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# cDNA structure and in situ localization of the *Aplysia californica* pro-hormone convertase PC2

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The complete cDNA structure of the *Aplysia californica* pro-protein and pro-hormone convertase PC2 (aPC2) was obtained from a cDNA library of the nervous system. The deduced amino acid sequence revealed that aPC2 exhibits an 85%, 61% and 62% sequence identity to the *Lymnaea stagnalis*, *Xenopus laevis* and mouse PC2 homologues, respectively. The deduced primary sequence suggested a protein of 653 amino acids which includes a 27- and 88-amino acid signal peptide and pro-segment. The signal peptide and the C-terminal segments are the least conserved regions. On Northern blots of nervous system we detected a transcript of 6.8 kb. The in situ hybridization histochemistry on the abdominal ganglion revealed intense labeling of the bag cells. Large peptidergic cells and clusters of sensory and motor neurons also contained high levels of aPC2 mRNA.

Pro-hormone convertase; PC2; *Aplysia*; cDNA cloning

## 1. INTRODUCTION

Peptides and neuropeptides are usually synthesized in the form of precursors which must undergo proteolytic cleavage at selected single or pairs of basic residues, in order to release the bioactive moiety [1]. Six enzymes belonging to a new family of subtilisin-like proteinases, known as convertases, have been recently characterized at the molecular level. These include furin [2], PC1 [3], PC2 [4,5], PACE4 [6], PC4 [7,8] and PC5 [9]. Furthermore, some of them have been shown to cleave pro-protein and pro-hormone precursors specifically at the pairs of basic residues known to be cleaved in vivo (for reviews see [10,11]). While furin and PACE4 have a rather ubiquitous distribution, PC5 has a more restricted one [9]. PC4 is exclusively expressed in testicular germ cells and ovaries [7,8]. On the other hand, PC1 and PC2 are mostly expressed within neural and endocrine cells of mammals [3–5,12].

Many neuropeptides of the marine mollusc *Aplysia californica* exist in the form of precursors that must be processed in order to become active. The pro-egg laying hormone (pro-ELH) represents one such extensively studied precursor. It undergoes a series of nine cleavages at specific tetra-, tri- and di-basic sites to produce ELH and various bag cell peptides [13]. Other regions of the abdominal ganglion contain precursors also known to be cleaved at pairs of basic residues [14]. It therefore seemed likely that the enzymes responsible for the processing of *Aplysia* precursors would belong to the same family of subtilisin-like serine proteases. This paper describes the molecular cloning of an *Aplysia* PC2-like convertase and the study of its distribution in identified neurons of the abdominal ganglion by in situ hybridization.

## 2. MATERIALS AND METHODS

### 2.1. *Animals*

Adult *Aplysia californica* (150–250 g) were purchased from Marine Specimen Unlimited (CA, USA) and kept under standard laboratory conditions (light–dark cycle: 12 h; fed with dry seaweed every other day; water temperature 14°C; pH 7.8–8.2).

### 2.2. *PCR, library screening and DNA sequencing*

Polymerase chain reactions were performed in a Perkin-Elmer/Cetus DNA cycler using degenerate oligonucleotides located in the highly conserved catalytic region. The sequences of the sense (OL1) and antisense (OL2) oligos are as follows:
*OL1*, 5′-TCGATATCTACAG(T/C)GC(A/C)AG(C/A)TGGGGCCC-3′;
*OL2*, 5′-TAGGATCGAG(A/G)TG(C/T)TGCATGTC(T/C)C(T/G)CC-AGGT-3′ [8,9].

Purified DNA from a cDNA library of the nervous system of *Aplysia* (lambda ZAPII, kindly provided by Dr. D. Solomon, Columbia U., N.Y.) was subjected to 30 cycles of amplification (1 min, 94°C; 2 min, 55°C; 3 min, 72°C). The expected 450bp band was subcloned (TA-Cloning Kit, Invitrogen, San Diego, CA), and sequenced. The PCR product was used as a probe to screen approximately 1 million clones of the cDNA library according to a modified method from [15]. Positive clones were purified by a second round of hybridization. DNA sequence analysis was performed automatically using an ALF DNA sequenator (Pharmacia) using 5′-end labeled fluorescent oligonucleotides as primers for the sequence analysis.

### 2.3. *Northern blots and in situ hybridization*

*Aplysia* nervous system total RNA preparation, and Northern blot

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analysis were carried out as previously described in [16]. The filter was hybridized to an antisense cRNA probe to aPC2.

For in situ hybridization, single ganglia were dissected under a stereomicroscope and freshly fixed in 4% paraformaldehyde. The paraffin-embedded 5-$\mu$m thick sections were hybridized with a $^{35}$S-labeled cRNA probe to aPC2 using a modified method described in [17].

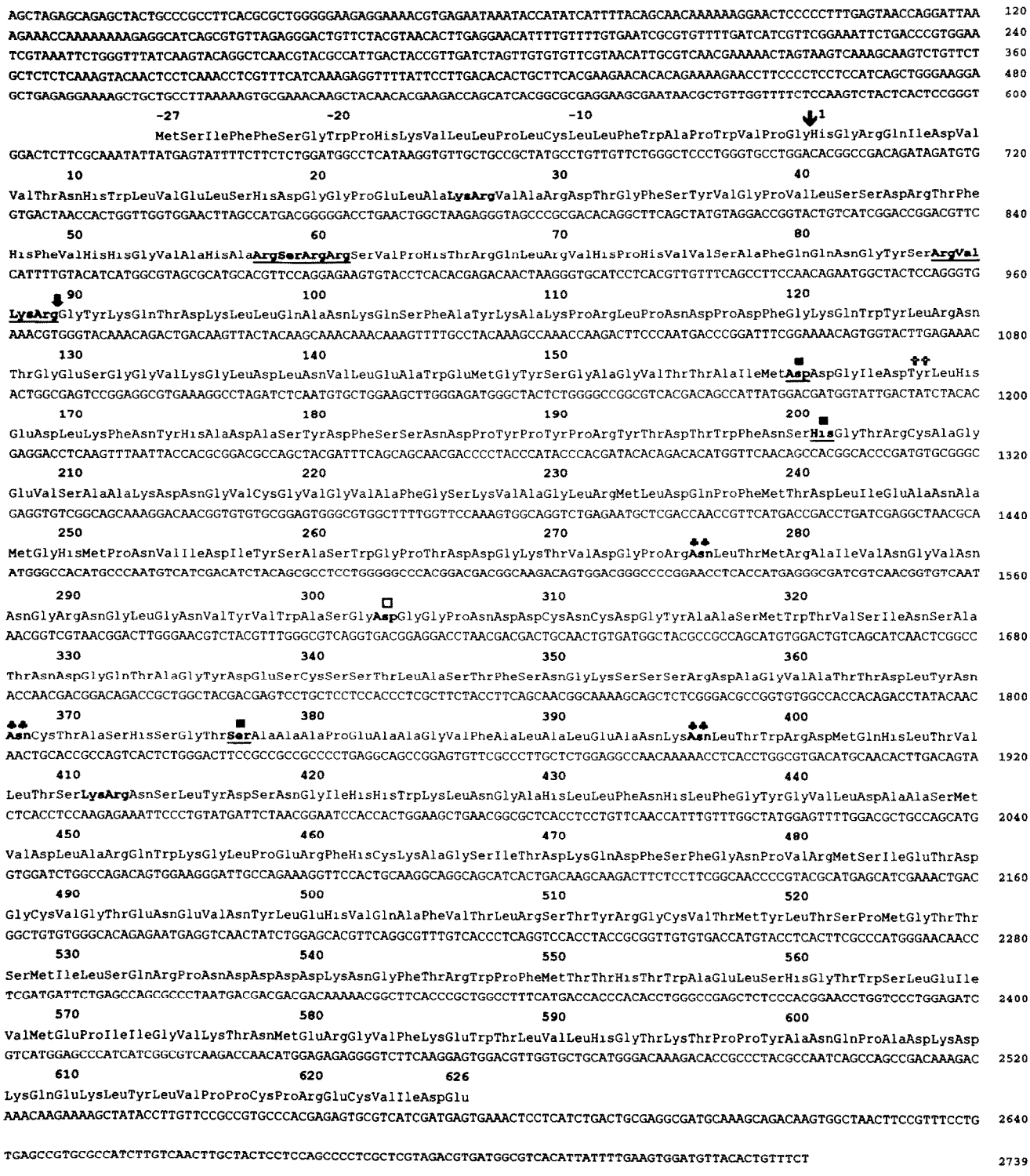

Fig. 1. Complete cDNA and the deduced amino acid sequence of aPC2. The active site Asp (■) His (■), and Ser (■), and the important Asp (□) residue, as well as the three potential N-glycosylation (♣♣) sites and the position of the sulfated Tyr ((++) sequence [19] are emphasized. The predicted sites of the signal peptidase cleavage and zymogen activation are depicted by different arrows, respectively. The 4 pairs of basic residues are in bold

## 3. RESULTS AND DISCUSSION

One purpose of this study was to isolate and characterize the cDNA structures of pro-hormone convertases in the nervous system of *Aplysia*. We thus started by generating a specific probe by PCR, which we subsequently used to screen a cDNA library. After the second round of screening, we obtained 20 positive clones. Two clones (#8-PC, #9-PC), containing inserts of 5 kb and 2.3 kb, respectively, were completely sequenced. While both clones contained identical sequences, only the longest of the two (#8-PC) gave us a full-length structure.

The cDNA sequence of the first 2739 bp of this 5 kb clone showed an open reading frame starting from nt. 619 up to 2577, coding for a protein containing 653 amino acids. This suggested the presence of a 618 nt-long 5′-untranslated region, which is unusually long and could be important in the regulation of aPC2 mRNA levels. The 3′ end of this clone was not fully sequenced as it represents a non-coding segment of the cDNA. The analysis of the sequence suggested the presence of a signal peptide of 27 amino acids (Fig. 1), the cleavage of which would result in a 626 amino acids-long proteinase. Comparison of this sequence and the deduced amino acid structure to those of the known convertases, suggested that this is an *Aplysia* PC2-like sequence (aPC2), as it resembles best this convertase in its structure. Thus, although it contains the **Asp-160**, **His-201** and **Ser-377** residues found in the catalytic triad of the serine proteinases of the subtilisin-type, it characteristically contains an Asp-303 in place of the usual catalytically important Asn found in subtilisins and in all the other convertases except for PC2 [4,5,10,11]. The calculated overall identity of sequence are 85%, 61% and 62% at the protein level and 73%, 58% and 58% at the nucleotide level with the sequences of *Lymnaea stagnalis* (ly) [18], *Xenopus laevis* (xen) [19] and mouse PC2 [4], respectively, with the catalytic segment (residues 90–493) being the most conserved and the N- and C-terminal sequences showing considerable variations.

Recently, we have shown that PC1 and PC2 are first synthesized as precursor enzymes (zymogens) which undergo post-translational modifications leading to the excision of their N-terminal pro-segment via the cleavage at a specific LysArg sequence [20,21]. Therefore, we expect that aPC2 would, like mammalian PC2s, be synthesized first as a zymogen with an 89-amino acid pro-segment which would then be cleaved, possibly within the trans-Golgi network [21] at the **ArgValLysArg-89↓** sequence, to release the active enzyme. As shown in Fig. 1, the mature enzyme would therefore contain 537 amino acids, three potential Asn-glycosylation sites and one potential Tyr sulfation site which is conserved in all PC2 structures [21]. Similar to lyPC2 [18], but different from xenPC2 [19], we notice in aPC2 the absence of the characteristic **ArgGlyAsp (RGD)** sequence found in all mammalian convertases. The equivalent sequence in aPC2 is **ArgGly**Cys (residues 513–516). It is interesting to note that the C-terminal sequence of aPC2 is quite different from any PC2-like structure known and that it contains two extra Cys residues and is not predicted to contain an amphipathic α-helix, as was suggested for mammalian PC2s [4,5,22]. We also noticed that aside from those present within the pro-segment of aPC2 (residues 28–89), only one pair of basic residues is found in the catalytic region of the aPC2 structure. This is in contrast to mammalian PC2s which contain a number of pairs of basic residues within both the catalytic and C-terminal segments of the molecule [3,5,22].

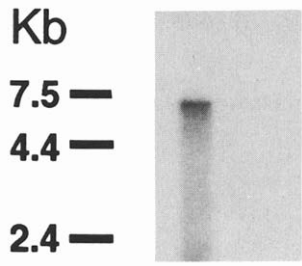


Fig. 2. Northern blot: total nervous system RNA (5 μg) hybridized to aPC2. The transcript is estimated at 6.8 kb.

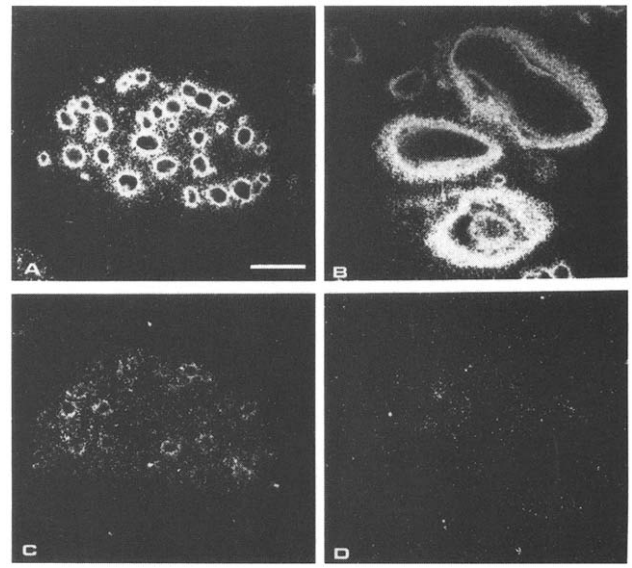


Fig. 3. Localisation of aPC2 mRNA in the bag cells cluster (A) and the large peptidergic neurons (R3-R14) of the right upper quadrant region of the abdominal ganglion (B). Control adjacent section of the bag cells cluster was hybridized to the sense probe (C). Another adjacent section was pretreated with RNAse and subsequently exposed to the antisense probe (D). The calibration mark for all sections is 100 μM.

Northern blot analysis revealed a single transcript of 6.8 kb in the nervous system (Fig. 2). In situ hybridization revealed the presence of aPC2 mRNA in the majority of neurons of the abdominal ganglion, where we focussed our attention. The reconstruction of the serial sections enabled us to identify specifically labelled cells.

The bag cells as well as large peptidergic neurons like the L2-L6 and R3-R14 [23,24] were among the highly responsive cells (Fig. 3). We also observed that regions containing the sensory clusters, interneurons and motor neurons of the gill and siphon withdrawal reflex contained many labelled cells [25]. The labelling of the neurons was not uniform and several cells were unresponsive; thus, the giant cell R2 does not seem to contain any aPC2 mRNA. While the presence of aPC2 mRNA is not surprising in the neurosecretory bag cells [13,26] or large peptidergic neurons, it is interesting to find a PC2 mRNA in the neurons mediating the gill and siphon withdrawal reflex which undergoes short and long-term behavioral modifications [27]. While these cells are known to contain classical neurotransmitters, the presence of aPC2 mRNA could suggest the presence of peptide precursors in these neurons. This would tend to confirm the generality of cotransmission [28] and suggest a physiological role of the convertases in the modulation of behavior. The possibility of using large identified neurons in the central nervous system of *Aplysia* will allow us to test the specific conditions under which the convertase activity could be amplified or attenuated. Moreover, the presence of aPC2 mRNA in identified cells could enable us to find potential substrates to this pro-hormone convertase. The identification of the other members of the family of convertases should allow us to examine the differential role of each convertase in the cellular and molecular basis of learning and memory.

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