Comparative peptidomics of the endocrine pancreas: islet hormones from the clawed frog *Xenopus laevis* and the red-bellied newt *Cynops pyrrhogaster*

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Abstract

Electrospray mass spectrometry coupled with reverse-phase HPLC was used to identify peptides in the molecular mass range 3000–6000 Da in extracts of the pancreata of the clawed frog *Xenopus laevis* (Anura: Pipidae) and the red-bellied newt *Cynops pyrrhogaster* (Caudata: Salamandridae). Amino acid sequences of insulins, peptides derived from the post-translational processing of proglucagons and pancreatic polypeptide were determined by automated Edman degradation. Three molecular forms of insulin were isolated from the tetraploid organism *X. laevis* that represent insulin-1 and insulin-2, as deduced from the nucleotide sequences of previously characterized cDNAs, and a third form which differed from insulin-2 by the single amino acid substitution Asp²¹→Glu in the B-chain. The amino acid sequence of *Xenopus* preproglucagons (genes 1 and 2) may be deduced from the nucleotide sequences of cDNAs but the pathways of post-translation processing of the precursors are not

known. Two molecular forms of glucagon with 36 amino acids, derived from genes 1 and 2 and representing glucagon-29 extended from its C terminus by different heptapeptides, and five molecular forms of glucagon-like peptide 1 (GLP-1) were isolated. The GLPs represent proglucagon-(77–113), -(122–158) and -(160–191) from gene 1, and proglucagon-(77–113) and -(160–191) from gene 2. A single molecular form of insulin, glucagon-36, a C-terminally α -amidated GLP-1 with 30 amino acid residues, a 33 amino acid residue GLP-2 and pancreatic polypeptide were isolated from the pancreatic extract of the diploid organism *C. pyrrhogaster*. This study has illustrated the power of electrospray mass spectrometry for the rapid and reliable identification of peptides in chromatographic fractions without the need to use radioimmunoassay, radioreceptor assay or bioassay.

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Introduction

The term ‘proteomics’ refers to the ambitious attempt to identify all the proteins/peptides, including post-translational modifications, present in a particular tissue of a particular organism with a view to understanding changes in the pattern of expression in response to physiological and pathophysiological stimuli. Until very recently, the goal of proteomics was unrealistic but elucidation of the nucleotide sequence of the human genome, methodological advances in the automation of high throughput techniques of two-dimensional electrophoresis coupled with mass spectrometry (Dongre *et al.* 2001, Liu *et al.* 2002, Rabilloud 2002) and a strong commitment to proteomics research by the biotechnology industry mean that we are in the midst of an explosion of data relating to human proteomics.

The field of vertebrate comparative genomics/proteomics is in its relative infancy. Attempts to characterize the genomes and/or proteomes of non-human species in a systematic fashion have been confined to those species that are commonly used in molecular research projects, such as the mouse (Klose *et al.* 2002), rat (Jacob & Kwitek 2002), zebrafish *Danio rerio* (Patton & Zon 2001), pufferfish *Takifugu rubripes* (Venkatesh *et al.* 2000) and medaka *Oryzias latipes* (Wittbrodt *et al.* 2002). These organisms are highly derived and the extent to which they are ‘representative’ of the class or order to which they belong is a matter for debate. With regard to the rest of the approximately 40 000 species of vertebrates, sequence information, whether at the nucleotide or amino acid level, has been obtained sporadically as investigators have focused upon a particular gene, cDNA or gene product on the basis of its known biological function, e.g. enzyme,

Figure 1 Reverse-phase HPLC on a Vydac C-18 column of an extract of the pancreas of *Xenopus laevis* after partial purification by gel permeation chromatography. The retention times of the islet hormones are denoted by: (1) glucagon-36 from genes 1 and 2, (2) insulin from gene 1, (3) insulin from gene 2, (4) insulin from gene 3, (5) GLP-1B from gene 1, (6) GLP-1C from gene 1, (7) GLP-1C from gene 2, (8) GLP-1A from genes 1 and 2 and (9) PP from genes 1 and 2. The broken line shows the concentration of acetonitrile in the eluting solvent. ABS_{280} , absorbance at 280 nm.

receptor, neurohormone, or have concentrated upon the mitochondrial genome for the purpose of phylogenetic analysis (Saccone *et al.* 2002).

The aim of the present study was to use electrospray mass spectrometry coupled with reverse-phase HPLC for the rapid identification and simultaneous purification of islet hormones (insulin, glucagon, glucagon-like peptide 1 (GLP-1), GLP-2 and pancreatic polypeptide (PP)) present in a pancreatic extract. The techniques are applied to the clawed frog *Xenopus laevis* (Anura: Pipidae), a tetraploid organism for which the nucleotide sequences of cDNAs encoding two preproinsulins (Shuldiner *et al.* 1989) and two preproglucagons (Irwin *et al.* 1997) are known, and to the Japanese red-bellied newt *Cynops pyrrhogaster* (Caudata: Salamandridae), a diploid organism whose islet preprohormones have not been characterized at the nucleotide sequence level.

Materials and Methods

Tissue extraction

All experiments involving *X. laevis* were approved by the University of Göteborg Animal Ethical Committee. All experiments involving *C. pyrrhogaster* were carried out in accordance with the Guide for the Care and Use of

Laboratory Animals prepared by Waseda University. Neither species is endangered. Pancreata (6.8 g) were removed from adult specimens of *X. laevis* of both sexes ($n=50$) by dissection immediately after they had been killed as previously described (Kim *et al.* 2001). Pancreata (1.35 g) were similarly removed from adult, male specimens of *C. pyrrhogaster* ($n=100$). The samples were stored at -80°C until the time of extraction. Tissues were separately homogenized with ethanol/0.7 M HCl (3:1 (v/v); 10 ml/g) using a Waring blender and stirred for 2 h at 0°C . After centrifugation (4000 *g* for 30 min), ethanol was removed from the supernatants under reduced pressure. After a further centrifugation (4000 *g* for 30 min), each extract was pumped at a flow rate of 2 ml/min through six Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0/29.9/0.1) and freeze-dried.

Purification of the *Xenopus* peptides

The pancreatic extract, after partial purification on Sep-Pak cartridges, was subjected to gel permeation chromatography on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden) under the conditions previously described (Kim *et al.* 2001). The fractions with K_{AV}

Xenopus laevis

	Observed M_r	Calculated M_r
Insulin (gene 1)	5800.2	5800.7
Insulin (gene 2)	5813.6	5813.7
Insulin (gene 3)	5827.5	5827.7
Glucagon-36 (gene 1)	4240.3	4240.6
Glucagon-36 (gene 2)	4168.3	4168.5
GLP-1A (gene 1)	4076.2	4076.5
GLP-1A (gene 2)	4099.8	4099.5
GLP-1B (gene 1)	4387.6	4387.0
GLP-1C (gene 1)	3681.9	3682.2
GLP-1C (gene 2)	3667.8	3668.1
PP (gene 1)	4342.3	4342.6
PP (gene 2)	4356.2	4356.6

Cynops pyrrhogaster

Insulin	5826.1	5826.4
Glucagon-36	4154.3	4154.6
GLP-1	3320.4	3320.7
GLP-2	3663.7	3664.1
PP	4214.2	4214.6

Figure 2 Molecular masses of the purified islet hormones from *X. laevis* and *C. pyrrhogaster* determined by electrospray mass spectrometry.

between 0.0 and 0.25 were pooled and pumped at a flow rate of 2 ml/min onto a 25 × 1 cm Vydac 218TP510 C-18 reverse-phase HPLC column (Separations Group, Hesperia, CA, USA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was increased to 21% over 10 min, followed by an increase to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. Aliquots of each fraction (20 µl) were subjected to electrospray mass spectrometry to determine the molecular masses of the major components in that fraction. Individual fractions of interest were separately rechromatographed on a 25 × 0.46 cm Vydac 214TP54 C-4 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 49% over 50 min using a linear gradient. *Xenopus* insulins and proglucagon-derived peptides were purified to apparent homogeneity by chromatography on a 25 × 0.46 cm Vydac 219TP54 phenyl column using the same elution conditions as those for the C-4 column. The purification of two molecular forms of *Xenopus* PP has been described previously (Kim *et al.* 2001).

Purification of the Cynops peptides

The pancreatic extract, after partial purification on Sep-Pak cartridges, was redissolved in 0.1% trifluoroacetic acid/water (5 ml) and injected onto a 25 × 1 cm Vydac 218TP510 (C-18) HPLC column 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 increased to 21% over 10 min, followed by an increase to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. Individual peptides were purified to near homogeneity by chromatography on Vydac C-4 and Vydac phenyl columns using the same elution conditions as for the *Xenopus* peptides.

Structural characterization

Xenopus insulin from gene 3 (approximately 1 nmol) and *Cynops* insulin (approximately 1 nmol) were separately reduced (dithiothreitol) and pyridylethylated (4-vinylpyridine) as previously described (Conlon *et al.* 1995). The derivatized A-chains and B-chains of the insulins were separated by reverse-phase HPLC on an analytical

Figure 3 Reverse-phase HPLC on a Vydac C-4 column of *Xenopus* (A) glucagon-36 from genes 1 and 2 (fraction 1 in Fig. 1), (B) GLP-1B from gene 1 (fraction 5), (C) GLP-1C from gene 1 (fraction 6), (D) GLP-1C from gene 2 (fraction 7) and (E) GLP-1A from genes 1 and 2 (fraction 8). The arrows show where peak collection began and ended. A_{214} , absorbance at 214 nm.

Vydac C-18 column under the conditions used for the purification of intact insulin.

The primary structures of the peptides were determined by automated Edman degradation using a Perkin Elmer model 491A sequenator (Perkin Elmer, Norwalk, CT, USA). Electrospray mass spectrometry was carried out using a Perkin Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was $\pm 0.05\%$.

Results

Purification of the Xenopus peptides

The fractions from Sephadex G-25 chromatography that were eluted at, and immediately after, the void volume of the column (K_{AV} between 0.0 and 0.25) were pooled and chromatographed on a semi-preparative Vydac C-18 reversed-phase HPLC column and the elution profile is shown in Fig. 1. The fractions were analyzed by

Figure 4 Reverse-phase HPLC on a Vydac C-18 column of an extract of the pancreas of *Cynops pyrrhogaster* after partial purification on Sep-Pak cartridges. The retention times of the islet hormones are denoted by: (1) insulin, (2) glucagon-36, (3) PP, (4) GLP-1 and (5) GLP-2. The broken line shows the concentration of acetonitrile in the eluting solvent.

electrospray mass spectrometry with a view to identifying those fractions that contain peptides in major abundance in the molecular mass range 3000–6000 Da. Nine fractions with the retention times shown in Fig. 1 were selected for further purification and the masses of the peptides present in these fractions are shown in Fig. 2.

As the same techniques were used to isolate all peptides, only the chromatograms illustrating the purification of the *Xenopus* proglucagon-derived peptides are provided (Fig. 3). The elution profiles on an analytical Vydac C-4 column of glucagon-36 (Fig. 3A), GLP-1B from gene 1 (Fig. 3B), GLP-1C from gene 1 (Fig. 3C), GLP-1C from gene 2 (Fig. 3D) and GLP-1A (Fig. 3E) are shown. It was not possible to resolve glucagon-36 from genes 1 and 2 or GLP-1A from genes 1 and 2 on the Vydac C-4 column but separation was accomplished on an analytical Vydac phenyl column.

Purification of the *Cynops* peptides

The elution profile on a semi-preparative Vydac C-18 column of the extract of *Cynops* pancreas, after partial purification on Sep-Pak cartridges, is shown in Fig. 4. Analysis of the fractions by electrospray mass spectrometry identified five fractions containing abundant peptides in the mass range 3000–6000 Da range. The observed masses of these components, subsequently shown to represent insulin (fraction 1), glucagon-36 (fraction 2), PP (fraction 3), GLP-1 (fraction 4) and GLP-2 (fraction 5), are shown

in Fig. 2. The peptides were purified to near homogeneity, as assessed by a symmetrical peak shape and by mass spectrometry, on analytical Vydac C-4 and phenyl columns.

Structural characterization

The primary structures of the islet hormones from *Xenopus* and *Cynops* were determined without ambiguity by automated Edman degradation and their amino acid sequences are shown in Fig. 5. In all cases, the proposed structures were confirmed by mass spectrometry (Fig. 2). In both species, glucagon was isolated as a C-terminally extended component with 36 amino acid residues. Analysis of the fractions from HPLC (Figs 1 and 4) by mass spectrometry for the presence of *Xenopus* glucagon-29 (calculated M_r 3483) and *Cynops* glucagon-29 (calculated M_r 3483) were unsuccessful, indicating that these components were not present in major abundance. Similarly, attempts to identify a component with the expected mass of *Xenopus* GLP-2 from preproglucagon gene 1 or a second peptide paralogous to *Xenopus* GLP-1B arising from gene 2 were unsuccessful.

Discussion

Previous studies designed to characterize islet hormones in non-mammalian vertebrates have focused upon the higher

<i>Xenopus laevis</i>				
Insulin A-Chain	Gene 1	GIVEQCCHST	CSLFQLESYC	N
	Gene 2	GIVEQCCHST	CSLFQLENYC	N
	Gene 3	GIVEQCCHST	CSLFQLENYC	N
Insulin B-Chain	Gene 1	LVNQHLGSH	LVEALYLVCG	DRGFFYYPKV
	Gene 2	LANQHLCGSH	LVEALYLVCG	DRGFFYYPKI
	Gene 3	LANQHLCGSH	LVEALYLVCG	ERGFFYYPKI
Glucagon-36 (gene 1)		HSQGTFTSDY	SKYLDSTRRAQ	DFVQWLMNTK RSGELS[RRN...]
Glucagon-36 (gene 2)		HSQGTFTSDY	SKYLDSTRRAQ	DFVQWLMNTK RSGGLS[RRN...]
GLP-1A (gene 1)		HAEGTFTSDV	TQQLDEKAAK	EFIDWLINGG PSKEIIS[RRN...]
GLP-1A (gene 2)		HAEGTFTSDV	TQHLDEKAAK	EFIDWLINGG PTKEIIS[RRN...]
GLP-1B (gene 1)		HAEGTYTNDV	TEYLEEKAAK	EFIEWLIKGR PKKIRYS[RHA...]
GLP-1C (gene 1)		HAEGTFTNDM	TNYLEEKAAK	EFVGWLIKGR PK[RNF...]
GLP-1C (gene 2)		HAEGTFTNDM	TNYLEEKAAK	EFVGWLINGR PK[RKD...]
PP-1		APSEPMHPGD	QASPEQLAKY	YDDWWQYITF ITRPRF
PP-2		APSEPMHPGD	QASPEQLAKY	YEDWWQYITF ITRPRF
<i>Cynops pyrrhogaster</i>				
Insulin A-chain		GIVEQCCHNT	CSLYQLETYC	N
Insulin B-chain		AITNQHLGCS	HLVEALYLVC	GDRGFFYYTPKG
Glucagon-36		HSQGTFTSDY	SKYLDNRRAQ	DFVQWLMSTK RSGGVV
GLP-1		HADGTLTSDI	SSYLEKQAAK	EFIAWLVNGR.NH ₂
GLP-2		HADGSFTSDI	NKALDTIAAK	EFLNWLINTK VTE
PP		APSEPEHPGD	DASPEELAKY	YSDLWQYITF VSRPRY

Figure 5 Primary structures of the islet hormones isolated from the pancreata of *Xenopus laevis* and *Cynops pyrrhogaster*. The residues in square brackets show the amino acids flanking the hormonal sequences in the preproglucagon precursors, as deduced from the nucleotide sequences of cDNAs.

teleost fishes as the complete or partial separation of endocrine and exocrine tissue in the Brockmann bodies (principal islets) of certain species has greatly facilitated the purification of the peptides. Mass spectrometry (the more cumbersome technique of fast-atom bombardment mass spectrometry in earlier studies) has been used primarily to confirm the identity of the purified islet hormones from extracts of the Brockmann bodies of the anglerfish *Lophius americanus* (Andrews *et al.* 1986, 1987, Nichols *et al.* 1988), catfish *Ictalurus punctatus* (Andrews & Ronner 1985, Mommsen *et al.* 2002), daddy sculpin *Cottus scorpius* (Conlon *et al.* 1987a,b), tilapia *Oreochromis nilotica* (Nguyen *et al.* 1995) and pacu *Piaractus mesopotamicus* (Ferraz de Lima *et al.* 1999). The present study has used electrospray mass spectrometry to identify insulins, proglucagon-derived peptides and PP in chromatographic fractions from extracts of the disseminated pancreatic glands of the amphibians *X. laevis* and *C. pyrrhogaster* without the

need for additional techniques of detection such as radioimmunoassay, radioreceptor assay or bioassay.

Consistent with the fact that *X. laevis* is a tetraploid organism (Kobel & Du Pasquier 1991), cDNAs encoding two non-allelic preproinsulins have been identified in a pancreatic cDNA library (Shuldiner *et al.* 1989). Mass spectrometry has identified both gene 1 and gene 2 insulins, whose primary structures are shown in Fig. 5, in the pancreatic extract but a third insulin was also purified and characterized that differs from insulin-2 by the single amino acid substitution Asp²¹→Glu in the B-chain. The identification of this additional component may be a consequence of a localized gene duplication within the *Xenopus* lineage occurring after the putative tetraploidization event or may reflect heterozygosity. Studies in the carp *Cyprinus carpio* suggest that allelic variability tends to increase after tetraploidization (Larhammar & Risinger 1994).

A-Chain			
Human	GIVEQCCTSI	CSLYQLENYC	N
<i>Cynops</i>	-----HNT	-----T--	-
<i>Amphiuma</i>	AR-----HNT	-----N-----	-
<i>Siren</i>	-----HNT	-----	-
B-Chain			
Human	FVNQHLCGSH	LVEALYLVCG	ERGFYTPKT
<i>Cynops</i>	AIT-----	-----	D-----G
<i>Amphiuma</i>	IT--Y-----	-----	D-----S--
<i>Siren</i>	VP-KP---A-	---VM-F---	D-----PSS-
Glucagon			
Human	HSQGTFTSDY	SKYLDSSRAQ	DFVQWLMNTK RNKNINIA
<i>Cynops</i>	-----	-----N-----	-----S-- --SGGVS
<i>Amphiuma</i>	-----	-----N-----	-----I---S-
GLP-1			
Human	HAEGTFTSDV	SSYLEGQAQK	EFIAWLVKGR G
<i>Cynops</i>	--D--L---I	---K---	-----N-- .NH ₂
<i>Amphiuma</i>	--D--L---I	--F--K--T-	-----S-- GRRQ
GLP-2			
Human	HADGSFSDM	NTILDNLAA	DFINWLIQTK
<i>Cynops</i>	-----TSDI	-KA--TI--K	E-L---N-- VTE
<i>Amphiuma</i>	-----TSDI	-KV--TI--K	E-L---S-- VTE
Pancreatic Polypeptide			
Human	APLEVPYPGD	NATPEQMAQY	AADLRRYINM LTRPRY
<i>Cynops</i>	--S--EH---	D-S--EL-K-	YS--WQ--TF VS----
<i>Amphiuma</i>	--K--EH---	D-S---LEK-	YQ--FQ--IF I-----
<i>Siren</i>	--S--EH---	--S-DEL-K-	YS--WQ--TF VG----

Figure 6 A comparison of the primary structures of the known islet hormones from three species of Caudata: the red-bellied newt *Cynops pyrrhogaster*, the three-toed amphiuma *Amphiuma tridactylum* and the lesser siren *Siren intermedia*, with their orthologs from the human. – denotes residue identity.

Nucleotide sequence analysis of a cloned cDNA encoding preproglucagon-1 from *X. laevis* has shown that, after removal of the signal peptide, the precursor comprises 246 amino acids and contains the sequences of three peptides with structural similarity to GLP-1 (termed GLP-1A, GLP-1B, and GLP-1C) arranged in tandem, as well as sequences corresponding to mammalian glucagon and GLP-2 (Irwin *et al.* 1997). In addition, the nucleotide sequence of a second *Xenopus* preproglucagon cDNA, encoding an identical glucagon but lacking the GLP-2 domain, has been deposited in the Gene Bank database (accession number AF004433.1) by the same investigators. The pathway of post-translational processing of the *Xenopus* preproglucagons is not known, so it is unclear which, if any, of the GLP-1 peptides are produced *in vivo*. The present study has shown that preproglucagons from gene 1 and 2 are not processed to glucagon-29, the primary gene product in the pancreata of mammals, but to a C-terminally extended component that corresponds to mammalian glucagon-37 (oxyntomodulin) (Tager & Steiner 1973). The concentrations of C-terminally

extended forms of glucagon are higher than the concentrations of glucagon-29 in the pancreata of several species of anurans that have been examined (*Rana catesbeiana* (Pollock *et al.* 1988), *Bufo marinus* (Conlon *et al.* 1998a), *Ceratophrys ornata* (White *et al.* 1999) and *Pipa pipa* (Matutte & Conlon 2000)). As shown in Fig. 5, processing of preproglucagon to generate glucagon-36 (gene 1 and 2), GLP-1A (gene 1 and 2) and GLP-1C (gene 2) involves proteolytic cleavage at the site of dibasic residues (Arg-Arg and Arg-Lys) whereas generation of GLP-1B (gene 1) and GLP-1C (gene 1) involves cleavage of a monobasic (single Arg) site.

The newt *C. pyrrhogaster* is classified taxonomically in the family Salamandridae which is grouped with the Amphiumidae (amphiumas), Plethodontidae (lungless salamanders), Ambystomatidae (axolotls), Proteidae (mudpuppies), Rhyacotritonidae (torrent salamanders) and Dicamptodontidae (Pacific giant salamanders) in the sub-order Salamandroidea. The Salamandroidea are sister-group to the Cryptobranchioidea (hellbenders) with the Sirenidae (sirens) as outgroup (Titus & Larson 1995). Consistent with this phylogenetic scheme, the primary structure of *Cynops* insulin shows greater structural similarity to insulin from the three-toed amphiuma *Amphiuma tridactylum* (Conlon *et al.* 1996) (eight substitutions) than to insulin from the lesser siren *Siren intermedia* (Conlon *et al.* 1997) (fourteen substitutions) (Fig. 6). However, *Cynops* insulin lacks the dipeptide extension to the N terminus of the A-chain that is found in amphiuma insulin, indicating that the pathway of post-translational processing of *Cynops* proinsulin is not anomalous. Those residues in human insulin that are believed to be important in maintaining the receptor-binding conformation (Kristensen *et al.* 1997, Conlon 2001) have been conserved in *Cynops* insulin.

The amino acid sequences of *Cynops* GLP-1 and GLP-2 also resemble closely the corresponding peptides from the amphiuma (Cavanaugh *et al.* 1996). In amphiuma proglucagon, the Arg-Arg dibasic residue sequence located at the C terminus of GLP-1 (Gly³¹-Arg³²-Arg³³-Gln³⁴) is not recognized by a processing enzyme, whereas in *Cynops* proglucagon this dibasic pair is proteolytically cleaved and the Gly³¹ residue acts as a substrate for peptidylglycine α -amidating mono-oxygenase to produce a C-terminally α -amidated GLP-1. Cleavage at dibasic residue sites by the prohormone convertases PC1 (SPC3) and PC2 is promoted when the site is situated adjacent to a β -turn or Ω -loop and inhibited when the site forms part of an α -helix (Steiner 1998). Substitution of the Ser²⁸ residue in amphiuma GLP-1 by Asn, a residue that destabilizes α -helices in proteins, in *Cynops* GLP-1 may be responsible for the different pathways of processing of the two preproglucagons.

The primary structure of PP has been poorly conserved among non-mammalian tetrapods and it has been proposed that the amino acid sequence of the peptide may be a useful molecular marker for inferring phylogenetic

relationships between species (Conlon *et al.* 1998b). In this light, the primary structure of *Cynops* PP appears to be anomalous. As shown in Fig. 6, *Cynops* PP differs from amphiuma PP by eight amino acids but from siren PP by only three amino acids. This is inconsistent with the proposed closer phylogenetic relationship between the newts and the amphiuma based upon morphological criteria and mitochondrial DNA sequences (Titus & Larson 1995). The physiological role of PP, if any, in amphibians is unknown.

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