Multiple Forms of Glucagon and Somatostatin Isolated from the Intestine of the Southern-Hemisphere Lamprey *Geotria australis*

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Accepted October 1, 1998

Current views on Agnathan phylogeny favor the hypothesis that the genera of holarctic lampreys belong to a single family (Petromyzontidae) and form an interrelated progression in which *Petromyzon* is near to *Ichthyomyzon* at the base of the phylogenetic tree and *Lampetra* is the most derived. A stock similar to that of contemporary *Ichthyomyzon* is considered to have given rise to the southern hemisphere lamprey *Geotria australis*, the sole member of the Geotriidae. In the present study, two molecular forms of glucagon were isolated from an extract of *G. australis* intestine that differed in structure by six amino acid residues. One form shows two amino acid substitutions (Leu¹⁴ → Met and Ala²⁹ → Ser) compared with the single molecular form of glucagon isolated from the sea lamprey *Petromyzon marinus* and the second form shows three substitutions (Asp¹⁵ → Glu, Ser¹⁶ → Ala, Ile²⁴ → Thr) compared with the single glucagon isolated from the river lamprey *Lampetra fluviatilis*. As *Petromyzon* and *Lampetra* glucagons differ by six amino acid residues, the data suggest that a duplication of the glucagon gene occurred prior to or early in lamprey evolution. Although both genes are strongly expressed in *G. australis*, the expression of one gene predominates in *P. marinus* while that of the other gene

predominates in *L. fluviatilis*. Previous work has shown that, in the islet organ of *G. australis*, preprosomatostatin is processed almost exclusively to somatostatin-33. However, the present study demonstrates that somatostatin-14 is the major molecular form in *G. australis* intestine with somatostatin-33 present only as a minor component. This result demonstrates a tissue-dependent pathway of posttranslational processing of preprosomatostatin in the *Geotria* enteropancreatic system. © 1999

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The lampreys (Petromyzontiformes) and the hagfishes (Myxiniiformes) are the only surviving groups of the agnathan (jawless) phase of early vertebrate evolution (Hardisty, 1982). Although these two groups were once classified collectively as the Cyclostomata, they are no longer considered to form a monophyletic group. Indeed, recent cladistic analysis has suggested that the lampreys are more closely related to gnathostomes (jawed vertebrates) than either are to the hagfishes (Forey and Janvier, 1993). Analyses based upon the comparison of the nucleotide sequence of several genes give an estimate of 564 ± 75 million years before present as the time of divergence of the lineage leading to the Petromyzontiformes from that leading to the Gnathostomata, which agrees well with the “classical”

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estimate based upon the limited fossil record (Kumar and Hedges, 1998). Present-day lampreys are separated into three families (Potter, 1980). All of the 34 species of holarctic lampreys currently recognized (Reynaud, 1997) are placed in the Petromyzontidae, whereas the four species of southern-hemisphere lampreys are placed in either the Mordaciidae (three species) or the Geotriidae (one species). On the basis of morphological characteristics and the patterns of distribution of contemporary lampreys, it has been proposed that *Mordacia* and the monospecific *Geotria* arose independently in the pre-Tertiary period from stocks similar to those represented today by the genus *Ichthyomyzon* (Hubbs and Potter, 1971; Potter and Hillard, 1987). *Ichthyomyzon* and the monospecific *Petromyzon* are considered to be closely related and ultimately to have given rise to *Lampetra*, the most derived of all holarctic genera.

Since an endocrine pancreas is already present in the Agnatha (Falkmer and Van Noorden, 1983), characterization of the peptide products of this organ is of particular importance in our understanding of the evolution of the islet hormones. However, immunohistochemical and immunocytochemical techniques have shown that the islet organs of adult lampreys contain only insulin (B-cell)-producing and somatostatin (D-cell)-producing cells (Youson and Elliott, 1989). Cells containing glucagon and glucagon-like peptide (A-cell) and pancreatic polypeptide (PP) family peptides (F-cell) are localized, together with another population of D-cells, in the intestine (Cheung *et al.*, 1990; Youson and Cheung, 1990; Youson and Potter, 1993). Our knowledge of the primary structures of enteropancreatic hormones of agnathans is accumulating but is still fragmentary. Previous studies have led to the purification and characterization of insulin from the islet organs of the holarctic lampreys, *Petromyzon marinus* (sea lamprey) (Plisetskaya *et al.*, 1988) and *Lampetra fluviatilis* (river lamprey) (Conlon *et al.*, 1995a), and of a partially processed form of proinsulin from the southern-hemisphere lamprey, *Geotria australis* (Conlon *et al.*, 1995b). Somatostatins, comprising between 33 and 37 amino acid residues, have been isolated from the islet organs of *P. marinus* (Andrews *et al.*, 1988), *L. fluviatilis* (Conlon *et al.*, 1995a), and *G. australis* (Conlon *et al.*, 1995b). Glucagon has been purified from extracts of the intestines of parasitic

phase *P. marinus* (Conlon *et al.*, 1993) and *L. fluviatilis* (Conlon *et al.*, 1995a). A peptide of the PP family, that is structurally related to mammalian peptide tyrosine-tyrosine (PYY), has also been isolated from *P. marinus* intestine (Conlon *et al.*, 1991). The structures of the enteropancreatic hormones of Myxiniiformes are known only for insulin (Peterson *et al.*, 1974) and two molecular forms of somatostatin with 34 and 14 amino acid residues purified from the islet organ of the Atlantic hagfish *Myxine glutinosa* (Conlon *et al.*, 1988).

The aim of the present study was to purify and characterize structurally the molecular forms of glucagon and somatostatin in an extract of the intestine of the southern-hemisphere lamprey *G. australis*. A comparison of this data with the amino acid sequences of the corresponding hormones from the holarctic species *P. marinus* and *L. fluviatilis* increases our understanding of the molecular evolution of the regulatory peptides of the enteropancreatic system of lampreys.

MATERIALS AND METHODS

Tissue Extraction

Adult upstream-migrant *G. australis* (113 animals of both sexes) were collected from the Donnelly River in southwestern Australia. The entire intestine was removed immediately after capture and washed and the tissues were stored at -70°C . The frozen tissue (92 g) was extracted with ethanol/0.7 M HCl (3/1, v/v; 900 ml) as previously described (Conlon *et al.*, 1991). After centrifugation ($1600 \times g$ for 1 h at 4°), 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). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0/29.9/0.1) and freeze-dried.

Radioimmunoassay

Glucagon-like immunoreactivity was measured with an antiserum directed against the central region of porcine glucagon as previously described (Conlon and Thim, 1985). Somatostatin-like immunoreactivity was measured using an antiserum raised against synthetic

somatostatin-14 that is directed against a site in the central region of the peptide and shows full-reactivity towards somatostatin-28 (McIntosh *et al.*, 1978).

Purification of *Geotria* Glucagon

The intestinal extract, after partial purification on Sep-Pak cartridges, was redissolved in 1 M acetic acid (4 ml) and chromatographed on a 2.5×90 -cm column of Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 1 M acetic acid. The column was eluted at a flow rate of 48 ml/h and fractions (8 ml) were collected. Absorbance was measured at 280 nm. The concentrations of glucagon-like immunoreactivity (GLI) and somatostatin-like immunoreactivity (SLI) in the fractions were determined at a dilution of 1:30. Fractions containing GLI were pooled 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, held at this concentration for 30 min, and then raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm. Fractions (2 ml) were collected and assayed for GLI at a dilution of 1:30.

The peak designated G (containing both *Geotria* glucagons) (Fig. 1A) was rechromatographed on a 0.46×25 -cm Vydac 214TP54 C-4 reversed-phase column equilibrated with acetonitrile/water/trifluoroacetic acid (210/789/1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 49% over 60 min using a linear gradient. *Geotria* glucagons were purified to near homogeneity by successive chromatographies on (A) 0.46×25 -cm Vydac 219TP54 phenyl, (B) 0.46×25 -cm Vydac 218TP54 C-18, and (C) 0.21×25 -cm Vydac 218TP52 columns under the same conditions used for the C-4 column except that a flow rate of 0.3 ml/min was used with the microbore column.

Purification of *Geotria* Somatostatins

Geotria somatostatin-33 (Fig. 1A) and *Geotria* somatostatin-14 (Fig. 2A) were purified to near homogeneity by successive chromatographies on 0.46×25 -cm Vydac 214TP54 (C-4), 219TP54 phenyl, and Vydac

218TP54 (C-18) columns under the same conditions used for the purification of glucagon except that the concentration of acetonitrile in the eluting solvent was raised from 17.5 to 38.5% over 45 min.

Structural Characterization

The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for online detection of phenylthiohydantoin amino acids under gradient elution conditions as previously described (Conlon *et al.*, 1991). Mass spectrometry was performed at the Novo/Nordisk Research Institute, Bagsvaerd, Denmark, using a API III LC/MS/MS triple quadrupole instrument (Sciex, Thornhill, Ontario, Canada). The accuracy of mass measurement was 0.02%.

RESULTS

Purification of *Geotria* Glucagons

The GLI in the extract of *Geotria* intestine, after partial purification on Sep-Pak cartridges, was eluted from a Sephadex G-25 gel permeation column as a single peak with K_{av} between 0.29 and 0.37. Fractions containing maximum immunoreactivity were pooled and chromatographed on a semipreparative Vydac C-18 column (Fig. 1A). The fractions containing GLI are denoted by the bar marked G. After rechromatography on an analytical Vydac C-4 column, the GLI was associated with the ascending shoulder of the major peak in the chromatogram as delineated by the arrows (Fig. 1B). This fraction was rechromatographed on an analytical Vydac phenyl column (Fig. 1C) and GLI was associated with a well-defined major peak. *Geotria* glucagon was purified to apparent homogeneity, as assessed by a symmetrical peak shape, by chromatography on an analytical Vydac C-18 column (Fig. 1D). As amino acid sequence analysis demonstrated that the peak contained two glucagon-related peptides, the material was subjected to the greater resolving power of a microbore Vydac C-18 column (Fig. 1E). Partial separation of the two components was achieved and subsequent analysis showed that the earlier-eluting

FIG. 1. Purification by reversed-phase HPLC on (A) semipreparative Vydac C-18 (B) analytical Vydac C-4 (C) analytical Vydac phenyl and (D) analytical Vydac C-18 and (E) microbore Vydac C-18 columns of glucagons in an extract of *Geotria* intestine after partial purification by gel permeation chromatography. The concentration of acetonitrile in the eluting solvent is shown by a broken line and the arrows show where peak collection began and ended. In A, G denotes the fraction containing glucagon-like immunoreactivity and S-33 denotes the fraction containing somatostatin-like immunoreactivity. In E, peak 1 contains glucagon I and peak 2 contains glucagon II.

FIG. 2. Purification by reversed-phase HPLC on (A) semipreparative Vydac C-18 (B) analytical Vydac C-4 and (C) analytical Vydac C-18 columns of somatostatin-14 (S-14) in an extract of *Geotria* intestine after partial purification by gel permeation chromatography. The bar denotes the fraction with somatostatin-like immunoreactivity.

shoulder contained glucagon I in a purity >95% (final yield approximately 800 pmol) and the later-eluting peak contained predominantly glucagon II with a purity of approximately 85% (final yield approximately 2 nmol).

Purification of *Geotria* Somatostains

The SLI in the extract of *Geotria* intestine, after partial purification on Sep-Pak cartridges, was eluted from a Sephadex G-25 gel permeation column as a major peak with K_{av} between 0.80 and 1.00 (containing somatostatin-14) and a minor peak with K_{av} between 0.29 and 0.37 (containing somatostatin-33). The approximate ratio of concentration of somatostatin-14 to so-

matostatin-33 was 10:1. Fractions from the later-eluting peak were pooled and chromatographed on a semipreparative Vydac C-18 column (Fig. 2A). SLI was associated with the single fraction denoted by the bar. *Geotria* somatostatin-14 was purified to near homogeneity by rechromatography on (A) an analytical Vydac C-4 column (Fig. 2B) and (B) an analytical Vydac C-18 column (Fig. 2C). The final yield of pure peptide was approximately 600 pmol. *Geotria* somatostatin-33 was eluted from a Sephadex G-25 gel permeation column in the same fractions as glucagon but the two peptides were completely separated by chromatography on a semipreparative Vydac C-18 column (Fig. 1A). *Geotria* somatostatin-33 was purified to near homogeneity by chromatography on analytical Vydac C-4 and phenyl

columns (chromatograms not shown). The final yield of pure peptide was 50 pmol.

Structural Characterization of *Geotria* Glucagons and Somatostatins

Despite the fact that *Geotria* glucagon was eluted from an analytical Vydac C-18 column as a sharp, symmetrical peak (Fig. 1D), automated Edman degradation revealed that the material was not pure. Two phenylthiohydantoin amino acid derivatives were detected during cycles 3, 5, 14, 16, 24, and 29 whereas a single derivative was identified during the other cycles. This result indicates that the material contain two structurally distinct glucagons that coeluted on HPLC. Partial separation of the peptides was achieved on a C-18 microbore column (Fig. 1E) and glucagon I and glucagon II were separately subjected to Edman degradation. The data indicated that glucagon I was >95% pure and its amino acid sequence was established without ambiguity as His-Ser-Gln-Gly-Ser-Phe-Thr-Ser-Asp-Tyr¹⁰-Ser-Lys-Tyr-Leu-Glu-Ala-Lys-Gln-Ala-Lys²⁰-Asp-Phe-Val-Thr-Trp-Leu-Met-Asn-Thr.

Glucagon II was approximately 85% pure and its amino acid sequence was established without ambiguity as His-Ser-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Tyr¹⁰-Ser-Lys-Tyr-Met-Glu-Asn-Lys-Gln-Ala-Lys²⁰-Asp-Phe-Val-Arg-Trp-Leu-Met-Asn-Ser.

The proposed structures were confirmed by electrospray mass spectrometry. Glucagon I gave a strong signal at 3383.8 amu compared with a calculated mass of 3384 amu. The observed molecular mass of glucagon II was 3530.1 amu compared with a calculated mass of 3531 for the methionine sulfoxide derivative of the peptide. A signal at 3383.8 representing the glucagon I contaminant was also observed.

Geotria somatostatin-14 was subjected to automated Edman degradation and its primary structure was established without ambiguity as Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys. This sequence represents the C-terminal tetradecapeptide of *Geotria* somatostatin-33 previously isolated from the islet organ (Conlon *et al.*, 1995b). There was insufficient pure peptide to determine the complete primary structure of *Geotria* intestinal somatostatin-33. The peptide was subjected to 10 cycles of automated Edman degradation and the primary structure at the N-terminus of the molecule was established as Ala-Val-Gln-Glu-Ala-Gly-

Gly-Ala-Ala-Met... This sequence is identical to the corresponding sequence in *Geotria* pancreatic somatostatin-33.

DISCUSSION

The amino acid sequence of glucagon has, in general, been better conserved during the evolution of the vertebrates than has been the sequence of insulin. For example, glucagon from the amphibian *Xenopus laevis* is identical to human glucagon (Irwin *et al.*, 1997) whereas the amino acid sequences of the two molecular forms of insulin contain eight and nine substitutions compared with human insulin (Shuldiner *et al.*, 1989). Although, the primary structure of glucagon has been less well conserved in nontetrapods (Plisetskaya and Mommsen, 1996), glucagon from the ray *Torpedo marmorata* (Elasmobranchii) differs from human glucagon by only three amino acid residues (Conlon and Thim, 1985) whereas the insulins differ by 16 residues (Conlon and Thim, 1986). *Geotria* insulin differs from *Petromyzon* insulin by 17 amino acid substitutions which is consistent with the proposed ancient (pre-Tertiary) divergence of the holarctic and southern-hemisphere lampreys (Conlon *et al.*, 1995b). It was unexpected, therefore, to discover that the insulins of the holarctic lampreys *P. marinus* and *L. fluviatilis* are identical whereas their glucagons differ by six amino acid residues (Fig. 3). The data obtained in the present study shed light upon this apparent paradox. We propose that the difference in structure between the glucagons from the two holarctic lampreys arises from the fact that they are the products of two different genes. The isolation from *Geotria* intestine of two molecular forms of glucagon, differing by six amino acid residues, suggests that a duplication of the gene encoding glucagon occurred either prior to or early in the evolution of the Petromyzontiformes lineage to give preproglucagon I and II. *Geotria* glucagon I shows three substitutions from *Lampetra* glucagon and *Geotria* glucagon II shows two substitutions from *Petromyzon* glucagon (Fig. 3). As only one molecular form of glucagon was isolated from the intestines of *P. marinus* (Conlon *et al.*, 1993) and *L. fluviatilis* (Conlon *et al.*, 1995a), we suggest that the preproglucagon I gene is expressed predominantly or exclusively in the intes-

tine of *Lampetra*, whereas the preproglucagon II gene is expressed predominantly or exclusively in the intestine of *Petromyzon*. It may thus be relevant that the exceptionally high diploid numbers found in lamprey species such as *Geotia* (Robinson and Potter, 1981) indicate that these species are polyploid in origin (Potter and Robinson, 1981). Expression of two preproglucagon genes is a feature of several species of teleost fish (Plisetskaya and Mommsen, 1996). For example, two glucagons, differing in primary structure by six residues, have been isolated from the Brockmann bodies of the anglerfish, *Lophius americanus* (Nichols *et al.*, 1988), and two glucagons differing by five residues have been extracted from the tilapia, *Oreochromis nilotica* (Nguyen *et al.*, 1995) (Fig. 3).

The genes and/or cDNAs-encoding preprosomatostatin have not yet been characterized for any agnathan. However, previous studies have led to the isolation of a single molecular form of somatostatin with 33 amino acid residues from an extract of the islet organ of *G. australis* (Conlon *et al.*, 1995b) and a single molecular form with 35 residues from the islet organ of *L. fluviatilis* (Conlon *et al.*, 1995a). A 34-residue somatostatin was the major molecular form isolated from the islet organ of *P. marinus* but minor components with 37 and 14 amino acid residues were also identified (Andrews *et al.*, 1988). All of these components were characterized by the substitution (Thr¹² → Ser) in the somatostatin-14 region at the C-termini of the peptides

Glucagon							
Petromyzon	HSEGT	FTSDY	SKYLE	NKQAK	DFVRW	LMNA	
Lampetra	--Q-S	-----	----D	S----	---I-	---T	
Geotria I	--Q-S	-----	-----	A----	---T-	---T	
Geotria II	-----	-----	---M-	-----	-----	---S	
Anglerfish I	-----	-SN--	-----	DRK-Q	E----	---N	
Anglerfish II	-----	-SN--	-----	TRR-Q	---Q-	-K-S	
Tilapia I	-----	-SN--	-----	DRK-Q	-----	---N	
Tilapia II	-----	-SN--	-----	TRR-Q	---Q-	-K-S	

FIG. 3. Comparison of the primary structures of glucagons from the southern-hemisphere lamprey *Geotria australis*, from the holarctic lampreys *Petromyzon marinus* (sea lamprey) and *Lampetra fluviatilis* (river lamprey), and from two species of teleost, the anglerfish *Lophius americanus* and the tilapia *Oreochromis nilotica*. (–) Denotes residue identity.

Somatostatin							
Geotria islet	AVQEA	GGA**	AMPFP	QQRDR	KAGCK	NFFWK	TFSSC
Lampetra islet	-AAAP	-A-GG	-QL-L	-N-E-	-----	-----	-----
Petromyzon islet	-AAV-	-SPQ*	QLL-L	---E-	-----	-----	-----
Geotria intestine					-----	-----	-----
Petromyzon brain					-----	-----	--T--
Lampetra brain					-----	-----	--T--
Mammals					-----	-----	--T--

FIG. 4. Comparison of the primary structures of somatostatins from the southern-hemisphere lamprey *Geotria australis*, the holarctic lampreys *Petromyzon marinus* (sea lamprey) and *Lampetra fluviatilis* (river lamprey), and somatostatin-14 from all mammals yet studied. (–) Denotes residue identity; (*) denotes residue deletion.

when compared with mammalian somatostatin-14 (Fig. 4). The data in the present study show that preprosomatostatin is processed predominantly to somatostatin-14 in *Geotria* intestine, with somatostatin-33 representing only a minor component. The pathway of posttranslational processing of preprosomatostatin in mammals is also markedly tissue dependent but, in this case, a larger molecular form (somatostatin-28) is the major component in the intestine whereas somatostatin-14 predominates in the pancreas (Conlon *et al.*, 1997). Somatostatin-14 has been isolated from extracts of the whole brain of *P. marinus* (Sower *et al.*, 1994) and *L. fluviatilis* (Conlon *et al.*, 1995a) but this peptide is identical in structure to mammalian somatostatin-14 (Fig. 4). This observation was interpreted as evidence that more than one gene encoding preprosomatostatin is expressed in the Petromyzontidae in a tissue-dependent manner. At this time, the nature of the molecular forms of somatostatin in *Geotria* brain have not been determined and it is not known whether more than one gene encoding preprosomatostatin is expressed in the tissues of southern-hemisphere lampreys.

The appreciable difference in primary structure between *Geotria* somatostatin-33 and *Petromyzon* somatostatin-34 provides an explanation for the observation that an antiserum raised against *Petromyzon* somatostatin-34 did not immunostain D-cells in the *Geotria* islet organ (Youson and Potter, 1993). However, in the anterior intestine of *G. australis*, one population of cells was immunostained with antisera to both *Petromyzon* somatostatin-34 and mammalian somatostatin-14

whereas a second population stained only with the antiserum to *Petromyzon* somatostatin-34. The data in the present study do not provide an immediate explanation for this observation. One possibility is that *Geotria* synthesizes a second somatostatin in the intestine that resembles *Petromyzon* somatostatin more closely in its N-terminal region but such a component was not purified in the present study. Alternatively, *Geotria* somatostatin-33 may adopt more than one conformation such that the epitope recognized by the antiserum to *Petromyzon* somatostatin-34 may be accessible in the intestinal form but not in the pancreatic form.

The physiological role of the gastroenteropancreatic hormones in the regulation of metabolism in Agnatha is unclear. Studies with *G. australis* are of particular importance in this regard as the species allows complete removal of the islet tissue without major damage to other tissues (Epple *et al.*, 1992). Total isletectomy resulted in an immediate rise in plasma glucose concentrations (from 5.0 to 11.6 mM) that remained elevated for at least 5 days indicating a possible glycogenolytic and/or gluconeogenic action of intestinally derived glucagon in the absence of insulin. However, the small size of the liver in *G. australis* (about 1% of the total body weight) suggests that this organ is unlikely to make a major contribution to circulating glucose levels (Plisetskaya and Kuzmina, 1972) and the site of glucose production is not known. The metabolic effects of somatostatin in southern-hemisphere lampreys have not been investigated but intraperitoneal injections of somatostatin-14 (mammalian sequence) into the larval and metamorphosing sea lamprey *P. marinus* resulted in elevated plasma fatty acid levels (Kao *et al.*, 1998)

ACKNOWLEDGMENTS

This work was supported by an award from the National Science Foundation (IBN-9806997) to J.M.C. and from the Australian Research Council to I.C.P. We thank Dr Stacia Sower, University of New Hampshire, U.S.A. for help in collection of lamprey tissues.

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