Characterization of insulins and proglucagon-derived peptides from a phylogenetically ancient fish, the paddlefish (*Polyodon spathula*)

Tan M. NGUYEN,* Thomas P. MOMMSEN,† Steven M. MIMS‡ and J. Michael CONLON*§

*Regulatory Peptide Center, Creighton University School of Medicine, Omaha, NE 68178, U.S.A., †Department of Biochemistry and Microbiology, University of Victoria, British Columbia, V8W 3P6, Canada and ‡Aquaculture Research Center, Kentucky State University, Frankfort, KY 40601, U.S.A.

The North American paddlefish, *Polyodon spathula* (Order Acipenseriformes) is an extant representative of a group of primitive Actinopterygian (ray-finned) fish that probably shared a common ancestor with present-day teleosts. Two molecular forms of insulin which differ by a single amino acid substitution, His or Asp at position 15 of the A chain, were isolated from the pancreas of the paddlefish. Paddlefish insulins show greatest structural similarity to insulin from the garfish (order Lepisosteiformes) and resemble mammalian insulins more strongly than do insulins from teleost fish. The primary structures of several proglucagon-derived peptides, two molecular forms of glucagon which differ by the single amino acid substitution Arg^{18}→Ser, and glucagon-like peptide, have been less well conserved during evolution. The paddlefish glucagons contain 31 amino acid residues, rather than the usual 29, and show several structural features, such as Met^{5}, Glu^{24} and Gly^{29}, not previously observed in glucagons from other species. In spite of considerable differences in structure between paddlefish and mammalian glucagons (10 or 11 amino acid substitutions), both paddlefish glucagons are equally as effective as bovine glucagon in stimulating glycogenolysis in dispersed hepatocytes from the teleost fish *Sebastes caurinus* (rockfish). However, the substitution Arg^{18}→Ser in the paddlefish glucagon results in a 6-fold decrease in potency in this system.

INTRODUCTION

The paddlefish occupies an important position in phylogeny as one of the most primitive living members of the Actinopterygii (ray-finned species), and recent years have seen an awakening of interest in the species as a commercially important food source (Mims et al., 1991). The family Polyodontidae comprises only two extant species, the North American paddlefish, *Polyodon spathula*, and the Chinese paddlefish, *Psephurus gladius*, which is found in the River Yangtze only. The Polyodontidae and Acipenseridae (sturgeons) are closely related families which are frequently classified together as Chondrosteans, but the fossil record shows that in the late Cretaceous period the two families were already clearly distinct from one another (Carroll, 1988). The American paddlefish is predominantly a plankton-feeder and possesses one of the most complex gastrointestinal tracts found in fishes (Weisel, 1927; Epple and Brinn, 1975). The pancreas of the paddlefish is similar to that of the sturgeons and is disseminated throughout the body cavity following blood vessels and bile ducts into the liver and spleen. Unlike many species of teleost fish, there is no segregation of the pancreatic islets and the exocrine parenchyma.

The phylogenetically ancient Actinopterygians include, in addition to the paddlefishes and sturgeons, the garfish (Order Lepisosteiformes), the bowfin (Order Amiiformes) and the reedfish and bichirs (Order Polypteriformes). The islet hormones from species of this group of fishes have been characterized only incompletely. Recent studies have led to the purification and characterization of insulin (Pollock et al., 1987) and glucagon (Pollock et al., 1988) from the alligator gar, *Lepisosteus spathula*, and the corresponding peptides from the bowfin, *Amia calva* (Conlon et al., 1991, 1993b) but structures of pancreatic hormones from chondrostean fish have not been reported. In this study, we describe the isolation and structural characterization of multiple forms of insulin, glucagon and glucagon-like peptide (GLP) from the pancreas of the paddlefish, *P. spathula*.

EXPERIMENTAL

Tissue extraction

Paddlefish (24 specimens of both sexes; age 14 months; 72–79 cm long; weight 1.2–1.8 kg) were collected at the Aquaculture Research Center, Frankfort, KY, U.S.A.) during June, 1993. Pancreatic tissue (44 g) was extracted with ethanol/0.7 M HCl (3:1, v/v; 800 ml) as previously described (Conlon et al., 1991). After centrifugation (10000 *g* for 1 h at 4 °C), ethanol was removed from the supernatant under reduced pressure. Peptide material was isolated from the extract using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:29:1, by vol.) and freeze-dried.

Radioimmunoassay

Insulin-like immunoreactivity was measured by using an antiserum raised against pig insulin as described previously (Bailey and Ahmed-Sorour, 1980). Glucagon-like immunoreactivity was measured using an antiserum raised against pig glucagon as described previously (Conlon and Thim, 1985).

Purification of paddlefish insulins

The pancreatic extract, after partial purification of Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid (5 ml) and chromatographed on a Sephadex G-25 gel permeation column (1.6 cm × 90 cm) (Pharmacia, Uppsala, Sweden) equili-

Abbreviations used: G, glucagon; GLP, glucagon-like peptide; I, insulin.

§ To whom correspondence should be addressed.

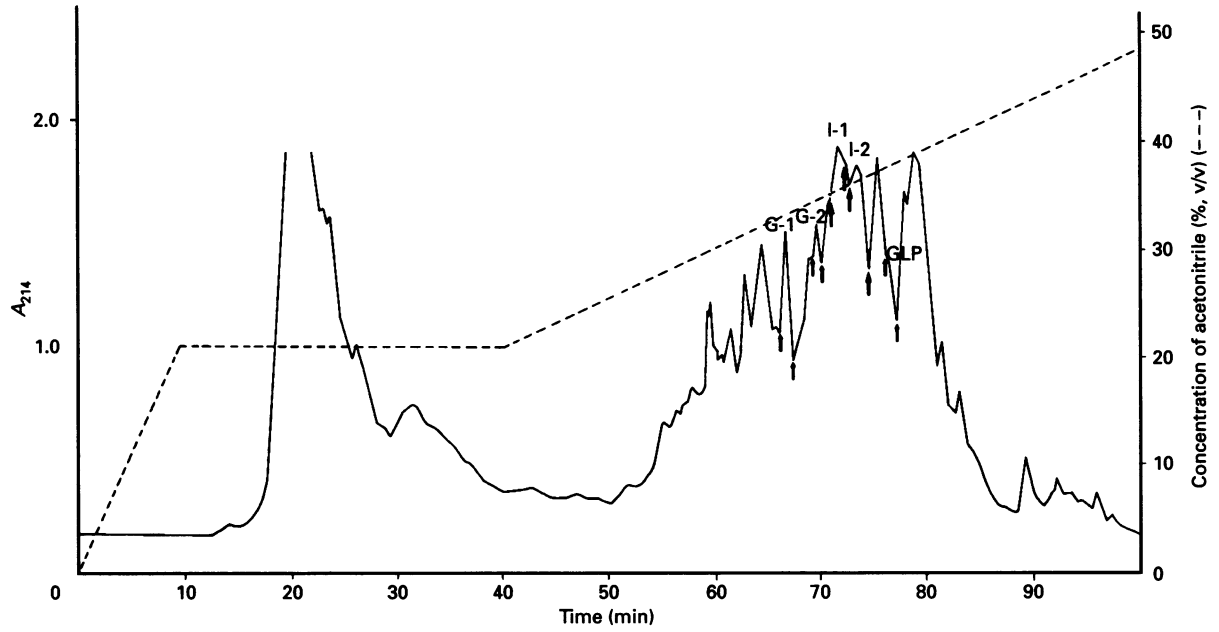

Figure 1 Fractionation of an extract of paddlefish pancreas by reversed-phase h.p.l.c.

The extract, after partial purification on Sep-Pak C_{18} cartridges and on a Sephadex G-25 gel permeation column, was chromatographed on a Vydac C_{18} semi-preparative h.p.l.c. column under the conditions described in the text. The fractions denoted by I-1 and I-2 contained insulin, G-1 and G-2 contained glucagon and GLP contained glucagon-like peptide. The broken line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended.

brated with 1 M acetic acid at a flow rate of 24 ml/h. Fractions (2 ml) were collected and assayed for insulin-like immunoreactivity at a dilution of 1:30. Fractions containing insulin were pooled and injected on to a Vydac 218 TP510 C_{18} reversed-phase h.p.l.c. column (1 cm × 25 cm) (Separations Group, Hesperia, CA, U.S.A.) equilibrated with 0.1 % (v/v) trifluoroacetic acid at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21 % (v/v) over 10 min and to 49 % (v/v) over 60 min with linear gradients. Absorbance was measured at 214 nm and 280 nm and individual peaks were collected by hand. The peaks designated I-1 and I-2 (containing insulin-like immunoreactivity) (Figure 1) were separately rechromatographed on a Vydac 214 TP54 C_4 reversed-phase column (0.46 cm × 25 cm) equilibrated with acetonitrile/water/trifluoroacetic acid (210:789:1, by vol.) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 40 min using a linear gradient. The paddlefish insulins were purified to near homogeneity, as assessed by peak symmetry, by successive chromatographies on a Vydac 219 TP54 phenyl column (0.46 cm × 25 cm) and on a Vydac 218 TP54 C_{18} column (0.46 cm × 25 cm) under the same conditions used for the C_4 column.

Purification of paddlefish glucagons and GLP

The peaks designated G-1 and G-2 (containing glucagons) and GLP (containing glucagon-like peptide) in Figure 1 were separately rechromatographed on Vydac C_4, phenyl and C_{18} reversed-phase columns (0.46 cm × 25 cm) under the same conditions used for the purification of paddlefish insulins.

Structural characterization of the paddlefish peptides

Paddlefish insulins I-1 and I-2 (~ 8 nmol) were reduced (dithiothreitol) and pyridylethylated (4-vinylpyridine) as previously described (Conlon et al., 1991). The derivatized A chain and B chain were separated by reversed-phase h.p.l.c. on a Vydac C_4 column under the conditions used for the purification of insulin (Figure 2a).

Amino acid compositions were determined in duplicate by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems model 420A derivatizer followed by reversed-phase h.p.l.c. with an Applied Biosystems model 130A separation system. Hydrolysis (24 h at 110 °C in 5.7 M HCl) of ~ 1 nmol of peptide was carried out. Cysteine and tryptophan residues were not determined. The primary structures of the paddlefish peptides (~ 3 nmol portions) were determined by automated Edman degradation in an Applied Biosystems model 471A sequenator modified for detection of phenylthiohydantoin amino acid derivatives under gradient elution conditions.

Glycogenolytic activity of paddlefish glucagon and GLP

The abilities of the paddlefish glucagons to stimulate glycogenolysis were measured in freshly prepared hepatocytes from adult copper rockfish, *Sebastes caurinus*. Full details of the experimental procedure have been provided in a recent article (Conlon et al., 1993b). The activities of bovine glucagon, paddlefish glucagon G-1 and paddlefish glucagon G-2 were compared in parallel incubations. Peptide concentrations were measured by amino acid analysis. All determinations were performed in triplicate for five independent experiments. Data are expressed as means ± S.E.M. and comparisons are made using Analysis of Variance (ANOVA); $P < 0.05$ was considered significant. Potency is defined as the concentration of peptide producing half-maximum activation of glucose production and effectiveness is the maximum response produced by the peptide. There was not sufficient pure peptide to determine the biological activity of paddlefish GLP.

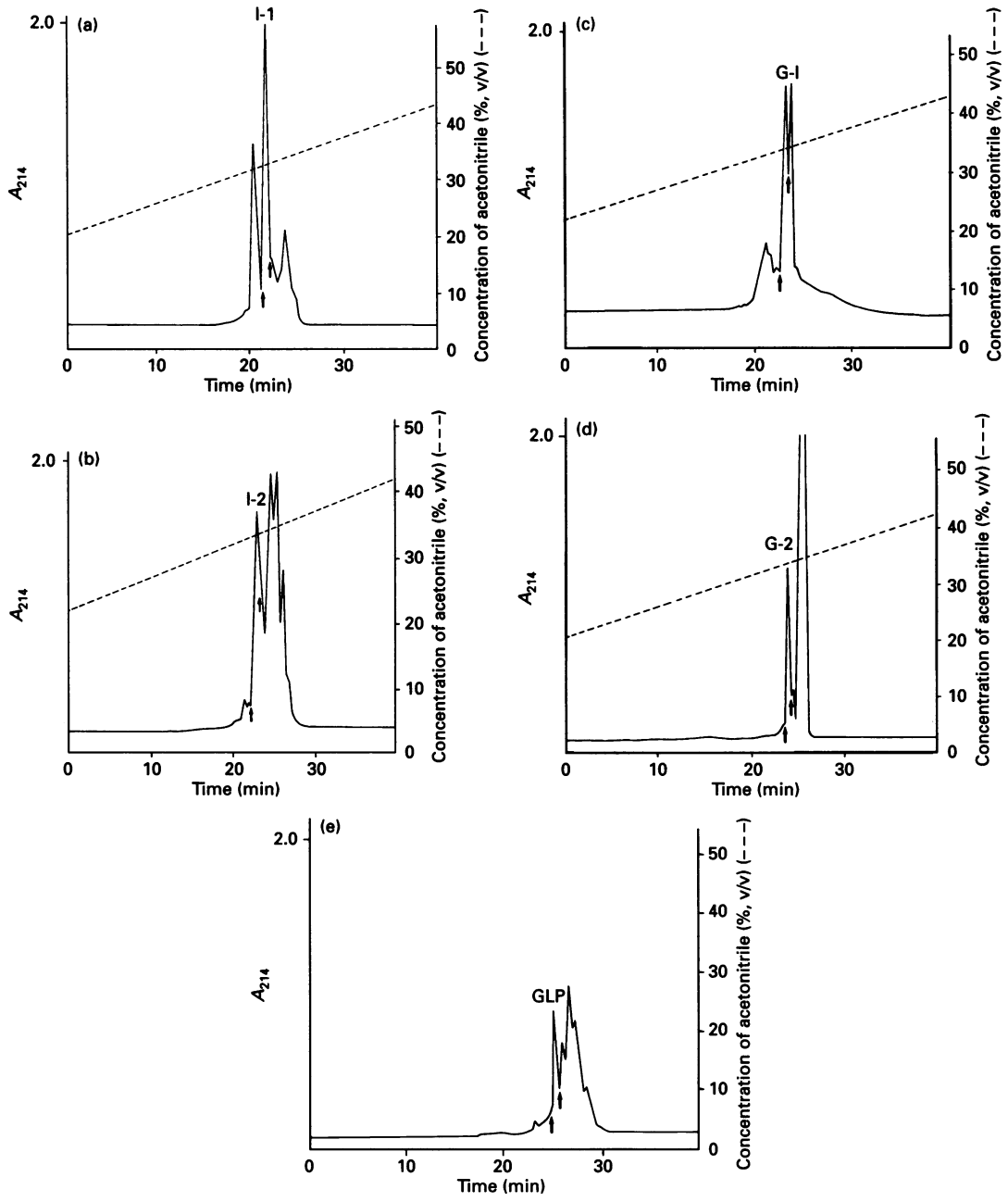

Figure 2 Purification of paddlefish insulins, glucagons and GLP by reversed-phase h.p.l.c.

Chromatography on an analytical Vydac C_4 column of (**a**) insulin I-1, (**b**) insulin I-2, (**c**) glucagon G-1, (**d**) glucagon G-2 and (**e**) GLP. The islet hormones were purified to near homogeneity (purity > 95%) by further chromatography on Vydac phenyl and C_{18} columns. The broken line shows the concentration of acetonitrile in the eluting solvent and the arrows show where peak collection began and ended.

RESULTS

Purification of paddlefish insulins

The insulin-like immunoreactivity in the pancreatic extract was eluted from a Sephadex G-25 gel permeation column as a broad, trailing peak immediately after the void volume of the column. Fractions with K_D between 0.1 and 0.4 were pooled and injected onto a semipreparative Vydac C_{18} reversed-phase h.p.l.c. column (Figure 1). The peaks designated I-1 and I-2 contained insulin-like immunoreactivity and were separately rechromatographed on an analytical Vydac C_4 column (Figures 2a and 2b). In each case, insulin-like immunoreactivity was associated with sharp, well resolved peaks. The peptides were purified to near homogeneity, as assessed by symmetrical peak shape, by chromatography on analytical Vydac phenyl and C_{18} columns (results not shown). The final yields of pure peptides (determined by amino acid analysis) were: I-1, 12 nmol; and I-2, 14 nmol.

Purification of proglucagon-derived peptides

Analysis by radioimmunoassay of the peak fractions shown in Figure 1 indicated that no peak was associated with glucagon-

Table 1 Determination of the primary structures of insulins from the paddlefish by automated Edman degradation

The values in parentheses show the yields of phenylthiohydantoin derivatives (pmol).

Cycle no.	Insulin I-1 A chain		Insulin I-1 B chain		Insulin I-2 A chain		Insulin I-2 B chain	
1	Gly	(3066)	Ala	(2473)	Gly	(2238)	Ala	(2490)
2	Ile	(2509)	Ala	(2539)	Ile	(1502)	Ala	(2352)
3	Val	(3175)	Asn	(1381)	Val	(2144)	Asn	(2685)
4	Glu	(2992)	Gln	(2170)	Glu	(2475)	Gln	(1691)
5	Gln	(2923)	His	(907)	Gln	(1688)	His	(1333)
6	Cys	(2673)	Leu	(2021)	Cys	(1571)	Leu	(2167)
7	Cys	(2684)	Cys	(1112)	Cys	(1790)	Cys	(1420)
8	His	(1984)	Gly	(1328)	His	(1314)	Gly	(1357)
9	Ser	(316)	Ser	(119)	Ser	(261)	Ser	(126)
10	Pro	(2001)	His	(516)	Pro	(1271)	His	(1032)
11	Cys	(1471)	Leu	(1182)	Cys	(1067)	Leu	(1040)
12	Ser	(158)	Val	(868)	Ser	(196)	Val	(983)
13	Leu	(1072)	Glu	(608)	Leu	(608)	Glu	(520)
14	Tyr	(1057)	Ala	(793)	Tyr	(818)	Ala	(451)
15	His	(651)	Leu	(1106)	Asp	(616)	Leu	(959)
16	Leu	(689)	Tyr	(688)	Leu	(467)	Tyr	(702)
17	Glu	(463)	Leu	(1045)	Glu	(395)	Leu	(713)
18	Asn	(617)	Val	(607)	Asn	(521)	Val	(592)
19	Tyr	(352)	Cys	(439)	Tyr	(356)	Cys	(450)
20	Cys	(180)	Gly	(547)	Cys	(192)	Gly	(429)
21	Asn	(171)	Glu	(545)	Asn	(97)	Glu	(318)
22			Arg	(356)			Arg	(334)
23			Gly	(422)			Gly	(322)
24			Phe	(441)			Phe	(277)
25			Phe	(521)			Phe	(338)
26			Tyr	(257)			Tyr	(209)
27			Thr	(63)			Thr	(67)
28			Pro	(151)			Pro	(117)
29			Asn	(237)			Asn	(102)
30			Lys	(82)			Lys	(45)
31			Val	(24)			Val	(16)

like immunoreactivity. The antiserum is directed against the central region of pig glucagon (Conlon and Thim, 1985), thus the results suggest that paddlefish glucagon is appreciably different from mammalian glucagons in this region of the molecule. The strategy employed to isolate paddlefish glucagons was to purify to near homogeneity all the component present in major abundance whose retention time on h.p.l.c. (Figure 1) was between 55 and 80 min. A total of 26 peptides were purified. The glucagons were then identified by determination of the amino acid composition of the purified peptides. Identification was facilitated by the fact that all known glucagons contain a single histidine residue and no proline or cysteine residues.

Following this approach led to the identification of two peaks in Figure 1 (designated G-I and G-2) that contained glucagons and one peak (designated GLP) that contained GLP. These components were purified to near homogeneity by successive chromatographies on analytical Vydac C_4 (Figure 2c–e), phenyl and C_{18} reversed-phase h.p.l.c. columns. The final yields of purified peptides were: G-I, 7 nmol; G-2, 8 nmol; and GLP, 3 nmol.

Characterization of paddlefish insulins, glucagons and GLP

The primary structures of the pyridylethylated A and B chains of paddlefish insulins I-1 and I-2 were determined by automated Edman degradation; the results are shown in Table 1. The data indicate that the B chains of both insulins are identical and that the A chain of insulin I-1 differs from the A chain of insulin I-2 by the single substitution ($His^{15} \rightarrow Asp$). The results of amino acid analysis (Table 2) are in good agreement with the sequence analysis data, indicating that the full sequence of the peptides had been obtained, and confirming that the A chain of insulin I-1 differs from that of I-2 by a single amino acid substitution. The data indicated that the peptides were > 95% pure.

Automated Edman degradation of glucagons G-I and G-2 (Table 3) demonstrated that both peptides comprise 31 amino acid residues and differ by the single substitution $Arg^{18} \rightarrow Ser$. This conclusion is supported by the results of amino acid analysis

Table 2 Amino acid compositions of insulins, glucagons and GLP from the paddlefish

Values in parentheses are the number of residues predicted from the proposed amino acid sequences

Amino acid	Relative amount (residues/molecule) I-1 A chain	I-1 B chain	I-2 A chain	I-2 B chain	G-1	G-2	GLP
Asx	2.1 (2)	2.1 (2)	3.1 (3)	2.1 (2)	2.7 (3)	2.8 (3)	3.2 (3)
Glx	3.1 (3)	3.1 (3)	2.8 (3)	2.8 (3)	4.6 (5)	4.7 (5)	4.0 (4)
Ser	1.9 (2)	1.0 (1)	2.0 (2)	1.0 (1)	2.6 (3)	3.6 (4)	3.6 (4)
Gly	1.0 (1)	2.9 (6)	1.1 (1)	2.8 (3)	2.0 (2)	2.1 (2)	2.4 (2)
His	1.8 (2)	1.8 (2)	1.0 (1)	1.9 (2)	1.0 (1)	1.0 (1)	1.1 (1)
Arg		1.0 (1)		1.1 (1)	1.0 (1)		1.1 (1)
Thr		1.0 (1)		0.9 (1)	1.0 (1)	1.2 (1)	2.1 (2)
Ala		2.8 (3)		2.7 (3)	1.0 (1)	1.2 (1)	3.8 (4)
Pro	1.2 (1)	1.0 (1)	1.1 (1)	1.0 (1)			
Tyr	1.8 (2)	1.9 (2)	1.9 (2)	1.9 (2)	1.8 (2)	2.0 (2)	0.9 (1)
Val	0.4 (1)	2.6 (3)	0.5 (1)	2.7 (3)	0.9 (1)	1.0 (1)	
Met					1.0 (1)	0.9 (1)	
Ile	0.4 (1)		0.5 (1)				1.0 (1)
Leu	1.9 (2)	3.6 (4)	2.0 (2)	3.6 (4)	1.9 (2)	2.1 (2)	1.9 (2)
Phe		2.0 (2)		1.9 (2)	1.6 (2)	1.9 (2)	1.8 (2)
Lys		0.9 (1)		1.0 (1)	4.3 (4)	4.0 (4)	1.8 (2)

Table 3 Determination of the primary structures of glucagons and GLP from the paddlefish by automated Edman degradation

The values in parentheses show yields of phenylthiohydantoin derivatives (pmol).

Cycle no.	G-1		G-2		GLP	
1	His	(1049)	His	(2216)	His	(1245)
2	Ser	(169)	Ser	(576)	Ala	(1395)
3	Gln	(1303)	Gln	(1734)	Asp	(740)
4	Gly	(1352)	Gly	(2102)	Gly	(1287)
5	Met	(1239)	Met	(1730)	Thr	(680)
6	Phe	(1608)	Phe	(1686)	Tyr	(1023)
7	Thr	(432)	Thr	(812)	Thr	(592)
8	Asn	(949)	Asn	(1401)	Ser	(151)
9	Asp	(941)	Asp	(1455)	Asp	(588)
10	Tyr	(713)	Tyr	(1313)	Ala	(719)
11	Ser	(98)	Ser	(213)	Ser	(108)
12	Lys	(705)	Lys	(1202)	Ser	(115)
13	Tyr	(530)	Tyr	(1203)	Phe	(420)
14	Leu	(635)	Leu	(1175)	Leu	(428)
15	Glu	(451)	Glu	(942)	Gln	(670)
16	Glu	(423)	Glu	(1157)	Glu	(303)
17	Lys	(536)	Lys	(1022)	Gln	(640)
18	Arg	(219)	Ser	(102)	Ala	(325)
19	Ala	(345)	Ala	(701)	Ala	(375)
20	Lys	(438)	Lys	(710)	Arg	(204)
21	Glu	(243)	Glu	(487)	Asp	(154)
22	Phe	(333)	Phe	(501)	Phe	(211)
23	Val	(305)	Val	(474)	Ile	(181)
24	Glu	(195)	Glu	(437)	Ser	(21)
25	Trp	(31)	Trp	(85)	Trp	(25)
26	Leu	(209)	Leu	(318)	Leu	(154)
27	Lys	(280)	Lys	(197)	Lys	(176)
28	Asn	(138)	Asn	(92)	Lys	(196)
29	Gly	(210)	Glu	(196)	Gly	(68)
30	Lys	(168)	Lys	(162)	Gln	(41)
31	Ser	(18)	Ser	(22)		

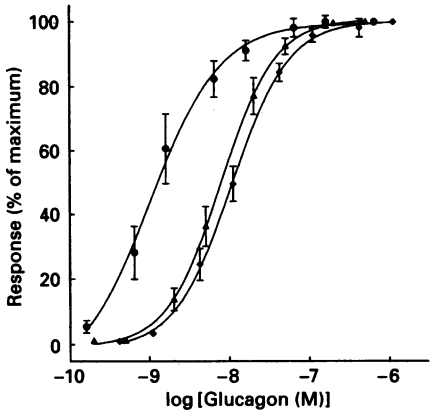

Figure 3 Comparison of the abilities of paddlefish and bovine glucagons to stimulate glycogenolysis in rockfish hepatocytes

Dispersed hepatocytes from fed copper rockfish (*S. caurinus*) were incubated with (■) paddlefish G-1 (●), paddlefish G-2 (◆) and bovine glucagon (▲) under the conditions described in the text. Data are presented as percentage of the maximum amount of glucose released into the medium. Each point represents mean ± S.E.M. of five independent experiments.

(Table 2). It was possible to identify without ambiguity phenylthiohydantoin derivatives of amino acids for 30 cycles of operation during sequence analysis of paddlefish GLP (Table 3). Agreement between the results of Edman degradation and amino acid analysis (Table 2) is good, demonstrating that the full sequence of the peptide had been obtained.

Glycogenolytic activity of paddlefish glucagons

Under the conditions of incubation, the rate of glucose release from endogenous glycogen in the rockfish hepatocytes treated with vehicle only was linear over 30 min. This rate (8.7 ± 1.0 μmol of glucose produced/h per g of cells) was used as the baseline value. Maximum stimulation of the rate of glucose release from rockfish hepatocytes was produced by 5×10^{-7} M concentrations of both paddlefish glucagons and bovine glucagon. The maximum stimulations produced by paddlefish G-1 (5.9 ± 0.3-fold greater than basal release), paddlefish G-2 (6.0 ± 0.4-fold) and bovine glucagon (6.5 ± 0.5-fold) were not significantly different, demonstrating that the peptides are equally effective in this system. The concentration dependences of the hormone-induced release are compared in Figure 3. The concentrations of paddlefish G-2 (1.0×10^{-8} M) and bovine glucagon (7.8×10^{-9} M) that produced half-maximum stimulation of glucose release were similar. The potency of paddlefish G-1 (1.6 ± 10^{-9} M) was significantly ($P < 0.05$) greater than that of paddlefish G-2.

DISCUSSION

Despite the fact that the line of evolution leading to paddlefish diverged from that leading to mammals at least 300 million years ago, the primary structures of paddlefish insulins are remarkably similar to that of human insulin (Figure 4). In particular, the amino acid residues at the surface of the molecule that comprise the putative receptor-binding region (A1–A3, A5, A19, A21 and B22–B26) together with residues involved in dimer (B12, B16 and B21) and hexamer (B6, B10, B14, B17, B20, A13 and A14) formation have been conserved between mammalian (Baker et al., 1988) and paddlefish insulins. A comparison of paddlefish insulins with insulins isolated from other classes of fish (Figure 4) reveals that the paddlefish insulins resemble most closely the peptide from the alligator gar (Pollock et al., 1988). This observation is consistent with the classical phylogenetic view, derived from morphological analysis and the fossil record, that the chondrostean fishes are more closely related to the Lepisosteiformes than to the Amiiformes (Carroll, 1988).

The isolation of two forms of paddlefish insulin, in approximately equimolar amounts, that differ by a single amino acid substitution suggests a recent gene duplication event. Among the mammals, rats (Lomedico et al., 1979) and mice (Wentworth et al., 1986) express two nonallelic preproinsulin genes. It has been proposed that insulin I arose from RNA-mediated transposition, as a functional gene, of a cDNA copy of an incompletely processed upstream transcript of insulin gene II into the ancestral murine germ line (Soares et al., 1985). Rat/mouse insulin I differs from rat/mouse insulin II by two amino acid residues. The nucleotide sequences of the preproinsulin genes are 90% identical, suggesting that the putative duplication event occurred 25–35 million years ago (Steiner et al., 1985). Isolation of two molecular forms of insulin with very similar primary structures has also been reported for a teleost fish, the toadfish (Smith, 1966), and for an amphibian, the clawed toad *Xenopus laevis* (Shulinder et al., 1989). The amino acid substitution between paddlefish insulin I-1 and I-2 (His for Asp at position A10) may be accomplished by a single nucleotide change in the gene. It is noteworthy that sequence analysis of a mutant human preproinsulin gene, isolated from cloned DNA from individuals

	A chain																			
Paddlefish I-1	G	I	V	E	Q	C	C	H	S	P	C	S	L	Y	H	L	E	N	Y	C	N
Paddlefish I-2	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-	-	-	-	-	-
Human	-	-	-	-	-	-	-	T	-	I	-	-	-	E	-	-	-	-	-	-	-
Salmon	-	-	-	-	-	-	-	K	-	-	N	I	F	D	-	Q	-	-	-	-	-
Gar	-	-	-	-	-	-	-	K	-	-	T	I	-	E	-	-	-	-	-	-	-
Bowfin	-	-	-	-	-	-	L	K	-	-	T	I	-	E	M	-	K	-	-	-	-
Torpedo	-	-	-	-	H	-	-	N	T	-	-	F	D	-	-	G	-	-	-	-	-
Ratfish	-	-	-	-	-	-	-	N	T	-	-	A	N	-	-	G	-	-	-	-	-
Lamprey	-	-	-	-	-	-	-	R	K	-	-	I	Y	D	M	-	-	-	-	-	-

	B chain																													
Paddlefish I-1	A	A	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	N	K	V
Paddlefish I-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human	F	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	T				
Salmon	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	N	-	K				
Gar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	N	-	-				
Bowfin	-	-	S	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-	-	-	S	-	-	N	-	-	S				
Torpedo	L	P	S	-	-	-	-	-	-	-	-	-	-	-	F	-	-	-	P	K	-	-	Y	-	L	-	K	A			
Ratfish	V	P	T	-	R	-	-	-	-	-	-	D	-	-	F	-	-	-	-	-	S	-	K	P	I						
Lamprey	-	G	G	T	-	-	-	-	-	-	-	-	V	-	-	-	D	-	-	-	-	-	S	-	T						

Figure 4 Comparison of the primary structures of paddlefish insulins with insulin from other classes of vertebrate

— denotes residue identity.

	Glucagon																													
Paddlefish G-1	H	S	Q	G	M	F	T	N	D	Y	S	K	Y	L	E	E	K	R	A	K	E	F	V	E	W	L	K	N	G	K	S
Paddlefish G-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-
Bovine	-	-	-	-	T	-	-	S	-	-	-	-	D	S	R	-	-	Q	D	-	-	Q	-	-	M	-	T				
Anglerfish 1	-	-	E	-	T	-	S	-	-	-	-	-	D	R	K	-	Q	-	-	-	R	-	-	M	-	N					
Anglerfish 2	-	-	E	-	T	-	S	-	-	-	-	-	T	R	-	-	Q	D	-	-	Q	-	-	-	S						
Gar	-	-	-	-	T	-	-	-	-	-	-	-	D	T	R	-	-	Q	D	-	-	Q	-	-	M	S	T				
Bowfin	-	-	-	-	T	-	-	-	-	-	-	-	M	D	T	R	-	-	Q	D	-	-	Q	-	-	M	S	T			
Dogfish	-	-	E	-	T	-	-	S	-	-	-	-	M	D	N	R	-	-	-	D	-	-	Q	-	-	M	S	T			
Ratfish	-	T	D	-	I	-	S	S	-	-	-	-	D	N	R	-	T	-	D	-	-	Q	-	-	L	S	T	K	R	N	G	A	N	T
Lamprey	-	-	E	-	T	-	-	S	-	-	-	-	-	N	-	Q	-	-	D	-	-	R	-	-	M	-	A				

	GLP																													
Paddlefish	H	A	D	G	T	Y	T	S	D	A	S	S	F	L	Q	E	Q	A	A	R	D	F	I	S	W	L	K	K	G	Q
Human GLP-1	-	-	A	E	-	F	-	-	-	V	-	-	Y	-	E	G	-	-	-	K	E	-	-	A	-	V	-	-	R	G
Human GLP-2	-	-	-	-	S	F	S	D	E	M	D	T	I	-	D	N	L	-	-	-	-	N	-	-	I	Q	T	K	I	T	D	R
Anglerfish 1	-	-	-	-	-	F	-	-	-	V	-	-	Y	-	K	D	-	-	I	K	-	-	V	D	R	-	-	A	-	Q	V	R	R	E
Anglerfish 2	-	-	-	-	-	-	-	-	-	V	-	-	Y	-	-	D	-	-	-	K	-	-	V	-	-	-	-	A	-	R	G	R	R	E
Gar	-	-	-	-	-	-	-	-	-	V	-	-	Y	-	-	D	-	-	-	K	K	-	V	T	-	-	-	Q	-	-	D	R	R	E
Bowfin	Y	-	-	A	P	-	I	-	-	V	Y	-	Y	-	-	D	-	V	-	K	K	*	*	*	-	-	-	S	-	-	D	R	R	E
Dogfish	-	-	-	E	-	-	-	-	-	V	D	-	L	S	D	Y	F	K	-	R	-	V	D	S	-	-	S	Y						
Ratfish	-	-	-	-	I	-	-	-	-	V	A	-	L	T	D	Y	L	K	S	K	R	-	V	E	S	-	S	N	Y	N	R	K	Q	N	D
Lamprey	-	-	-	-	-	F	-	N	-	M	T	-	Y	-	D	A	K	-	-	-	-	V	-	-	A	R	S	D	K	S				

Figure 5 Comparison of the primary structures of paddlefish glucagons and GLP with the corresponding peptides from other classes of vertebrate

— denotes residue identity; * denotes residue deletion.

in a family with inherited hyperproinsulinaemia, revealed a single nucleotide substitution in the codon for residue B10 of insulin (CAC→GAC) which predicts the same amino acid substitution (Asp→His) seen in paddlefish insulin (Chan et al., 1987).

Nucleotide sequence analysis of cDNAs and/or genomic fragments encoding preproglucagons from several mammalian species has shown that glucagon is cosynthesized with two structurally related peptides, GLP-1 and GLP-2 (Bell, 1986). In the anglerfish, *Lophius americanus*, however, the nucleotide sequence of the preproglucagon cDNA has revealed that a region corresponding to mammalian GLP-2 is not encoded by the gene (Lund et al., 1983). GLP, along with glucagon, has been isolated from the pancreas of several species of teleost fish, from the bowfin and gar, from the elasmobranch *Scyliorhinus canicula* (dogfish), from the holocephalan fish *Hydrolagus colliei* (ratfish) and from the Agnathan *Petromyzon marinus* (lamprey) (reviewed by Conlon et al., 1993a).

The primary structures of the two molecular forms of glucagon and GLP from the paddlefish are compared with those from other classes of fish in Figure 5. The structure of glucagon has been strongly conserved among tetrapods (reviewed by Conlon and Hicks, 1990). The data in Figure 5, however, show that the amino acid sequences of the proglucagon-derived peptides have been less well conserved among fish than those of insulin. The paddlefish glucagons show 10 amino acid substitutions compared with alligator gar glucagon (Pollock et al., 1988) and the peptides contain several unusual substitutions, e.g. Met^{5}, Glu^{24} and Gly^{29}, not previously encountered in glucagons from other species. In common with the ratfish (Conlon et al., 1987), the paddlefish glucagons are extended from their C-terminus by additional amino acid residues. In those proglucagon molecules that have been characterized (Bell, 1986), the glucagon sequence is followed by a Lys-Arg pair of residues that acts as a recognition site for a processing enzyme. We speculate that in the paddlefish, the substitution Arg^{31}→Ser in this processing site has led to adoption of an alternative pathway of post-translational processing. Despite the presence of these unusual substitutions, both molecular forms of paddlefish glucagon are equally effective as bovine glucagon in stimulating glycogenolysis in dispersed hepatocytes from a teleost fish. However, the unusual substitution Arg^{18}→Ser in paddlefish G-2 results in a 6-fold decrease in potency relative to paddlefish G-1, suggesting that the dibasic residue site in the central region of the molecule may be involved in the interaction of the peptide with its receptor. In mammalian glucagon, Arg^{17}-Arg^{18} may represent a site of post-translational processing to yield a biologically active fragment (Mallat et al., 1987). This dibasic residue site is retained in most fish glucagons but it has been shown that glucagon-(1–17)-peptide does not stimulate glycogenolysis in teleost hepatocytes (T. P. Mommsen and A. Jardim, unpublished work).

The data in Figure 5 support our previous conclusion (Conlon et al., 1993a,b) that the primary structure of GLP has been even more poorly conserved than glucagon during evolution of the vertebrates. Consistent with the known close phylogenetic re-

lationship between the Polyodontidae and the Lepisosteiformes, paddlefish GLP is most similar in structure to gar GLP but similarity to elasmobranch, holocephalan and agnathan GLPs is confined to the domain at the N-terminus of the peptide.

In common with many species of teleost fish [reviewed by Cutfield and Cutfield (1993)], the paddlefish pancreas produces two molecular forms of glucagon, and the existence of two non-allelic genes encoding distinct preproglucagons has been demonstrated for the anglerfish (Lund et al., 1983). Anglerfish G-1 differs from anglerfish G-2 by six amino acid substitutions, and a comparison of the nucleotide sequences of the corresponding preproglucagon cDNAs has led to the proposal that the two genes arose from a duplication event occurring ~ 160 million years ago (Lopez et al., 1984). The close structural similarity between the amino acid sequences of the two paddlefish glucagons suggests that a similar gene duplication event probably took place more recently. In the present study, only one molecular form of GLP was isolated from the pancreatic extract but we cannot exclude the possibility that a second form was present but not identified.

This work was supported by grants from the National Science Foundation (U.S.A.), the United States Department of Agriculture Capacity Grants Programme and the Natural Sciences and Engineering Research Council (Canada). We thank Julia A. Clark for help with collection of the tissues and Berndt Fritzsch for helpful discussions.

REFERENCES

Bailey, C. J. and Ahmed-Sorour, H. (1980) Diabeteologia **19**, 475–481

Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Hodgkin, D. M. C., Hubbard, R. E., Isacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N. and Vijayan, N. M. (1988) Phil. Trans. R. Soc. London B **319**, 369–456

Bell, G. I. (1986) Peptides **7** (Suppl. 1), 27–36

Carroll, R. L. (1988) in Vertebrate Paleontology and Evolution, pp. 98–102, W. H. Freeman, New York

Chan, S. J., Seino, S., Gruppuso, P. A., Schwartz, R. and Steiner, D. F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 2194–2197

Conlon, J. M. and Hicks, J. W. (1990) Peptides **11**, 461–466

Conlon, J. M. and Thim, L. (1985) Gen. Comp. Endocrinol. **60**, 398–405

Conlon, J. M., Dafgard, E., Falkmer, S. and Thim, L. (1987) Biochem. J. **208**, 851–856

Conlon, J. M., Youson, J. H. and Whittaker, J. (1991) Biochem. J. **276**, 261–264

Conlon, J. M, Nielsen, P. F. and Youson, J. H. (1993a) Gen. Comp. Endocrinol. **91**, 93–104

Conlon, J. M., Youson, J. H. and Mommsen, T. P. (1993b) Biochem. J. **295**, 857–861

Cutfield, S. M. and Cutfield, J. F. (1993) Gen. Comp. Endocrinol. **91**, 281–286

Epple, A. and Brinn, J. E. (1975) Gen. Comp. Endocrinol. **27**, 320–349

Lomedico, P., Rosenthal, N., Efstratiadis, A., Gilbert, W., Kolodner, R. and Tizard, R. (1979) Cell **18**, 545–558

Lopez, L. C., Li, W.-H., Frazier, M. L., Luo, C.-C. and Saunders, G. F. (1984) Mol. Biol. Evol. **1**, 335–344

Lund, P. K., Goodman, R. H., Montminy, M. R., Dee, P. C. and Habener, J. F. (1983) J. Biol. Chem. **258**, 3280–3284

Mallat, A., Pavoine, C., Dufort, M., Lotersztajn, S., Baille, D. and Pecker, F. (1987) Nature (London) **325**, 620–622

Mims, S. D., Clark, J. A. and Tidwell, J. H. (1991) Aquaculture **99**, 69–82

Pollock, H. G., Kimmel, J. R., Hamilton, J. W., Rouse, J. B., Ebner, K. E., Lance, V. and Rawitch, A. B. (1987) Gen. Comp. Endocrinol. **67**, 375–382

Pollock, H. G., Kimmel, J. R., Ebner, K. E., Hamilton, J. W., Rouse, J. B., Lance, V. and Rawitch, A. B. (1988) Gen. Comp. Endocrinol. **69**, 133–140

Shulinder, A. R., Bennett, C., Robinson, E. A. and Roth, J. (1989) Endocrinology **125**, 469–477

Smith, L. F. (1966) Am. J. Med. **40**, 662–666

Soares, M. B., Schon, E., Henderson, A., Karathanasis, S. K., Cate, R., Zeitlin, S., Chirgwin, J. and Efstratiadis, A. (1985) Mol. Cell. Biol. **5**, 2090–2103

Steiner, D. F., Chan, S. J., Welsh, J. M. and Kwok, S. C. M. (1985) Annu. Rev. Genet. **19**, 463–484

Weisel, G. F. (1972) J. Morphol. **140**, 243–256

Wentworth, B. M., Schaefer, I. M., Villa-Komaroff, L. and Chirgwin, J. M. (1986) J. Mol. Evol. **23**, 305–312

Received 16 November 1993; accepted 10 January 1994