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Molecular characterization of frog chromogranin B reveals conservation of selective sequences encoding potential novel regulatory peptides[1]

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Abstract Chromogranin B (CgB) is a member of the granin family of neuroendocrine secretory proteins, which has been proposed to play a role in secretory granule biogenesis and as a precursor to bioactive peptides. The cloning of CgB in a phylogenetically distant vertebrate, the frog *Rana ridibunda*, reveals a modest overall homology (35–40%) with mammalian CgB. However, the sequences of the N- and C-terminal regions are more highly conserved (57–65% amino acid identity) and may give rise to novel regulatory peptides. In frog, intense expression of CgB mRNA was observed in particular structures of the brain and in the distal lobe of the pituitary.

Key words: Chromogranin B; Secretory protein; Peptide precursor; Neuroendocrine cell; Molecular phylogeny

1. Introduction

The granin protein family includes several acidic glycoproteins that are localized in secretory granules of endocrine, neuroendocrine and neuronal cells. Chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII) are members of this family whose biochemical and biological properties have been extensively studied in mammals [1,2]. Granins have been first described as major proteins co-stored and co-secreted with hormones and neurotransmitters upon activation of the regulated secretory pathway in endocrine and neuroendocrine cells [3].

The primary structure of granins has been determined through molecular cloning of the corresponding cDNAs [1,2,4]. These studies have revealed several commonalties between the granin protein family members, including a high proportion of acidic amino acids (up to 30%) and the occurrence of numerous pairs of basic amino acids which represent potential cleavage sites for prohormone convertases. Indeed, processing of granins within secretory vesicles is now well evidenced and gives rise to a variety of peptides that are also secreted upon stimulation [1–4]. The biochemical and biological characteristics of the granin family indicate that these proteins may play fundamental roles in the biogenesis of neuroendocrine secretory granules and may serve as precursors to several biologically active peptides [1–4].

We have previously characterized two members of the granin family, namely SgII and CgA, in a submammalian species, the frog *Rana ridibunda*, and we have observed that the sequences of discrete regions within these proteins flanked by dibasic cleavage sites have been highly preserved from amphibians to mammals [5]. Some of these conserved potential peptides have been actually characterized in different neuroendocrine tissues and have been shown to exert biological activities in the central nervous system or in peripheral organs [6,7].

Despite intense investigation on granins, the sequence of CgB has only been determined in mammalian species [1,2]. Determination of the primary structure of CgB and characterization of the expression pattern of its gene in a phylogenetically distant species may help to define the function of this protein. In the present study, we have cloned CgB in frog in order to compare its sequence and its distribution to those of its mammalian homologs.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) cloning

Frog brain, pituitary and adrenal RNA (5 μg) was reverse transcribed at 42°C for 50 min with 200 U of Moloney murine leukemia virus RNase H^- superscript in the presence of 0.5 μg oligo$(dT)_{12–18}$ in the buffer supplied with the enzyme (Life Technologies, Cergy-Pontoise, France). PCR was performed on an aliquot of the reverse transcriptase (RT) reaction in the presence of the degenerate sense primer CGB5, 5′-GA(A/G)TT(C/T)CC(A/C/G/T)GA(C/T)TT(C/T)TA(C/T)-GA(C/T)-3′ and antisense primer CGB6, 5′-AA(C/T)TT(C/T)TC(A/C/G/T)AT(C/T)TT(C/T)TG-3′, corresponding respectively to the amino acid sequences EFPDFYD and QKIAEKF in the C-terminus, and 2.5 U Taq DNA polymerase (Life Technologies). Amplification was achieved through 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min in a GeneAmp PCR System 9700 (Applied Biosystems, Norwalk, USA). PCR products were electrophoresed on a 2% agarose gel and a DNA fragment with the expected size (165 bp) was purified and ligated into the pGEMT vector (Promega, Charbonnières, France). Sequencing revealed that this PCR product was highly homologous to the corresponding C-terminal sequence of mammalian CgB.

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[1] The sequence reported in this paper has been deposited in the GenBank database (accession number 453421).

Abbreviations: CgA, chromogranin A; CgB, chromogranin B; RT-PCR, reverse transcriptase-polymerase chain reaction; SgII, secretogranin II

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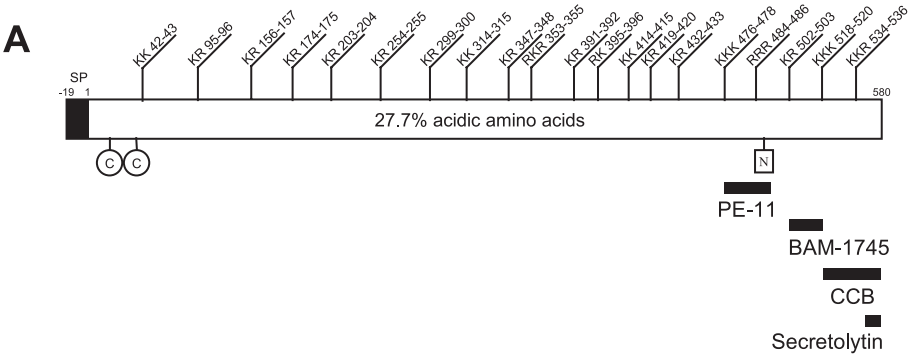
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KKK 476-478
RRR 484-486
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C
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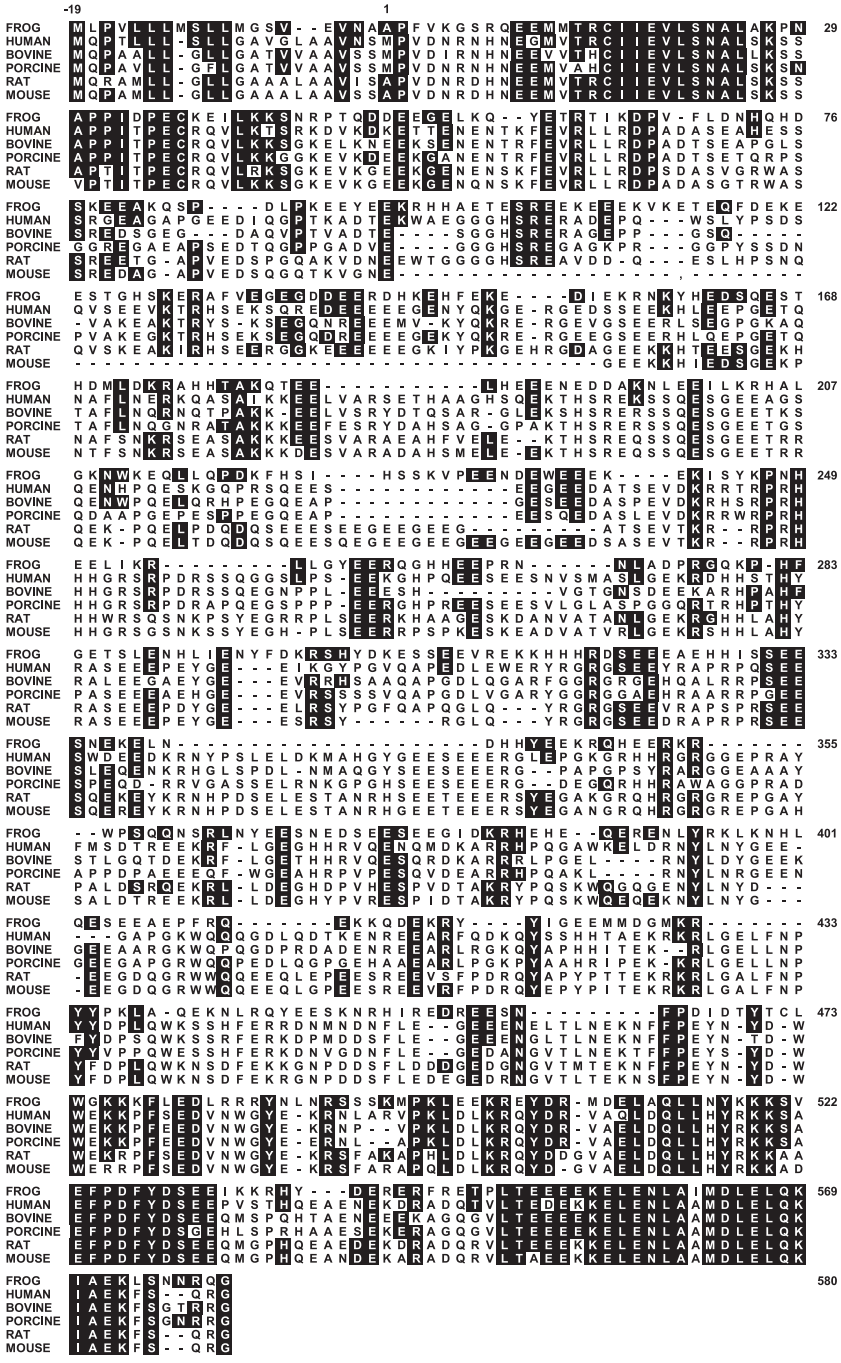
PE-11
BAM-1745
CCB
Secretolytin

B


-19
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FROG
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433
473
522
569
580

Fig. 1. Primary structure and sequence alignment of frog CgB. A: The frog prepro-CgB protein contains a 19 amino acid signal peptide (SP) and a 580 amino acid mature protein that encompasses 27.7% acidic residues. The positions of the di- or tribasic residues are indicated, the two cysteines forming the disulfide bridge are circled and the potential *N*-glycosylation site is boxed. The CgB-derived peptides PE-11 [22], secretolytin [24], CCB [20] and BAM-1745 [21] that have been described in mammals, and their locations are also indicated. B: The frog CgB amino acid sequence was aligned with the human (GenBank accession number AAH00375), bovine (accession number AAC48720), porcine (accession number AAG13399), rat (accession number AAB72089) and mouse (accession number CAA37199) sequences. The amino acids of frog CgB that are conserved in the other species are in dark boxes. The hyphens represent gaps that have been introduced for the alignment. The numbers refer to the frog sequence.
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2.2. cDNA library screening and sequence analysis

A frog (*R. ridibunda*) pituitary cDNA library made in the CDM7/amp plasmid was screened with the oligonucleotide CGB7 5′-AGCGATCTTCTGCAACTCCAGGTCCATAATTGCCAGGTTC-TCCAATTC-3′, whose sequence was deduced from the PCR DNA fragment. This oligonucleotide was 3′ end-labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Les Ulis, France) using terminal deoxynucleotide transferase (Promega). After prehybridization, filters were hybridized with ^{32}P-labeled CGB7 as described previously [8]. A positive clone, FPL22$_1$, was sequenced on both strands on a LI-COR 4000L DNA sequencer (ScienceTec, Les Ulis, France) using the Thermosequenase kit (Amersham Pharmacia Biotech), fluorescent universal primers and internal specific primers (MWG Biotech, Ebersberg, Germany). Nucleotide and deduced amino acid sequences were analyzed using the DNASIS v2.1 software (Hitachi, Olivet, France). Prediction of the secondary structures of the frog and human CgB proteins was performed at the NPS@ server (npsa-pbil.ibcp.fr) which allows to determine consensus secondary structures of a protein based on several predictions achieved by different algorithms (PHD, HNNC, Predator, SIMPA96, SOPM, etc.).

2.3. Northern blot analysis

Total RNA from frog tissues was prepared by the acid guanidinium thiocyanate-phenol-chloroform method [9] using the Tri reagent (Sigma, Saint-Quentin Fallavier, France). RNA (10–20 μg) was dissolved in denaturing buffer, heated at 65°C for 15 min, and fractionated on a formaldehyde-agarose gel. After staining with ethidium bromide, the gel was blotted on Hybond NX membrane (Amersham Pharmacia Biotech) that was subsequently hybridized at 42°C with a ^{32}P-labeled random primed (Prime-a-Gene labeling System, Promega) probe as described previously [10].

2.4. RT-PCR

Approximately 5 μg of total RNA isolated from various frog tissues was reverse transcribed by using an oligo(dT)$_{12-18}$ primer and the superscript II reverse transcriptase RNase H$^-$ (Life Technologies). PCR amplification was performed with 5 U of Taq DNA polymerase (Life Technologies), and 50 pmol of the sense (CGB-S, 5′-GC-AAGGTCATCACGAAGA-3′) and antisense (CGB-AS, 5′-AGCT-GAGCCAGTTCATCC-3′) oligonucleotides in the GeneAmp PCR System 9700 (Applied Biosystems) for 30 cycles of denaturation at 94°C for 40 s, annealing at 50°C for 1 min and extension at 72°C for 40 s.

2.5. In situ hybridization histochemistry

Frogs were anesthetized with MS 222 (Sigma) and perfused transcardially with 4% paraformaldehyde. Brains were embedded in O.C.T. Tissue Teck (Nussloch, Germany) and frontal sections (12 μm thick) were cut in a cryostat (Frigocut, Reicher-Jung, Germany). Tissue sections were hybridized with 10^7 cpm/ml ^{35}S-labeled riboprobe as previously described [11]. Sense and antisense riboprobes were prepared by in vitro transcription of a *Nco*I/*Spe*I fragment (nucleotides 950–1705) subcloned into pGEMT (Promega), in the presence of [^{35}S]UTP (Amersham Pharmacia Biotech) and T7 or SP6 RNA polymerase (Promega). Tissue sections were dehydrated and exposed onto Hyperfilm β max (Amersham Pharmacia Biotech) for 4 days. Anatomical structures were identified by staining tissue slices with hematoxylin and eosin.

3. Results

3.1. Characterization and analysis of the frog CgB sequence

Several pairs of degenerate oligonucleotides deduced from mammalian CgB sequences were used in order to amplify by PCR a DNA fragment from reverse transcribed RNA. One pair of primers deduced from the C-terminal sequence of mammalian CgB proteins allowed to amplify a DNA fragment of the expected size from frog adrenal gland reverse transcribed RNA (data not shown). Cloning and sequencing revealed that this PCR product encodes an amino acid sequence that is highly homologous to the mammalian CgB C-terminal sequence.

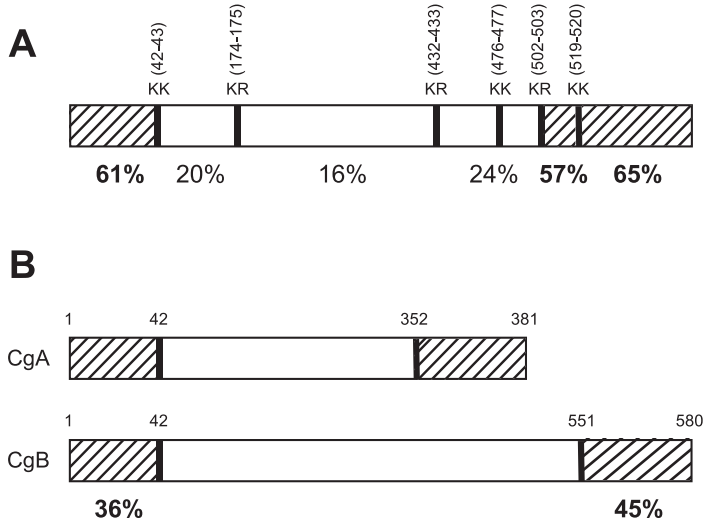

Fig. 2. Comparison of the sequence of the frog CgB protein with those of the human CgB protein and frog CgA protein. A: Schematic representation of CgB showing the percentages of amino acid identity between conserved dibasic residues in the frog and human proteins. B: Schematic representation of frog CgA and CgB showing the percentages of identity in the N- and C-terminal regions.

Based on the PCR product sequence, an oligonucleotide was synthesized and used to isolate full-length clones from a frog pituitary cDNA library.

Analysis of a positive clone revealed that the cDNA contained an open reading frame of 1797 bp encoding a 599 amino acid pre-protein (Fig. 1A). The first 19 amino acids correspond to the signal peptide and the remaining 580 amino acids represent the mature frog CgB protein with a predicted molecular mass of 70 kDa. The calculated p*I* is 5.32 which reflects the abundance of acidic residues (27.7%) in the CgB sequence, consisting of 21.2% Glu and 6.5% Asp. Frog CgB contains a putative *N*-glycosylation site at position 490 and two cysteine residues located at positions 16 and 37 which are known to form a disulfide bridge in the mammalian protein [2]. It is interesting to note that the number of amino acids separating the two cysteines (20 residues) is identical in all CgB sequences characterized to date. The amino acid sequence of frog CgB also comprises 20 di- or tribasic potential cleavage sites (Fig. 1A and B).

Sequence alignment of frog CgB with its mammalian orthologues shows an overall amino acid sequence identity of only 35–40% (Fig. 1B). However, a much higher degree of similarity was observed in the N- and C-terminal regions of the protein (Fig. 1B). These conserved domains exhibit 60–65% amino acid identity between the frog and human sequences, whereas the central region of the molecule shows less than 25% similarity (Fig. 2A). It is worth noting that the conserved regions are delimited by preserved pairs of basic residues (Fig. 2A). The comparison of the sequences of frog CgB and frog CgA, that we have previously characterized [8], showed some degree of homology in the N- and C-terminal regions which exhibit 36 and 45% sequence identity, respectively (Fig. 2B).

3.2. Tissue distribution of frog CgB mRNA

The expression and abundance of CgB mRNA in different frog tissues was studied by Northern blot and RT-PCR analysis. A single CgB transcript of approximately 2.5 kb was detected by Northern blot analysis in the brain, spinal cord and pituitary (Fig. 3A). The expression of CgB mRNA was much higher in the brain and pituitary than in the spinal cord (Fig. 3A). RT-PCR analysis revealed the presence of the CgB transcript also in the adrenal gland, stomach, pancreas and intestine (Fig. 3B). Unexpectedly, CgB mRNA was also detected in non-neuroendocrine tissues such as heart, liver, spleen and kidney (Fig. 3B). When reverse transcriptase was omitted in the RT-PCR experiments, no DNA amplification was observed in any of the tissues examined (Fig. 3B).

3.3. Localization of CgB mRNA in the frog brain

In situ hybridization histochemistry revealed the widespread distribution of CgB mRNA in the frog central nervous system (Fig. 4). In the telencephalon, intense labeling was observed in the lateral pallium and the postolfactory eminence, while the internal granular layer of the olfactory bulb and the median pallium contained a lower concentration of CgB transcript (Fig. 4A, B). The dorsal striatum and the medial amygdala were more strongly labeled than the ventral striatum and the medial or lateral septum (Fig. 4C, D). In the diencephalon, an intense hybridization signal was observed in the anterior preoptic area (Fig. 4C), the dorsal hypothalamic nucleus and the nucleus of the periventricular organ (Fig. 4E). A moderate signal was observed in the ventral hypothalamic nucleus and

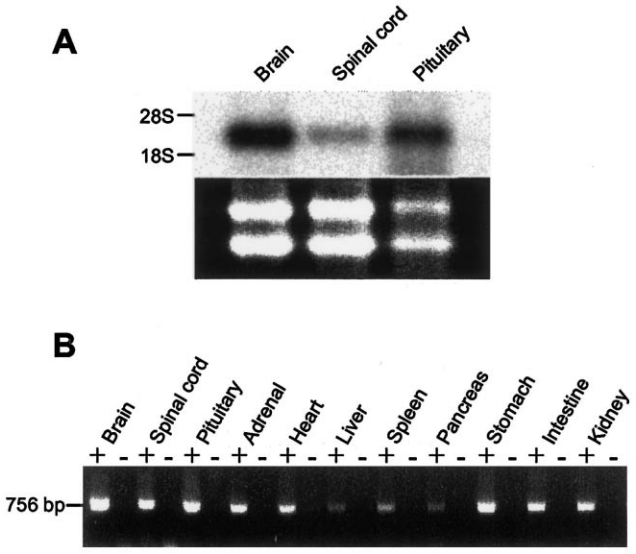

Fig. 3. Tissue distribution of frog CgB mRNA. A: Northern blot analysis was performed on total RNA extracted from the brain, spinal cord and pituitary that was hybridized to a ^{32}P-labeled CgB probe. The positions of 28S and 18S ribosomal RNA are indicated and their staining by ethidium bromide is shown under the autoradiogram. B: RT-PCR analysis of CgB mRNA expression in different frog tissues. RNA from these tissues was analyzed in the presence (+) or absence (−) of RT.

the thalamic nuclei where only a few cells were strongly labeled (Fig. 4E, F). In the mesencephalon, CgB mRNA was abundant in the optic tectum and in the anteroventral tegmental nucleus, whereas the anterodorsal tegmental nucleus and the torus semicircularis displayed only background hybridization signal (Fig. 4G). In the pituitary, intense expression was observed in the distal lobe, while the intermediate lobe was totally devoid of CgB mRNA (Fig. 4G, H). In the spinal cord, both the dorsal and ventral horns were labeled (Fig. 4I, J). When a sense probe was used, no hybridization signal was observed (Fig. 4K).

4. Discussion

This study has described the first molecular characterization of CgB in a non-mammalian vertebrate, the frog *R. ridibunda*. The predicted frog CgB protein exhibits the common characteristics of granins such as the presence of multiple pairs of basic residues (20 dibasic motifs) and a high content of acidic amino acids (27.7%). Alignment of the frog CgB protein with its mammalian homologs [12,13] revealed a rather low overall amino acid sequence identity (~35%). However, the N- and C-terminal regions were more highly conserved (~60%) than the internal region which exhibited a remarkable sequence variation (~20%). In mammalian species, the N- and C-terminal portions of CgB exhibit ~90% sequence identity and the central region shows ~60% sequence similarity [2]. It thus appears that the N- and C-terminal extremities of CgB have been selectively conserved during evolution while the middle portion of the protein has diverged more rapidly. In addition, a bioinformatic analysis revealed that consensus secondary structures predicted by different algorithms in the N- and C-terminal domains of the mature frog CgB protein are preserved in the human CgB protein, whereas the secondary structures predicted in the central portion of the molecule

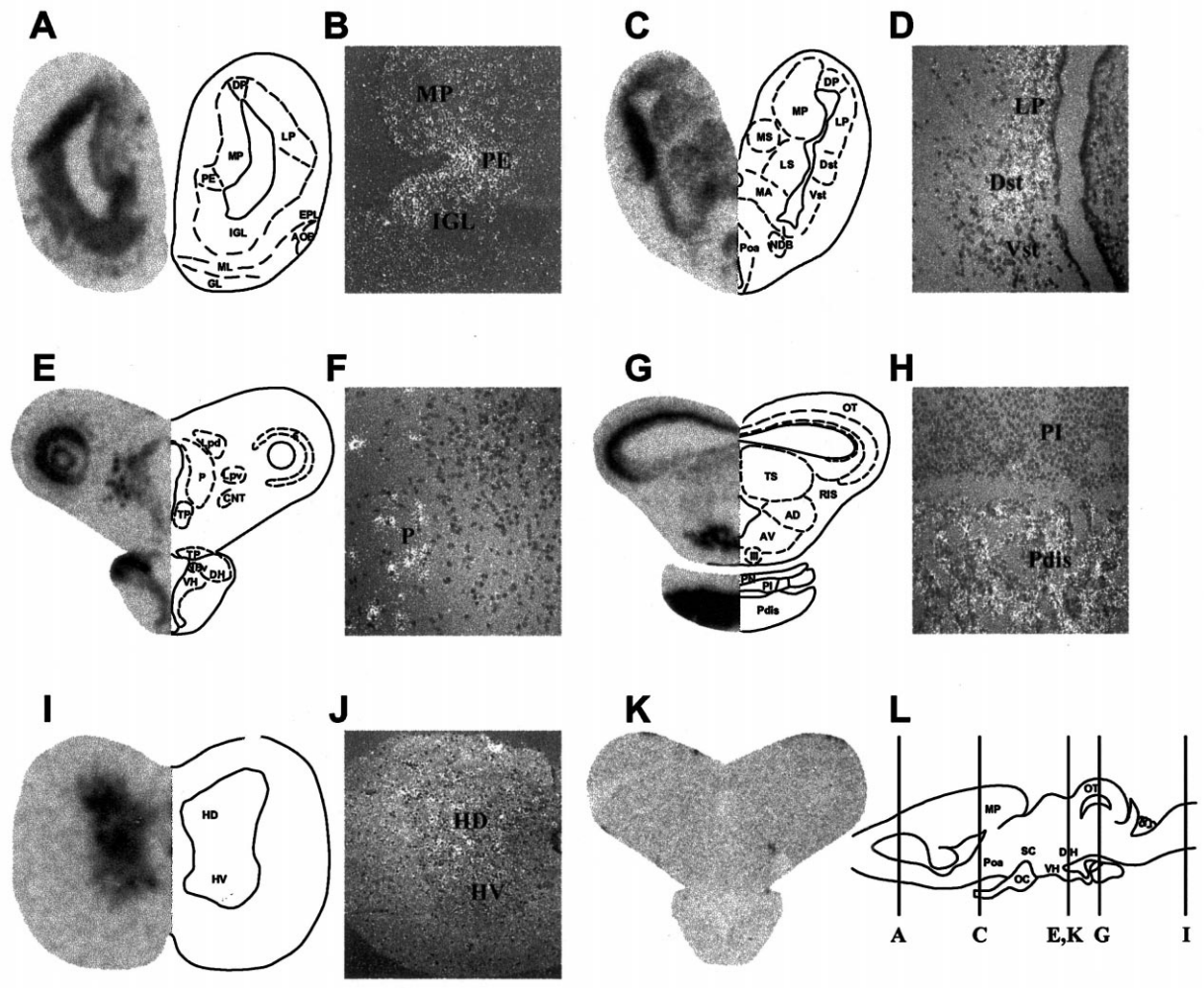

Fig. 4. In situ hybridization analysis of CgB gene expression in the frog central nervous system. The autoradiograms obtained from frontal brain slices at the level of the telencephalon (A), telencephalon/diencephalon (C, E), mesencephalon (G) and spinal cord (I) are shown. Hybridization with the sense probe is shown on a section at the level of the telencephalon/diencephalon (K). L: Schematic drawing of a parasagittal view of the frog brain showing the levels of the frontal sections. The anatomical structures are indicated beside each section. Emulsion photomicrographs of selected areas of sections in A, C, E, G and I are shown in B, D, F, H and J, respectively. Abbreviations: AD, anterodorsal tegmentum; AOB, accessory olfactory bulb; AV, anteroventral tegmentum; CNT, central thalamic nucleus; DH, dorsal hypothalamic nucleus; DP, dorsal pallium; Dst, dorsal striatum; HD, dorsal horn; HV, ventral horn; IGL, intragranular layer of the olfactory bulb; LP, lateral pallium; LS, lateral septum; MA, medial amygdala; MP, medial pallium; MS, medial septum; NPv, nucleus of the periventricular organ; P, posterior thalamic nucleus; Pdis, pars distalis; PE, postolfactory eminence; PI, pars intermedia; POA, anterior preoptic area; TS, torus semicircularis; Vst, ventral striatum.

markedly differ in the two proteins (not shown). In frog as in mammals, the N-terminal portion of CgB exhibits two α-helices separated by a random coil structure and the C-terminal region displays a predominant α-helical structure. In contrast, while the central region of the frog protein is enriched in α-helices that alternate with random coils, the internal portion of human CgB mainly consists of random coil secondary structures. Consequently, the overall content of α-helix and random coil structures are 58 and 36%, respectively, for the frog protein and 31 and 62%, respectively, for the human protein. The strong conservation of the sequence and the secondary structure of the N- and C-terminal regions of CgB in vertebrates reinforces the idea that these segments are critical for the function of this protein.

The N-terminal region of CgB and the conserved disulfide-bonded loop it contains, have been shown to be important for the interaction of this protein with *trans*-Golgi network membranes and its sorting to secretory granules [14]. It has recently been shown that reduction of CgB expression does not affect secretory granule formation while depletion of CgA provokes the disappearance of secretory vesicles in neuroendocrine cells, indicating that CgA, but not CgB, plays a critical role in the biogenesis of dense-core secretory granules [15] and that CgB cannot compensate for the absence of expression of CgA although the two granins exhibit structural and biochemical similarities. However, CgB may play a different role in secretory granule and neuroendocrine cell biology. For instance, it has been shown that CgB and CgA in heterotetramers can interact with the intraluminal loop of the inositol 1,4,5-triphosphate (IP_3) receptor/calcium channel allowing the release of calcium which is bound to the granins in secretory vesicles [16]. This interaction involves the N-terminal domains of CgA and CgB which are conserved between frog and mammals.

The cloning of frog CgB also revealed the conservation of the C-terminal sequence and the dibasic cleavage sites it con-

tains, suggesting that this region can be processed to generate peptides which may exert biological activities in different species as it has been shown for SgII- or CgA-derived peptides [6,7,17–19]. In fact, most of the CgB-derived peptides that have been identified so far are derived from the C-terminal part of the protein (see Fig. 1). These include two peptides named GAWK and CCB that have been isolated from the human pituitary gland [20], a pyroglutamyl peptide named BAM-1745 that has been purified from bovine adrenochromaffin cell secretory granules [21] and an 11 amino acid peptide named PE-11 that has been identified in the rat and the human brain [22,23]. To date, no function has been attributed to these four peptides. Another peptide corresponding to the sequence 614–626 of CgB has been characterized from the bovine adrenal medulla [24]. This 13 amino acid peptide exerts antibacterial activity and has been named secretolytin. The sequence of secretolytin as well as those of BAM-1745 and CCB have been particularly well conserved during evolution, suggesting that these peptides may play important biological functions.

A single mRNA species encoding CgB has been characterized in frog and is highly expressed in the brain and pituitary. High concentrations of CgB mRNA were observed in several areas of the frog brain including the lateral pallium, the dorsal hypothalamic nucleus and the anteroventral tegmentum, in agreement with the widespread expression of CgB mRNA that has been reported in the mammalian brain [2]. In frog, CgB mRNA was also detected in various neuroendocrine tissues including the adrenal, the pancreas or the stomach. In particular, intense expression of CgB mRNA was observed in the distal lobe but not in the intermediate lobe of the pituitary, suggesting that CgB and/or derived peptides may play a role in specific hypophysial activities. Expression of the CgB transcript was also detected in other frog peripheral organs including the heart, kidney and spleen. In mammals, chromogranin immunoreactivity has been described in the rat heart [25] where it seems to localize to muscle cells of the atria [26] and in paraganglionic cells present in the human kidney [27]. Further studies with more sensitive techniques are required to identify and confirm the types of cells expressing CgB mRNA in non-endocrine tissues including the liver, spleen and kidney.

In conclusion, this study has described the characterization of a non-mammalian CgB, revealing the high conservation of the N- and C-terminal regions of the protein. These conserved domains may represent structural determinants underlying an intravesicular role of CgB. Alternatively, these regions may give rise to peptides that exert biological activities after release from endocrine, neuroendocrine and neuronal cells where the CgB gene is widely expressed.

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