Molecular Cloning and Expression of Prohormone Convertases PC1 and PC2 in the Pituitary Gland of the Bullfrog, *Rana catesbeiana*

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ABSTRACT—We cloned cDNAs encoding PC1 and PC2 from a cDNA library constructed for the anterior pituitary gland of the bullfrog (*Rana catesbeiana*) and sequenced them. The bullfrog PC1 cDNA consisted of 2972 base pairs (bp) with an open reading frame of 2208 bp and encoded a protein of 736 amino acids, including a putative signal peptide of 26 amino acids. The protein showed a high homology to *R. ridibunda* PC1 (95.1%) and mammalian PC1 (72.6%). The bullfrog PC2 cDNA consisted of 2242 bp with an open reading frame of 1914 bp and encoded a protein of 638 amino acids, including a putative signal peptide of series protein and a high homology to *R. ridibunda* PC2 (84.8%). The catalytic triad of serine proteinases of the subtilisin family was found at Asp-168, His-209, and Ser-383 in the PC1 protein and at Asp-167, His-208, and Ser-384 in the PC2 protein. *In situ* hybridization staining revealed that PC2 mRNA was detected in corticotrope cells of the tadpoles, but not in those of the adults. In the adult, only PC1 mRNA was detected in the pars distalis but both PC1 and PC2 mRNAs were detected in the pars intermedia. The data also showed that PC1 mRNA was expressed in gonadotrope cells.

Key words: PC1, PC2, mRNA expression, pituitary gland, bullfrog

INTRODUCTION

In mammals, adrenocorticotropin (ACTH)-related peptides in corticotrope cells in the pars distalis and α -melanocortin-stimulating hormone (a-MSH)-related peptide in melanotrope cells in the pars intermedia are known to be produced post-traslationally by intracellular proteolytic cleavage of the large precursor molecule known as proopiomelanocortin (POMC). Nevertheless, the processing of POMC differs between these 2 lobes: in corticotrope cells, ACTH, β -lipotropic hormone (β -LPH), and a 16-kDa fragment are the major end products, whereas in melanotrope cells, ACTH is processed further into α -MSH and cortcotropin-like intermediate peptide (CLIP), and β -LPH is processed almost completely into β -endorphin (Eipper and Mains, 1980; Rosa et al., 1980; Chretien et al., 1989). In mammals, 2 mammalian prohormone convertase, PC1 (also called PC3) and PC2, have been characterized by cloning and sequencing of

* Corresponding author: Tel. +81-54-238-4783; FAX. +81-54-238-0986. E-mail: sbstana@ipc.shizuoka.ac.jp their cDNA (Seidah et al., 1990, 1991; Smeekens et al., 1990, 1991; Hakes et al., 1991). In the pituitary, PC1 is expressed in both the pars distalis and intermedia and cleaves POMC mainly at the paired basic sites flanking the ACTH sequence, whereas PC2 was expressed mainly in the pars intermedia and cleaved POMC in concert with PC1 to yield joining peptide, α -MSH, and β -endorphin (for review see, Seidah and Chretien, 1992). The proteolytic processing of POMC in the pars intermedia of amphibians is essentially the same as that in the mammalian pars intermedia and is considered to be different from POMC processing in the corticotrope cells in the pars distalis. In the anuran amphibian pituitary gland, the presence of PC1 and PC2 has been demonstrated immunohistochemically (Kurabuchi and Tanaka, 1997). In the pars distalis, however, immunoreactivity of the convertases showed a different pattern among the anuran amphibians examined: either PC1 or PC2 was found in the corticotrope cells in several species, whereas both PC1 and PC2 were observed in the corticotrope cells in R. brevipoda porosa; although PC2-immunopositive cells did not express α -MSH (Kurabuchi and Tanaka, 1997).

Immmunohistochemistry often yields also results because the antibody used recognizes pseudo epitopes in different molecules. Therefore, to define expression of a molecule, the mRNA of the molecule should also be detected using the *in situ* hybidization method.

However, cloning of cDNAs encoding PC1 and PC2 and/or determination of the primary structure of amphibian PC1 and PC2 have been accomplished only in *Xenopus laevis* (PC2: Braks *et al.*, 1992) and *R. ridibunda* (PC1: Gangnon *et al.*, 1999, PC2: Vieau *et al.*, 1998). Therefore, we sought to obtain cDNAs encoding the bullfrog PC1 and PC2 to deduce the amino acid sequence of these two proteins. Using each cDNA as a probe, we examined the expression of the mRNAs in the pituitaries by *in situ* hybridization.

MATERIALS AND METHODS

Animals

Adult male bullfrogs (*Rana catesbeiana*) and tadpoles at stage VII (Taylor and Kollros, 1946) were purchased from Ouchi (Misato, Japan). They were acclimated under normal laboratory conditions for at least 1 week before use. The animals were fed pieces of porcine liver or boiled spinach twice a week. Pituitary glands dissected under anesthesia with MS-222 (Nacalai tesque, Kyoto, Japan) were used for histochemical examination and RT-PCR analysis. All animal experiments were in compliance with the Guide for Care and Use of Laboratory Animals established by Shizuoka University.

Cloning of bullfrog PC1 and PC2

Total RNA was extracted from 86.2 μ g of the anterior pituitaries of bullfrogs using TRIZOL RNA extraction reagent (Life Technologies, Inc., Rockville, MD), and then 5.0 µg polyadenylated RNA was separated from about 255 µg of the total RNA using Oligotex-dT30 super (Takara, Kyoto, Japan). We constructed a λZAP cDNA library $(4.6 \times 10^5 \text{ pfu/}\mu\text{g of arms})$ from the polyadenylated RNA using a ZAP-cDNA synthesis kit and a Gigapack III Gold cloning kit (Stratagene, La Jolla, CA), in accordance with the manufacture's instructions. Purified DNA from a bullfrog anterior pituitary cDNA library was amplified by the polymerase chain reaction (PCR) in a thermal cycler (ASTEC, Fukuoka, Japan). The procedure for the PCR amplification was an initial denaturation step of 95°C for 5 min followed by denaturation (94°C, 90 sec), annealing (54°C, 90 sec), and extension (72°C, 150 sec) for 30 cycles, using degenerate oligonucleotides (Sawady Technology, Tokyo, Japan) designed based on the conserved regions of PC1 and PC2 from other species. The sequences of sense (primer 1) and antisense (primer 2) primers were as follow: PC1 primer 1, 5'-TGGTAY(C/T)TTGM(A/ C)R(A/G)AGAY(C/T) ACM(A/C)AG-3'; PC1 primer 2, 5'-GCN(A/C/G/ T)GAK(G/T)GTK(G/T)CCH(A/C/T)GTR(A/G)TGB(C/G/T)GT-3'; PC2 primer 1, 5'-TAY (C/T)AGY(C/T)GCM(A/C)AGCTGGGGB(C/ G/T)CC-3'; PC2 primer 2, 5'-TGY(C/T)TGCAW(A/T)GTCY(C/T)CK (G/T)CCAD(A/G/T)GT-3'. The amplified PCR products were electrophoresed on a 2% agarose gel and the 754-bp fragment (the expected size based on the known R. ridibunda PC1 cDNA sequence) and the 440-bp fragment (the expected size based on the known Rana ridibunda PC2 cDNA sequence) were subcloned directly into the pGEM-3Z vector (Promega, Madison, WI) and sequenced. We synthesized DNA probes with sequences identical to those of the PCR products described above using a digoxigenin (DIG)-High Prime kit (Roche Mol. Biochem., Meylan, France) and used them to screen approximately 1.25×10⁵ plaques of the bullfrog cDNA library under high stringency hybridization. Five positive clones for PC1 and 2 positive clones for PC2 were obtained, purified by a second screening, and sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA). The sequencing reactions were analyzed with an Applied Biosystems DNA sequencer model 377 (PE Applied Biosystems).

Protein sequence analysis

We used the ScanProsite (http://kr.expasy.org/tools/scanprosite/) to analyze the protein sequence.

RT-PCR of bullfrog tissues

The tissue expressions of PC1 and PC2 mRNAs were analyzed by RT-PCR. Using TRIZOL reagent, total RNA was prepared from various adult bullfrog organs (pars distalis, neurointermediate lobe, brain, heart, liver, pancreas, lung, kidney, spleen, stomach, intestine, testis, ovary, and skeletal muscle). After treatment of 20 µg of total RNA with DNase I (4 U; Takara), a 10-µg aliquot of the former was reverse-transcribed in 20 µl of reaction buffer containing a 1 mM concentration of each dNTP, 9.9 U of RAV-2 reverse transcriptase (Takara), 20 U of RNase inhibitor (Toyobo, Osaka, Japan), and 7.5 mM oligo-dT(19)primer (Life Technologies, Inc., Rockville, MD) at 42°C for 1 hr, and then at 52°C for 30 min. PCR was then performed by the same method, basically as described above, using the following homologous primers: PC1 sense, 5'-GTAGGAGGCATTCGGATGTTA-3' (809-829 b); and antisense, 5'-GAAGATTGAGCCTTTTCCATTT-3' (994-1015 b); PC2 sense, 5'-TCTTCCACTTTAGCCTCTACAT-3' (1197-1218 b); and antisense, 5'-CTCTAGGGCTAATGCAAACA-3' (1333-1352 b). Bullfrog β-actin was used as an internal standard during detection of PC1 and PC2 mRNA expressions. The *β*-actin cDNA was amplified by using a set of primers designed to amplify a β-actin fragment of 96 bp (Yaoi et al., 2003). The RT-PCR products were analyzed on a 2% agarose gel containing ethidium bromide (EtBr; 0.5 μ g/ml) with Marker 6 (λ / Sty1 digest; Wako Pure Chemicals, Osaka, Japan) molecular weight markers. The gels were subsequently transferred onto a nylon membrane (Roche) and subjected to Southern blot analysis using bullfrog PC1 or PC2 cDNAs as probes.

In situ hybridization histochemistry

DIG-labeled antisense and sense cRNA probes were prepared from the full coding region of PC1 and PC2 cDNAs by in vitro transcription, as described previously (Saito et al., 2002). Bullfrog pituitary glands were fixed with 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4, overnight at 4°C. After fixation, the tissues were dehydrated through a graded alcohol series, cleared in methyl benzoate-celloidin, and embedded in Paraplast. Sections were cut at a 4-µm thickness and mounted on silane-coated slides. In situ hybridization was carried out according to a method described previously (Saito et al., 2002). Briefly, deparaffinized sections were digested with 5 µg/ml proteinase K for 20 min, fixed in 4% PFA for 20 min, and then incubated with the DIG-labeled cRNA at 50°C for 15 hr. After hybridization, the sections were treated with 1 µg/ml RNase solution for 30 min and then incubated with alkaline phosphatase-conjugated sheep anti-DIG Fab antibody (Roche) for 15 hr. The label was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Roche).

Dual mRNA and protein staining

After the mRNA had been stained as described above, the sections were washed with PBS and incubated with guinea pig antibody against bullfrog POMC (Berghs *et al.*, 1997), rabbit anti- α -MSH (Tanaka and Kurosumi, 1986) or mouse monoclonal antibody against bullfrog LH β (Park *et al.*, 1987) overnight, followed by Cy3-labeled donkey anti-guinea pig IgG, FITC-labeled donkey anti-rabbit IgG or FITC-labeled donkey anti-mouse IgG (Jackson Immunore-search, West Grove, PA) for 2 hr. The sections were washed with PBS, then mounted in PermaFluor (Immunon, Pittsburgh, PA), and

examined under an Olympus BX50 microscope equipped with a BXepifluorescence attachment (Olympus Optical Co., Tokyo, Japan).

RESULTS

cDNA cloning of bullfrog PC1 and PC2 Fig. 1 shows the full cDNA sequence of bullfrog PC1

-103 -90 1 1	$\label{eq:construct} CGCGGCCGCGGACGGCGCGGACGGCGGACGGGGGGGGGG$	FCG -91 3AA -1 CGA 90 R 30
91 31	TATGTGAATGGACTGCAGAGAATCCCTGGAGGTCCAGAGGAAGCACTGGCATGACCGGATGAACTGGGCTATGACTACGGTCGGGCYVVNEWAAEIPGGGCGGGGAGGAAGCACTGGCATGACCGGCGATGAACTGGGCTATGACCAGGGGGGGG	CAG 180 Q 60
181 61	$ \begin{array}{cccc} ATTGGATCGCTTCCAAACCATTTTTTTTTCAAACATAGGGATCATCCCAGGAGATCACGGAGAAGTGCCCCCACACATTACAAAGCGGGAGATCACCGGAGAAGTGCCCCCACACATTACAAAGCGGGAGATCACCGGAGAAGTGCCCCCACACATTACAAAGCGGGAGATCACCGGAGAAGTGCCCCCACACACA$	FTG 270 L 90
271 91	TATGATGATAACCGGGCTCTCATGGGCAGAACAGCAGTATCTCAAGCAAAGGACTAAACGAGGCTATGTGATGAATACAGACTCAGAACAGACTCAGAACAGACTCAGAACAGACTAAGCAAGACTAAGCAAGACTAAGCAAGACTAAGACTAAGACTAAGAATACAGAACTCAGAACAGACTAAGCAAGACTAAACGAGGCTATGTGATGAATACAGAACTCAGAACAGACTAAAGCAAGACTAAACGAGGCTATGTGATGAATACAGAACTCAGAACAGACTAAACGAGGCTAAGCAAGACTAAACGAGGCTAAGCAAGAACAGAACAGAACAGAACAGACTAAACGAGGCTAAGCAAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGACTAAACGAGGCTAAGCAAGAACAGAACAGAACAGAACGAGCAATACAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAAGA	GAC 360 D 120
361 121	CTTTTTAACGATCCTTTGTGGAAAAATCACTGGTATTTGAGGAGACACAAGAGTCAACCCAAAGTTGCCAAAGCTGGATTTACATGTC/ L F N D P L W K N Q W Y L R D T R V N P K L P K L D L H V	ATA 450 I 150
451 151	$\begin{array}{c} \hline \hline CCAGTCTGGAGAAAGGGAATAACTGGCAAAGGCAGTGTTGTAACCGTTCTTGATGATGGTCTGGAATAGGAACCATACAGATATCTATG P V W R K G I T G K G S V V T V L Q D G L E W N H T D I Y \\ \hline \end{array}$	3TA 540 V 180
541 181	aactatgatccagaagcaagttatgactttaatgataatgacaagaccagacccgttcccaagatatgacattacaaatgagaacaagacaa \mathbf{N} \mathbf{X} \mathbf{D} \mathbf{P} \mathbf{E} \mathbf{A} \mathbf{S} \mathbf{X} \mathbf{D} \mathbf{F} \mathbf{N} \mathbf{D} \mathbf{N} \mathbf{D} \mathbf{F} \mathbf{P} \mathbf{R} \mathbf{X} \mathbf{D} \mathbf{I} \mathbf{T} \mathbf{N} \mathbf{E} \mathbf{N} \mathbf{K} \mathbf{H}	GA 630 G 210
631 211	acaagatgtgcaggagaagttgccatggttgccaaacaaccaccacatgtggagttggtggtggttgctttttaatgccaaagtaggaggcattg T R C A G E V A M V A N N H K C G V G V A F N A K V G G I	CGG 720 R 240
721 241	ATGTTAGATGGAGTTGTACTGATGCCATTGAAGCCAGCTCTATTGGATTTAATCCTCAGCATGTAGATATTTACAGTGCCAGCTGGG M L D G V V T D A I E A S S I G F N P Q H V D I Y S A S W	GA 810 G 270
811 271	CCAAATGATGGAGAAAAACAGTAGAAGGCCCTGGAAGATTAGCAGAAAAGGCCCTTTGAGTATGGCATCAAACAGGGGCGAAATGGA P N D D G K T V E G P G R L A E K A F E Y G I K Q G R N G	чаа 900 к 300
901 301	GGCTCAATCTTCGTTTGGGCTTCCGGTAATGGTGGCAGGAGAGAGGATAACTGTGACTGCGATGGATACACAGAGAGAG	ATC 990 I 330
991 331	TCAATCAGCAGTGCGTCACAACAAGGACTATCCCTTGGTATGCAGAAAAATGTTCTTCCACATTAGCACAGCAATACAGCAGTGGAGAS S S S S S S S S S S S S S S S S S	GAC 1080 D 360
1081 361	P rimer 2 TACACTGACCAGAGAATTGTCAGTGCTGATCTACATAATGACTGCCACATACTGGCACCTCTGCTTCCGCTCCATTGGCT Y T D Q R I V S A D L H N D C T E T H T G T <u>S</u> A S A P L A	GCT 1170 A 390
1171 391	$\begin{array}{cccc} GGGATCTTCGCTCTGGACAAAATCCTAACCTCACATGGAGGAGGATATGCAACACCTGGTTGTCTGGACAAGTGAGTATGATGATGATGATGATGATGATGATGATGAT$	CT 1260 P 420
1261 421	CTAGCAAATAATCCCGGCTGGAAGAAGAATGGAGCAGGTCTTATGGTCAACAGTCGCTTTGGGTTTGGACTACTGAATGCTAAGGCCC L A N N P G W K K N G A G L M V N S R F G F G L L N A K A	CTT 1350 L 450
1351 451	GTGGATTTAGCTGATCCTAAAACATGGAAAAACTGTACCTGAGAAAAAAATCTGCATTATTAAAGACAGTGACTTCACCCCCAGGCTT V D L A D P K T W K T V P E K K I C I I K D S D F T P R L	FTT 1440 F 480
1441 481	I LIGATCTGTGGGAGGAAATTACGATTGAGATCCCCACAAAGGCATGTGAAGGCCAAGATAATTATATATA	CTG 1530 L 510
1531 511	CAAGCCACTATCGAGTATACACGTCGAGGAGATCTCCACATTACACTAATTTCCCCATCAGAACCAAGACAGTCCTCCTGACTGA	AGA 1620 R 540
1621 541	GAAAGAGACACATCGACCAATGGTTTCAAAAACTGGGGGTTTATGTCTGTC	FTC 1710 V 570
1711 571	AAAATCACTGATGTGTCAAAAAAGATTGGAAAATGAAGGAAG	JAC 1800 D 600
1801 601	CACATGACAAATCCCAGAGTGTATACTTCCTACAATGTGGTTCAGAATGACCGAAGAGGTGTGGAGAAGTTAACAAACA	JAC 1890 D 630
1891 631	TCTTCCAATGAACAAATGAACAAAAGCAAAAGCAAAAGCAAAAGCAAAAGCAAAAGCGATGCTGCATCTTC S S N E Q I V T E K P T E N E E P E D P V K A K A M L H L	CTG 1980 L 660
1981 661	ANGANTGCTTTTGACAGAGAAGGGGCGCGCATTGCAGAAGAACAGGCCTAAAAATTCCTAAGACCCATTATTATCACGCTCTGCAAAAAG K N A F D R E G A A F A E E Q A K I P K T H Y Y H A L Q K	CTG 2070 L 690
2071 691	TACAAGCAGTCAGGTGCAAAGGACAAAGGGAACAATCTATACAATGACTATATTGACCGATTCTATAAAGAAGACCCCTATAAGCATA Y K Q S G A K D K G N N L Y N D Y I D R F Y N R R P Y K H	AGA 2160 R 720
2161 721	GATGATCGATTATTGCAAGCTCCCCGAATATTGTAGACAAGGATTCGTAGGATTAAATTTATCTTACAGTTGGAAACATGCCTCCAXDDR R L L Q A L L N I V D K D S *	ICC 2250 736
2251 2341 2431 2521 2611 2701 2791	$\label{eq:starter} AATACATTTTGATCTCCATAACCCTAGATTTCCATACAATTCGTTAAAATGACAAAAATTAATT$	AA 2340 FGA 2430 FTC 2520 ATC 2610 ATG 2700 AGG 2790 2869

Fig. 1. Nucleotide and deduced amino-acid sequences of bullfrog PC1 cDNA. The predicted amino acid is shown below the nucleotide sequence (DDBJ/EMBL/GenBank accession no. AB105175). The asterisk indicates the termination codon. Polyadenylation signal region is boxed. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by solid and dotted boxes, respectively. Triangles indicate putative *N*-glycosylation sites. Canonical integrin binding sequence indicates by a black background. Diamonds and white circles indicate Asp, His, and Ser of active site and tyrosine sulfation sites, respectively.

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Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	signal peptide 1: MEGGCWPYKYIALVS-VFSCCLGFVAPUERRYVNEWA-AEI-PG-GPEEALALADELGY-DYGGQIGSLPNHFLFKHRDHPRR-SRSAPHI 1:RCA, SI, CTAPAFCNW,A, NS, KAK, QF,,, VI,, A,, GT,, R, Q,, A, 1:RCA, SLQCTAFVLFCW,A, NS, KAK, QF,,,, Q, A, S, I, E,, LL,, E, Y,, KN,, F, 1:RCA, SLQCTAFVLFCW,A, NSVKAK, QF,,, Q, A, S, I, E,, LL,, E, Y,, KN,, F, 1:KQRG, TLQCTAFFLFCVW,A, NSVKAK, QF,,, A, S, I, E,, LL, E, Y,, KN,, L, 1:KQRG, TLQCTAFTLFCVW,A, NSVKAK, QF,,, A, S, I, E,, LL, E, Y,, KN,, L, 1:KQRG, TLQCTAFTLFCVW,A, NSVKAK, QF,,, A, S, I, E,, LL, E, Y,, KN,, L, 1:KQRG, TLQCTAFTLFCVW,A, S, SSYR-, Q, L,, -V,, A, S, I, E,, LL, E, Y,, KN,, L, 1:KQRG, TLQCTAFTLFCVW,A, -NSVKAK, QF,,, A, GCT, I, K, D, -QLVR, A, ED,, N, S, -MK, D, 1:CRG, TLQCTAFTLFCVW,A, -NSVKAK, QF,,, A, GCT, I, K, D, -QLVR, A, ED,, N, S, -MK, D, 1:CRFYAMULAIVAFS-S-H-GRAEDCEDQEDQFH-FT, T,, A, D, D, L, LDH, ENL, O,, N, S, -MK, D, 1:NL, S-LALLVLTVSCSDHIVRTISAQDDDGH-L,, V, -Q, -Q, -ESH, ESV, AQH, -TLVRALK, I, D, YVLRRS, T, H,, H, H 1: NYR, IYRRRYVFULLLVAVVNI, YGWTVLKNKYK,LSPSGVEKVRKHLSRKYVASRNNTQTF-KKHYFSNTWAV, IDPPDN, VAD, IAKKHGFTN	86 86 86 86 89 1 86 1 87 1 99
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	prosegnent 87: TKRL-YDDNRVSWAEQ-QYLKQRTKRGYVMNTDSEDLFNDPL#KNQWYLRDTRVNELPKLDLHYIPV@RKGITGKGS 87:SD.IE.E.SSALRDSAL-NM.NQQMTAAQV 87:SD.TE.E.SSVQKDSAL-NM.NQQMTAAQV 87:SD.TE.E.SSVQKDSAL-NM.NQQMTAAQV 87:SD.TE.E.SSVQKDSAL-NM.NQQMTAAQV 87:SD.TE.E.R.N.SVQKDSAL-NM.NQQMTASQV 87:SD.IE.E.R.N.ASVQKDSAL-NM.NQQMTASQV 87:SD.LE.R.N.ASVQKDSAL-NM.NQQMTASQV 87:SD.LE.R.N.ASVQKDSAL-NM.NQQTSSSQN.V 87:SD.LE.R.N.ASVQKDKXN.M.NQQTSSSQN.V 87:S	162 162 161 161 161 161 168 168 178 196
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	163: Image: Construction of the construc	262 262 261 261 261 268 268 268 278 295
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	263: DIVSASWGPNDDGKTWEGPGRIDAEKAFEYGI KQGRNCK-GSH FVWASGNCGRQCDNCDCDCWTDS HYTH SISSASQQGLSPWYAEKCSSTDATAY-SSG 263:	359 358 358 358 358 365 365 365 365 375 394
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	360:	456 455 455 455 462 461 472 490
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	P-domain *** 457: KTWKTVPEKKICIIKDSDFTPRLFRSVDEITIEIPTKACEGQDNYIKSLEHLQLEATIEYTRRGDLHITLISPSGTKT-VLLTERERDTSTNGFKNM 457: N. A 457: N. A 456: R. RS EVV. N. E. ALKANG.VI R. RS 456: R. RS EVV. N. E. ALKANG.VI R. RS 456: R. RS EVV. N. E. ALKANG.VIV. R 845: R. RN EVV. N. E. ALKANG.VIV. R 845: R. RN EVV. N. E. ALKANG.VIV. R 845: R. RN 845: R. RN 845: R. RN 84: R.	552 551 551 551 555 555 555 555 570 584
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	553: AFMSVHSWGEDPAGTWTVK ITDVSKRLENEGRI VNWKLVLHGTSTCPDHMTNPRVYTSYNVVQNDRRGVEKLTNIDE-DSSNEQI-V-T-E 553: T	: 639 639 649 649 649 657 654 635 1 674
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Amphioxus Aplysia Hydra	640:KPTENEEPEDPVK-AKAMLHLLKNAFDR-EGAA-FAEEQA-KIP-KTHYHALQKLYKQSGAKDKGNNLYNDYIDRFYNRF 640:T	2 715 715 732 732 731 753 748 2 688 2 771
Bullfrog Rana ridibunda Human Mouse Rat Anglerfish Anghioxus Aplysia Hydra	716: PY-KHRD-DR-L-LQALLNIVD-KDS 716:	736 753 753 752 775 774 703 793

Fig. 2. Comparison of the predicted amino-acid sequences of the bullfrog PC1 with those of other vertebrate PC1s. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by black and gray boxes, respectively. Diamonds indicate Asp, His, and Ser of active site of catalytic region. Canonical integrin binding sequence indicates by asterisks. The amino acid residues that match those of bullfrog PC1 are shown as dots. Gaps, indicated by dashed lines, have been introduced to obtain maximum homology. The sequences for *Rana ridibunda* (Gangnon *et al.*, 1999), human (Creemers *et al.*, 1992), mouse (Seidah *et al.*, 1991), rat (Hakes *et al.*, 1991), anglerfish (Roth *et al.*, 1993), *Amphioxus* (Oliva *et al.*, 1995), *Aplysia* (Gorham *et al.*, 1996), and Hydra (Chan *et al.*, 1992) PC1s are shown.

and the deduced amino acids. The cDNA consisted of a 5'untranslated region of 103 bp and a 3'-untranslated region of 658 bp followed by a poly (A) tail. An open reading frame of 2208 bp encoded a protein of 736 amino acids (with a calculated Mr of 82876 and an isoelectric point of 5.86), consisting of a signal peptide of 26 amino acids and a mature peptide of 710 amino acids. The 3'-noncoding sequence contained a consensus polyadenylation signal (AATAAA)

and a poly (A) tail. The protein contained the Asp-168, His-209, and Ser-383 residues found in the catalytic triad of serine proteinases of the subtilisin family. There were 2 putative *N*-linked glycosylation sites, at Asn-174 and Asn-402, in the predicted amino acid sequence of the bullfrog PC1. The PC1 contained a canonical integrin binding sequence (Arg-Gly-Asp⁵²¹). Also, putative sulfation sites were found at Tyr-54, Tyr-182, Tyr-188, Tyr-201, Tyr-323,

-155 -90	AGGI	AGG	CGC	GGA	GGA	.GCC	CCA	CCG	CG ACA	CGG ACT	CCG ACA	CGT CAG	CGA CTC	CGG CGG	CAT	CAG. CTC	AAG CTC	TAA' TCC.	TCT ACT	CAC	ATA CTC	CAT CCC	AGA TCA	GCG CCT	GAG(FCC(CGG	AGA ACA	CATO	GTA FGA	.CTG .GAG	-91 -1
1 1	ATGA M	AGA R	GCA A	AGC S	TCC S	CCG P	CTC L	CGG R	GCT A	GTG V	CTG L	GCG A	GCA A	TTGL	CTG L	CTC. L	ATA I	CAG' Q	TAC. Y	ATCO I	GCC' A	TCG S	ACC T	CAC' H	rcco S	GCT А	CTC L	CTCI L	ACC T	CAA Q	90 30
91 31	CAA'I Q	PAC Y	TTG L	GTC V	GAC D	TTA L	.caa Q	CCA P	GGA G	.GGA G	GAG E	CCG P	ACA T	GAC D	GCC(A	GCA A	CAA Q	CTC L	GCC A	CAA(Q	CAG' Q	ГАТ Ү	GGA' G	TTC. F	ACT(T	GGG G	GCC. A	AGG2 R	AAG K	CTA L	180 60
181 61	CCC1 P	FTT	TCG S	GAI D	'AGT S	TTA L	TAC Y	CAT H	TTC F	TAT Y	GAC. D	AGT S	'GGA G	IGCA A	ACT/ T	AAG K	TTC. F	AGG. R	AGG. R	AAA K	CGA/ R	AGC S	CTC. L	AAC. N	AGT/ S	AAG K	AAT N	CAC' H	ΓTΑ L	TCC S	270 90
271 91	ATGO M	CAC H	CCC P	AAC K	GTT V	CGG R	AAA K	GTT V	GTT V	CAG Q	CAA Q	GAA E	.GGT G	TTT F	GACI D	AGA. R	AAG. K	AAA K	CGA R	GGG' G	TAC Y	AGG R	GAC. D	ATC. I	AAT(N	GAC. D	ATC I	GACI D	ATC I	AAT N	360 120
361 121	ATG M	AT N	GAT D	P P	CTA L	TTT F	ACA T	AAA K	CAG Q	TGG' W	TAT Y	CTĜ L	ATC I	AAC N	ACA(T	GGT G	CAA Q	GCA A	GAT(D	GGG; G	ACT(T	CCA P	GGT G	CTT L	GAT' D	FTG L	AAT N	GTT(V	GCT A	GAA E	450 150
451 151	GCC1 A	rgg W	GAG E	TTC L	GGA G	TAC Y	ACG T	GGG. G	AGA R	GGA G	GTT. V	ACC T	ATA I	IGCA A	ATT I	ATG M	GAT	GAT(D	GGAJ G	ATA I	GAT' D	TAC Y	CTT L	CAC H	CCA(P	GAT D	CTT L	GCC' A	ГСА S	AAT N	540 180
541 181	TATA Y	AAT N	GCA A	GAG E	GCA A	AGC S	TAT Y	GAC D	TTT F	AGC. S	AGC. S	AAT N	GAC D	CCT P	TAT(Y	CCA P	TAT Y	CCC P	CGC' R	TACI Y	ACT(T	GAT D	GAC' D	TGG' W	FTT F	AAC. N	AGC S	CAT(H	GGG G	ACA T	630 210
631 211	CGC1 R	C C	GCG A	GGG G	GAA E	GTA V	TCT S	GCT A	GCT A	GCA. A	AAC. N	AAT N	'AAT N	'ATT I	TGC(C	GGG G	GTG V	GGA(G	GTT(V	GCT' A	TATZ Y	AAC' N	TCT. S	AAA K Pr	GTT(V	GCT A	GGT. G	ATT(I	CGC R	ATG M	720 240
721 241	CTAC L	BAC D	CAG Q	CCC P	TTT F	ATG M	ACA T	GAT. D	ATA I	ATT I	GAA E	GCA A	TCT S	'TCA S	ATC/ I	AGT S	CAT. H	ATG M	CCA(P	CAA) Q	ATTZ I	ATA I	GAC. D	ATC I	TAC Y	AGC S	GCC. A	AGC' S	rgg W	GGG G	810 270
8 1 1 271	CCC# P	ACA T	GAC D	GAI D	'GGC G	AAG K	ACA T	GTG V	GAT D	GGG G	CCC. P	AGA R	.GAA E	CTG L	ACC' T	TTA L	CAG Q	GCC. A	ATG M	GCA(A	GAT(D	GGT G	GTA. V	AAT. N	AAG(K	G G	CGC(R	GGT(G	GGA G	AAA K	900 300
901 301	GGAA G	AGT. S	ATT I	TAT Y	GTC V	TGG W	GCG A	TCT S	GGA G	GAT D	GGA G	GGA G	AGT S	TAT Y	GAT(D	GAC' D	TGT. C	AAC' N	rgt(C	GAT(D	GGT' G	TAT Y	GCA A	TCC. S	AGC/ S	ATG M	TGG. W	ACC/ T	ATC I	TCC S	990 330
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991 331	ATAA I	AAC' N	TCT S	GCT A	TTA' T	AAT N	GAT D	GGT G	CGG R	ACT T	GCA A	CTA L	TAC Y	GAT D	GAAJ E	AGC' S	TGC' C	TCT' S	FCC S	ACT" T	TTA L	GCC A	TCT. S	ACA' T	FTC F	AGC. S	AAT N	GGA/ G	AGA R	AAA K	1080 360
991 331 1081 361	ATAA I CGAA R	AAC' N AAT' N	TCT S CCT P	GCT A GAA E	I I GCT A	AAT N GGC G	GAT D GTG V	GGT G GCT. A	CGG R ACA T	ACT T ACA T	GCA A GAT D	CTA L TTA L	TAC Y TAT Y	'GAT D 'GGA G	GAAI E AAC' N	AGC S TGC. C Pri	TGC C ACT T	TCT S TTA L	FCC S CGT R	ACT T CAC H	TTAC L TCAC S	GCC A GGA G	TCT. S ACA' T	ACA' T TCT S	FTCZ F GCG(A	AGC S GCA A	AATO N GCAO A	GGAZ G CCA(P	AGA R GAA E	AAA K GCA A	1080 360 1170 390
991 331 1081 361 1171 391	ATAA I CGAA R GCTO A	AAC N AAT N GGG	TCT S CCT P GTG V	GCT A GAA E TTT F	IGCT A GCA A	GGC G TTA L	GAT D GTG V .GCC A	GGT G GCT. A CTA L	CGG R ACA T GAG E	ACT T ACA T GCA	GCA A GAT D AAT N	CTA L TTA L CCA P	TAC Y TAT Y .GGT G	GAT D GGA G CTC L	GAA E AAC' N ACT' T	AGC S TGC. C Pri TGG. W	TGC C ACT T Mer AGG R	TCT S TTA L S GAC D	FCC S CGT R FTG L	ACT T CAC H CAAC	TTAC L TCAC S CAC H	GCC A G G ITA L	TCT. S ACA' T ACA' T	ACA' T TCT S GTG V	F F GCG(A CTAJ L	AGC. S GCA A A A CA T	AATO N GCAO A TCC. S	GGAJ G CCAC P AAAJ K	AGA R GAA E AGG R	AAA K GCA A AAC N	1080 360 1170 390 1260 420
991 331 1081 361 1171 391 1261 421	ATAZ I CGAZ R GCTO A CAGO Q	AAC N AAT N G G CTG L	TCT S CCT P GTG V CAC H	GCI A GAA E TTT F GAI D	GCT A GCA CGCA A CGAA E	GGC G TTA L GTT V	GAT D GTG V GCC A CAT H	GGT G GCT. A CTA L AAG K	CGG R ACA T GAG E TGG W	ACT T ACA T GCA A CGT. R	GCA A D AAT N AGG. R	CTA L TTA L CCA P AAT N	TAC Y TAT Y 	GAT D GGA CTC L GTT V	GAA E AAC' N ACT' T GGT' G	AGC S TGC. C Pri TGG. W TTG	TGC C T T AGG R GAG E	TCT S TTAC L SAC D TTT F	FCC2 S CGT0 R FTG0 L AAC0 N	ACT T CAC H CAA Q CAC H	TTAC L TCAC S CAC H TTG	GCC A G TTA L F F	TCT. S ACA' T ACA T GGC' G	ACA T TCT S GTG V TAT Y	F F GCG(A CTA L GGT(G	AGC. S GCA A ACA T GTA V	AATO N GCAO A TCC: S CTGO L	GGAZ G CCAC P AAAZ K GATC D	AGA R GAA E AGG R GCT A	AAA K GCA A AAC N GGC G	$1080 \\ 360 \\ 1170 \\ 390 \\ 1260 \\ 420 \\ 1350 \\ 450 \\ $
991 331 1081 361 1171 391 1261 421 1351 451	ATAZ I CGAZ R GCTO A CAGO Q TCTZ S	AAC'N NAAT'N GGGG G CTG L ATG M	TCT S GTG V CAC H GTT V	GCT E TTT F GAT D AAA K	GCT A GCA CGCA A GAA E ATG M	GGC G G TTA L GTT V GCA A	GAT D GTG V GCC A CAT H CGA R	GGT GCT A CTA L AAG K GAA E	CGG R ACA T GAG E TGG W TGG	ACA T GCA A CGT R AAAA K	GCA A D AAT N AGG. R ACT T	CTA L CCA P AAT N GTT V	TAC Y GGT GGT G CCA P	GAT GGA G CTC L GTT V GTT V GAA E	GAA E AAC' N ACT' T GGT' G G AGA' R	AGC S TGC. Pri TGG. W TTG L TTG F	TGC C T T AGG R GAG E CAC H	TCT S TTAC L GAC D TTT F TGT C	FCC2 S CGTC R FTGC L AACC N AATTC I	ACT T CAC H CAAC Q CAC H GGTC G	TTAC L TCAC S CAC H TTG L GGA G	GCC G G ITTA L ITTT F ICA S	ACA' T ACA' T ACA T GGC' G G G	ACA T TCT S GTG V TAT Y CAG	F GCGC A CTAL CTAL G G G G G G G G G G G G G G G G G G G	AGC. S ACA T STA V CCA P	AATO N GCAO A TCC. S CTGO L AGG. R	GGAI G CCAC P AAAA K GATC D AAAA K	AGA R GAA E AGG R GCT A ATA I	AAA K GCA A AAC N GGC G CCT P	1080 360 1170 390 1260 420 1350 450 1440 480
991 331 1081 361 1171 391 1261 421 1351 451 1441 481	ATAZ I CGAZ R GCTC A CAGC Q TCTZ S	AAC'N NAAT'N GGG CTG L XTG M SAT'D	TCT S CCT P GTG V CAC H GTT V GGA G	GGCT A GAA E TTTT F CGAT D AAA K K	GCT A GCA A CGAA E ATG M TTG L	AAT N GGCC G TTA L GTT V GCA A GCA M	GAT D GTG V GCC A CAT H CCAT R CCAC L	GGT G GCT A CTA L AAG K GAA E GAA T	CGG R ACA T GAG E TGG W TGG W CTT L	ACT T ACA T GCA A CGT R AAA K ACA T	GCA A GAT D AAAT N AAGG. R ACA T ACA	CTA L TTA L CCA P AAT N GTT V GAT D	TAC Y TAT Y GGT G CCA P GCT A	GGAT D GGAA G CTCC L U GGAT V GGAA E TGT C	GAAC N ACT GGT GGT R AGA R GAAC E	AGC' S TGC. C Pri TGG. W TTG L TTTG F GGA. G	TGC' C T T AGGG R GAG' E CAC' H AAGG K	TTTA L 2 GAC' D TTTT: F TTTT: C GAAA E	ICC/ S CGTV R ITGG L AAACO N AAACO N	ACT T CAC H CAA Q CAC H G G TTTC F	L TCAC S CAC: H TTG L GGA: G GTTC V	GCC A G TTA C TTA F F TCA S CGA R	TCT. S ACA T ACA T GGC' G ATA I I TACY Y	ACA T TCTC S GTG V TAT Y CAG Q CTTC L	FTC/ F GCG(A CTAA L GGT(G G G G G G G G G G G G G G G G G	AGC. S GCA A ACA T STA V CCA P CAT H	AAT N GCA A TCC S CTG L AGG R GTCC V	GGAA G CCAC P AAAAA K GATC D AAAAA K CAAC Q	AGA R GAA E AGG R GCT A TA I GCG A	AAA K GCA A AAC N GGC G CCT P GTT V	1080 360 1170 390 1260 420 1350 450 1440 480 1530 510
991 331 1081 361 1171 391 1261 421 1351 451 1441 481 1531 511	ATAZ I CGAZ R GCTC A CAGC Q TCTZ S TCTZ S ATAZ I	AAC N AAT N GGG G CTG L XTG M SAT D	TCT S CCT P GTG V CAC H GTT V GGA G G G TA V	GCT PGAA E TTT F GGAT D CAAA K AAAC K AAAC	GCT I GCT A GCA A GCA A C GCA A C GCA A C C C A C C C C	AAT N GGCC G TTA GTT V GCA A GCA M ATG M Y ACA T	GAT D GTG V GCCA H CAT H CGA R CTC L AGG R	GGT GCTA L AAG K GAA E ACA T CGT	CGG R ACA T GAG E TGG W TGG W TGG W CTT L GGA G	ACT T ACA T GCA A CGT R AAAA K ACA T GAC	GCA A GAT D AAAT N AGG. R ACT T T ACA T T I, I,	CTA L TTA L CCA P AAT N GTT V GAT D AAC N	TAC Y TAT Y GGT G G CCA P GCT A CCA I ATC I	GAT D GGAA G CTC L V GTT V GAA E TGT C C AAT	GAAJ E AACT T GGT G GGT R AGAA R GAAA E ATGJ	AGC'S S TGC. C Pri TGG. W TTG TTG GGA. G GGA. G ACA' T	TGC' C ACT'' T AGGG R GAG' E CAC' H AAAG' K S	TCT'S STACL C GAC'D TTTT: F GAAJ E CCCG; P	ICC. S CGTV R ITTG/ L AAAC(N AATT(N AATG(M	ACT" T CAC' H CAAC Q CAC' G G G G G G G G G G G G G G G G G G G	TTAC L TCAC S CAC H TTG G G G G G G G G G T C CAC L C C C C C C C C C C C C C C C C	GCC A G G TTA L TTT F F S CGA R AAAA K	TCT. S ACA' T GGC' G GGC' I TAC' Y TAC' S	ACA'T TCT S GTG V TAT Y CAGG Q CTT L L ATC I	FTC/ F GCG(A CTA/ L GGT(G G GAG(E CTC(C L	AGC. S GCA ^I A ACA T STA ^I V CCA P H CCA H	AAT N GCA A TCC: S CTG L AGG R GTC V V AGT S	GGAJ G CCAC P AAAAJ K GATC D K CAAC Q CGCC R	AGA R GAA E AGG R GCT A I GCG R CGT R	AAA K GCA A AAC N GGC G CCT P GTT V CCA P	1080 360 1170 390 1260 420 1350 450 1440 480 1530 510 1620 540
991 331 1081 361 1171 391 1261 421 1351 451 1441 481 1531 511 1621 541	ATAF I CGAA R GCTC A CAGC Q TCT7 S TCT7 S TCT7 S I I AGGC R	AAC N N GGG CTG L CTG CTG M SAT D SAT T D SAC D	TCT S CCT P GTG V CAC H GTT V GGA G GAT D	GCT A GAA E TTTI F GAT D CAAA K AAG K AAG K AAG K AAG K O CAC D	GCA GCA A GCA A GCA A GCA A GAA E M TTG L TTG L TCT S	AAT N GGCC G TTA L GTT V GCA ATG AATG M AATG M AAAA K	GAT D GTG V GCC A CAT H CCAT H CCAT CCAT CCAT R CCAC R GTT V	GGT G GCT. A CTA L AAG K GAA E ACA T GGT GGT G	CGG R ACA T GAG E TGG W TGG W CTT L GGA C TTT F	ACT T GCA CGT. R AAAA. K ACA. T GAC D	GCA A GAT D AAT N AGG. R ACT T T ACA T T T T C AAAA K	CTA L TTA L CCA P AAT N GTT V GTT D AAC N TGG W	TAC Y TAT Y GGT G GGT A Y CCA P GCT A Y ATC I Y CCA P	GGAT D GGGA G GTT V GTT V GGAA E TGT C C C AAT N F	GAAL E AACT T GGT GGT GGT R GAAC E ATGI M ATGI M	AGC S TGC. C Pri TGG. W TTG L TTT F GGA. G ACA T ACA. T	TGC' C T T AGGG R GAG' E CAC' H AAAG K TCAC S S ACT' T	TCT S TTA L S GAC' D TTT F TGT. C GAAJ E CCCG. H	ICC2 S CGTV R PTG L AACC N AATTC N AATCG M AACC T	ACT" T CAC' H CAAC Q CACC H G G G G G G G G G G G G G G G G G G	FTAC L TCAC S CAC H TTG G G G G G G T C G G C C C C C C C C	GCC A G G TTA C G TTT F F C G A A A A A A A A A A A A A C G A C G A C C A C C C A C C C C	ACA' T ACA' T GGC' G ATA' I TAC' Y TCC. S GAC' D	ACA'T TCTO S GTGO V TATO Q CAGO Q CTTO L ATCO I CCAL P	FTC/ F GCG(A CTAI L GGT(G G G G G G G G G G G G G CTC(C L CTC(R CTC) C CTC(C C C C C C C C C C C C C C C C	AGC. S GCA A ACA T STA V CCA P CAT H CTG. L GGA G	AAT N GCA A TCC. S CTG L AGG. R GTC V AGT S AGT AGT	GGAJ G CCAC P AAAAJ K GATC D AAAAJ K CAAC Q CGCC R IGGC W	AGA R GAA AGG R GCT A TA ATA I GCG R CGT R V	AAA K GCA A AAC N GGC G CCT P GTT V CCA P CCTT L	1080 360 1170 390 1260 420 1350 450 1440 480 1530 510 1620 540 1710 570
991 331 1081 361 1171 391 1261 421 1351 451 1441 481 1531 511 1621 571	ATAF I CGAP R GCTC A CAGC Q TCTF S TCTC S ATAF I AGGC R R GAGF E	AAC'N NAAT'N SGGG G CTG'C L XTG' D SAT' D SAT' D SAC'I D	TCT S CCT P GTG V CAC H GTT V GGA G GTA V GGA T D GGT G G T C CAC H	GCT A E TTTT F CGAT D AAAA K AAAC K AAAT N GAC D	GCT GCA CGAA CGAA CGAA CTTG CTCT S CTCT S CGTT V	AAT N GGC G TTA L GTT V GCA A T AATG M AACA T AAAA K GGGG	GAT D GTG V GCC A CAT H CGA R CCA R CCA C CAT H CCA C CAT C CA C CAT C CA C CA	GGT G GCT. A CTA' L GAAG K GAAG K T GGAA T CGT' R GGT G G T G GT G	CGG R ACA T GAG E TGG W TGG W CTT L GGA C TTT F CCA	ACA T GCA. A CGT. R AAAA K ACA. T GAC. D GAA. D	GCA A GAT D AAT N AGG. R ACA T T T T G. L AAA K K AAG K	CTA L ITA L CCA P AAT N GAT D AAC N TGG W GGT GGT	TAC Y TAT Y GGT GGT A P GCTA P GCTA P CCAA P CCAA P GTA V	GGAT D CGGA G CTC L C GAA E TGT C C AAAT N M TTC F TTC L	GAAJ E AACT T GGT G GGT R GAAG E ATGJ M ATGJ M AAAA(K	AGC'S S TGC. C Pri FGG. W TTG L TTT'F GGA. G ACA' T ACA. T GAA'	TGC' C T T GACT' T GAGG R GAGG R CACC H H TCAC' T T CAC' T T CAC' T T GG C CAC'	TCT S TTA L C GAC D TTTT C C GAAJ E CCCGJ P CACC H ACCC T	ICC. S CGT(R ITG(L AAC(N AATT(I AATT(M AATG(M AATG(T T CTG, L	ACT" T CAC' H CAAC Q CAC' H G G G G G G G G G G G G G G G G G G	ITAC L ICAC S CAC: H ITG G G G G G G G C T G G A CAC A CAC T G G C T C C C C C C C C C C C C C C	GCC'A GGA G TTA L TTT F CGA R CGA K GAAA E CAT H	TCT. S ACA' T GGC' G G TACA' T TACA' Y TCC. S GAC' D GGAC D GGAAG	ACA T TCT S GTG V TAT Y CAGG Q CTT L ATC I CCA P ACT	FTCZ F GCG(A CTAJ L GGT(G GAG(E GAG(E CTCC L CTCC R CAAJ Q	AGC. S GCA A T STA V CCA P CAT H CTG. L GGA G	AAT(N GCA(A TCC: S CTG(L AGG; R GTC(V AGT(S AGT(T CCC(A	GGAJ G CCAC P AAAAJ K GATC D K CAAA Q CGCC R IGGC W CCCC P	AGA R GAA E AGG R GCT A TA CGT R GCG A CGT R CGT R CGT R CGT R CGT R	AAA K GCA A AAAC N GGC G GTT V CCT P GTT V CCA P CTT L L ATA I	$1080 \\ 360 \\ 1170 \\ 390 \\ 1260 \\ 420 \\ 1350 \\ 450 \\ 1440 \\ 480 \\ 1530 \\ 510 \\ 1620 \\ 540 \\ 1710 \\ 570 \\ 1800 \\ 600 \\ 1$
991 331 1081 361 1171 391 1261 421 1351 451 1441 481 1531 511 1621 571 1801 601	ATAZ I CGAZ R GCTC A CAGC Q TCTZ S ATAZ I CAGC R GACGQ R GACCQ D	AAC N N GGG CTG CTG CTG L CTG D CTG D SAC D SAC D SAC I CAA Q	TCT S CCT P GTG V CAC H GTT V GGA G GTA D GGT G G GTA I I	GCT A GAA E TTTI F GAT D CAAA K AAAG K AAAG C C D TTTI F GAC D TTTI F	GCT GCA GCA GCA C GCA C GCA C C GCA C C C C	AAT N GGCC G TTA L GTT V GCA A T AATG M AATG M AACA T AAAA K GGGG G GAT D	GAT D GTG V GCC A CAT H CCAT R CCAT R CCAT R CCAT R CCAT R CCAT S CTC L S CTC C C T C C C C T C C C C C C C C C	GGT GCT. A CTA L AAG K GAA T CGT R GGT G GGT G ATG M CAG Q	CGG R ACA T GAG E TGG W TGG W CTT L GGA G TTTT F CCA P TCA S	ACT T GCA A CGT. R AAAA K ACA. T GACA D GAA. D GAA. CGT. K	GCA A GAT D AAT N AGG R ACT T T ACA T T T C AAAA K K AAAG K T T TA	CTA L L CCA P AAT V GAT D GAT D AAC N GGT G G G G G CT A	TAC Y TAT Y GGT G GGT CCA P GGT A CCA P GCT A V V TATG M	GAT D GGA G CTC L GTT V GAA E TTGT C TTGT F TTG L L TTC S	GAAJ E N AAC ^T T GGT ^T GGT ^T GAAG E AAGA ^T M ATGJ M AAGA ^T K	AGC'S S TGC. C Pri TGG. W TTG L TTT'F F GGA. G ACA'T T CAAAG K	TGC' C T T AGG R GAG' R GAG' H AAGG K T CAC' H T CAC' T T CAC' C ACT' T T GAG' C ACT' T T GAG' C ACT' T	TCT S TTAC C GAC' TTT. C GAAJ E CCCGJ H CACC T C C C C C C C C C C C C C C C C	ICC, S CGTV R FTG R FTG AACC N AATG N AATG M AACC T T CTG L	ACT T CAC H CAC Q CAC H G G G G G G G G G G G G G G G G G G	ITTAC ICAC S CACT H ITTC G G G TTC G G G CTTCC G G CTTCC G CTTCC G CTTCC G CTTCC G C CTTCC G C C C C	GCC A G G TTA L TTT F F CGA K SAAA K GAAA K GAAA K GAAA K	TCT. S ACA' T ACA' T GGC' G GC' G GCC G GGA. G CTG L	ACA T TCT S GTG V TAT V CAG Q Q CTT L CCA P CCA T CCA S ACC D	FTC2 F GCGQ CTA2 CTA2 G G G G G G G G G G G G G G G G CTCQ C CA2 C C C C C C C C C C C C C C C C C	AGC. S GCA' A T STA' V CCA P CCCA P CCCA P CCCA CCCA S G G G G G G G G CCTA S G CCTA S CCA CCCA CCCA CCCA CCCA CCCA CCC	AAT N GCA TCC. S CTGC L AGGC V AGT S GTCC V AGT GCCC A GTCC V	GGAJ G CCAC P AAAAJ K GATC D AAAAJ K CAAC Q CCCCC P GAAAJ E	AGA R GAA E AGG R GCT A TAC ATA I GCG R GTT V V FAC Y AGA R	AAA K GCA A AACC N GGC G CCTT P CCTT V CCA P CTTT L ATA I AGC S	$1080 \\ 360 \\ 1170 \\ 390 \\ 1260 \\ 420 \\ 1350 \\ 450 \\ 1440 \\ 480 \\ 1530 \\ 510 \\ 1620 \\ 540 \\ 1710 \\ 570 \\ 1800 \\ 600 \\ 1890 \\ 630 \\ 1890 \\ 630 \\ 1890 \\ 630 \\ 1800 \\ 600 \\ 1890 \\ 630 \\ 1800 \\ 1800 \\ $
991 331 1081 361 1171 391 1261 421 1351 451 1441 481 1531 511 1621 571 1801 601 1891 631	ATAA I CGAA R GCTC A CAGC Q TCTA S TCTC S ATAA I AGGC R GAGGA E GAGCQ D TTGZ L	AAC N N GGG G CTG G CTG C L M CTG D C T G D C T G D C T G C T G C T G C T G C T G C T G C T G C T G C C C C	TCT S CCT P GTG CAC H GTT V GGT GGT GGT D GGT D GGT A TA A GT S	GCT A E TTT F CGAT D CAAA K A CAAA K A CAAA K A C C C TTTT F GTT V V CTAL	ATT I GCT A GCAA A GGAA E GGAA E ATG L TTTG S GTT V V AGAA R L	AAT N GGCC G TTA L GTT V GCA A T GGCA M AATG M AATG G A T C GGG G G T D D C C T	GAT D GCC A CAT H CCAT H CCAT CAT CCAT C A CCAT C CAT C CAT C CAT C CAT C CAT C CAT C CAT C CAT C CAT C CAT C CAT C C C C	GGT A CTA L AAG K GAA E GAA T GGA T GGT G CAG Q Q AAC N	CGG R ACA T GAG E TGG W TGG W CTT L GGA CTT F CCA F CCA F CCA F CCA TTCA S	ACT T GCA CGT R AAAA K ACA T GAC D GAA E AAAA K CAC	GCA A GAT D AAT N AGG R ACT T T ACA T T AACA T L AAAA K AAG K L CGC	CTA L L CCA P AAT N GTT D AAC N GGT G G G G G G C T A	TAC Y TAT Y GGT G G G G G G C CCA P G G C CCA P G G C CCA P G G C C A C C A C C A C G G G G G G G G	GGAT GGA G GGGA G GGAA E TGT C C C C C C C C C C C C C	GAAJ E AAC ^T T GGT ^T GGT ^T R GAAG E ATGJ M AAAG K K CTAG	AGC'S S TGC. C Pri TGG. W TTG L TTTG GGA. T T GGA. T C C C T C C C C C C C C C C C C C C	TGC' C T T AGCT' T GAG' E CAC' H CAC' H CAC' T T CAC' T TGG. W GAGG E TAT.	TTTA L C C C C C C C C C C C C C	PCC, S CGTV R FTG R ATTG I AACC N ATTG N ATTG M CTG CTG L CTGG L FTC	ACT" T CAC' H G G G G G G G G G G G G G G G G G G	FTAC L S CAC' S TTG' G G TTG' G G G G G G C TGG G C TGGAC E TTC'	GCC G G ITTA ITTA ITTA ITTA ITTA ITTA ITTA	TCT. S ACA' T GGC' G G G ATA' T ATA' T ATA' T ATA' S GAC' D GGA G G C TGC L ATT'	ACA T TCTV S GTG V V TATC Q CTTV L ATCC I CCAL P ACT T GACC	FTC2 F GCG0 A CTA2 G G G G G G G G G G A G C TC0 C C A G C C C C C C C C C C C C C C C C	AGC. S GCA A ACA T STA V CCA P CAT H CTG L SGA G G S GCT A ATA	AATC N GCA A TCC: S CTG L AGG: R GTCC V A GTCC V A GTCC V TCT C TCT	GGAJ G P AAAAJ K GATC D AAAAJ K CAAC Q CCCC P CCCC P GAAAJ E TTCC	AGA R GAA E AGG R GCT A ATA I GCG A CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R CGT R C A A A G A A A G A A C C A A C A A C C A A C C A A C C A A C C A A C C A C C A C C C A A C C C A C C C A C C C C A C C C A C C C C C A C	AAA K GCA A AAAC N GGC G G T V V CCA P CTT L ATA I AAC S CAC	1080 360 1170 390 1260 420 1350 450 1440 480 1530 510 1620 540 1710 570 1800 600 1890 630

Fig. 3. Nucleotide and deduced amino-acid sequences of bullfrog PC2 cDNA. The predicted amino acid is shown below the nucleotide sequence (DDBJ/EMBL/GenBank accession no. AB105176). The asterisk indicates the termination codon. Polyadenylation signal region is boxed. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by solid and dotted boxes, respectively. Triangles indicate putative *N*-glycosylation sites. Canonical integrin binding sequence indicates by a black background. Diamonds and white circles indicate Asp, His, and Ser of active site and tyrosine sulfation sites, respectively.

	signal poptide	
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	1: <u>MRAS-SPLRAVLA_ALLLIQ-YIASTHSALLTQQYLVDLQPGGEPTDAAQLAQQYGFTGARKLPFSDSLYHFYDSGATKFRRKRSI</u> 1: .KGL 1: .KGL 1: .KGCVTVW-M 1: .KGCVSOWKAAGFFCVMVF.AERPVFNHFE.HKEDK.R.V.AEHVAEGHN.LA.AK.R 1: .EGGCSOWK-AGFFCVMVF.AERPVFNHF.E.HKEDK.R.V.AEHVAEGHN.LA.AK.R 1: .EGGCSOWK-AGFFCVMVF.AERPVFNHF.E.HKEDK.R.V.AEHVAEGHN.LA.AK.R 1: .KGGCSOWK-AGFFCVMVF.AERPVFNHF.E.HK.AEL.RK.VEHVAEGHN.LA.AK.R 1: .KVPVGWFLLRVQYLV.AEGHN.LA.AK.R 1: .VKPVGWFLLRVQYLV.AELATLVWYGACNSAPPY-TNDFAVQIRD.K.DT.EL.RK.VLMLGQIREQRDRH.VPHV.R.A 1: .SIFF.GWPHKVLLPC-LFWAPWVPGHGRQIDVVTNHW.E.SHD.G.EL.KRV.RDT.SYVGPVLS.RTF.VHH.VAHA.SR.M 1: .NSFFLGWSRKVLVSC-L	83 81 84 83 82 82 82 82 82 82 82 82 82 82 82 82 82
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	prosegment 84:NSKNHLSMHPKVRKVVQQEG-FDRKKRGY-RDINDI-DINMN-DP-LFTKQWYLINTGQADGTPGLDLNVAEAWELGYTGRGWT1AIMDC 82:K	<pre>169 167 167 170 169 168 168 168 168 188 189 189</pre>
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	170: TO: TO: D. 168:	269 267 270 269 268 268 268 268 276 288 289 289
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	270: GETDDCKTVDGPREITIQAMACUNKGRGEGESIVWASGDCCSVDDCNCDGYASSMWTISINSAINDGRTAFYDESCSSTFASTSSNGRKRNPEAGVA 268: 270: N. DV. 270: N. DV. 269: N	369 . 367 . 370 . 369 . 368 . 368 . 368 . 368 . 376 . 388 . 389 . 389
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	370: TOUYCHTERGUTSAAADEAAQVFALALSANEGLTWRDLQHL T T 368: T T T 371: S T T 370: L M. T 369: LD M. T 369: VD M. T 377: K Q. N. 377: K Q. N. 389: H Y. S V. 390: N. AS M. S.Y. 390: N. AS RN M. S.Y. 390: N. AS RN M. S.Y. 390: N. AS RN M. S.Y. S.Y.	1 467 . 465 . 468 . 467 . 466 . 466 . 474 3 486 . 489 . 489
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	P-domain *** 468: CIGGSIQEPRKIPSDCKLMLTTTDACEGKENFVRYLEHVQAVITVNSTRRGDLNINMTSPMCTKSILLSRRPRDDDS-KVGFDKWPFMTTHTWGEDPRC 466: N. 466: V. N. 467: V. V. A. 467: V. V. 475: T. M.SARP. 475: T. M.SARP. 487: EA.RTTKQYI. T. EV. 487: EA.RTKQYI. T. E. 487: EA.RTKQYI. T. E. 487: EA.RTKQYI. T. E. 490: KA. TVSAEKEFTFGKP.RMSIES.G. T. 490: KA. TVSAEKEFTFGKP.RMSIES.G. T.	 566 564 567 565 565 573 584 588 588
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	567: TWVLEIG-FVGSMPEKGVLKE-WTLMLHGTQSAPYIDQIVRDYQSKLAMSKK-EEL EEELDEAVE 565: 566: TL. 566: 566: 5674: D 574: D 586: 586: 566: 567: 566: 5674: D 574: D 585: S 585: S 585: S 589: S 589: 589: 589: 589: 589: 589: 589: </td <td>- 628 - 629 - 629 - 627 - 627 - 627 P 667 - 639 - 647 - 647</td>	- 628 - 629 - 629 - 627 - 627 - 627 P 667 - 639 - 647 - 647
Bullfrog Rana ridibunda Xenopus Human Mouse Rat Amphioxus Ascidian Aplysia Snail	629:-RS-LKSLL-TK-N 627:SS 629:Q.IR 628:Q.IR 628:63:KKEGGS.W.SV.P.YMIGL 648:SGVIDE 648:SGVVQE	638 639 638 637 637 688 642 653 653

Fig. 4. Comparison of the predicted amino-acid sequence of the bullfrog PC2 with those of other vertebrate PC2s. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by black and gray boxes, respectively. Diamonds indicate Asp, His, and Ser of active site of catalytic region. Canonical integrin binding sequence indicates by asterisks. The amino acid residues that match those of bullfrog PC2 are shown as dots. Gaps, indicated by dashed lines, have been introduced to obtain maximum homology. The sequences for *Rana ridibunda* (Vieau *et al.*, 1998), *Xenopus* (Braks *et al.*, 1992), human (Smeekens *et al.*, 1990), mouse (Seidah *et al.*, 1991), rat (Hakes *et al.*, 1991), *Amphioxus* (Oliva *et al.*, 1995), ascidian (AB086187), *Aplysia* (Chun *et al.*, 1994) and snail (Smit *et al.*, 1992) PC2s are shown.

and Tyr-501. From the high degree of identity between the amino acid sequence of this protein and those sequence of *R. ridibunda* (95.6%; Gangnon *et al.*, 1999), human (62.7%; Creemers *et al.*, 1992), mouse (67.2%; Seidah *et al.*, 1991), rat (66.8%; Hakes *et al.*, 1991), anglerfish (68.1%; Roth *et al.*, 1993), *Amphioxus* (56.1%; Oliva *et al.*, 1995), *Aplysia* (53.8%; Gorham *et al.*, 1996) and *Hydra* (41.5%; Chan *et al.*, 1992) PC1, we concluded that this cDNA encoded the bullfrog PC1 protein (Fig. 2).

Fig. 3 shows the full cDNA sequence of bullfrog PC2 and its deduced amino acid sequence. The cDNA consisted of a 5'-untranslated region of 155 bp and a 3'-untranslated region of 170 bp followed by a poly (A) tail. An open reading frame of 1914 bp encoded a protein of 638 amino acids (with a calculated Mr of 70632 and an isoelectric point of 5.83), consisting of a signal peptide of 23 amino acids and a mature peptide of 615 amino acids. The 3'-noncoding sequence contained a consensus polyadenylation signal (AATAAA) and a poly (A) tail. The protein contained the Asp-167, His-208, and Ser-384 residues found in the catalytic triad of serine proteinases of the subtilisin family. There were 3 putative *N*-linked glycosylation sites, at Asn-375,

а

Asn-514, and Asn-524, in the predicted amino acid sequence of bullfrog PC2. The PC2 contained a canonical integrin binding sequence (Arg-Gly-Asp⁵²⁰). In addition, putative sulfation sites were seen at Tyr-172 and Tyr-314. As there was a high degree of identity between the amino acid sequence of this protein and those of *R. ridibunda* (95.5%; Vieau *et al.*, 1998), *Xenopus* (90.4%; Braks *et al.*, 1992), human (85.9%; Smeekens *et al.*, 1990), mouse (84.2%; Seidah *et al.*, 1991), rat (84.2%; Hakes *et al.*, 1991), *Amphioxus* (71.6%; Oliva *et al.*, 1995), ascidian (64.6%; AB086187), *Aplysia* (60.2%; Chun *et al.*, 1994) and snail (61.0%; Smit *et al.*, 1992) PC2, we concluded that this cDNA encoded the bullfrog PC2 protein (Fig. 4).

Expression distribution of bullfrog PC1 and PC2 mRNAs in various organs

To investigate the tissue distribution of bullfrog PC1 and PC2 mRNA expression, we performed RT-PCR using total RNA from various tissues. Both PC1 and PC2 mRNAs were detected in the pars distalis, neurointermediate lobe, brain, and pancreas (Fig. 5a). PC1 mRNA was detected in the stomach and intestine, and PC2 mRNA was found in the

testis ovary skeletait

intestin



Fig. 5. RT-PCR (a) and Southern blot (b) analysis of PC1 and PC2 mRNAs in adult bullfrog tissue extracts. RT-PCR products obtained by using the primers described in Materials and Methods were separated on a 2% agarose gel and stained with ethidium bromide. The Southern blot of the gel was performed using bullfrog PC1 or PC2 cDNAs as a probe.

testis. No distinct bands of PC1 or PC2 mRNAs were detected in the heart, liver, lung, kidney, spleen, ovary, or skeletal muscle. These RT-PCR results were confirmed by Southern blot analysis (Fig. 5b).

Distribution of PC1 and PC2 mRNAs in the pituitary gland

We determined the sites of PC1 mRNA expression in the pituitary gland by *in situ* hybridization histochemistry with a DIG-labeled antisense cRNA probe. As shown in Fig. 6a, the hybridization signal for PC1 mRNA was distributed throughout the pars distalis, and the most intense staining was seen in the rostral region. Strong signals were also detected in the pars intermedia, whereas weak signal was noted in the pars nervosa. The hybridization signal was confined to the cytoplasm: the nucleus remained unstained (Fig. 6b). The positive cells were often round or ovoid. The number and intensity of reactions varied among the hybridization-positive cells, probably reflecting differences in mRNA expression. On the other hand, a different distribution of hybridization signals was observed when the PC2 cRNA probe was used. There was intense signal for the PC2 mRNA in the intermediate lobe, but not in the pars distalis or the pars nervosa (Fig. 6c, d). When the tissue section was incubated with sense PC1 or PC2 probes, no hybridization signal was detected (data not shown). In the tadpoles, both PC1 and PC2 mRNAs were expressed in the partes distalis, intermedia, and nervosa (Fig. 7).

To identify cells that express PC1 mRNA in the pars distalis, we applied fluorescence staining with guinea pig anti-bullfrog POMC, rabbit anti- α -MSH or mouse monoclonal antibody against bullfrog LH β to the same sections. In



Fig. 6. Light micrographs showing localization of PC1 mRNA (a, b) and PC2 mRNA (c, d) in the adult pituitary gland. PC1 mRNA is seen in the pars distalis, intermedia, and nervosa, whereas PC2 mRNA is detected in the pars intermedia and nervosa. PD: pars distalis, PI: pars intermedia, PN: pars nervosa. Bar: a, c=100 μm; b, d=10 μm



Fig. 7. Light micrographs showing localization of PC1 and PC2 mRNAs in the pituitary gland of the tadpoles. Both PC1 (a) and PC2 (b) mRNAs are expressed in the pars distalis, intermedia, and nervosa. Bar=100 μ m



Fig. 8. Light micrographs showing triple-staining for POMC (a, d), PC1 mRNA (b), α -MSH (c, f) and PC2 mRNA (e) in the adult pars distalis. PC1 mRNA-expressing cells correspond to POMC-immunopositive cells containing α -MSH (a, b, c). Cells co-expressing both POMC and α -MSH do not express PC2 mRNA (d–f). Arrowheads indicate the corresponding cells. Bar=10 μ m



Fig. 9. Light micrographs showing triple-staining for POMC(a), PC1 mRNA (b), and LH β (c) in the adult pars distalis. Some PC1 mRNA-expressing cells correspond to POMC-immunopositive cells (arrows); and others, to LH β -immunopositive cells (arrowheads). Bar=10 μ m

the adult bullfrog, we observed PC1 mRNA in POMC-immunopositive cells in the pars distalis (Fig. 8a, b). These cells were also reactive with anti- α -MSH (Fig. 8a, c); but cells positive for both POMC and α -MSH were not reactive with the PC2 antisense probe (Fig. 8d–f). In addition, PC1 mRNA was expressed in LH β -immunopositive cells (Fig. 9). In the pars distalis of tadpoles, PC1 mRNA was expressed in POMC-immunopositive cells that were also α -MSH positive (Fig. 10a–c); and PC2 mRNA was likewise detected in such cells (Fig. 10d–f).

DISCUSSION

The present study describes the sequences of mRNAs encoding PC1 and PC2 from the bullfrog pituitary. Both PCs were structurally characterized by having a signal peptide, a prosegment, a catalytic region, a P-domain, and a variable C-terminal region. The predicted amino acid sequence of these PCs showed high homology with those of various other species. The bullfrog PC1 cDNA was predicted to encode a 736-amino acid protein with a putative 26-residue signal peptide, and the PC2 cDNA, a 638-amino acid protein, with a putative 23-residue signal peptide. It has been shown that PCs are first synthesized as inactive precursor enzymes, which undergo autocatalytic excision or furindependent cleavage of their N-terminal prosegment via cleavage at a specific Arg-Arg-Ser-Arg-Arg and Arg-Ser-Lys-Arg in PC1 protein, and Lys-Arg-Arg-Arg and Arg-Lys-Lys-Arg in PC2 protein (Muller and Lindberg, 1999). Although there are 2 possible cleavage sites in the prosegment of both bullfrog PCs, the actual sites are considered to be Arg-Thr-Lys-Arg¹¹⁰ in the PC1 protein and Arg-Lys-Lys-Arg¹⁰⁹ in the PC2 protein, because these sites correspond to the cleavage sites of prosegment in the mammalian PCs (Benjannet et al., 1992; Zhou and Lindberg, 1993). The mature PC1 protein contains 626 amino acids with 2 putative N-glycosylation sites, whereas the mature PC2 protein contains 529 amino acids with 3 such sites. The catalytic domain is well conserved, especially in the regions surrounding the catalytic triad of PC1 (Asp¹⁶⁸, His²⁰⁹, and Ser³⁸³) and of PC2 (Asp¹⁶⁷, His²⁰⁸, and Ser³⁸⁴). In the Pdomain of both PCs, the canonical integrin binding Arg-Gly-Asp sequence was also present, as found in all mammalian convertases except PC7 (Seidah and Cretien, 1992; Seidah et al., 1996) though this sequence was absent in the PC2 protein of R. ridibunda (Vieau et al., 1998).

In this study, we investigated the expression of PC1 and PC2 mRNAs by using RT-PCR. Both PC1 and PC2 mRNAs were expressed in the pars distalis, pars neurointermedia, brain, and pancreas. This result is mostly consistent with previous reports (Vieau *et al.*, 1998; Seidah *et al.*, 1990; Gangnon *et al.*, 1999). It is of interest that only PC1 mRNA was expressed in the stomach and intestine, whereas PC2 mRNA was the only type in the testis. The expression of PC1 mRNA may be involved in the processing of the intestinal type of proglucagon (Dhanvantari *et al.*, *al.*, *al.*,



Fig. 10. Light micrographs showing triple-staining for POMC (a, d), PC1 mRNA (b), α -MSH (c, f), and PC2 mRNA (e) in the pars distalis of the tadpoles. PC1 mRNA-expressing cells correspond to POMC-immunopositive cells containing α -MSH (a, b, c). PC2 mRNA-expressing cells also co-express POMC and α -MSH (d–f). Arrowheads indicate the corresponding cells. Bar=10 μ m

1996) and in the processing of progastrin in the stomach (Macro *et al.*, 1996), whereas the PC2 may have some effect on the post-translational processing in the bullfrog testis. However, considering that only PC4 has been identified in the mammalian testis (Nakayama *et al.*, 1992; Seidah *et al.*, 1992; Mbikay *et al.*, 1997; Li *et al.*, 2000), it is possible that the PC related with PC4 is present in the bullfrog testis. Further studies are necessary to identify another type of PC in the frog testis, and to compare substrate-specificity between PC2 and PC4.

In mammals, heterologous gene transfection studies have indicated that PC1 and PC2 play an important role in the tissue-specific processing of POMC; PC1 alone cleaves POMC in the pars distalis, whereas both PC1 and PC2 are required to carry out POMC processing in the pars intermedia (Benjannet *et al.*, 1991; Thomas *et al.*, 1991). Earlier biochemical studies using *in situ* hybridization and Northern blot analysis showed that the corticotrope cells in the adult rat pituitary predominantly expressed PC1 mRNA but rarely PC2 mRNA (Seidah *et al.*, 1991; Day *et al.*, 1992). In the present in situ hybridization, PC1 mRNA was shown to be expressed in the pars distalis and the pars intermedia, whereas PC2 mRNA was detected only in the pars intermedia, of the adult bullfrog. Similar results were obtained with R. ridibunda (Vieau et al., 1998; Gangnon et al., 1999). An in situ hybridization experiment with the antisense RNA of Xenopus PC2 also revealed that PC2 mRNAs were predominantly expressed in the pars intermedia of the Xenopus pituitary (Braks et al., 1992). The expression of PC1 and PC2 mRNAs in the pituitary is also in good agreement with previous immunohistochemical findings showing that the pars distalis had PC1 protein, and the pars intermedia contained both PC1 and PC2 proteins, in the bullfrog pituitary (Kurabuchi and Tanaka, 1997). Consequently, although PC2 mRNA was detected in the adult pars distalis by the present RT-PCR, very little PC2 mRNA may have been translated there. However, Iwamura et al. (1992) obtained a considerable amount of N-terminal peptide of POMC not containing γ -MSH (NPP) from the pars distalis of the adult bullfrogs. If this peptide was generated in the same way as in the pars intermedia (Ekman *et al.*, 1982), PC2 would have to be present in the bullfrog pars distalis. Further studies are needed to reach a definite conclusion.

In the present study, we showed that PC1 mRNAexpressing cells corresponded to corticotrope cells in the pars distalis. This finding implies that proteolytic cleavage of POMC by PC1 would produce ACTH (1-39) in the pars distalis. On the other hand, both PC1 and PC2 mRNAs were expressed in the pars intermedia. Therefore, ACTH 1-39 liberated from POMC would be further cleaved into α -MSH and CLIP in this part of the pituitary. Also, the degree of expression of PC2 in the pars intermedia was higher than that of PC1. This finding is consistent with results from mammals (Day et al., 1992). Interestingly, the present study revealed that PC2 mRNA was expressed in the α-MSH-positive corticotrope cells of the tadpoles. In mammals, it is known that PC2 mRNA is also expressed in corticotrope cells during development until neonatal week 3, thereby producing α -MSH in the pars distalis (Marcinkiewicz *et al.*, 1993). The α -MSH is considered to have stimulatory effects on intrauterine growth (Swaabet et al., 1976) and growthstimulating effects on the adrenal zona glomerulosa (Robba et al., 1986). Similarly, in Ambystoma, corticotrope cells produce α -MSH during larval period (Dores *et al.*, 1989, 1990, 1993). The production of α -MSH during the neonatal period or larval period is considered to be a general phenomenon, and it is accepted that the α -MSH production varies in accordance with the expression of PC2. Thus, the situation in the frogs is nearly consistent with that of POMC cells in mammalian pituitary (Marcinkiewicz et al., 1993). However, in the present study, we did not observe expression of PC2 mRNA in corticotrope cells in the adult bullfrogs, although α -MSH-immunoreactivity was detected in the corticotrope cells. This implies that either very little PC2 mRNA is translated or that α -MSH, produced in the tadpoles, remains stored in the secretory granules.

The present study also showed that PC1 mRNA was expressed in the gonadotrope cells. It is conceivable that proprotein processing does not take place in these cells. However, it is possible that other proteins such as granin family proteins with proteolytic cleavage sites are contained in the secretory granules and that their proteins are cleaved by PC1. Indeed, we have shown that both PC1 and PC2 are expressed in the rat pituitary gonadotrope cells, suggesting that these convertases may be involved in the processing of secretogranin II and chromogranin A (Uehara *et al.*, 2001).

Taken together, the data from this comparative study provide further information about the molecular mechanism underlying proteolytic cleavage of POMC in the pituitary.

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