C2PA, a new protein expressed during mouse spermatogenesis

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Abstract C2PA is a novel protein that contains a C2 membrane binding domain, a PDZ protein/protein interaction domain, and an ATP/GTP binding domain. C2PA is expressed during embryogenesis from 8.5 days post-coitum (dpc) until birth. After birth, C2PA expression is mainly observed in the post-natal and adult testis. During spermatogenesis, C2PA transcripts are specifically observed in the spermatocytes, whereas spermatogonia and spermatids are negative. Taken together, these results suggest that C2PA might be involved in cell signaling pathways occurring during spermatogenesis.

Key words: C2PA; Spermatogenesis; C2 domain; PDZ domain

1. Introduction

During spermatogenesis haploid spermatids are formed from precursor cells [1]. Spermatogenesis can be divided in three phases: the proliferative, meiotic and differentiation phases. In the proliferative phase, the diploid spermatogonia undergo rapid successive divisions. In the meiotic phase, the genetic material of the diploid primary spermatocytes is segregated in order to give rise to the haploid secondary spermatocytes and spermatids. In the differentiation or spermiogenic phase, the spermatids undergo transformation to spermatozoa which are structurally equipped to reach and fertilize the egg.

Post-natally, during the first wave of spermatogenesis, all the germ cells are synchronized in their differentiation [2]. This temporal appearance of the successive cell types is well characterized. Thus, in the mouse, by day 3, most of the germ cells are dividing spermatogonia, by day 13 the most advanced cells are pachytene spermatocytes, by day 21, meiosis is complete and early spermatids appear, and, by day 35, mature spermatozoa are produced [3].

In the present study, we have identified and characterized a mouse cDNA that encodes a novel protein, C2PA. We have studied C2PA expression in embryonic and adult animals, during post-natal development of the testis, and during spermatogenesis. Together, our data provide evidence that C2PA might be involved in cell signaling events occurring during spermatogenesis.

2. Materials and methods

2.1. Cloning of cDNA, sequencing and sequence analysis

C2PA cDNAs subcloned in pBluescript vector (Stratagene Inc., La Jolla, CA, USA) were purified with RNase A treatment (10 μg/ml; 30 min, 37°C) followed by PEG/NaCl precipitation (0.57 vol; 20%, 2 M) and ethanol washing. Vacuum-dried pellets were resuspended at 200 ng/ml in T10E1 [4]. Double-stranded DNA templates were then sequenced with Taq polymerase, using either pBluescript universal primers and/or internal primers, and dye-labeled dNTPs for detection on an Applied Biosystems 373A automated sequencer.

PCR amplification was performed on cDNA reverse-transcribed from 4 weeks old testis RNA (35 cycles: 30 s 94°C; 30 s 68°C; 2 min 72°C) (see primers on Fig. 1A).

Protein sequence analysis was performed using the PileUp program provided by the Genetic Computer Group (GCG) package [5].

2.2. Northern blot analysis

Embryos were collected at days 7.5–16.5 post-coitum (dpc). Testes were collected at birth, 8 days old mice, and from mice of ages 16–21 days. Adult tissues were from 3 months old animals. Total RNA was isolated by using guanidinium isothiocyanate extraction [4], and was fractionated on 1% agarose gels in the presence of 2.2 M formaldehyde, and transferred to nylon membranes (Hybond N, Amersham Corp., Arlington Heights, IL, USA). Hybridization was done by using 5×SSC, 50% formamide, at 42°C for 36–48 h with ^{32}P-labeled probe corresponding to C2PA mouse cDNA fragment (Fig. 1, nucleotides 818–2068). Washing with 0.1×SSC, 0.1% SDS at 60°C was performed twice. Blots were autoradiographed 2 days at −80°C.

2.3. In situ hybridization

Tissues were fixed in 4% paraformaldehyde in PBS at 4°C for 1–2 h, equilibrated in 20% sucrose in PBS at 4°C for 8–10 h, and mounted in 7.5% gelatin, 15% sucrose in PBS at 37°C, frozen on dry ice and cut into 14 μm thick sections. Thawed sections were hybridized using digoxigenin-labeled probes in a buffer containing 50% formamide, 200 mM sodium chloride, 1×Denhardt's, 1 mg/ml yeast tRNA and 10% dextran sulfate at 65°C overnight [6]. After washing in 50% formamide, 150 mM sodium chloride, 15 mM sodium citrate, 0.1% Tween 20 at 65°C, the sections were incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (AP) (Boehringer Mannheim), washed and processed for AP activity with NBT/BCIP.

The C2PA antisense and sense probes were synthesized from a pBluescript vector containing an internal fragment (Fig. 1, nucleotides 818–4712) of C2PA, using the T7 and T3 RNA polymerases, respectively.

2.4. Immunohistochemistry

After in situ hybridization, the sections were washed in PBS, incubated with a 1:500 dilution of an anti-CREMτ polyclonal antibody (IgG subtype; a generous gift of Paolo Sassone-Corsi, IGBMC, Illkirch, France), generated by injecting the CREMτ recombinant protein into rabbits ([7] and references therein). Sections were then rinsed three times in PBS, and revealed using biotinylated anti-rabbit immunoglobulin antibody and avidin–biotin–peroxidase reagents (Vectastain kit Vector). Slides were stained 5 min with a peroxidase detection kit DAB (Vector, Burlingame, CA, USA), counterstained in methyl green, dehydrated and mounted [6].

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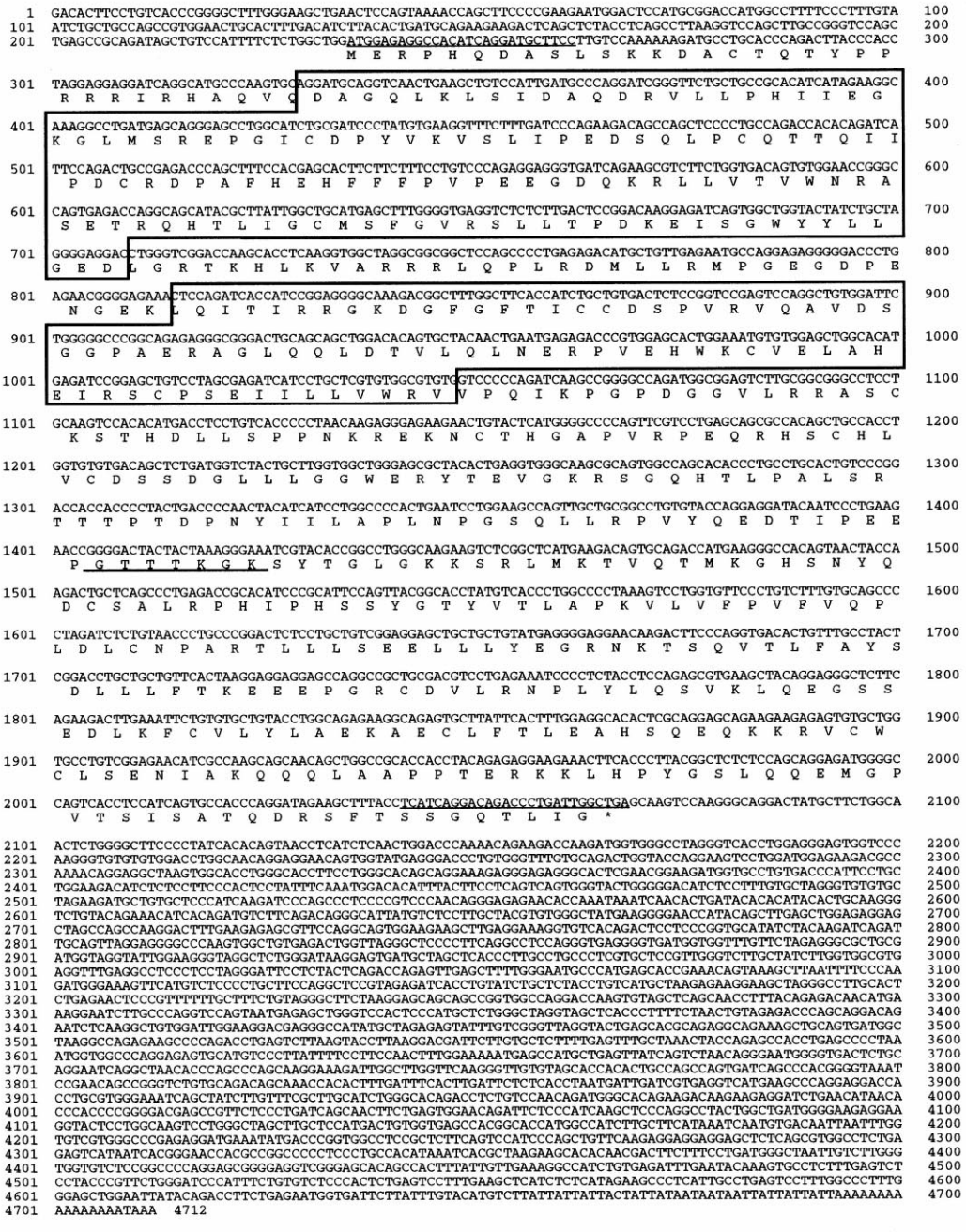
A



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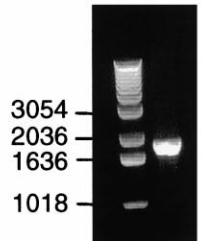

Fig. 1. cDNA and deduced amino acid sequences of mouse C2PA. A: Nucleotides are numbered from 5′ to 3′ and amino acids in the open reading frame are designated by the one letter code. The C2 (residues 33–158) and PDZ (residues 194–271) domains are boxed. The ATP binding motif (residues 391–398) is underlined in bold. The asterisk indicates the stop codon. 5′- and 3′-primers used for RT-PCR amplification are underlined. B: Gel electrophoresis of RT-PCR product (right) and molecular weight markers (left).

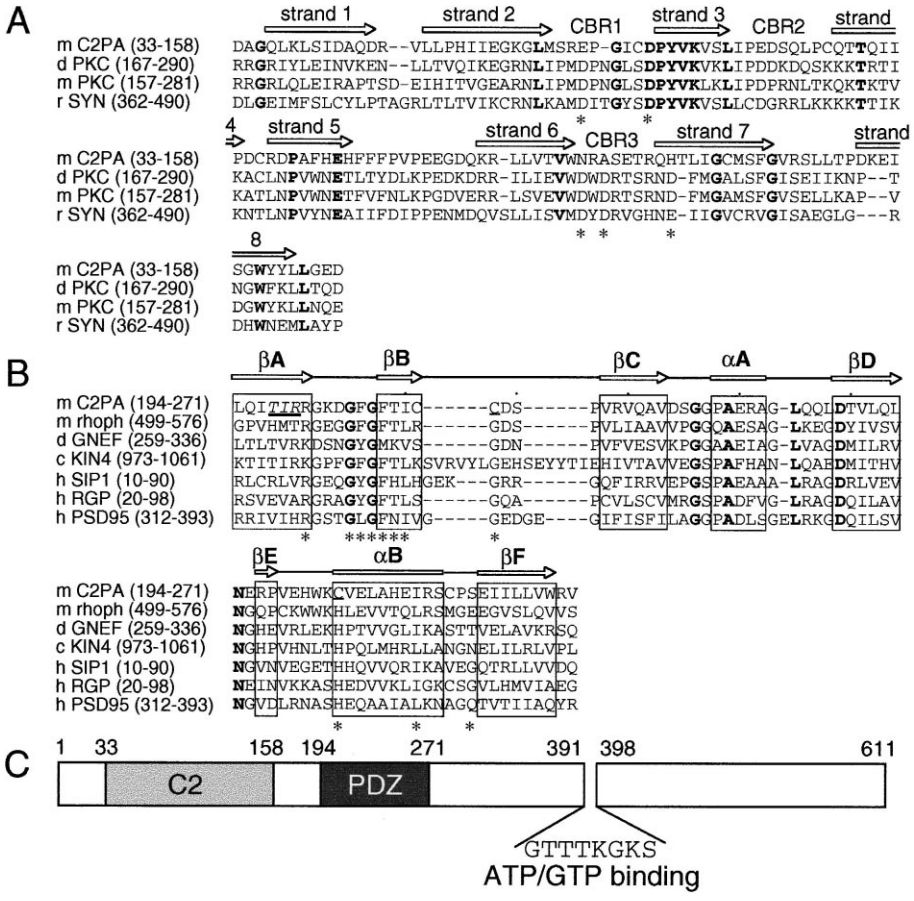

Fig. 2. Domain organization of the C2PA protein. Primary sequences of the C2PA C2 (A) and PDZ (B) domains were aligned with those from various proteins of various species. Bracket numbers indicate the respective positions of the motif in each protein. Residues identical in all sequences are bold-typed. Gaps (−) are introduced to optimize alignment. h, Homo; m, Mus; d, Drosophila; r, Rat; c Caenorhabditis. PKC, protein kinase C; SYN, synaptotagmin IV; rhoph, rhophilin; GNEF, guanine nucleotide exchange factor; KIN4, kinesin-4; SIP1, SRY interacting protein; RGP, regulator of G protein signaling 12; PSD95, synaptic density protein. The secondary structural elements are shown as arrows (β-sheets), bars (α-helices) and lines (connecting loops). A: C2PA C2: the five calcium-coordinating acidic residues conserved in the C2 domain of PKC and SYN are marked by asterisks. B: C2PA PDZ: the PSD95 residues involved in the binding of target peptides are indicated with asterisks. Cysteines 211 and 250 that replace conserved glycine and histidine are underlined. The PKC phosphorylation site (TIR) is in italics and underlined. C: Schematic representation of the C2PA protein organization. The C2 (gray) and PDZ (black) domains are boxed, and the conserved ATP/GTP binding loop sequence is indicated.

3. Results

3.1. cDNA and the predicted amino acid sequence of C2PA

We have previously identified a new cDNA containing 197 base pairs (bp) (nucleotides 818–1014 of the sequence presented in Fig. 1A) (J.-L. Linares, unpublished results). In order to clone the complete corresponding cDNA, we used this fragment as a probe to screen a mouse cDNA library established in the pASV3 vector. This library contains random cDNA fragments derived from 9.5–12.5 dpc mouse embryo RNAs ([8] and references therein). Identified cDNA fragments were subcloned into pBluescript, and both sense and antisense strands were sequenced. Some of them were further used as probes to identify more 5′- or 3′-cDNA fragments. The complete mouse C2PA cDNA sequence (Fig. 1A; EMBL/GenBank/DDBJ accession number AJ250999) was established from overlapping clones. The C2PA cDNA contains 4712 bp and the first ATG codon (bp 236–238) has a favorable context for initiation of translation [9]. A stop codon, located at bp 2066–2068, indicates that the cDNA contains a 3′-untranslated region of 2647 bp and encodes a putative protein of 611 residues.

The complete C2PA open reading frame was amplified using RNA isolated from the testes of 4 weeks old mice. Gel analysis of RT-PCR product showed an intense band at an approximate size of 1.8 kb (Fig. 1B).

3.2. Domain organization of C2PA

Multiple sequence alignment analysis (PileUp program) showed that, from its amino (N)- to its carboxy (C)-terminal part, C2PA protein shares several domains with known proteins.

Residues 33–158 share 28–35% identity and 53–60% similarity with the C2 domain of the mouse (EMBL p05130) and Drosophila (EMBL p05697) protein kinase C (PKC), and the rat synaptotagmin (EMBL Q62746) (Fig. 2A). The C2 domain is about 130 residues long and consists of a β-sandwich of two four-stranded β-sheets. Sequence alignment with the C2 domains of a wide variety of proteins suggests that all known C2 domains exhibit either a type I or type II topology,

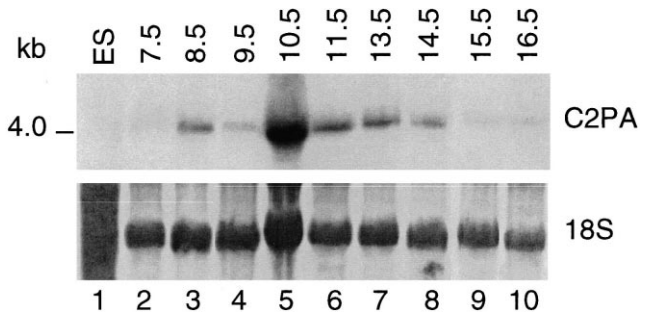

Fig. 3. Northern blot analysis of C2PA mRNA during mouse development. Each lane contained 10 μg of total RNA. From left to right, RNA samples from ES cells and 7.5 to 16.5 dpc embryos, as indicated. Hybridization was carried out using a ^{32}P-cDNA probe for C2PA. The 4.0 kb C2PA transcript was observed from 8.5 to 14.5 dpc (lanes 3–8). 18S RNA was used as an internal control of gel RNA loading.

differing slightly in their β-strand connectivity [10]. The C2 domain of C2PA shows the highest homology with those of PKC and synaptotagmin that are of the type I. The calcium binding sites of the C2 domain usually consist of three Ca^{2+} binding loops named calcium binding regions 1, 2, and 3 (CBR1, CBR2, CBR3), and involve five calcium-coordinating acidic residues located on CBR1 and CBR3 [11]. Only two of them, located on CBR1 (residues 62 and 67), are conserved in C2PA. A region spanning residues 194–271 showed homology (28–41% identity, 52–62% similarity) with the PDZ domain [12,13] of several proteins. The PDZ domain is a globular structure mainly composed of six stranded β-sandwiches (βA–βF), flanked by two α-helices (αA and αB). PDZ domains fall into two classes that bind to the Ser/thr-X-Val-COOH and Phe/tyr-X-Phe/Val/Ala-COOH motifs, respectively [14]. The PDZ domain of C2PA resembles the PDZ domains of mouse rhophilin (EMBL Q61085), Drosophila guanine nucleotide exchange factor (EMBL O44113), Caenorhabditis KIN4 (EMBL P90744), human SIP1 (EMBL O00272), human regulator of G protein signaling 12 (EMBL CAB55859), and human PSD95 (EMBL P78352) (Fig. 2B), and, thus, can be classified putatively as a class I type. However, two amino acids (Gly and His) known to be involved in the protein binding are not conserved in the C2PA PDZ, but replaced by two Cys (residues 211 and 250). Modeling analysis showed that these two Cys lie in a configuration that allows for the formation of a disulfide bond (data not shown). Consequently, the tertiary structure of the PDZ domain might be conserved. Moreover, we note the presence of a PKC phosphorylation site (consensus S/T-X-R/K) within this PDZ domain (TIR, residues 197–199).

Finally, C2PA contains an ATP/GTP binding site motif A (residues 391–398) that is shared by several proteins that bind ATP or GTP [15].

Thus, from its N-terminus to its C-terminus, the C2PA contains a C2, a PDZ and an ATP binding domain (Fig. 2C).

3.3. Expression of C2PA in mouse embryos and in adult tissues

C2PA expression during mouse embryogenesis was studied by Northern blot analysis of total RNA extracted from embryonic stem (ES) cells corresponding to embryos at 3.5 dpc and daily from whole embryos from 7.5 to 16.5 dpc. The murine C2PA mRNA detected was approximately 4 kb, a size consistent with the nucleotide sequence established previously (Fig. 1A). The gene was found to be expressed during mouse embryonic development from 8.5 dpc until 16.5 dpc, but not in ES cells nor 7.5 embryos. The highest levels of expression were observed from 8.5 dpc to 14.5 dpc (Fig. 3).

A variety of mouse adult tissues including colon, small intestine, stomach, kidney, liver, lung, salivary gland, heart, muscle, skin, placenta, mammary gland, uterus, ovary, seminal vesicle, thyson gland, testis, spleen and thymus was tested for the expression of C2PA using Northern blot analysis. No expression was observed in most of the tissues, except for the

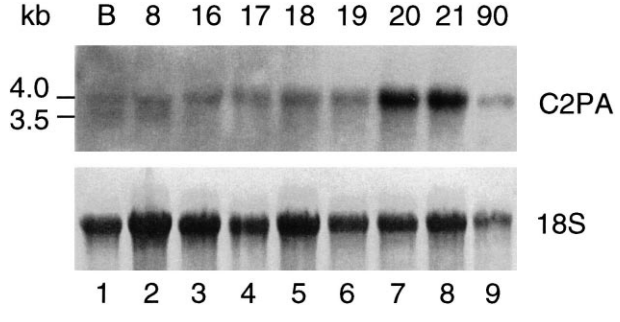

Fig. 4. Northern blot analysis of expression of C2PA during post-natal development of mouse testis. From left to right, RNA samples (10 μg) from newborn (B), 8, 16–21, and 90 days old mice, as indicated. Hybridization was carried out using a ^{32}P-cDNA probe for C2PA. C2PA is expressed throughout post-natal testis development (lanes 1–8), and in the adult testis (lane 9).

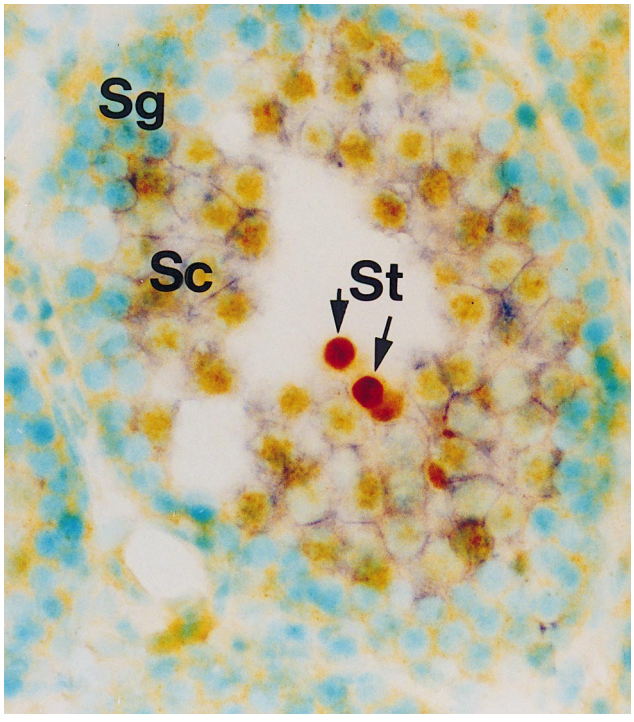

Fig. 5. Cellular localization of C2PA mRNA and CREMτ protein in 21 days old mouse testis. In situ hybridization of testicular sections using C2PA antisense RNA probe showed that its expression (violet) was restricted to the spermatocytes (Sc). Immunohistochemistry of the same sections using anti-CREMτ antibody showed that CREMτ (brown) is present in the nuclei of spermatids (St). Spermatogonia (Sg) were negative for the expression of the two genes. Cell nuclei were counterstained in green.

testis (Fig. 4, lane 9) and lung (data not shown) which showed moderate and low level expression, respectively.

3.4. C2PA expression during post-natal development of mouse testis

The expression of C2PA was investigated during post-natal testis development, from birth to 21 days of age. Using Northern blot analysis, C2PA mRNA was observed in the mouse testis at all the days tested (Fig. 4). A peak of expression was observed at 20–21 days of age (Fig. 4, lanes 7 and 8). In addition, a second smaller size mRNA with an approximate size of 3.5 kb was detected, during the earlier stages of development, from birth to 8 days of age (Fig. 4, lanes 1 and 2).

3.5. Cellular localization of C2PA

In the testis, germ cells representing different developmental stages can be distinguished by their morphological features and typical position in the tubular epithelium. From the periphery to the center of the tubule, three cell types can be discerned: the spermatogonia, spermatocytes and spermatids [2,3].

To determine which early germ cells were expressing C2PA, tissue sections from the testes of 21 days old mice were examined using in situ hybridization. C2PA mRNA was observed in the spermatocytes, whereas spermatogonia and spermatids were totally negative (Fig. 5). Since the cAMP-responsive element modulator CREMτ has been reported to be specifically expressed in the spermatids [7], CREMτ immunostaining of the same sections was performed in order to identify this cell type. Intense nuclear labeling of round cells corresponding to early spermatids was observed (Fig. 5).

4. Discussion

In the present study, we have identified and characterized a cDNA encoding for a novel protein which we have named C2PA. It contains, from the N-terminus to the C-terminus, a C2, a PDZ and an ATP binding domain. Database searches show that, at present, there are only three proteins, namely GAP (BCR-like GTPase activator protein) [16], Rim (Rab3 effector protein) [17] and aczonin [18], which contain both C2 and PDZ domains, but in a reversed manner since they are located at the N- and C-terminus, respectively. These proteins are mostly expressed in the brain where they accumulate in synaptic junctions. Thus, C2PA defines a new type of C2 and PDZ domain-containing proteins.

The C2 domain is involved in calcium-dependent phospholipid binding, and confers membrane binding activity to a wide variety of proteins involved in signal transduction and membrane trafficking events [19]. Five acidic residues located in CBR1 and CBR3 are involved in the Ca^{2+}-dependent membrane binding activity [20]. Several of them are not conserved in C2PA, and the ability of this structure to bind to Ca^{2+} remains to be evaluated. Finally, C2PA might also bind to membrane in a Ca^{2+}-independent manner, as is the case for the second C2 domain of synaptotagmin II [21].

The PDZ domain is a multi-functional protein–protein interaction module that plays important roles in clustering membrane proteins, organizing signal transduction complexes, and maintaining cell polarity [12,13]. The PDZ domain of C2PA is closely related to the class I type PDZ domain that interacts with the Ser/Thr-X-Val peptide at the C-terminus of target proteins [14]. Although two residues important for this interaction are not conserved, the tertiary structure elements are conserved in the C2PA PDZ, suggesting that it might be functional. During mouse embryogenesis, C2PA is continuously expressed from 8.5 dpc to birth, indicating that it might be involved in developmental process(es) shared by various organs at different times of development. In adult tissues, C2PA expression is strongly regulated since it is mainly observed in the testis. This result prompted us to investigate the possible involvement of C2PA during testis post-natal development and spermatogenesis.

During testis post-natal maturation, C2PA expression was first detected in the newborn and increased to reach the highest levels at 20–21 days of age. Thus, C2PA participates throughout post-natal testis development. Surprisingly, a lower size C2PA mRNA was detected during the first week of neo-natal testis development. During this period, immature type A spermatogonia enlarge and initiate the cycle of spermatogenesis that actively begins by 9 days after birth [3]. The significance of this lower size mRNA remains to be studied.

Spermatogenesis begins by the mitotic division of germ cell spermatogonia to give rise to diploid primary spermatocytes, which themselves replicate their DNA content before undergoing the two successive meiotic divisions that result in the production of haploid secondary spermatocytes and round spermatids [2,3]. The nature of the germ cells that express C2PA during spermatogenesis was defined during the first wave of spermatogenesis, at 21 days of age. During this process, C2PA was present in the spermatocytes, but not in the spermatogonia or the spermatids. The spermatocyte stage-specific C2PA expression suggests a role for C2PA during the phase of spermatogenesis involving this cell type.

The cellular function of C2PA is still unknown. All of the three other proteins associating both the C2 and PDZ domains are involved in cellular events occurring at the proximity of the plasma membrane. GAP proteins are known to be mediators of signals generated by the ras family of proteins [22]. Rim participates in the formation of GTP-dependent complexes between synaptic plasma membranes and docked synaptic vesicles [17]. Finally, aczonin is involved in the structural organization of the synaptic active zones and regulates neurotransmitter vesicle trafficking [18]. Although the interacting molecules remain to be determined, the presence of a C2 membrane binding domain and a PDZ protein/protein interaction domain in C2PA suggests that C2PA could behave as an adapter molecule in pathway(s) involved in cell signaling.

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