horned frog *Ceratophrys ornata* (Leptodactylidae) represents an interesting animal model with which to study the effects of nutritional stimuli and neuroendocrine peptides upon adaptation of the gastrointestinal tract. The animal estivates underground for months during the dry season and then feeds voraciously after emerging during the rainy season (Abe, 1995). Preliminary data indicate that, as in the Burmese python *Python molurus* (Secor and Diamond, 1995, 1997a,b), feeding results in profound and rapid adaptive changes in the gastrointestinal tract such as increases in mucosal mass (3-fold), rates of intestinal nutrient uptake (5- to 10-fold), oxygen consumption rate (10-fold), and intestinal brush border oligopeptidase activity (4-fold) (Secor and Diamond, 1996). The role of neuroendocrine factors in mediating these changes is not understood and so our laboratories have embarked upon a program of studies to purify regulatory peptides from *Ceratophrys* gastroenteropancreatic tissues in order to investigate their possible involvement in the post-prandial responses. In the present study, we describe the isolation and characterization of insulin, glucagon, and glucagon-like peptide-1 (GLP-1) from an extract of *Ceratophyrs* pancreas. The data provide further insight into the molecular evolution of the islet hormones within Amphibia.

MATERIALS AND METHODS

Tissue Extraction

Pancreatic tissue (1.2 g) from horned frogs (12 adult specimens of both sexes; 7 fasted) was homogenized with ethanol/0.7 M HCl (3:1 v/v; 30 ml) using a Waring blender and stirred for 2 h at 0°C as previously described (Cavanaugh *et al.*, 1996). After centrifugation (4000*g* for 30 min), ethanol was removed from the supernatant under reduced pressure. After a further centrifugation (4000*g* for 30 min), the extract was pumped at a flow rate of 2 ml/min through 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0/29.9/0.1) and freeze-dried.

Radioimmunoassay

Insulin-like immunoreactivity was measured using an antiserum raised against pig insulin as previously described (Flatt and Bailey, 1981). Glucagon-like immunoreactivity was measured with an antiserum directed against the central region of porcine glucagon as previously described (Conlon and Thim, 1985).

Purification of the Peptides

The pancreatic extract, after partial purification on Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid/water (5 ml) and injected onto a 1 × 25-cm Vydac 218TP510 C-18 reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min and to 49% over 60 min with linear gradients. Absorbance was measured at 214 nm and 280 nm and fractions (1 min) were collected. The fractions designated I (containing insulin-like immunoreactivity) and G (containing glucagon-like immunoreactivity (Fig. 1) were rechromatographed on a 0.46 × 25-cm Vydac

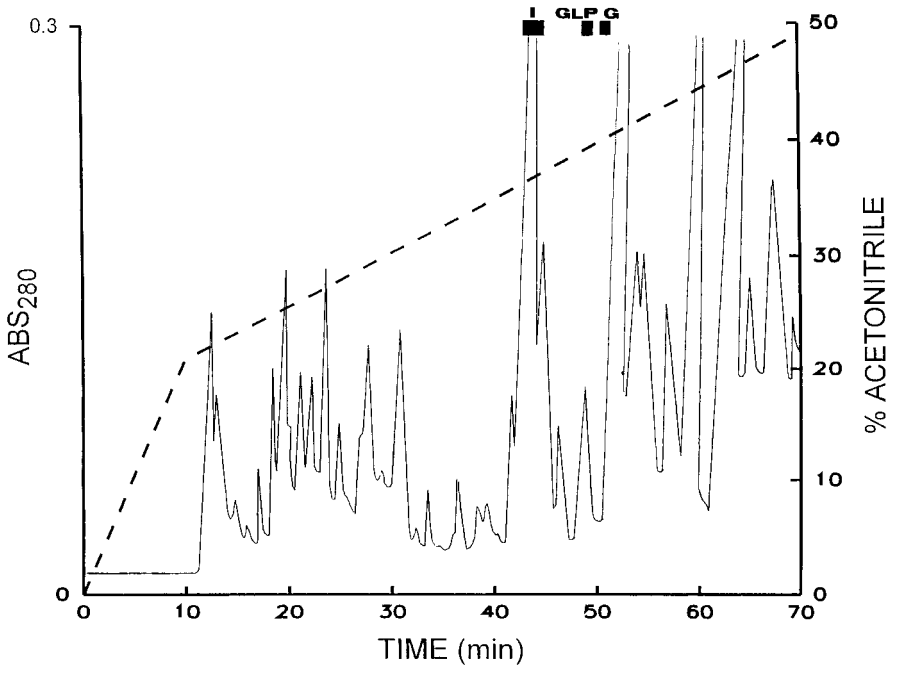


FIG. 1. Reversed-phase HPLC of an extract of the pancreas of *Ceratophrys ornata* after partial purification on Sep-Pak cartridges. I denotes the fraction containing both insulin and glucagon-36, G denotes the fraction containing glucagon-29, and GLP denotes the fraction containing GLP-1. (---) shows the concentration of acetonitrile in the eluting solvent.

214TP54 C-4 reversed-phase HPLC column equilibrated with acetonitrile / water / trifluoroacetic acid (21.0 / 78.9 / 0.1) at a flow rate of 1.5 ml / min. The concentration of acetonitrile in the eluting solvent was raised to 42% over 40 min using a linear gradient. *Ceratophrys* insulin, glucagon-36, and glucagon-29 were purified to near homogeneity, as assessed by peak symmetry, by chromatography on a 0.46 × 25-cm Vydac 219TP54 phenyl column under the same conditions used for the C-4 column.

As radioimmunoassays for GLP-1 or GLP-2 were not available in the laboratory, all fractions with retention times between 41 and 68 min from the semi-preparative Vydac C-18 column (Fig. 1) were individually chromatographed on analytical Vydac C-4 and phenyl columns under the same experimental conditions used for the purification of *Ceratophrys* glucagon. Purified peptides that were isolated in relatively high abundance were subjected to electrospray mass spectrometry.

Structural Characterization

Ceratophrys insulin (approximately 2 nmol) was incubated for 3 h at room temperature with dithiothreitol (2 mg) in 0.1 M Tris–HCl / 6 M guanidine hydrochloride buffer, pH 7.5 (0.4 ml) under an atmosphere of argon. Cysteine residues were derivatized by addition of 4-vinylpyridine (3 μl) and the pyridylethylated A- and B-chains of insulin were separated on a 0.46 × 25-cm Vydac 218TP54 C-4 column under the conditions used for the purification of intact insulin (Fig. 2A).

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems Model 471A sequenator modified for on-line detection of phenylthiohydantoin amino acids under gradient elution conditions. Electrospray mass spectrometry was carried out using a Perkin–Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was ±0.02%.

RESULTS

Purification of Ceratophrys Insulin and Glucagon

The tissue extract, after partial purification on Sep-Pak cartridges, was chromatographed on a semi-

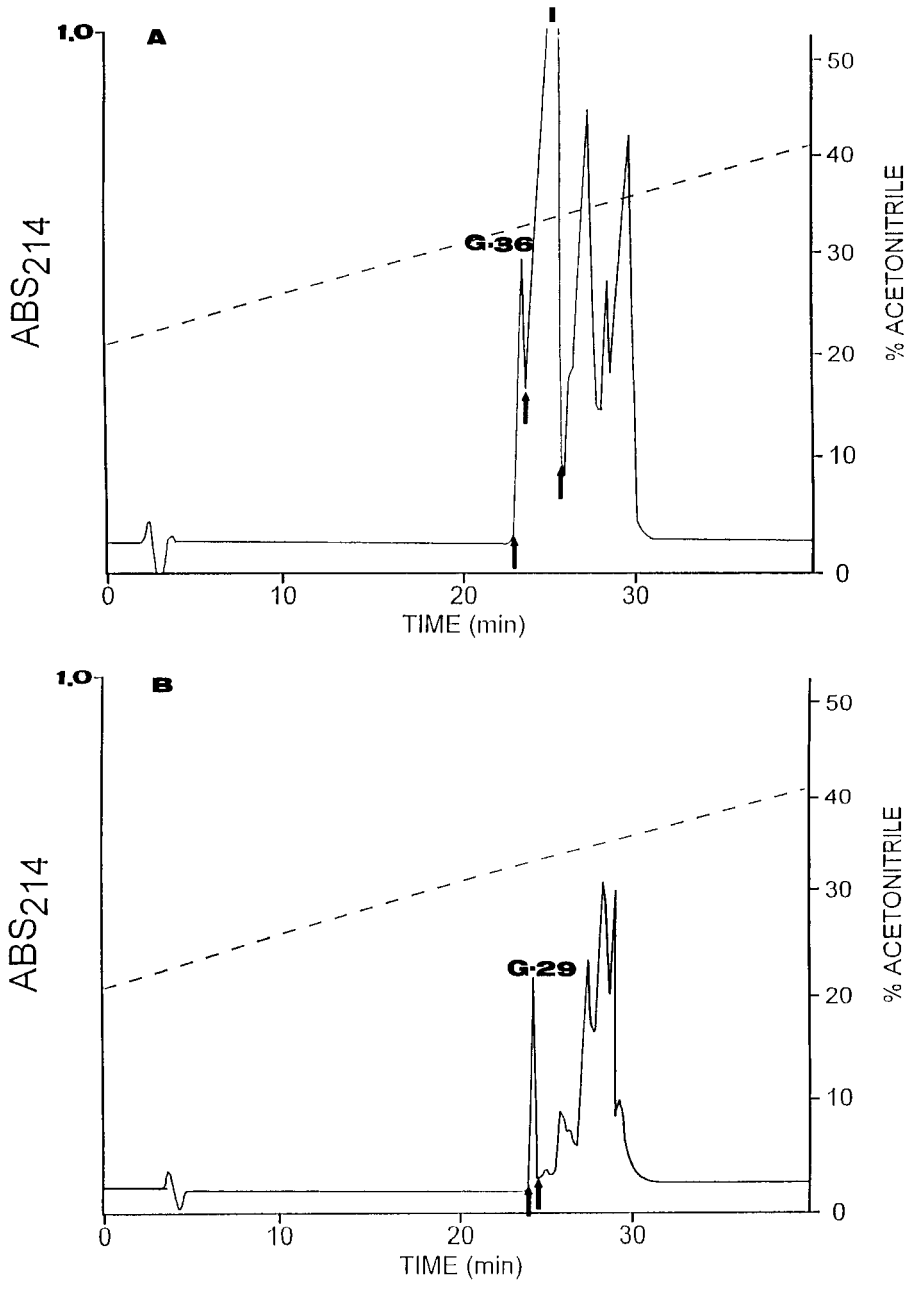


FIG. 2. Purification by reversed-phase HPLC on an analytical Vydac C-4 column of *Ceratophrys* (A) insulin (peak I) and glucagon-36 (peak G-36), (B) glucagon (peak G-29), and (C) GLP-1. The arrows show where peak collection began and ended.

preparative Vydac C-18 column and the elution profile is shown in Fig. 1. The prominent peak designated I was associated with both insulin-like immunoreactivity and glucagon-like immunoreactivity. Rechromatography of this fraction on an analytical Vydac C-4 column (Fig. 2A) revealed that the material was heterogeneous and the insulin-like immunoreactivity was associated with the major peak designated I and delineated by the arrows and the glucagon-like immunoreactivity was associated with the early eluting peak designated G-36. *Ceratophrys* insulin was purified to near homogeneity (as assessed by symmetrical peak

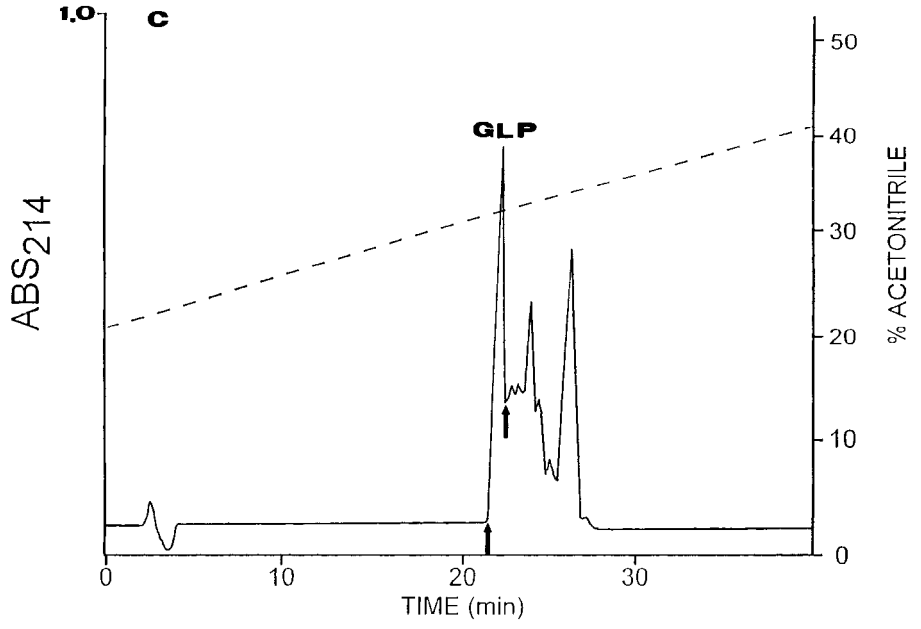


FIG. 2—*Continued*

shape) by a final chromatography on a Vydac phenyl column (Fig. 3A). The final yield of the pure peptide was approximately 12 nmol. Glucagon-36 was purified to apparent homogeneity under the same conditions of chromatography (Fig. 3B) and the final yield of the pure peptide was approximately 2 nmol.

After chromatography on a semi-preparative Vydac C-18 column (Fig. 1), the fraction designated G was also associated with glucagon-like immunoreactivity. *Ceratophrys* glucagon-29 was purified to near homogeneity by successive chromatographies on analytical Vydac C-4 (Fig. 2B) and Vydac phenyl (Fig. 3C) columns and the final yield of pure material was 1.5 nmol.

Purification of Ceratophrys GLP-1

The strategy employed to isolate *Ceratophrys* GLP-1 was based upon that used to isolate the corresponding peptide from a extract of amphiuma pancreas (Cavanaugh *et al.,* 1996) and involves purification to near homogeneity of most of the components present in major abundance that were eluted from a semipreparative C-18 HPLC column with a retention time between 41 and 68 min (Fig. 1). The peptides were subjected to electrospray mass spectrometry and those peptides with a molecular mass between 3000 and 5000 D were identified by amino acid sequence analysis. A total of 21 fractions (volume 2 ml) were individually chromatographed on an analytical Vydac C-4 column under the same conditions used for the purification of glucagon. Peaks of major abundance (a total of 17) were rechromatographed on an analytical Vydac phenyl column and 19 peptides were isolated in pure form, as assessed by peak symmetry. Subsequent characterization of these peptides indicated that GLP-1 was eluted from the semipreparative C-18 column in the fraction designated GLP-1 (Fig. 1). The purification of the peptide on the analytical C-4 (Fig. 2C) and phenyl (Fig. 3D) columns is shown. The final yield of pure *Ceratophrys* GLP-1 was 4 nmol. Attempts to identify a peptide with structural similarity to GLP-2 were unsuccessful.

Structural Characterization

The primary structures of the pyridylethylated A-chains and B-chains of *Ceratophrys* insulin were determined by automated Edman degradation and the results are shown in Fig. 4. It was possible to identify without ambiguity phenylthiohydantoin-coupled amino acids for 21 cycles of operation of the sequena-

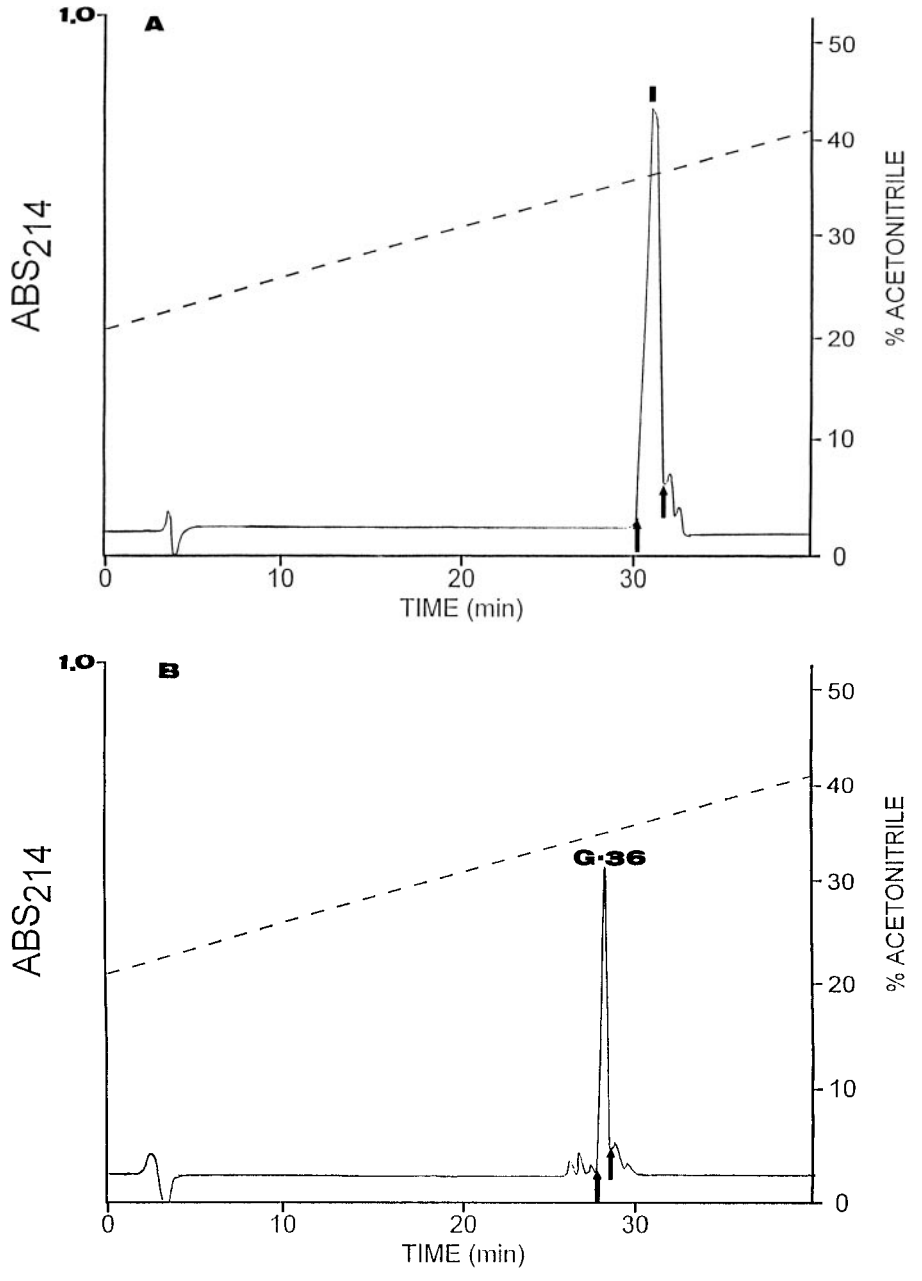


FIG. 3. Purification by reversed-phase HPLC on an analytical Vydac phenyl column of *Ceratophrys* (A) insulin, (B) glucagon-36, (C) glucagon-29, and (D) GLP-1.

tor during sequence analysis of the A-chain and for 30 cycles during analysis of the B-chain. The proposed amino acid sequence of *Ceratophrys* insulin was confirmed by electrospray mass spectrometry. The observed molecular mass of A-chain of *Ceratophrys* insulin was 2828.8 a.m.u. compared with a calculated average mass of 2828.7 a.m.u. and the observed molecular mass of the B-chain was 3664.0 a.m.u. compared with a calculated average mass of 3664.1 a.m.u.

The primary structures of *Ceratophrys* glucagon-29, glucagon-36, and GLP-1 were determined without ambiguity by automated Edman degradation (Fig. 4).

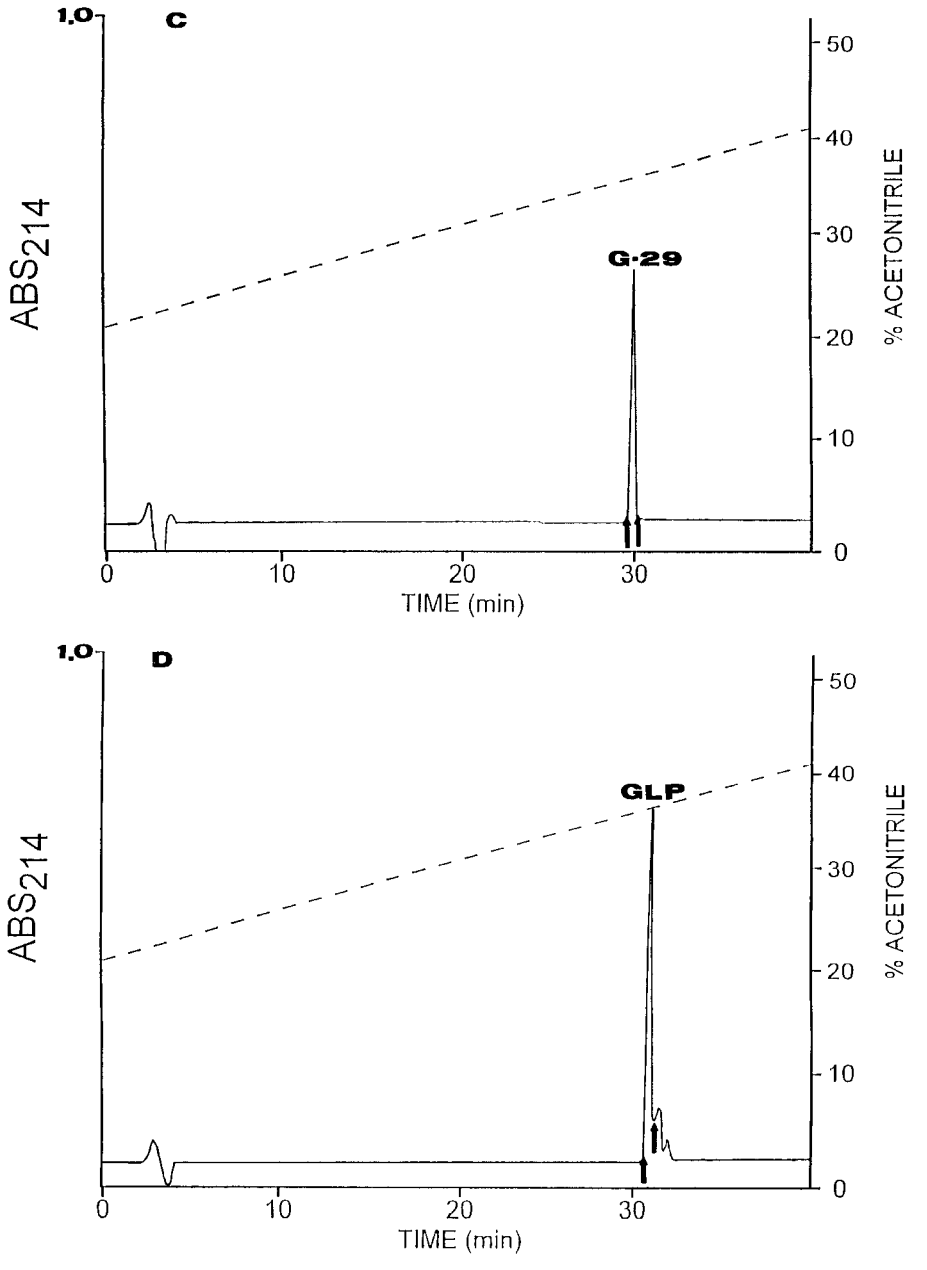


FIG. 3—*Continued*

The proposed structures were confirmed by electrospray mass spectrometry (glucagon-29: observed molecular mass 3468.1 a.m.u., calculated average mass 3468.8 a.m.u.; glucagon-36: observed molecular mass 4172.1 a.m.u., calculated average mass 4172.6 a.m.u.; GLP-1: observed molecular mass 4306.3 a.m.u., calculated average mass 4306.9 a.m.u.).

DISCUSSION

The amino acid sequences of the amphibian insulins are compared with human insulin in Fig. 5. *Ceratophrys* insulin is identical in structure to the protein previously isolated from *B. marinus* pancreas (Conlon *et al.*,

Insulin A-Chain

Gly-Ile-Val-Glu-Gln-Cys-Cys-His-Ser-Thr^{10}-Cys-Ser-Leu-Tyr-Glu-Leu-Glu-Asn-Tyr-Cys^{20}-Asn

Insulin B-Chain

Leu-Ala-Asn-Gln-His-Leu-Cys-Gly-Pro-His^{10}-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly^{20}-Glu-Arg-Gly-Phe-Tyr-Tyr-Tyr-Pro-Lys-Val^{30}

Glucagon-29

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr^{10}-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln^{20}-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Ser

Glucagon-36

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr^{10}-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-Ala-Gln^{20}-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Ser-Lys^{30}-Arg-Ser-Gly-Gly-Met-Ser

Glucagon-like Peptide-1

His-Ala-Asp-Gly-Thr-Tyr-Thr-Asn-Asp-Val^{10}-Thr-Gln-Phe-Leu-Glu-Glu-Lys-Ala-Ala-Lys^{20}-Glu-Phe-Ile-Asp-Trp-Ile-Leu-Lys-Gly-Lys^{30}-Pro-Lys-Lys-Gln-Arg-Leu-Ser

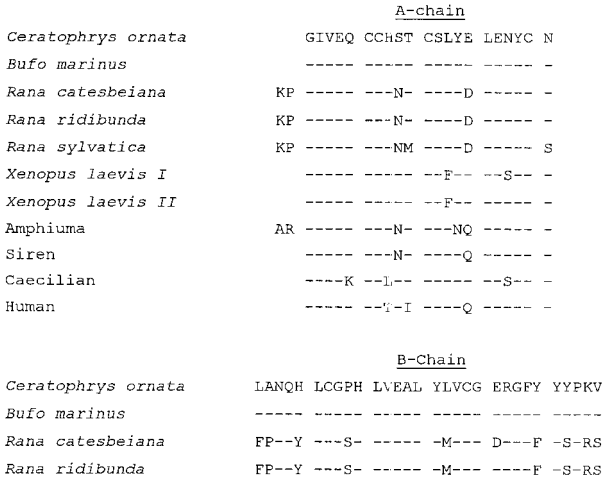
FIG. 4. Amino acid sequences of insulin, glucagon-29, glucagon-36, and glucagon-like peptide-1 from the horned frog *Ceratophrys ornata.*

1998a). The genera *Bufo* and *Ceratophrys* are classified together in the Neobatrachia (often referred to as the "higher" frogs). This group includes 95% of the known species of frogs but phylogenetic relationships between the major lineages are not well resolved so that the evolutionary connection between the Leptodactylidae and the Bufonidae is unclear (Duellman and Trueb, 1994). Our data are consistent with the hypothesis that the Leptodactylidae and the Bufonidae are more closely related to each other than either are to the Ranidae but previous analysis has shown that the amino acid sequence of insulin is not always a reliable molecular marker with which to infer phylogenetic relationships between species (Conlon, 1999).

The traditional view, based primarily on X-ray crystallographic data, is that the amino acid residues at positions B12, B16, B23–B26, A1–A5, A19, and A21 in the insulin molecule comprise the receptor-binding domain (Baker *et al.,* 1988). However, more recently, it has been proposed that the conformation adopted by insulin in the crystal structure is an inactive one and therefore distinct from the receptor-binding conformation (Derewenda *et al.,* 1991). The results of alanine-scanning mutagenesis studies (Kristensen *et al.,* 1997) have suggested the alternative model in which the receptor-binding domain of human insulin consists of five residues (IleA2, ValA3, TyrA19, GlyB23, and Phe24) forming a patch on the surface of the molecule, with residues LeuB6, GlyB8, LeuB11, GluB13, and PheB25, although not part of the binding epitope, being important in maintaining the overall receptor-binding conformation. A comparison of the structures of the amphibian insulins supports this revised view as the data indicate that evolutionary pressure has acted to conserve all these residues, which are postulated to be important in determining the receptor-binding conformation of insulin, with the exception of the conservative substitution (Phe → Tyr) at B25. In contrast, resi-

dues such as Gln at A5 and Asn at A21 that are important in constituting and maintaining the crystal structure of insulin have not been conserved among the amphibia. All the amphibian insulins, with the exception of that from the caecilian, contain a histidine residue at position A8. This amino acid is postulated to stabilize the receptor-binding conformation relative to the inactive conformation (Derewenda *et al.*, 1991), thereby accounting for the observations that *Bufo* (Conlon *et al.*, 1998a) and *Xenopus* (Shuldiner *et al.*, 1989) insulins are appreciably more potent than human insulin in inhibiting the binding of radiolabeled insulin to the human insulin receptor.

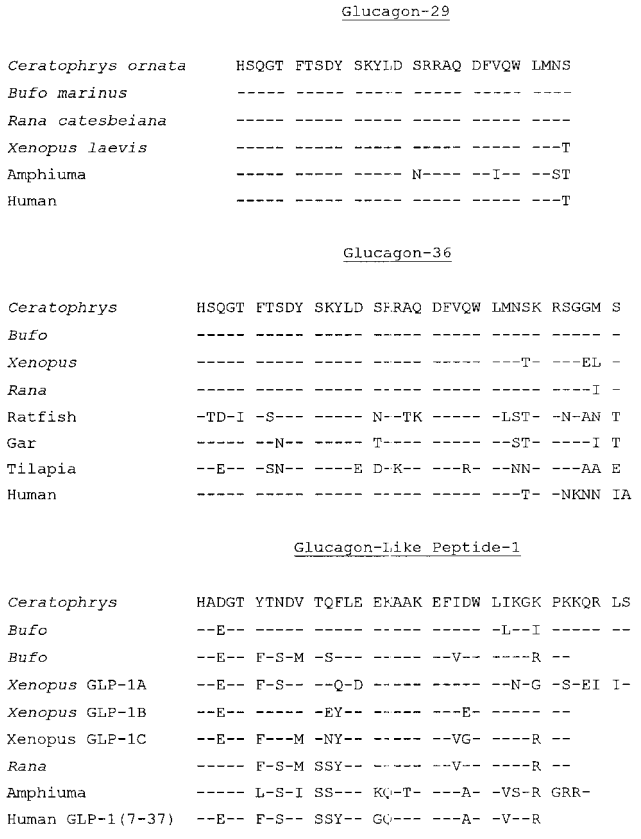
The amino acid sequences of *Ceratophrys* glucagons and GLP-1 are compared with the corresponding peptides from other amphibian species in Fig. 6. Consistent with the observation that the primary structure of glucagon-29 has been very strongly conserved among tetrapods (Mommsen and Plisetskaya, 1993), *Ceratophrys* glucagon is identical to *Bufo* glucagon (Conlon *et al.*, 1998a) and to glucagon from the bullfrog *R. catesbeiana* (Pollock *et al.*, 1988a) and shows only one amino acid substitution (Thr^{29} → Ser) compared with glucagons from the human and from *Xenopus* (Irwin *et al.*, 1997). The significance of the C-terminally extended glucagon (glucagon-36) isolated in relatively high yield from *Ceratophrys* pancreas is unclear. It is probable that this peptide is derived from the same preproglucagon as glucagon-29 by an alternative pathway of post-translational process but the possibility that more than one glucagon gene is expressed cannot be excluded. A 37-amino acid residue C-terminally extended glucagon, termed oxyntomodulin, has been isolated from extracts of the small intestine of several mammals and is 10–20 times more potent than glucagon in inhibiting pentagastrin-stimulated gastric acid secretion in the rat (Jarrousse *et al.*, 1985). However, oxnytomodulin is present in the pancreata of mammals only in low concentration and, as shown in Fig. 6, the C-terminally extended glucagons isolated from the pancreata of *Ceratophrys*, *Rana*



	A-chain				
Ceratophrys ornata	GIVEQ	CCHST	CSLYE	LENYC	N
Bufo marinus	-----	-----	-----	-----	-
Rana catesbeiana	KP -----	---N-	----D	-----	-
Rana ridibunda	KP -----	---N-	----D	-----	-
Rana sylvatica	KP -----	---NM	----D	-----	S
Xenopus laevis I	-----	-----	--F--	--S--	-
Xenopus laevis II	-----	-----	--F--	-----	-
Amphiuma	AR -----	---N-	---NQ	-----	-
Siren	-----	---N-	----Q	-----	-
Caecilian	----K	--L--	-----	--S--	-
Human	-----	--T-I	----Q	-----	-

	B-Chain					
Ceratophrys ornata	LANQH	LCGPH	LVEAL	YLVCG	ERGFY	YYPKV
Bufo marinus	-----	-----	-----	-----	-----	-----
Rana catesbeiana	FP--Y	---S-	-----	-M---	D---F	-S-RS
Rana ridibunda	FP--Y	---S-	-----	-M---	----F	-S-RS
Rana sylvatica	FP---	---S-	--D--	-M---	D---F	-S-RS
Xenopus laevis I	-V--Y	---S-	-----	-----	D---F	-----
Xenopus laevis II	----Y	---S-	-----	-----	D---F	----I
Amphiuma	IT--Y	---S-	-----	-----	D---F	-S--
Siren	VP-KP	---A-	---VM	-F---	D---F	-PSST
Caecilian	I----	---S-	-----	----A	D---F	-T--S
Human	FV---	---S-	-----	-----	----F	-T--T

FIG. 5. A comparison of the primary structures of insulins from species of Anura, Caudata, and Gymnophiona with human insulin. (-) denotes residue identity.



	Glucagon-29					
Ceratophrys ornata	HSQGT	FTSDY	SKYLD	SRRAQ	DFVQW	LMNS
Bufo marinus	-----	-----	-----	-----	-----	----
Rana catesbeiana	-----	-----	-----	-----	-----	----
Xenopus laevis	-----	-----	-----	-----	-----	---T
Amphiuma	-----	-----	----- N	----	--I--	--ST
Human	-----	-----	-----	-----	-----	---T

	Glucagon-36							
Ceratophrys	HSQGT	FTSDY	SKYLD	SRRAQ	DFVQW	LMNSK	RSGGM	S
Bufo	-----	-----	-----	-----	-----	-----	-----	-
Xenopus	-----	-----	-----	-----	-----	---T-	---EL	-
Rana	-----	-----	-----	-----	-----	-----	----I	-
Ratfish	-TD-I	-S---	-----	N--TK	-----	-LST-	-N-AN	T
Gar	-----	--N--	-----	T----	-----	--ST-	----I	T
Tilapia	--E--	-SN--	----E	D-K--	---R-	--NN-	---AA	E
Human	-----	-----	-----	-----	-----	---T-	-NKNN	IA

	Glucagon-Like Peptide-1							
Ceratophrys	HADGT	YTNDV	TQFLE	EKAAK	EFIDW	LIKGK	PKKQR	LS
Bufo	--E--	-----	-----	-----	-----	-L--I	-----	--
Bufo	--E--	F-S-M	-S---	-----	--V--	----R	--	
Xenopus GLP-1A	--E--	F-S--	--Q-D	-----	-----	--N-G	-S-EI	I-
Xenopus GLP-1B	--E--	-----	-EY--	-----	---E-	-----	--	
Xenopus GLP-1C	--E--	F---M	-NY--	-----	--VG-	----R	--	
Rana	-----	F-S-M	SSY--	-----	--V--	----R	--	
Amphiuma	-----	L-S-I	SS---	KQ-T-	---A-	-VS-R	GRR-	
Human GLP-1(7-37)	--E--	F-S--	SSY--	GQ---	---A-	-V--R		

FIG. 6. A comparison of the primary structures of glucagon-29, glucagon-36, and GLP-1 from species of Anura and Caudata with the corresponding peptides from fish and with human oxyntomodulin (glucagon-37). (-) Denotes residue identity.

(Pollock *et al.*, 1988a), and *Bufo* (Conlon *et al.*, 1998a) and deduced from the nucleotide sequence of *Xenopus* proglucagon (Irwin *et al.*, 1997) show no structural similarity to oxyntomodulin in the C-terminal region. On the other hand, C-terminally extended glucagons have been isolated from the pancreata of fish [the Alligator gar *Lepisosteus spatula* (Pollock *et al.*, 1988b); the Pacific ratfish *Hydrolagus colleie* (Conlon *et al.*, 1987); and the tilapia *Oreochromis nilotica* (Nguyen *et al.*, 1995)] that resemble the frog peptides more closely (Fig. 6). The physiological role of these C-terminally extended glucagons in either fish or amphibia is unknown.

In *Ceratophrys* pancreas, as in the pancreata of the cane toad, bullfrog, and amphiuma, GLP-1 is stored in the mature, fully processed form that corresponds to human intestinal GLP-1 (7–37). The data in Fig. 6 support the previous assertion that the primary structure of GLP-1 has been very poorly conserved during vertebrate evolution (Conlon *et al.*, 1994). *Ceratophrys* GLP-1 shows closest structural similarity with the 37-amino acid residue form of *Bufo* GLP-1 (3 amino acid substitutions) but contains 11 substitutions compared with the corresponding peptide from *Xenopus* (GLP-1A) and 15 substitutions compared with GLP-1 from the amphiuma. Among the nontetrapods, the structure of GLP-1 has been even more poorly conserved. Only three residues (Ala^{2}, Asp^{9}, and Leu^{26} corresponding to residues 8, 15, and 32 in human GLP-1) are found in the same position in all fish GLPs (Conlon *et al.*, 1994). Several structure–activity studies have used alanine-scanning mutagenesis to demonstrate that residues His^{7}, Gly^{10}, Tyr^{12}, Thr^{13}, Asp^{15}, Tyr^{19}, Glu^{21}, Phe^{28}, Ile^{29}, and Leu^{32} in human GLP-1(7–36)amide are important for high-affinity binding by cells expressing the rat pancreatic GLP-1 receptor (Adelhorst *et al.*, 1994; Gallwitz *et al.*, 1994; Parker *et al.*, 1998). The data in Fig. 6 indicate that residues at positions 7, 10, 13, 15, 28, and 32 have been conserved during the radiation of the amphibia but substitutions are seen at positions 12, 19, 21, and 29.

In mammals, the truncated form of GLP-1 [GLP-1(7–36)amide] is the most potent insulinotropic peptide yet discovered (Mojsov *et al.*, 1987), whereas in fish the peptide is not insulin releasing but stimulates hepatic glycogenolysis and gluconeogenesis (Mommsen and Moon, 1989). The physiological role of GLP-1 in amphibians is unknown. Preliminary data suggest that it is reasonable to assume that amphibian pancreatic islets also respond to human GLP-1(7–36)amide with increased release of insulin (Mommsen and Moon, 1994). Similarly, the GLP-related peptides from *Bufo* pancreas produced concentration-dependent increases in insulin release from glucose-responsive rat insulinoma-derived BRIN-BD11 cells (Conlon *et al.*, 1998a) and synthetic replicates of the *Xenopus* GLP-1 peptides stimulated insulin release from the rat pancreas (Irwin *et al.*, 1997). Preliminary data indicate that human GLP-1(7–36)amide does not stimulate glycogenolysis in frog hepatocytes (Mommsen and Moon, 1994).

A peptide with appreciable structural similarity to either human GLP-2 or to the GLP-2 peptides isolated from the pancreata of the bullfrog (Pollock *et al.*, 1988a) and the amphiuma (Cavanaugh *et al.*, 1996) was not identified in the extract of *Ceratophrys* pancreas despite a thorough search for the peptide using mass spectrometry. It has been demonstrated that cDNAs encoding preproglucagons isolated from chicken and trout intestines contain both the GLP-1 and the GLP-2 regions, but an alternative RNA splicing mechanism generates a preproglucagon mRNA in the pancreas that lacks the region encoding the GLP-2 sequence (Irwin and Wong, 1995). It is possible, therefore, that the failure to detect GLP-2 in extracts of the pancreata of *Ceratophrys* and *Bufo* may be a consequence of the fact that the pancreatic proglucagon mRNA does not encode the GLP-2 region. Nucleotide sequence analysis studies are required to test this hypothesis. In the light of the observation that GLP-2 may play a physiologically important role as an intestinal trophic factor in mammals (Drucker *et al.*, 1996), the peptide released from the intestine may be involved in mediating the dramatic intestinal adaptive responses to feeding seen in *Ceratophrys* (Secor and Diamond, 1996).

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