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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 23 amino acids. This protein showed a high homology to *R. ridibunda* PC2 (95.5%) and mammalian 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 (α-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 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).

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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 µg 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 (4.6×10^5 pfu/µg 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 1.25×10^5 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 µg/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 LHβ (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 Immunoresearch, 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 BX-epifluorescence attachment (Olympus Optical Co., Tokyo, Japan).

RESULTS

cDNA cloning of bullfrog PC1 and PC2

Fig. 1 shows the full cDNA sequence of bullfrog PC1

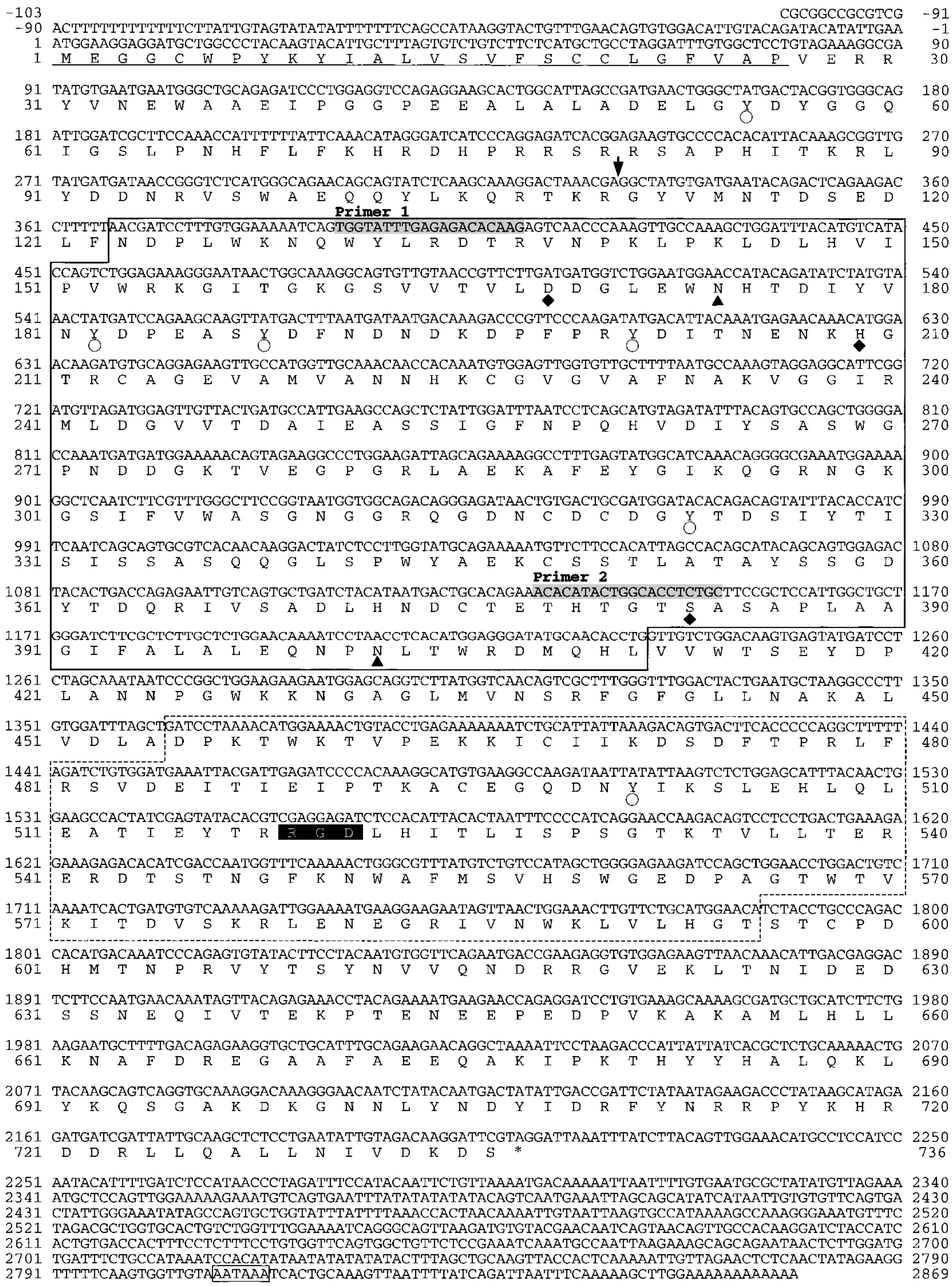

Fig. 1. Nucleotide and deduced amino-acid sequences of bullfrog PC1 cDNA. The predicted amino acid is shown below the nucleotide sequence (DDBJ/EMBL/GenBank accession no. AB105175). The asterisk indicates the termination codon. Polyadenylation signal region is boxed. The underlined letters indicate the 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.

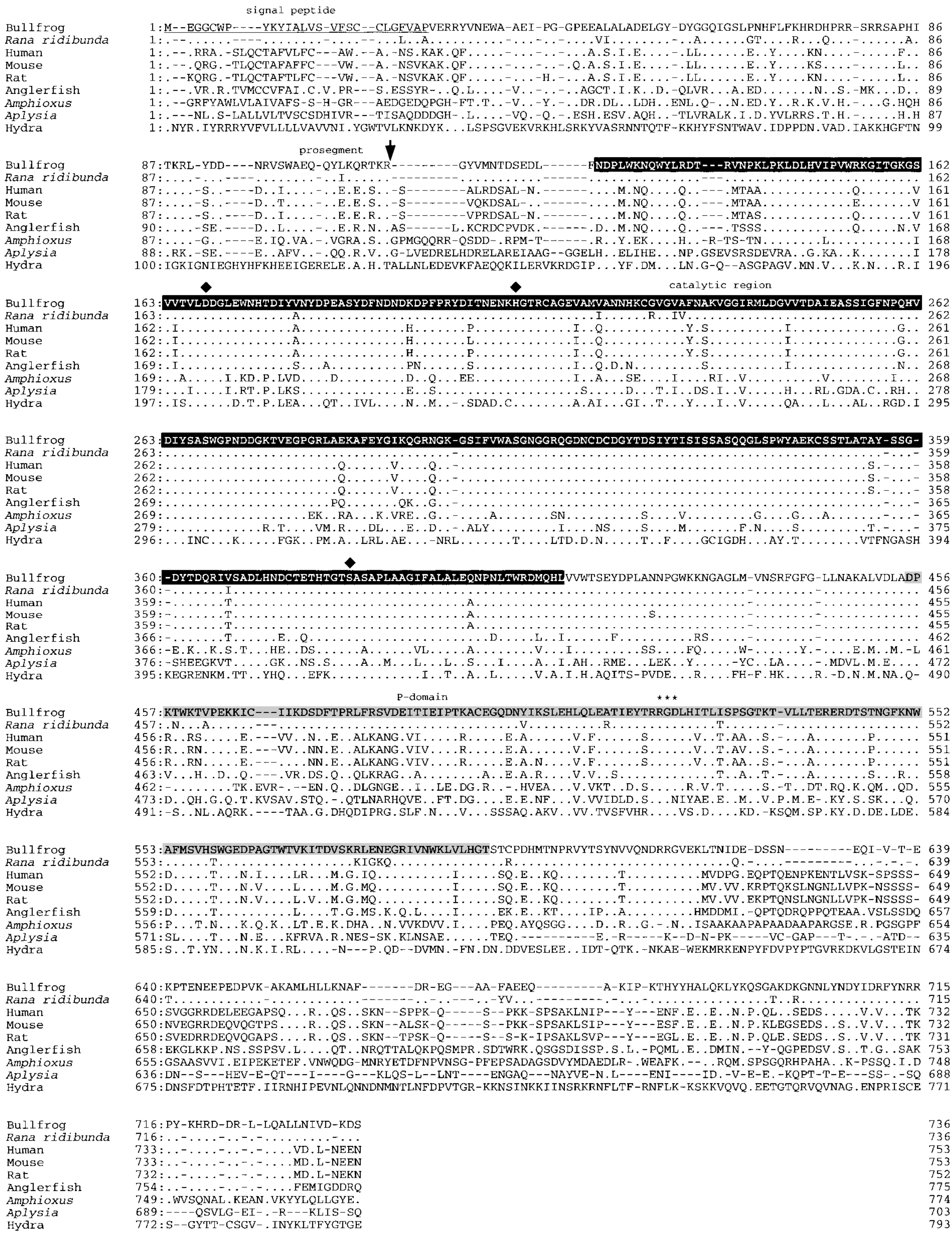

Fig. 2. Comparison of the predicted amino-acid sequences of the bullfrog PC1 with those of other vertebrate PC1s. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed 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-Asp[521]). Also, putative sulfation sites were found at Tyr-54, Tyr-182, Tyr-188, Tyr-201, Tyr-323,

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-155                                 CGCGGCCGCGTCGACGGCATCAGAAGTAATCTCACATACATAGAGCGGAGCGGAGACATGTACTG   -91
 -90 AGGAGGCGCGGAGGAGCCCCACCGACAACTACACAGCTCCGGCATCTCCTCTCCACTCCTCTCCCCTCACCTTCCCACACAAAGTGAGAG    -1

   1 ATGAGAGCAAGCTCCCCGCTCCGGGCTGTGCTGGCGGCATTGCTGCTCATACAGTACATCGCCTCGACCCACTCCGCTCTCCTCACCCAA    90
   1  M  R  A  S  S  P  L  R  A  V  L  A  A  L  L  L  I  Q  Y  I  A  S  T  H  S  A  L  L  T  Q    30

  91 CAATACTTGGTGGACTTACAACCAGGAGGAGAGCCGACAGACGCCGCACAACTCGCCCAACAGTATGGATTCACTGGGGCCAGGAAGCTA   180
  31  Q  Y  L  V  D  L  Q  P  G  G  E  P  T  D  A  A  Q  L  A  Q  Q  Y  G  F  T  G  A  R  K  L    60

 181 CCCTTTTCGGATAGTTTATACCATTTCTATGACAGTGGAGCAACTAAGTTCAGGAGGAAACGAAGCCTCAACAGTAAGAATCACTTATCC   270
  61  P  F  S  D  S  L  Y  H  F  Y  D  S  G  A  T  K  F  R  R  K  R  S  L  N  S  K  N  H  L  S    90

 271 ATGCACCCCAAGGTTCGGAAAGTTGTTCAGCAAGAAGGTTTTGACAGAAAGAAACGAGGGTACAGGGACATCAATGACATCGACATCAAT   360
  91  M  H  P  K  V  R  K  V  V  Q  Q  E  G  F  D  R  K  K  R  G  Y  R  D  I  N  D  I  D  I  N   120

 361 ATGAATGATCCCCTATTTACAAAACAGTGGTATCTGATCAACACAGGTCAAGCAGATGGGACTCCAGGTCTTGATTTGAATGTTGCTGAA   450
 121  M  N  D  P  L  F  T  K  Q  W  Y  L  I  N  T  G  Q  A  D  G  T  P  G  L  D  L  N  V  A  E   150

 451 GCCTGGGAGTTGGGATACACGGGGAGAGGAGTTACCATAGCAATTATGGATGATGGAATAGATTACCTTCACCCAGATCTTGCCTCAAAT   540
 151  A  W  E  L  G  Y  T  G  R  G  V  T  I  A  I  M  D  D  G  I  D  Y  L  H  P  D  L  A  S  N   180

 541 TATAATGCAGAGGCAAGCTATGACTTTAGCAGCAATGACCCTTATCCATATCCCCGCTACACTGATGACTGGTTTAACAGCCATGGGACA   630
 181  Y  N  A  E  A  S  Y  D  F  S  S  N  D  P  Y  P  Y  P  R  Y  T  D  D  W  F  N  S  H  G  T   210

 631 CGCTGTGCGGGGGAAGTATCTGCTGCTGCAAACAATAATATTTGCGGGGTGGGAGTTGCTTATAACTCTAAAGTTGCTGGTATTCGCATG   720
 211  R  C  A  G  E  V  S  A  A  A  N  N  N  I  C  G  V  G  V  A  Y  N  S  K  V  A  G  I  R  M   240
                                                                                  Primer 1
 721 CTAGACCAGCCCTTTATGACAGATATAATTGAAGCATCTTCAATCAGTCATATGCCACAAATTATAGACATCTACAGCGCCAGCTGGGGG   810
 241  L  D  Q  P  F  M  T  D  I  I  E  A  S  S  I  S  H  M  P  Q  I  I  D  I  Y  S  A  S  W  G   270

 811 CCCACAGACGATGGCAAGACAGTGGATGGGCCCAGAGAACTGACCTTACAGGCCATGGCAGATGGTGTAAATAAGGGTCGCGGTGGAAAA   900
 271  P  T  D  D  G  K  T  V  D  G  P  R  E  L  T  L  Q  A  M  A  D  G  V  N  K  G  R  G  G  K   300

 901 GGAAGTATTTATGTCTGGGCGTCTGGAGATGGAGGAAGTTATGATGACTGTAACTGTGATGGTTATGCATCCAGCATGTGGACCATCTCC   990
 301  G  S  I  Y  V  W  A  S  G  D  G  G  S  Y  D  D  C  N  C  D  G  Y  A  S  S  M  W  T  I  S   330

 991 ATAAACTCTGCTATTAATGATGGTCGGACTGCACTATACGATGAAAGCTGCTCTTCCACTTTAGCCTCTACATTCAGCAATGGAAGAAAA  1080
 331  I  N  S  A  I  N  D  G  R  T  A  L  Y  D  E  S  C  S  S  T  L  A  S  T  F  S  N  G  R  K   360

1081 CGAAATCCTGAAGCTGGCGTGGCTACAACAGATTTATATGGAAACTGCACTTTACGTCACTCAGGAACATCTGCGGCAGCACCAGAAGCA  1170
 361  R  N  P  E  A  G  V  A  T  T  D  L  Y  G  N  C  T  L  R  H  S  G  T  S  A  A  A  P  E  A   390
                                                    Primer 2
1171 GCTGGGGTGTTTGCATTAGCCCTAGAGGCAAATCCAGGTCTCACTTGGAGGGACTTGCAACACTTAACAGTGCTAACATCCAAAAGGAAC  1260
 391  A  G  V  F  A  L  A  L  E  A  N  P  G  L  T  W  R  D  L  Q  H  L  T  V  L  T  S  K  R  N   420

1261 CAGCTGCACGATGAAGTTCATAAGTGGCGTAGGAATGGTGTTGGTTTGGAGTTTAACCACTTGTTTGGCTATGGTGTACTGGATGCTGGC  1350
 421  Q  L  H  D  E  V  H  K  W  R  R  N  G  V  G  L  E  F  N  H  L  F  G  Y  G  V  L  D  A  G   450

1351 TCTATGGTTAAAATGGCACGAGAATGGAAAACTGTTCCAGAAAGATTTCACTGTATTGGTGGATCAATACAGGAGCCAAGGAAAATACCT  1440
 451  S  M  V  K  M  A  R  E  W  K  T  V  P  E  R  F  H  C  I  G  G  S  I  Q  E  P  R  K  I  P   480

1441 TCTGATGGAAAGTTGATGCTCACACTTACAACAGATGCTTGTGAAGGAAAGGAAAACTTTGTTCGATACCTTGAACATGTCCAAGCGGTT  1530
 481  S  D  G  K  L  M  L  T  L  T  T  D  A  C  E  G  K  E  N  F  V  R  Y  L  E  H  V  Q  A  V   510

1531 ATAACTGTAAATTCTACAAGGCGTGGAGACTTGAACATCAATATGACATCACCGATGGGAACAAAATCCATCCTCCTGAGTCGCCGTCCA  1620
 511  I  T  V  N  S  T  R  R  G  D  L  N  I  N  M  T  S  P  M  G  T  K  S  I  L  L  S  R  R  P   540

1621 AGGGACGATGACTCTAAAGTTGGTTTTGATAAATGGCCATTCATGACAACTCACACCTGGGGAGAAGACCCAAGAGGAACTTGGGTTCTT  1710
 541  R  D  D  D  S  K  V  G  F  D  K  W  P  F  M  T  T  H  T  W  G  E  D  P  R  G  T  W  V  L   570

1711 GAGATCGGTTTTGTTGGGAGCATGCCAGAAAAGGGTGTATTGAAAGAATGGACCCTGATGCTGCATGGAACTCAAAGTGCCCCCTACATA  1800
 571  E  I  G  F  V  G  S  M  P  E  K  G  V  L  K  E  W  T  L  M  L  H  G  T  Q  S  A  P  Y  I   600

1801 GACCAAATAGTTAGAGATTACCAGTCAAAATTAGCTATGTCTAAGAAGGAGGAGCTGGAGGAAGAACTGGACGAAGCTGTGGAAAGAAGC  1890
 601  D  Q  I  V  R  D  Y  Q  S  K  L  A  M  S  K  K  E  E  L  E  E  E  L  D  E  A  V  E  R  S   630

1891 TTGAAAAGTCTATTAACCAAGAACTAGCACCGCATTGCATGTCTACCTTATATTTCTTTTTCAGAATTTTCAGCATATCTTTCTAACAC  1980
 631  L  K  S  L  L  T  K  N  *                                                                   638

1981 TTAAATTTCTGTATAGAATACAACAGCCTCTTGGTACCATATGTTCTAAATATTTATAGTCATCTGTTCCTTTGTTTGGAATCAAATAAA  2070
2071 TATATATCTATATAAAA                                                                            2087
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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.

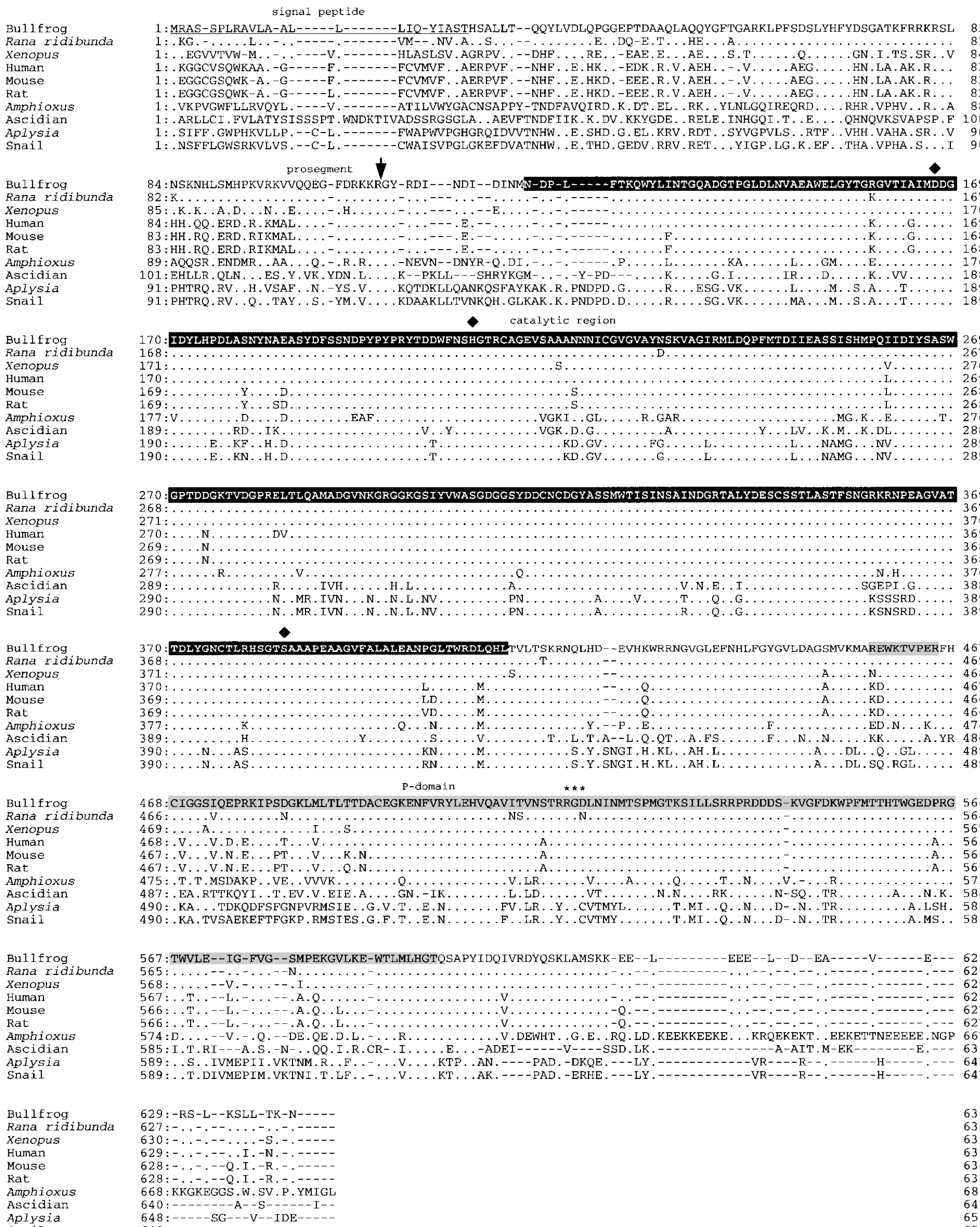

Fig. 4. Comparison of the predicted amino-acid sequence of the bullfrog PC2 with those of other vertebrate PC2s. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed 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, Asn-514, and Asn-524, in the predicted amino acid sequence of bullfrog PC2. The PC2 contained a canonical integrin binding sequence (Arg-Gly-Asp^{520}). 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

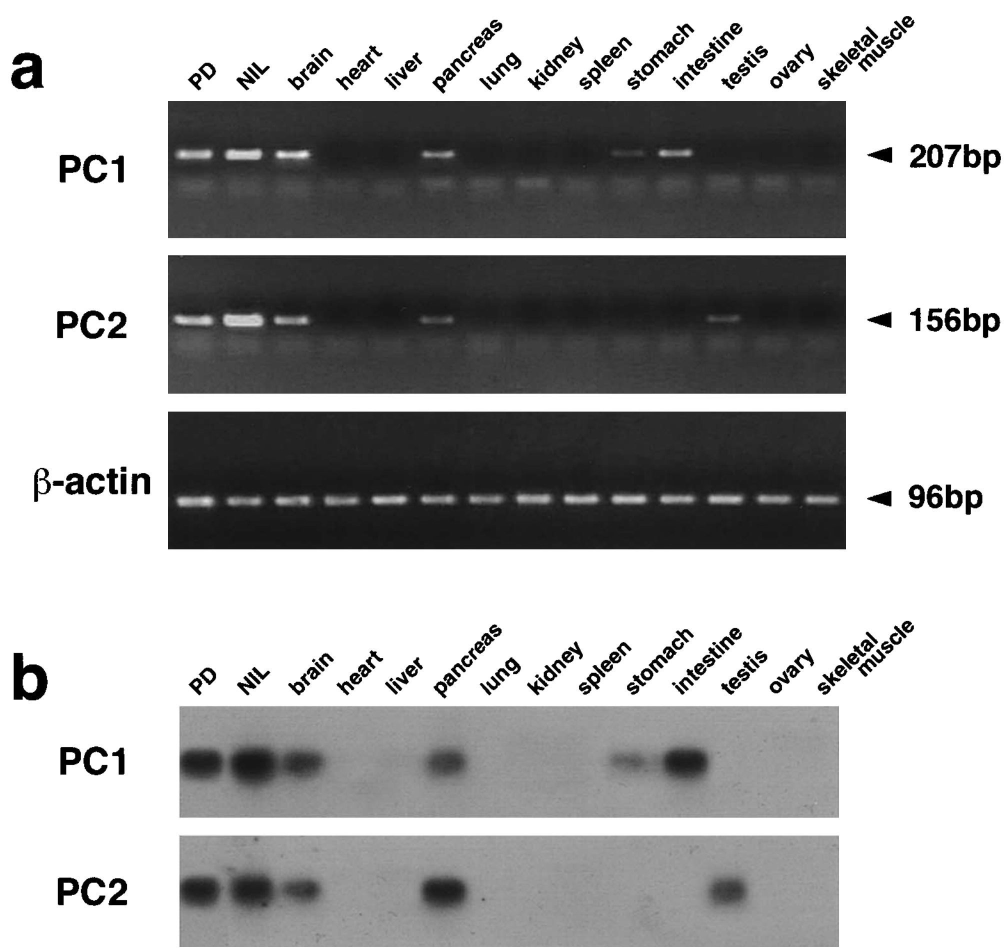

Fig. 5. RT-PCR (a) and Southern blot (b) analysis of PC1 and PC2 mRNAs in adult bullfrog tissue extracts. RT-PCR products obtained by using the primers described in Materials and Methods were separated on a 2% agarose gel and stained with ethidium bromide. The Southern blot of the gel was performed using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

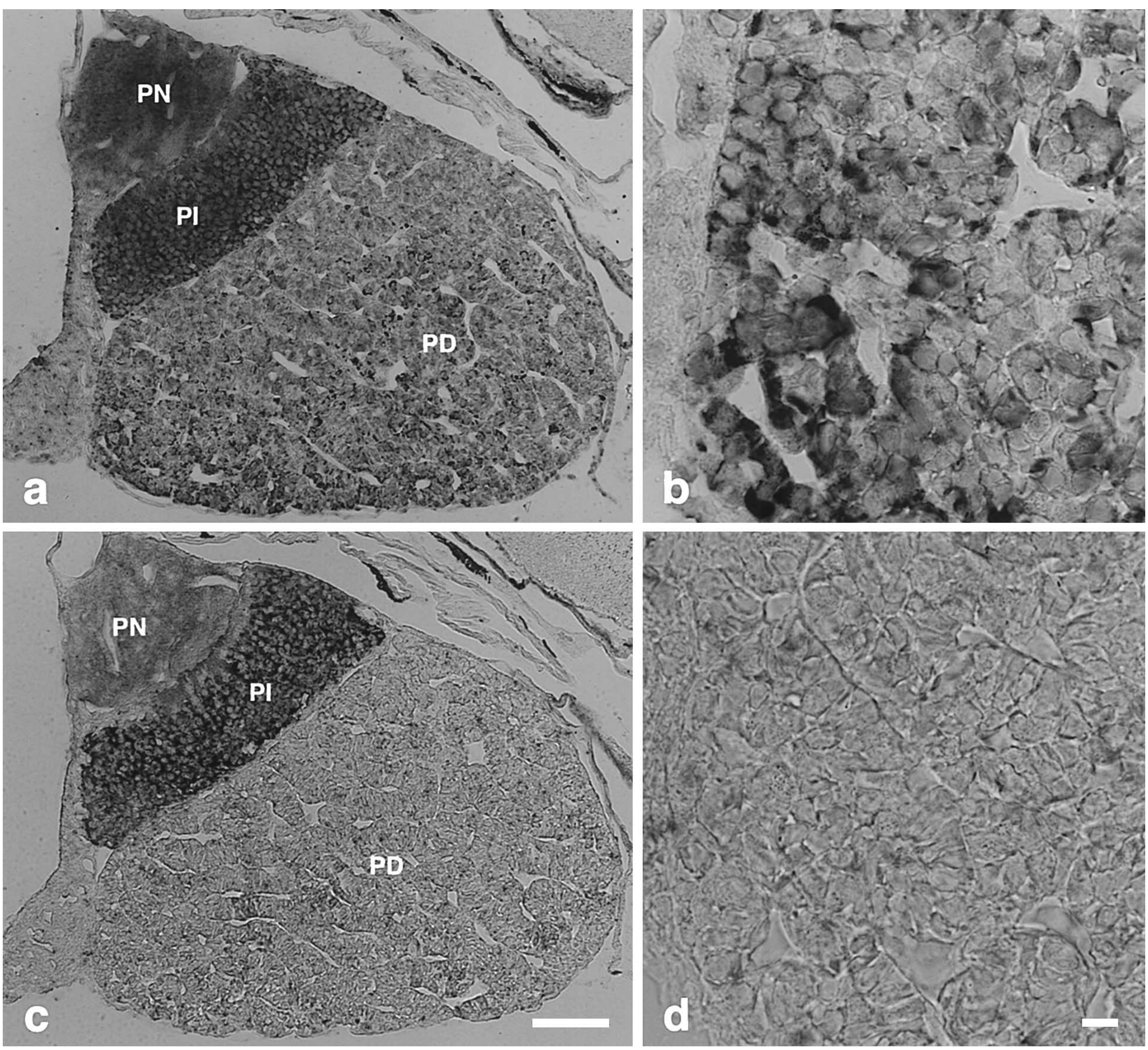

Fig. 6. Light micrographs showing localization of PC1 mRNA (a, b) and PC2 mRNA (c, d) in the adult pituitary gland. PC1 mRNA is seen in the pars distalis, intermedia, and nervosa, whereas PC2 mRNA is detected in the pars intermedia and nervosa. PD: pars distalis, PI: pars intermedia, PN: pars nervosa.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

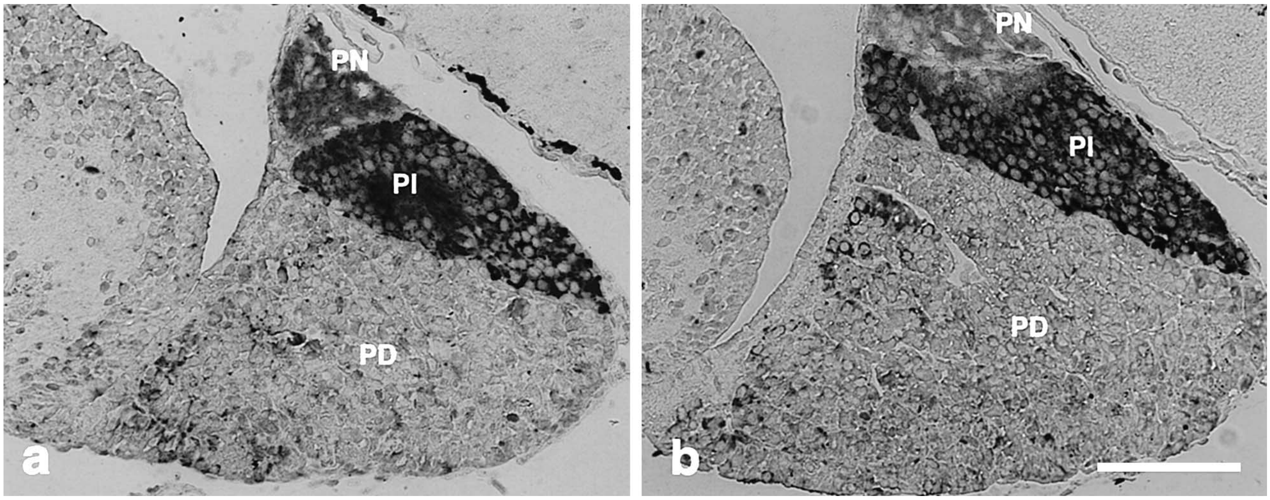

Fig. 7. Light micrographs showing localization of PC1 and PC2 mRNAs in the pituitary gland of the tadpoles. Both PC1 (a) and PC2 (b) mRNAs are expressed in the pars distalis, intermedia, and nervosa. Bar=100 µm

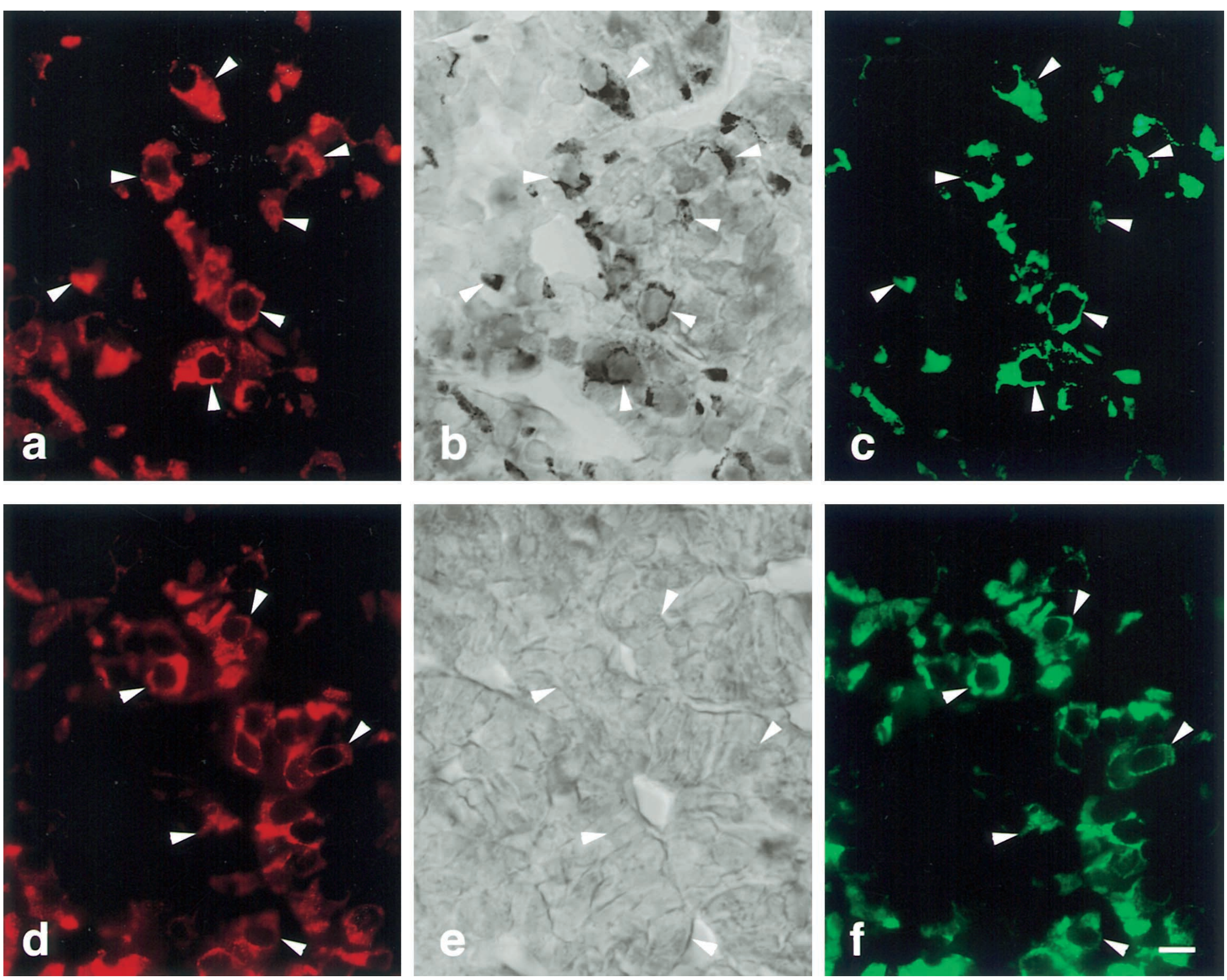

Fig. 8. Light micrographs showing triple-staining for POMC (a, d), PC1 mRNA (b), α-MSH (c, f) and PC2 mRNA (e) in the adult pars distalis. PC1 mRNA-expressing cells correspond to POMC-immunopositive cells containing α-MSH (a, b, c). Cells co-expressing both POMC and α-MSH do not express PC2 mRNA (d–f).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

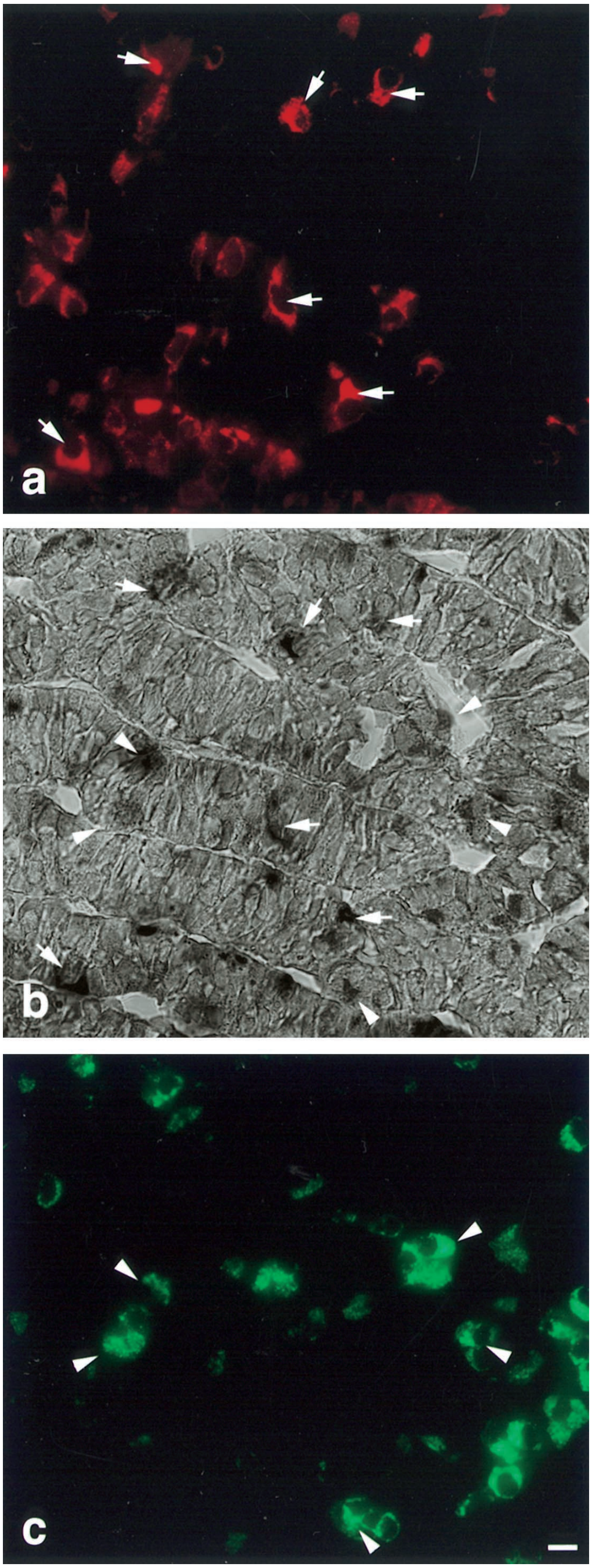

Fig. 9. Light micrographs showing triple-staining for POMC(a), PC1 mRNA (b), and LHβ (c) in the adult pars distalis. Some PC1 mRNA-expressing cells correspond to POMC-immunopositive cells (arrows); and others, to LHβ-immunopositive cells (arrowheads). Bar=10 μm

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 LHβ-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 furin-dependent 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-Arg^{110} in the PC1 protein and Arg-Lys-Lys-Arg^{109} 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 (Asp^{168}, His^{209}, and Ser^{383}) and of PC2 (Asp^{167}, His^{208}, and Ser^{384}). In the P-domain 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.*,

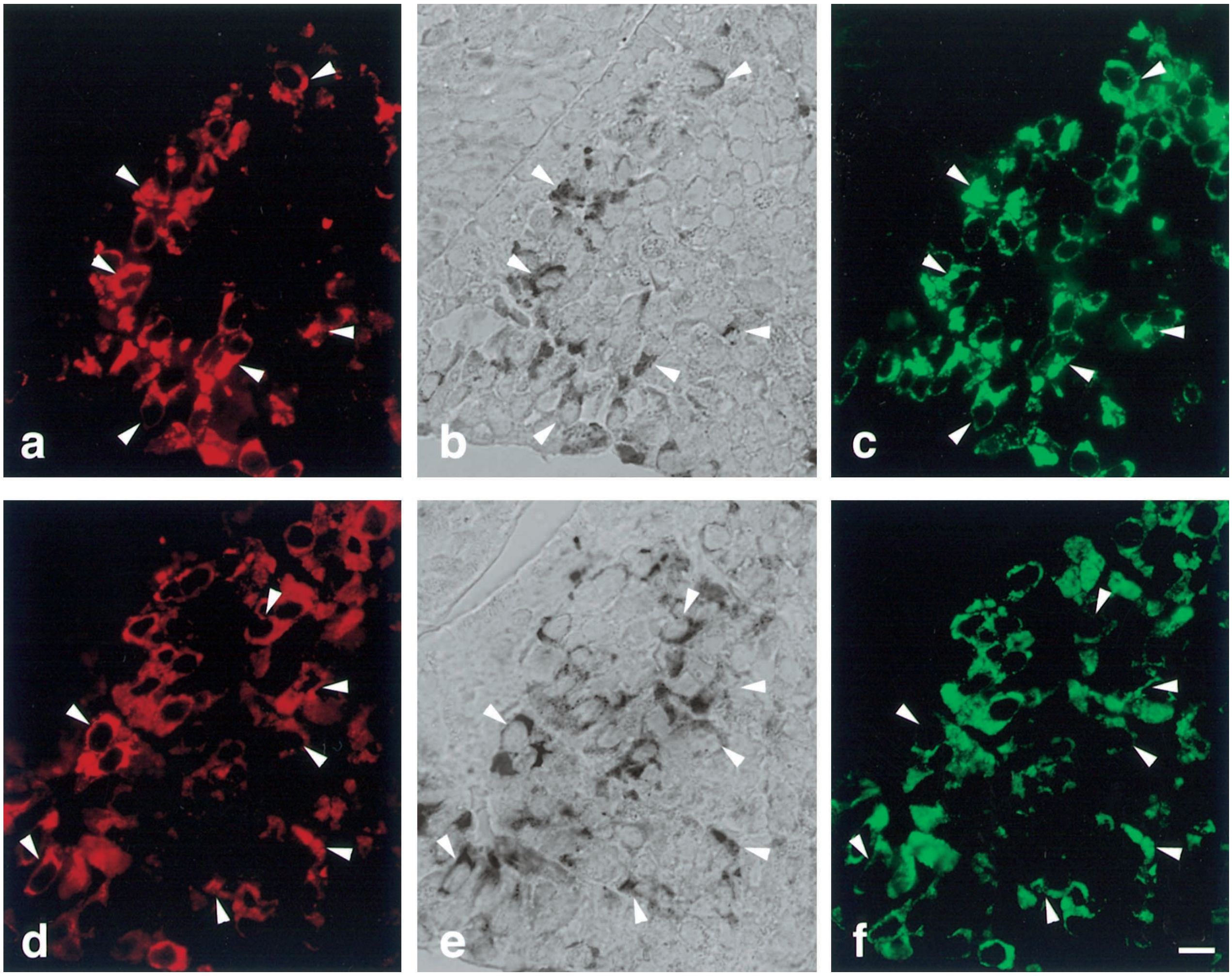

Fig. 10. Light micrographs showing triple-staining for POMC (a, d), PC1 mRNA (b), α-MSH (c, f), and PC2 mRNA (e) in the pars distalis of the tadpoles. PC1 mRNA-expressing cells correspond to POMC-immunopositive cells containing α-MSH (a, b, c). PC2 mRNA-expressing cells also co-express POMC and α-MSH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

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 mRNA-expressing 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 growth-stimulating 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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