GENERAL AND COMPARATIVE ENDOCRINOLOGY 84, 440–446 (1991)

Primary Structure of Pancreatic Polypeptide from Four Species of Perissodactyla (Przewalski's Horse, Zebra, Rhino, Tapir)

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Accepted February 12, 1991

Pancreatic polypeptide (PP) has been purified from extracts of the pancreas of four species of odd-toed ungulates (Perissodactyla): Przewalski's horse, mountain zebra, white rhinoceros, and mountain tapir. The amino acid sequence of Przewalski's horse pancreatic polypeptide was established as Ala-Pro-Met-Glu-Pro-Val-Tyr-Pro-Gly-Asp[10]-Asn-Ala-Thr-Pro-Glu-Gln-Met-Ala-Gln-Tyr[20]-Ala-Ala-Glu-Leu-Arg-Arg-Tyr-Ile-Asn-Met[30]-Leu-Thr-Arg-Pro-Arg-Tyr · NH_2. Zebra PP was identical to Przewalski's horse PP, rhinoceros PP contained three substitutions relative to the horse (Ser for Ala[1], Leu for Met[3], and Glu for Gln[16]), and tapir PP contained one substitution relative to the horse (Leu for Met[3]). On the basis of morphological characteristics and the fossil record, the rhinocerotids are classified with the tapirids in the suborder Ceratomorpha, whereas the horse and zebra belong to a separate suborder, Hippomorpha. On the basis of structural similarity of the PP molecules, however, it would appear that the tapir is more closely related to the horse than to the rhinoceros. These observations provide a further example of the need for extreme caution when inferring taxonomic or phylogenetic relationships between species from the structures of homologous peptides.

The Perissodactyla, or odd-toed ungulates, are represented today by only 16 species classified into 5 genera despite the fact that there are 152 extinct genera of the order known from the fossil record (Morris, 1965). With the exception of domestic horse and domestic donkey, all surviving species of the order are approaching extinction. On the basis of morphological characteristics, the extant Perissodactyla are classified into the suborder Hippomorpha, which includes the horses, wild asses, and zebras (superfamily Equoidea), and the suborder Ceratomorpha, which includes the rhinoceroses (superfamily Rhinocerotoidea) and tapirs (superfamily Tapiroidea). This group of mammals has not been studied extensively by molecular endocrinologists.

Pancreatic polypeptide (PP) was first identified as a contaminant in preparations of insulin (Kimmel *et al.*, 1968; Chance *et al.*, 1979) and was isolated in pure form from the chicken pancreas (Kimmel *et al.*, 1975). Subsequent work has shown that PP is present in the islet cells of all mammalian species yet studied (Epple and Brinn, 1987), but its precise physiological role remains an enigma. The primary structure of the peptide has been determined for several mammalian species (reviewed in Schwartz *et al.*, 1989) including three species of Artiodactyla (pig, ox, sheep). PP is a member of a family of homologous peptides that includes neuropeptide tyrosine (NPY) and peptide tyrosine tyrosine (PYY). All members of the PP family that have been characterized comprise 36 amino acid residues and terminate in an α-amidated tyrosine residue.

This study describes the purification and

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0016-6480/91 $1.50

structural characterization of PP from four Perissodactyla species: Przewalski's horse (*Equus przewalskii*), the mountain zebra (*Equus zebra*), the mountain tapir (*Tapirus pinchaque*), and the white rhinoceros (*Ceratotherium simum*). The taxonomic relationship between the species inferred from the structures of these homologous peptides is compared with the relationship deduced from classical morphological and phylogenetic analysis.

MATERIALS AND METHODS

Tissue extraction. Pancreata were obtained postmortem (up to 12 hr after death) from single adult female specimens and were stored at −20°. Tissue (horse, 184 g; zebra, 245 g; rhino, 202 g; tapir, 149 g) was homogenized at 4° with 9 vol ethanol/0.7 *M* HCl (3/1, v/v) using a Waring blender. Peptides were isolated from the extract using Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) as described (Conlon *et al.*, 1991). Bound material was eluted with acetonitrile/water/trifluoroacetic acid (80/19.9/0.1) and lyophilized (Savant Speed Vac).

Radioimmunoassay procedure. PP-like immunoreactivity was measured by radioimmunoassay using antiserum PP221 directed against the COOH-terminal region of human PP in a procedure that has been described previously (O'Hare *et al.*, 1983). The antiserum requires the presence of an α-amidated COOH-terminal tyrosine residue in PP for reactivity. Concentrations are expressed relative to a human PP standard from Peninsula Laboratories (Belmont, CA).

Purification of PP. The same procedure was used for the purification of PP from each species and so only the method used for the isolation of the peptide from Przewalski's horse is described in detail. The extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid (10 ml) and an aliquot (2 ml) was injected onto a 1 × 25-cm Vydac 218TP510 (C-18) reversed-phase HPLC column (Separations Group, Hesperia, CA) equilibrated with 0.1% 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 5 min and to 49% (v/v) over 40 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. PP-like immunoreactivity was measured at a dilution of 1:100. The fraction containing maximum immunoreactivity was rechromatographed on a 1 × 25-cm Ultrapore 6RPSC288 (C-3) reversed-phase HPLC column (Beckman, Duarte, CA) equilibrated with 0.1% 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 5 min and to 44% over 45 min using linear gradients. Peaks of material with absorbance at 214 nm were collected manually. Horse PP was purified to apparent homogeneity by chromatography on a 0.46 × 25-cm Vydac 214TP54 (C-4) column equilibrated with 0.1% trifluoroacetic acid at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 27% (v/v) over 5 min and to 45% (v/v) over 35 min using linear gradients.

Structural characterization. Amino acids were determined by precolumn derivatization with phenylisothiocyanate using an Applied Biosystems Model 420A derivatizer and a Model 130A separation system as previously described (Conlon *et al.*, 1990). Hydrolysis was carried in the absence of phenol and so the values for tyrosine have been multiplied by 1.2 to correct for degradation. Automated Edman degradation was performed using an Applied Biosystems Model 471A sequenator modified for on-line detection of phenylthiohydantoin (PTH) amino acids under gradient elution conditions. The detection limit for the PTH derivatives was 0.5 pmol. Californium-252 plasma desorption time-of-flight mass spectrometry was carried out using a BIO-ION BIN-20K instrument. Spectra were recorded at 16 kV for 10^6 primary fission events. The accuracy of the mass determinations was ±0.1%.

RESULTS

Purification of PP from Przewalski's horse. The elution profile on a C-18 reversed-phase HPLC column of an aliquot (approximately 20% of the total) of the extract of horse pancreas is shown in Fig. 1. PP-like immunoreactivity was associated with a single fraction that corresponded in retention time with the prominent peak denoted by PP. The peaks denoted I1 and I2 contained insulin-like immunoreactivity. The PP-containing peak was chromatographed on a C-3 reversed-phase column (Fig. 2) and the PP-like immunoreactivity was associated with the single peak shown. Przewalski's horse PP was purified to apparent homogeneity by a final chromatography on an analytical C-4 reversed-phase column (Fig. 3), and the final yield of pure peptide was 14 nmol.

Purification of PP from the zebra, rhino, and tapir. PP peptides were isolated from pancreatic extracts from the zebra, rhino, and tapir under the same conditions of

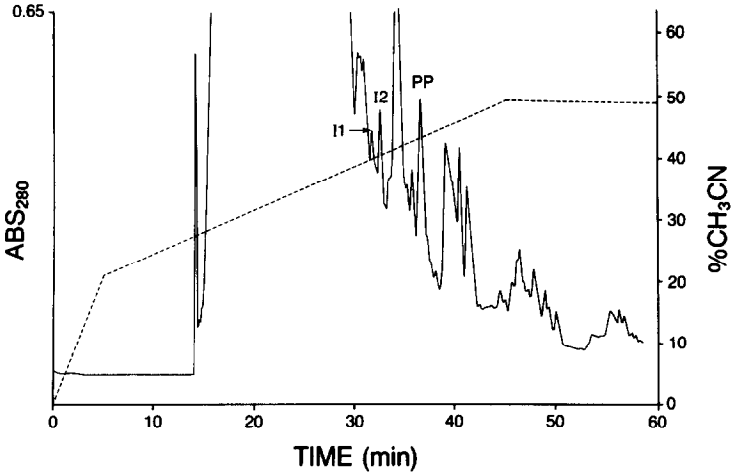

FIG. 1. The elution profile on a Vydac C-18 reversed-phase HPLC column of an extract of pancreas from Przewalski's horse after partial purification on Sep-Pak C-18 cartridges. The peak denoted by PP contained pancreatic polypeptide-like immunoreactivity and the peaks denoted by I1 and I2 contained insulin-like immunoreactivity. The dashed line shows the concentration of acetonitrile in the eluting solvent.

chromatography used for the purification of horse PP. The approximate final yields of pure peptides were zebra, 9 nmol; rhinoceros, 7 nmol; and tapir, 6 nmol. As the tissues were collected postmortem, these amounts probably do not accurately reflect the actual concentrations in the functioning pancreas.

Structural characterization. The amino acid compositions of the perissodactyl PPs are shown in Table 1. The data indicate that the peptides probably comprise 36 residues excluding possible tryptophan and cysteine residues. The results of automated Edman degradation are shown in Table 2. It was possible to assign without ambiguity PTH–

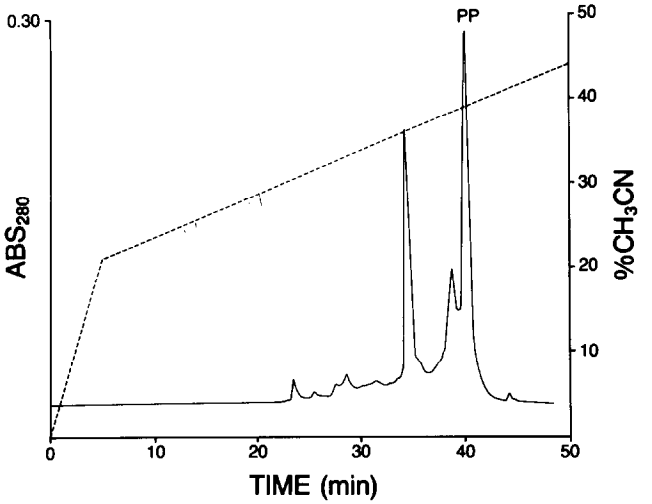

FIG. 2. Elution profile on an Ultrapore C-3 reversed-phase HPLC column of Przewalski's horse pancreatic polypeptide. The peak denoted by PP contained PP-like immunoreactivity and was purified further.

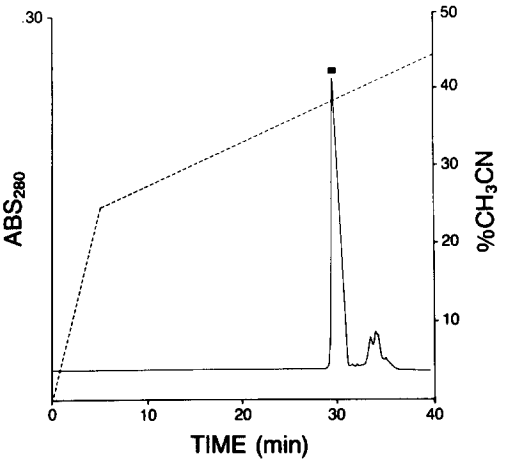

FIG. 3. Purification to apparent homogeneity of pancreatic polypeptide from Przewalski's horse. The pure peptide, denoted by ■ was subjected to automated Edman degradation.

amino acid derivatives during 36 cycles of operation of the sequenator and there was no trace of a PTH–glycine derivative during cycle 37 of any run. The agreement between the sequence analysis and the composition data was good, demonstrating that the full sequence of the peptides had been obtained. It is proposed that the COOH-terminal residue of the PP peptides is α-amidated on the basis of the strong reactivity with an antiserum directed against the common COOH-terminal hexapeptide region that requires an α-amidated tyrosine[36] residue for recognition. The primary structures of the perissodactyl PPs were confirmed by mass spectrometry. The observed molecular mass of Przewalski's horse PP was 4213.7 ± 4.2 compared with a theoretical value of 4217 for the α-amidated form of the proposed sequence. The corresponding values for PPs from the other species were zebra observed mass, 4216.6 ± 4.2 (calculated mass 4217); and rhino observed mass, 4213.5 ± (calculated mass 4214). The mass spectrum of tapir PP was not recorded, but the amino acid sequence of the peptide was confirmed by a second automated Edman degradation.

TABLE 1
AMINO ACID COMPOSITIONS OF PANCREATIC POLYPEPTIDE FROM FOUR SPECIES OF PERISSODACTYLA

Amino acid	Residues/mol peptide			
	Horse	Zebra	Rhinoceros	Tapir
Asx	2.9 (3)	3.1 (3)	3.1 (3)	3.2 (3)
Thr	1.9 (2)	2.1 (2)	1.9 (2)	1.9 (2)
Ser	0.0 (0)	0.0 (0)	1.0 (1)	0.0 (0)
Glx	4.8 (5)	5.2 (5)	5.1 (5)	5.3 (5)
Pro	5.2 (5)	5.4 (5)	5.1 (5)	4.6 (5)
Gly	1.2 (1)	1.3 (1)	1.2 (1)	1.3 (1)
Ala	5.0 (5)	4.9 (5)	4.0 (4)	5.0 (5)
Val	1.0 (1)	1.1 (1)	1.0 (1)	1.3 (1)
Met	2.9 (3)	2.5 (3)	2.1 (2)	1.9 (2)
Ile	1.0 (1)	1.3 (1)	1.1 (1)	1.3 (1)
Leu	2.1 (2)	2.2 (2)	3.0 (3)	3.3 (3)
Tyr	4.4 (4)	3.8 (4)	4.3 (4)	3.5 (4)
Arg	4.3 (4)	4.2 (4)	4.2 (4)	4.1 (4)

Note. The data represent the means of two determinations. Numbers in parentheses are the values predicted from the proposed structures.

DISCUSSION

This report describes the first structural characterization of PP from species of Perissodactyla (odd-toed ungulates) and the amino acid sequences of the peptides are compared with those from three species of Artiodactyla (even-toed ungulates) in Table 3. In general, evolutionary pressure to conserve the amino acid sequence of members of the PP family, even among the mammals, has not been very strong. Rat PP, for example, contains eight amino acid substitutions relative to pig PP (Kimmel *et al.*, 1984). Evolution has acted, however, to conserve overall tertiary structural features in the molecule (Schwartz *et al.*, 1989; Conlon *et al.*, 1991). The three-dimensional structure of turkey PP has been determined by X-ray diffraction analysis (Wood *et al.*, 1977). The molecule comprises a polyproline helix (residues 1–8) that is connected to an amphiphilic α-helix (residues 15–36) by a β-turn region. The two helices are held in a stable folded conformation (the "PP-fold") by non-bonded interactions, whereas the COOH-terminal hexapeptide (residues 31–36) is relatively mobile. In the perissodactyl PPs, the proline residues at positions 2, 5,

TABLE 2
AUTOMATED EDMAN DEGRADATION OF PANCREATIC POLYPEPTIDE FROM FOUR SPECIES OF PERISSODACTYLA

Cycle no.	Amino acid			
	Horse	Zebra	Rhinoceros	Tapir
1	Ala (3558)	Ala (2170)	Ser (517)	Ala (1417)
2	Pro (2925)	Pro (1022)	Pro (1801)	Pro (513)
3	Met (2234)	Met (1308)	Leu (1891)	Leu (540)
4	Glu (2294)	Glu (739)	Glu (833)	Glu (380)
5	Pro (2441)	Pro (659)	Pro (1243)	Pro (271)
6	Val (1268)	Val (726)	Val (1209)	Val (405)
7	Tyr (1771)	Tyr (732)	Tyr (893)	Tyr (375)
8	Pro (2083)	Pro (659)	Pro (1282)	Pro (345)
9	Gly (1799)	Gly (842)	Gly (691)	Gly (342)
10	Asp (722)	Asp (428)	Asp (201)	Asp (431)
11	Asn (1591)	Asn (804)	Asn (322)	Asn (283)
12	Ala (1998)	Ala (903)	Ala (471)	Ala (371)
13	Thr (290)	Thr (116)	Thr (140)	Thr (58)
14	Pro (523)	Pro (367)	Pro (413)	Pro (221)
15	Glu (356)	Glu (141)	Glu (293)	Glu (179)
16	Gln (457)	Gln (179)	Glu (371)	Gln (108)
17	Met (568)	Met (176)	Met (392)	Met (135)
18	Ala (634)	Ala (278)	Ala (303)	Ala (247)
19	Gln (755)	Gln (265)	Gln (212)	Gln (159)
20	Tyr (902)	Tyr (155)	Tyr (299)	Tyr (198)
21	Ala (892)	Ala (422)	Ala (317)	Ala (202)
22	Ala (970)	Ala (471)	Ala (453)	Ala (262)
23	Glu (223)	Glu (133)	Glu (137)	Glu (144)
24	Leu (357)	Leu (116)	Leu (268)	Leu (151)
25	Arg (336)	Arg (121)	Arg (223)	Arg (111)
26	Arg (541)	Arg (244)	Arg (349)	Arg (150)
27	Tyr (750)	Tyr (131)	Tyr (312)	Tyr (153)
28	Ile (312)	Ile (102)	Ile (219)	Ile (127)
29	Asn (286)	Asn (136)	Asn (182)	Asn (121)
30	Met (301)	Met (89)	Met (220)	Met (70)
31	Leu (291)	Leu (85)	Leu (223)	Leu (66)
32	Thr (27)	Thr (12)	Thr (22)	Thr (12)
33	Arg (263)	Arg (102)	Arg (228)	Arg (105)
34	Pro (159)	Pro (49)	Pro (126)	Pro (97)
35	Arg (323)	Arg (129)	Arg (232)	Arg (116)
36	Tyr (320)	Tyr (42)	Tyr (103)	Tyr (67)

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

and 8 have been conserved despite other substitutions in the NH_2-terminal region and so it is probable that the conformation of the polyproline helix has been maintained. Similarly, the substitution of Gln by Glu at position 16 of rhino PP will not disrupt the α-helix.

Table 3 demonstrates that tapir PP is more similar in structure to horse and zebra PP than to PP from the rhinoceros. The result is unexpected as the fossil record indicates that present day tapirs and rhinoceroses probably evolved from a common ancestor that was distinct from the ancestor of the horses (Prothero and Schoch, 1989). This is reflected in the fact that the tapirids and rhinocerotids are classified in the same suborder, Ceratomorpha, whereas the horses and zebra are placed in a different suborder, Hippomorpha. The theoretical

TABLE 3
A COMPARISON OF THE PRIMARY STRUCTURES OF PANCREATIC POLYPEPTIDE FROM SEVERAL UNGULATE SPECIES

	5	10	15	20	25	30	35
Horse	APMEP	VYPGD	NATPE	QMAQY	AAELR	RYINM	LTRPR Y · NH_2
Zebra	----------	----------	----------	----------	----------	----------	---------- -
Rhino	S-L-----	----------	----------	E---------	----------	----------	---------- -
Tapir	----L----	----------	----------	----------	----------	----------	---------- -
Pig	----L----	----------	D---------	----------	----------	----------	---------- -
Ox	----L----	E---------	----------	----------	----------	----------	---------- -
Sheep	-SL-----	E---------	----------	----------	----------	----------	---------- -

Note. (-) Denotes conservation of the amino acid residues.

basis for using molecular structures to assign taxonomic or phylogenetic relationships between species is the evolutionary clock hypothesis (Wilson *et al.*, 1977). According to the theory, the difference in structure of two homologous proteins is directly proportional to the time of divergence of the species. Several examples from the field of molecular endocrinology have called into question the validity of this assumption (Schwabe, 1986). The primary structures of relaxins from closely related species, e.g., spiny dogfish (*Sqalus acanthias*) and sand tiger shark (*Odontaspis taurus*), differ markedly, whereas relaxin from the whale (*Balaeonoptera edeni*) is almost identical to that from the pig (Schwabe and Bullesbach, 1990). Insulins from the hystricomorph rodents have undergone an accelerated rate of mutation so that a phylogenetic tree based solely upon the structures of vertebrate insulins would indicate that these species diverged from the line of evolution leading to mammals at the same time as the agnatha (Bajaj *et al.*, 1984). Similarly, the fossil record indicates that the crocodilians are more closely related to birds than the chelonians, but insulin from the turtle is identical to insulin from the chicken, whereas alligator insulin contains three amino acid substitutions (Conlon *et al.*, 1990). In the case of insulins from the Perissodactyla, however, there is no discrepancy between molecular and classical taxonomic classification. Przewalski's horse insulin is the same as insulins from the zebra and the domestic horse, whereas insulins from the tapir and the rhinocerous are identical and are of the same structure as insulin from the pig (unpublished data). Nevertheless, this study has emphasized the need for extreme caution when inferring phylogenetic or taxonomic relationships between species on the basis of the structures of homologous peptides.

ACKNOWLEDGMENTS

The authors thank Dr. Lars Thim, Novo Nordisk Research Institute, Bagsvaerd, Denmark, for mass spectrometry measurements and Dr. Mairead O'Hare, Queen's University of Belfast, North Ireland, for a gift of antiserum.

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