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Localization of Group IIc Low Molecular Weight Phospholipase A_2 mRNA to Meiotic Cells in the Mouse

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Abstract We use in situ hybridization to demonstrate that the testicular expression of a novel, mouse, low molecular weight phospholipase A_2 (PLA_2 Group IIc) mRNA is specific to cells undergoing meiosis. A complete cDNA (1421 bp) encoding the mouse *Pla2g2c* gene was generated with reverse transcription-PCR (RT-PCR) and 5′ and 3′ RACE (rapid amplification of cDNA ends) RT-PCR, and its nucleotide sequence was determined. Northern blots of RNA from different tissues revealed a single 1.6 kb transcript only in testis. In situ hybridization indicated that this mouse gene is transcribed mainly in pachytene spermatocytes, secondary spermatocytes, and round spermatids. Expression of the gene is seen in all stages of the seminiferous epithelium, especially in stages VI–VII. J. Cell. Biochem. 64:369–375.

Key words: testis; phospholipase A_2; cDNA sequence; in situ hybridization; mouse; *pla2g2c*; spermatocytes; meiosis

Phospholipase A_2s (PLA_2; phosphatide 2-acylhydrolase, EC 3.1.1.4) are a class of acyl hydroxylases or esterases that hydrolyze the sn-2-acyl ester bond in glycerophospholipids [Dennis, 1983]. To date, two classes of unrelated, calcium-dependent PLA_2s and their genes have been reported [Mayer et al., 1993; Dennis, 1994]. One is a family of low molecular mass (approximately 14 kDa) PLA_2s (Groups I–III and V) which are characterized by a catalytic requirement for Ca^{2+} and a rigid three dimensional structure maintained by disulfide bridges [Heinrikson, 1991; Tischfield et al., 1996]. The second is a high molecular mass, 85 kDa, intracellular PLA_2 (Group IV) found in the cytosolic fraction in the absence of calcium but associated with cellular membranes at calcium concentrations from 0.1 to 10 μM [Clark et al., 1991; Sharp et al., 1991].

The low molecular weight (14 kDa) PLA_2s rank among the best characterized enzymes, with complete primary sequences available for more than 80 PLA_2s from organisms such as snakes, bees, and mammals [Heinrikson, 1991; Davidson et al., 1990]. In mammals, these enzymes are distributed in a wide variety of tissues [Kudo et al., 1993]. Based on selected structural determinants, 14 kDa PLA_2s have been, thus far, classified into four groups [Heinrikson et al., 1977; Davidson et al., 1990; Tischfield et al., 1996]. Group I enzyme is found in cobra and krait venoms (Group Ia) and in pancreatic juice of mammals (Group Ib). Group II enzyme is observed in rattlesnake and viper venoms (Group IIa) and primarily in inflamed sites and inflammatory and cytokine-stimulated cells of mammals (Group IIa), as well as in Gaboon viper venom (Group IIb). Group III enzyme is thus far restricted to the bee and certain lizards. The gene for Group V enzyme has been described from man [Chen et al.,

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1994a] and rat [Chen et al., 1994c] is most actively expressed in heart.

We have also reported the cDNA sequence of another low molecular weight, Ca^{2+} dependent, rat PLA_2 (RPLA2-8) [Chen et al., 1994b]. The deduced protein sequence contains the six amino acid carboxyl terminal extension and the cys 50-cys carboxyl terminal disulfide bridge characteristic of group II PLA_2s. This protein, however, is distinguished from the two previously identified group II subtypes [Heinrikson, 1991] in that it contains 16 cysteines, two more than each of the group II enzymes previously reported. We thus suggested that it be classified as a novel subgroup, Group IIc[1] [Tischfield et al., 1996]. Northern blot analysis of different rat tissue RNA, and RNA from whole testis of different developmental stages, suggested that *Pla2g2c* may only be expressed at significant levels in testis, and that it is developmentally regulated. Here, we report the cellular localization of the mRNA within mouse testis and the cloning and sequencing of the complete cDNA of mouse *Pla2g2c*.

METHODS

RT-PCR and RACE-RT-PCR

Total RNA was prepared with the RNAgents kit (Clontech, Palo Alto, CA) and RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer Corp., Norwalk, CT). 5′ RACE-RT-PCR was performed with the 5′ RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Grand Island, NY). 3′ RACE-RT-PCR was performed as previously de-

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1       TATACGTCAGCAACTCCAGCCACTTCACCGCGTTTCCCAACAGCTTTGAGTTTTGGAGGC    60
61      CGGCAGCCTGACTGCTTTCTCAGAAGCTATGGTCCACTACCTCAGCCATTCTGTTGGAGC   120
121     CAAACTGGCAAATGAAGGTGAAATCCCCGAACCGTGGACCTCCTGGTCACCTCGGGAATG   180
                                                          Met
181     AAGGGCATTGCCATCTTCCTTGTCTTCATCTTCTACTGGACAACCTCCACCCTCAGCAGT   240
        LysGlyIleAlaIlePheLeuValPheIlePheTyrTrpThrThrSerThrLeuSerSer
241     TTCTGGCAGTTCCAGAGGATGGTCAAACACGTCACAGGGCGCAGCGCCTTCTTCTCCTAT   300
        PheTrpGlnPheGlnArgMetValLysHisValThrGlyArgSerAlaPheSerTyr
301     TACGGATATGGCTGCTACTGTGGGCTTGGGGGCAAAGGGCTCCCTGTGGATGCCACAGAC   360
        TyrGlyTyrGlyCysTyrCysGlyLeuGlyGlyLysGlyLeuProValAspAlaThrAsp
361     AGGTGCTGCTGGGCTCATGACTGCTGCTACCACAAGCTTAAGGAATATGGCTGCCAACCC   420
        ArgCysCysTrpAlaHisAspCysCysTyrHisLysLeuLysGluTyrGlyCysGlnPro
421     ATCTTGAATGCCTACCAGTTCACCATTGTCAATGGGACGGTGACCTGTGGATGCACCGTG   480
        IleLeuAsnAlaTyrGlnPheThrIleValAsnGlyThrValThrCysGlyCysThrVal
481     GCCAGCAGCTGCCCCTGCGGGCAGAAGGCCTGTGAGTGTGACAAACAGTCTGTGTACTGC   540
        AlaSerSerCysProCysGlyGlnLysAlaCysGluCysAspLysGlnSerValTyrCys
541     TTCAAGGAGAACCTGGCCACCTACGAGAAGGCCTTCAAGCAGCTCTTCCCAACCAGGCCT   600
        PheLysGluAsnLeuAlaThrTyrGluLysAlaPheLysGlnLeuPheProThrArgPro
601     CAGTGTGGCAGGGACAAACTCCAGTGCTAGGAAGGCCACCCTCGTCCCTCCATCACCTGC   660
        GlnCysGlyArgAspLysLeuGlnCysEnd
661     TCTAGTCCCGAGCCCCTTCTGAAGAGCCTTGCTGTCTGGGGAGTGTCTTTTCAGTCATTA   720
721     AGGGACTGGAGGTGGAGAATTCCTGCCCTGGAAACAAACCGTGGGTATCTGGCCATTAAG   780
781     AGATACAGTCTGTCAGTAGGAAACAAGGACATAAGGGAGCAGGGATGGGGGGCATCCTGC   840
841     AATCCCCAGCATTTTGAGACATGGAGGCAGGAGAGGAAGGAGAGGCAGGAGGCCAGGGGC   900
901     AACCTCCACTATACGGTAAGTTCAAGGCTAGCCTGAGCTACTTTAGACCCTGCCTTGAAA   960
961     AATTTTTTAAACATTTTTTAAAGGAAAATAAAACAGGGGAACACAGGGGTTGAGCTGAAA  1020
1021    GGTGCTCTCAAACCCTTCTCCCCAGAAGAGCAGAGAGCCCCAGGATCCAGCCTAAACTCC  1080
1081    CCTGTACCCTCTATCCTGGTCAGGATGTGTGTCTGACTGGGGAACCAAGTCATCTACCCA  1140
1141    GGTCAGGCTGGGAAGTGAGCCGGGATCCTAGAAAACACAGCCCAGGAGCCTCGCCTGGAA  1200
1201    TCTCCTCATTTGCACCAGGTCTGACCTCTCCAGCAGGATGCAGGCTGCACCCCTGTCTCA  1260
1261    GAAGGTGGGGCACTGTCAGAAAAATGGTGTGCACCAGTGCCACAAAGATGTCACTGGTTA  1320
1321    AGATGGCATCAAGAAATGGAAAGAGGATGTTCGGGACAGGGGGTCCAAGGCACCCAAAAT  1380
1381    CCTTACCCCAATTTAGAAGCCTTTGGTCCTGTAAGACTTAATTCTAGTAAACGAGGAAGG  1420
1421    TCT
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Fig. 1. cDNA and deduced amino acid sequence of mouse *Pla2g2c*. The putative polyadenylation signal is underlined.

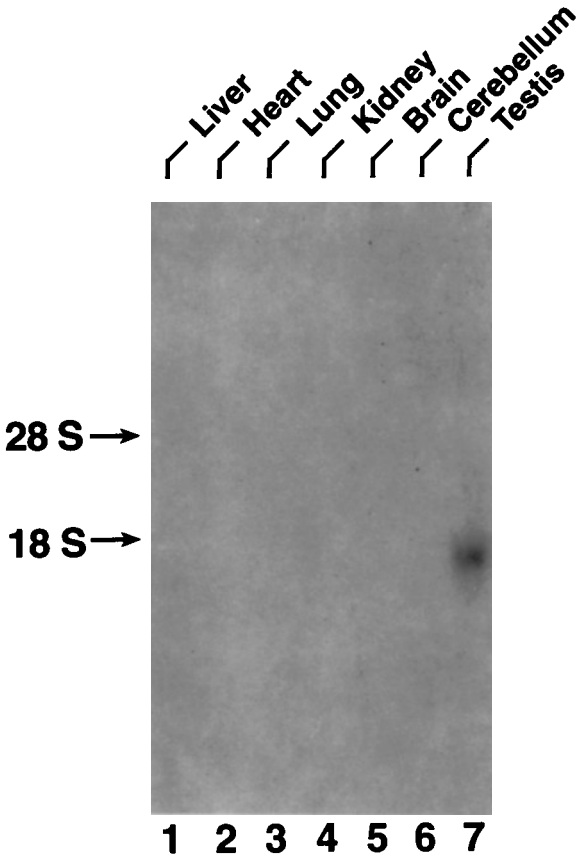

Fig. 2. Northern blot of total RNA from different mouse tissues. RNA was electrophoresed in a 1.2% agarose formaldehyde gel and transferred to a Nytran membrane using a Turboblotter. The membrane was hybridized to a random-primed ^{32}P-labeled probe generated by RT-PCR (bp 421-709).

scribed [Chen et al., 1994b]. Primers PLA8-1 (TTCTGGCAGTTCCAGAGGATGG) and PLA8-11 (AAGACACTCCCTAGACAGCAA) were used for RT-PCR. Primers 129 (GCGCAGCG-CCTTCTTCTCCTAT) and 137 (AGACAG-GTGCTGCTGGGCTCAT) were used for 3′ RACE-RT-PCR. Primers C3 (CTTGAAG-GCCTTCTCGTAGGTG) and 138 (CAGGTT-CTCCTTGAAGCAGTAC) were used for 5′ RACE-RT-PCR.

Northern blots

Ten mg of total RNA from different adult mouse tissues was subjected to electrophoresis in 1.2% agarose-formaldehyde gels [Sambrook et al., 1989] and transferred to a Nytran membrane using a Turboblotter (Schleicher & Schuell, Keene, NH). Membranes were prehybridized at 42°C for 2 h in 50% formamide, 5 x SSPE, 5 x Denhardt's solution, 0.1% SDS, and 100 mg/ml salmon sperm DNA. Hybridization to the ^{32}P-labeled probe (1.8 x 10^6 cpm/ml) was carried out under the same conditions for 16 h. The 469 bp probe was generated by RT-PCR using primers PLA8-1 and PLA8-11. Final wash conditions were 0.5 x SSC, 0.1% SDS at 62°C for 45 min. The membrane was exposed to X-ray film at −70°C for 72 h.

In Situ Hybridization

In situ hybridization histochemistry was carried out essentially as described [Bondy et al., 1993]. The sense and antisense ^{35}S-labeled riboprobe were synthesized from a 150 bp mouse *Pla2g2c* cDNA insert (bp 241 to 399, see Fig. 1) cloned in pT7/T3 (Ambion, Austin, TX), using an in vitro transcription kit (Ambion) according to manufacturer's instruction. Fresh frozen tissue sections from adult mice were used for in situ hybridization experiments. Testes (n = 10) were immediately removed from decapitated animal, snap frozen on dry ice, and stored at −70°C. Cross sections of 16 mm thickness were cut at −15°C, thaw-mounted onto poly-L-lysine-coated slides, and stored at −70°C until hybridization.

Prior to hybridization, tissue sections were warmed to 25°C, fixed in 4% formaldehyde, and acetylated in 0.25% acetic anhydride, 0.1 M triethanolamine hydrochloride, and 0.9% NaCl. Tissue was dehydrated through an ethanol gradient, delipidated in chloroform, rehydrated, and air dried. ^{35}S-labeled cRNA probes were added to fresh hybridization buffer (10^7 cpm/ml) composed of 50% formamide, 0.3 M NaCl, 20 mM Tris HCl (pH 8), 5 mM EDTA, 500 mg of tRNA/ml, 10% dextran sulfate, 10 mM DTT and 0.02% each of BSA, ficoll, and polyvinylpyrroldone. Hybridization buffer was added to the sections, which were then covered with glass coverslips and placed in humidified chambers overnight (14 h) at 55°C. Slides were washed several times in 4 x saline-sodium citrate (SSC) to remove coverslips and hybridization buffer, dehydrated, and immersed in 0.3 M NaCl, 50% formamide, 20 mM Tris HCl, and 1 mM EDTA at 60°C for 15 min. Sections were then treated with RNase A (20 mg/ml; Boehringer Mannheim) for 30 min at 37°C and passed through graded salt solutions, followed by a 15 min wash in 0.1 x SSC at 50°C. Slides were dehydrated, air dried, and apposed to Hyperfilm-beta Max (Amersham) for 4 days. Then they were dipped in Kodak NTB2 nuclear emulsion, stored with desiccant at 4°C for 12 days, devel-

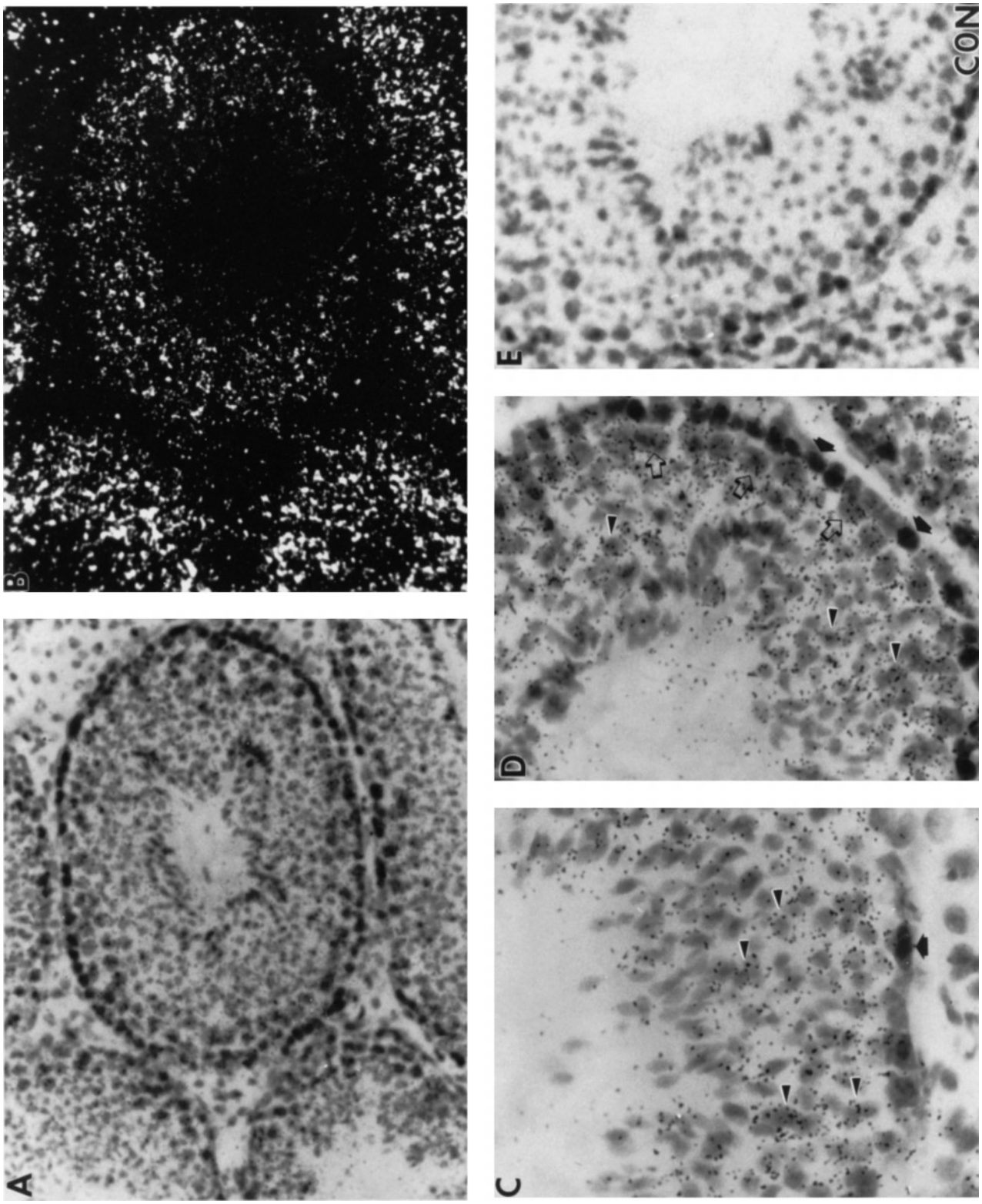

Figure 3.

oped, and stained with hematoxylin and eosin for microscopic evaluation. The level of nonspecific signal, as determined by hybridization of parallel tissue sections with a sense probe was minimal.

RESULTS

Two primers, PLA8-1 located in exon II and PLA8-11 located in exon IV, were used to RT-PCR amplify adult mouse brain total RNA as previously described [Chen et al., 1994b]. A 570 bp and a 450 bp product were obtained. Each of the two PCR fragments was cloned into pUC19 and sequenced. The sequence of the 570 bp fragment is highly homologous (see below) to rat *Pla2g2c* [Chen et al., 1994b]. There is no significant homology between the 450 bp fragment and *Pla2g2c*, nor to any other known PLA_2 cDNA sequence. The 450 bp fragment is most likely a nonspecific PCR artifact not seen in RT-PCR of rat RNA [Chen et al., 1994b]. 5′ and 3′ RACE-RT-PCR techniques were used to obtain the full-length cDNA from total adult mouse testis RNA [Chen et al., 1994b]. The entire cDNA sequence of mouse *Pla2g2c*, is shown in Figure 1.

The mouse *Pla2g2c* cDNA predicts a 130 amino acid mature peptide (Mr = 14,663) preceded by a 20 residue prepeptide. The deduced amino acid sequence, like that of its rat homologue, contains 16 cysteines. Rat and mouse *Pla2g2c* share 91% homology at the amino acid level and 92% DNA sequence homology within the protein coding region.

To determine which mouse tissues express *Pla2g2c* transcripts, a Northern blot was performed using total RNA isolated from adult mouse brain, cerebellum, liver, kidney lung, heart, and testis. As shown in Figure 2, a transcript (1.6 kb) was detected only in testis. Thus, the tissue expression of mouse *Pla2g2c* is essentially the same as that of the rat, except that the rat exhibits two transcripts (2.3 kb and 5 kb) in testis [Chen et al., 1994b] rather than only the one detected in mouse testis.

To determine which testicular cell type expresses *Pla2g2c*, in situ hybridization was carried out using a ^{35}S-labeled antisense RNA probe and histologic sections of adult mouse testis. As shown in Figure 3, *Pla2g2c* mRNA was detected in the seminiferous tubules. The dense area of hybridization is restricted to the germ cell layers. Only background radioactivity is visualized in the area between adjacent seminiferous tubules, where Leydig cells are located. Higher magnification of sections revealed that *Pla2g2c* is expressed mainly in pachytene and secondary spermatocytes and round spermatids, and predominates in stage VI–VII tubules. It is not highly expressed in spermatogonia, Sertoli cells, or elongating spermatids (Fig. 3).

Fig. 3. Localization of *Pla2g2c* mRNA in mouse testis by in situ hybridization. Preparation of testis sections and hybridization were performed as described in Materials and Methods. **A,B:** Low power brightfield and darkfield photomicrographs, respectively, showing hybridization within seminiferous tubules. **C,D**: Higher magnification photomicrographs, respectively, of seminiferous tubules. Filled arrowheads point to round spermatids; empty arrows point to pachytene spermatocytes; filled arrows point to spermatogonia (negative). **E** shows lack of hybridization with a sense-strand control probe.

DISCUSSION

A cDNA encoding the mouse testicular *Pla2g2c* has been cloned. Sequence analysis demonstrated a high level of homology to that of rat. It has been previously shown that levels of rat testicular *Pla2g2c* mRNA are developmentally regulated. The rat mRNA was not detected with Northern blots until about 4 weeks postpartum, and steady-state levels were not attained until 6–7 weeks [Chen et al., 1994b].

PLA_2 activity has been investigated from the sperm of humans [Thakkar et al., 1984; Fry et al., 1992], mice [Thakkar et al., 1983], and hamsters [Llanos et al., 1982], as well as from the seminal plasma of several different animals and man [Kunze et al., 1974; Wurl et al., 1985]. PLA_2 activity has also been found in rat epididymis, rat testis germinal cells, and rat interstitial cells [Abayasekara et al., 1990; Ellis et al., 1981].

Most of the PLA_2 activities cited above were observed to be Ca^{2+}-dependent and of low molecular weight. It has been reported that 90% of human seminal plasma PLA_2 activity is attributable to the group II enzyme [Takayama et al., 1991]. A new low molecular weight PLA_2, for which only the N-terminal sequence is known (YNYQFGLMIVITKGHFAMV), has been demonstrated in human spermatozoa [Langlais et al., 1992]. There is no significant homology between these 19 amino acids and the first 19 amino acids, or any other amino acid sequence, of mouse or rat PLA_2 Group IIc.

PLA_2s are believed to play a key role in the sperm acrosome reaction [Thakkar et al., 1983,

1984], the fusion of sperm and oocyte plasma membranes [Fry et al., 1992], the production of free fatty acids for sperm energy metabolism [Ellis et al., 1981], the regulation of luteinizing hormone-stimulated testosterone production in rat testis Leydig cells [Abayasekara et al., 1990], and the synthesis of seminal fluid prostaglandins [Takayama et al., 1991]. In testis, prostaglandin production is necessary for the contractions of the seminiferous tubules [Ellis et al., 1981].

In situ hybridization results in our study indicate that the mouse PLA_2 Group IIc gene is transcribed mainly in pachytene spermatocytes, secondary spermatocytes, and round spermatids. Expression of the gene is seen in all stages of the seminiferous epithelium, but predominates in stages VI–VII. It seems likely that the mRNA is translated within the testis tubule and that the protein is then sequestered for use at a later time, perhaps during sperm transport and fertilization.

We have recently demonstrated that the genes for human and mouse Groups IIa, IIc, and V lie within tight clusters on syntenic regions of chromosome 1p34–p36.1 and the distal part of chromosome 4, respectively. Further, we show that human *PLA2G2C* is missing part of an exon or frequently appears to contain a nonsense mutation. These data combined with our inability to detect *PLA2G2C* transcripts in adult testis or any other tissue suggest that *PLA2G2C* is a pseudogene in humans [Tischfield et al., 1996]. It is not known whether another PLA_2 gene product compensates for the absence of PLA_2 Group IIc in humans or if this enzyme activity is dispensable. The clustering of these genes raises the possibility of regulatory cross-talk and the compensatory up-regulation of one of the two other functional genes within the human gene cluster.

It was also recently reported that several strains of mice contain a frameshift mutation in *Pla2g2a* and produce no PLA_2 Group IIa enzyme [Kennedy et al., 1995, MacPhee et al., 1995]. This mutation mapped to the *Mom1* locus which is known to increase the *Min*-induced tumor number in mouse intestine [MacPhee et al., 1995]. No mutations in *PLA2G2A*, or *PLA2GV* were found in humans with attenuated adenomatous polyposis coli [Spirio et al., 1996]. These data from mice are difficult to interpret as no studies have been done to determine if the expression of the PLA_2 Group IIc or Group V genes is increased in a compensatory manner in any tissues of *Mom1* mice. Furthermore, it is likely that mutations in either *Pla2g2c* or *Pla2g5* would also map to the *Mom1* locus such that these genes should also be tested for mutations in *Mom1* mice.

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