Identification of a Molluscan Homologue of the Neuroendocrine Polypeptide 7B2*

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In vertebrates, interaction of prohormone convertase 2 (PC2) with the highly conserved polypeptide 7B2 is essential for transport and maturation of proPC2 in the regulated secretory pathway. In vitro, 7B2 displays a strong inhibitory activity toward PC2. Here, we characterize a cDNA encoding the first invertebrate 7B2-related protein (L7B2) from the brain of the mollusc Lymnaea stagnalis. The overall amino acid sequence identity between L7B2 and its vertebrate counterparts is surprisingly low (29%) and is restricted to a few small stretches of amino acid residues. Of particular interest are a conserved proline-rich region in the middle portion of the L7B2 sequence and a repeated conserved region in the carboxyl-terminal domain. Synthetic peptides corresponding to the carboxyl-terminal regions inhibit Lymnaea PC2 enzyme activity in extracts of insulin-producing neurons, in which both L7B2 and Lymnaea PC2 are abundantly expressed. Moreover, the peptides inhibit mouse PC2 enzyme activity. Our cloning of invertebrate 7B2 helps to delineate residues that are important for 7B2-PC2 interaction.

7B2 is a highly conserved neuroendocrine-specific protein of the regulated secretory pathway in vertebrates (1, 2), PC2 interacts transiently and specifically with PC2, thereby functioning as a chaperone; i.e. it is required for transport, as well as maturation, of proPC2 (3). In vitro, recombinant 7B2 is a potent inhibitor of PC2 enzyme activity (4–6). PC2 cleaves prohormones at pairs of basic amino acid residues (7) and is, like 7B2, exclusively expressed in peptidergic neurons and endocrine cells (8). The human 7B2 protein (185 amino acids; calculated molecular mass, 20.8 kDa) consists of two functional domains, namely an amino-terminal (NT) domain (154 amino acids, 17.3 kDa) that displays chaperone activity (2, 3) and a carboxyl-terminal (CT) domain (31 amino acids, 3.5 kDa) that is inhibitory (5, 9).

7B2 is strongly conserved among vertebrates. This applies particularly to the NT, which shows 84–86% amino acid sequence identity between Xenopus laevis and human PC2. Our cloning of invertebrate 7B2 helps to identify conserved residues that might be crucial for binding of 7B2 to PC2 or for inhibition of PC2 activity. We have now cloned a cDNA encoding an invertebrate 7B2 protein from the mollusc Lymnaea stagnalis. We find that Lymnaea 7B2 (L7B2) displays a remarkably low (29%) degree of conservation between vertebrate and invertebrate 7B2, which is predominantly restricted to only a few small stretches of residues in both the NT and the CT of L7B2. Two regions in the CT of L7B2, designated LCT1 and LCT2, appear to inhibit LPC2 (13) enzyme activity in extracts of the Lymnaea neuroendocrine insulin-producing cells and recombinant purified mouse PC2 activity (14).

MATERIALS AND METHODS

PCR Analysis—Degenerate oligonucleotides were synthesized based on amino acid sequences conserved among vertebrates 7B2 proteins (10–12). Oligos OL1 (5'-CGGAAATTCCAGGATCCTCCGAAACGCTAGCTCTACG-3') and OL2 (5'-CAAGCTTGGNACN(GC)(AT)(CT)(CT)TTNGCNACNAC-3') were used as random primed probe, labeled with [α-32P]dATP (specific activity, >10⁶ cpm/μg). Membranes were hybridized in 6 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.2% SDS, 5 × Denhardt’s solution, and 10 μg/ml herring sperm DNA at 65 °C for 18 h. The filters were washed in 0.2 × SSC, 0.2% SDS at 65 °C for 30 min and autoradiographed.

Subcloning and Nucleotide Sequence Analysis—L7B2 PCR products and pBluescript II L7B2 cDNA generated by in vitro excision were sequenced in both orientations according to the dyeoxy chain termination method (15), using T7 DNA polymerase (U. S. Biochemical Corp.). Following sequencing from universal primer sites present in the vectors, the sequence information was used to design new primers and sequencing was continued. Sequence alignments were made using the program Clustal V.

In Situ Hybridization and Immunocytochemistry—Specific [α-35S]-UTP (DuPont)-labeled cRNA probes were made on 200 ng of linearized cDNA of LPC2 (nucleotides 1521–2632 (13)) and L7B2 (nucleotides 1–462). Separate in vitro transcription reactions were performed at 37 °C, using either T3 or T7 RNA polymerase (Boehringer Mannheim) containing 1 mCi G/ACTP and 3 μl [α-35S]UTP as described by Smit et al.

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‡ The abbreviations used are: PC, prohormone convertase; L7B2, Lymnaea 7B2; LPC, Lymnaea prohormone convertase; PCR, polymerase chain reaction; AMC, amino methyl coumarin; NT, amino-terminal; CT, carboxyl-terminal; LGC, light green cell.
Identification of Molluscan 7B2 cDNA

Fig. 1. Nucleotide sequence and deduced amino acid sequence of L7B2 cDNA. The number of nucleotides is indicated at the end of each line. Amino acid sequence numbering starts at the predicted amino-terminal residue (arrow) and is indicated above the sequence. The consensus for polyadenylation is shown in bold. A potential site for N-linked glycosylation is overlined. Putative endoproteolytic cleavage sites are boxed. The horizontal arrows indicate positions of oligonucleotides OL1 and OL2.

al. (16). Both antisense and sense (control) cRNA probes had specific activities between 1 × 10⁶ and 1 × 10⁷ cpm/μg of RNA. Serial 7-μm sections of 1% paraformaldehyde/1% acetic acid-fixed cerebral ganglia were used for in situ hybridization. After pretreatment and prehybridization of the slides (16), hybridization was carried out overnight, at 50 °C, by applying 35S-labeled probe at a final activity of 1.5 × 10⁸ cpm per slide. Slides were rinsed at 2 × SSC/50% formamide at 50 °C (16). Finally, radioactivity was visualized by dipping slides in melted, diluted; 70 pmol/h in the control reactions of LPC2 immunodepleted LGC extracts not containing peptide was 1 and 70 pmol/h in the control reactions of LPC2 immunodepleted LGC extracts. Linear regression, using the program SYSTAT, was used to determine the IC₅₀ of enzymatic inhibition.

Peptides—Synthetic peptides were purified by high performance liquid chromatography (TANALABORATORY). LCT1 and LCT2 correspond to Leu₂⁰³-Lys²⁰⁴ and Ser²⁰⁵-Leu²⁰⁶ of L7B2, respectively. A control peptide not related to L7B2 (Arg-Ser-Asn-Leu-Lys-Tyr-Lys-Gly-Gln-Ile-Leu-Met) was tested.

RESULTS AND DISCUSSION

Cloning of Lymnaea 7B2 cDNA—Because a PC2 convertase is present in various types of peptidergic neurons in the Lymnaea brain (13), we hypothesized that the activation of LPC2, like that of vertebrate PC2, requires interaction with a protein related to 7B2. Because vertebrate 7B2 proteins show a high degree of sequence identity, in principle many possibilities for the design of primers toward regions of interphyllum sequence conservation exist. Therefore, a total of 10 degenerate oligonucleotide primers corresponding to different conserved parts of the vertebrate 7B2 sequences were designed and tested with PCR amplifications in 20 different combinations of primer sets. Only by using primers OL1 and OL2 was a PCR product of the expected size found. This PCR product was cloned and sequenced, and appeared to encode a sequence similar to that of the vertebrate 7B2 (data not shown).

We used the PCR fragment to screen 80,000 independent clones of a cDNA library of the cerebral ganglia of L. stagnalis. From 50 positive hybridization signals, the clone containing the most 5'-extended sequence (clone L7B2) was isolated and sequenced; it comprised 1529 nucleotides. The largest open reading frame (819 nucleotides) encodes a 273-amino acid polypeptide. Several sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford). Sections were exposed for 5–10 days in the dark at 4°C, and autoradiographs were developed in Kodak D19 (1:3) Ilford K5 emulsion (Ilford).
positions -13 and -20, indicating that the coding region is complete at the 5' end (Fig. 1). Translation of the mRNA is therefore likely to be initiated at methionine residue 1. Northern blot analysis showed a transcript of ~1.6 kilobase pairs (data not shown), indicating that the cDNA clone is indeed full-length.

Sequence Analysis of L7B2 Demarcates Only a Few Small Evolutionarily Conserved Regions— The predicted L7B2 protein is organized similarly to vertebrate 7B2 (Figs. 1 and 2), with a hydrophobic leader sequence, an NT domain, and a CT PC2 inhibitory domain (see below). Cleavage of the signal peptide most likely occurs after residue Ala-17 (19), providing a signal sequence that is shorter than that of vertebrate 7B2 (20, 21) (Fig. 2A). The L7B2 protein (calculated molecular mass, 28 kDa) is considerably larger than vertebrate 7B2 (calculated molecular mass, 20.8 kDa). The 7B2 sequences characterized in vertebrates show a high amino acid sequence identity, overall ranging from 71 to 99%. The sequence alignment of L7B2 with vertebrate 7B2 (Fig. 2) reveals a remarkably low degree of amino acid sequence identity (29%) and similarity (46–48%). Nevertheless, based on the structural organization and the overall degree of sequence conservation, we conclude that cDNA clone L7B2 encodes Lymnaea 7B2.

The deduced amino acid sequence reveals that in L7B2 various pairs of basic residues are present that are putative sites for endoproteolytic cleavage (7) (Lys201-Arg202, Lys208-Lys209, Lys215-Arg216, and Lys238-Lys239). Vertebrate 7B2 proteins contain three pairs of basic residues, except for salmon 7B2, which contains only two pairs, and mouse and rat 7B2, each of which contains four pairs. Three of the four pairs in L7B2 align with sites at analogous positions in vertebrate 7B2 (Fig. 2), but none are consensus sites for furin enzyme activity, a situation different from mammalian and Xenopus 7B2 but similar to salmon 7B2 (12). Mammalian 7B2 proteins are cleaved at two sites during their transport through the secretory pathway; e.g. porcine 7B2 is cleaved after Arg150-Arg-Lys-Arg-Arg154 and after Lys171-Lys172 (numbering of 7B2 proteins refers to that used in Fig. 2A) (20, 21). Processing of L7B2 to NT and CT domains might occur at Lys201-Arg202, a site corresponding to the 7B2 cleavage site in Xenopus (22).

Of special interest are two CT domains in L7B2, namely CT1, comprising Leu203 to Arg217 and CT2, from Ser218 to His236 (Fig. 2, B and C). The region in vertebrate 7B2 corresponding to CT2 displays a substantial degree of sequence identity with
these domains, whereas the region in vertebrate 7B2 corresponding to CT1 has diverged. The vertebrate 7B2 region analogous to CT2 is indeed a potent inhibitor of PC2 enzyme activity (4–6, 9), whereas the NT domain containing the CT1 region fails to be active (5). In particular, the Lys\(^{171}\)-Lys\(^{172}\) pair present in the inhibitory CT2 domain is essential (5, 9). The Lymnaea CT1 and CT2 domains may be internally cleaved because each contains a Lys-Lys pair (Lys\(^{208}\)-Lys\(^{209}\) and Lys\(^{238}\)-Lys\(^{239}\) for CT1 and CT2, respectively) (Figs. 1 and 2). As shown below, both LCT1 and LCT2 inhibit LPC2 enzyme activity.

The sequence identity between the NT domain of L7B2 and the vertebrate counterparts is predominantly restricted to a 10-amino acid proline-rich region (residues Pro\(^{90}\)-Thr\(^{99}\), rat Lymnaea brain. The corresponding to CT1 has diverged. The vertebrate 7B2 region analogous to CT2 is indeed a potent inhibitor of PC2 enzyme activity (4–6, 9), whereas the NT domain containing the CT1 region fails to be active (5). In particular, the Lys\(^{171}\)-Lys\(^{172}\) pair present in the inhibitory CT2 domain is essential (5, 9). The Lymnaea CT1 and CT2 domains may be internally cleaved because each contains a Lys-Lys pair (Lys\(^{208}\)-Lys\(^{209}\) and Lys\(^{238}\)-Lys\(^{239}\) for CT1 and CT2, respectively) (Figs. 1 and 2). As shown below, both LCT1 and LCT2 inhibit LPC2 enzyme activity.

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The sequence identity between the NT domain of L7B2 and the vertebrate counterparts is predominantly restricted to a 10-amino acid proline-rich region (residues Pro\(^{90}\)-Thr\(^{99}\), rat 7B2; Pro\(^{108}\)-Thr\(^{117}\), L7B2) and several conserved scattered residues (Fig. 2). It has been suggested that the region with sequence similarity with a portion of the 60 kDa subclass of molecular chaperones (residues 1–90) might represent the region involved in PC2–7B2 interaction (2). However, unlike the total NT domain and total 7B2, the vertebrate 7B2 1–90 NT domain has no influence on proPC2 maturation (3). Also, residues 90–185 do not support the maturation of proPC2 (3), indicating that in addition to the strongly conserved proline-rich region (see “Addendum”) (residues 90–99), other residues, e.g. those conserved between L7B2 and vertebrate 7B2, might be of importance for 7B2–PC2 complex formation.

**LPC2 Enzyme Activity Is Inhibited by LCT1 and LCT2**—To test the inhibitory effect of L7B2 CT1 and CT2 on LPC2 enzyme activity, the soluble fraction of extracts of the insulin-related peptide-producing neuroendocrine LGC was used as an enriched source of LPC2. In situ hybridization on alternate sections of the Lymnaea brain revealed the cellular colocalization of LPC2 and L7B2 in the LGC of the cerebral ganglia (Fig. 3). Therefore, enzyme activity present in the LGC extract was used to hydrolyze the fluorogenic substrate Pyr-Arg-Thr-Lys-Arg-AMC in the presence or absence of synthetic LCT1, LCT2, and a control peptide not structurally related to L7B2. In the LGC, the furin-like convertase Lfur1 (13) is expressed at low levels, whereas Lfur2 (23) expression is not detectable. In contrast to the LGC, the salivary gland is devoid of Lfur2 (12) but expresses Lfur2 (23), and therefore it served as a control for endoprotease activity not related to LPC2.

Dose-response analysis revealed that in the LGC extract, the inhibition of protease activity by LCT2 displays a biphasic character (Fig. 4A). To discriminate between the inhibition of LPC2 and of non-LPC2 activity, LPC2 was specifically removed from the cell extract by immunoprecipitation (Fig. 4B). The high affinity inhibition by LCT2 was lost after LPC2 immunodepletion, whereas the remaining activity was inhibited only at a high dose (IC\(_{50}\) of 31 ± 3 \(\mu\)M). The inhibition profile on LPC2 activity was determined by subtraction of non-LPC2
activity from the activity in the LGC extract (Fig. 4C). A high affinity inhibition of LCT2 is found at 1.3 ± 0.3 nM, which is in the same range as the IC_{50} of vertebrate CT on PC2 (5, 9). Because LCT2 shows no inhibition toward Ca^{2+}-dependent proteases present in the soluble fraction of the salivary gland (Fig. 4D) and because previous experiments revealed that the CT domain is not an inhibitor of PC1/3 activity (5), the non-molar inhibition of LCT2 very likely involves LPC2 activity, whereas the inhibition at high concentrations concerns other, as yet unidentified enzymes. Addition of 100 mM EDTA to either the LGC extract or the salivary gland extract resulted in a conversion of 10%, displaying the residual activity of Ca^{2+}-independent proteases. Although LCT1 is much less potent than LCT2, it also shows a biphasic curve, and upon LPC2 immunodepletion from the LGC extracts, the residual activity is inhibited at 40 ± 3 μM (Fig. 4, A and B). The IC_{50} of LCT1 (~2.5 ± 0.2 μM) toward LPC2 activity was determined by subtraction of non-LPC2 activity from the activity in the LGC extract (Fig. 4C).

**Interspecies Conservation of PC2–7B2 Interaction**—To examine an interspecies functional conservation of the inhibitory LCT, both LCT1 and LCT2, as well as the control peptide, were tested on recombinant purified mouse PC2 and PC1/3 (14, 18). LCT2 inhibits mouse PC2 with an IC_{50} of 36 ± 3 μM, and the less active LCT1 inhibits mouse PC2 with an IC_{50} of 154 ± 4 μM, whereas mouse CT inhibits PC2 activity at 45 ± 3 nM. PC1/3 enzyme activity is not inhibited by either of these peptides (data not shown). Thus, the LCT1 and LCT2 peptides inhibit LPC2 at low micromolar and nanomolar concentrations, respectively, whereas they inhibit mouse PC2 only at higher concentrations. This is likely due to sequence divergence and reflects the evolutionary distance between vertebrates and invertebrates (~600 million years). So the region that resembles the carboxyl terminus of vertebrate 7B2 most (i.e. LCT1) has the least potency with respect to the inhibition of (L)PC2 activity. This finding demonstrates that carboxyl-terminal regions of L7B2 with quite different amino acid sequences are able to inhibit the catalytic site of (L)PC2. Our results reveal that during evolution the inhibitory action of the CT domain on PC2 activity has been conserved from vertebrates to invertebrates.

**Addendum**—In a recent publication, Zhu et al. (24) suggest the importance of a proline-rich region in 7B2-PC2 interaction. Interestingly, all prolines in this region are conserved in L7B2.

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