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Characterization and localization of the rat, mouse and human testicular phosphatidylethanolamine binding protein

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Abstract. A cytosolic 23kDa protein was initially purified from bovine brain and shown to bind phosphatidylethanolamine. Later, it was also characterized in rat and human brain, and it is now known to be widespread, having been found in numerous tissues in several species. Here, we report the high level of mRNA and phosphatidyl ethanolamine binding protein expression in rat testis and to a lesser extent in mouse testis. In human testis, although it was not detectable by Northern blot analysis, the mRNA was shown to be present when PCR amplification was performed. Immunohistochemical experiments revealed that the testicular phosphatidylethanolamine binding protein (tPBP) is principally expressed in the elongated spermatids of both rat and mouse testis. This finding, and the association of tPBP with cellular membranes, suggest its possible implication in membrane remodelling during spermatid maturation.
Key words. 21-23kDa protein; phosphatidylethanolamine binding protein; spermatogenesis; testis development.

A 23kDa protein was purified from bovine brain cytosol[1]. The protein was reported to bind hydrophobic ligands and more especially phosphatidylethanolamine[2], suggesting its involvement in lipid metabolism. In 1987, we determined the amino acid sequence of the bovine brain 23kDa protein and compared it with sequences from protein data banks[3]. The comparison showed few significant sequence homologies and it was concluded that, from a structural point of view, the 23kDa protein might represent a new type of protein. Moreover, the amino acid sequence determination revealed the correct molecular weight to be 21kDa; thus, afterwards the protein was called the 21kDa protein or 21-23kDa protein. Here, we will use the latter term to designate the bovine brain protein and its related protein family. Immunochemical analyses, employing a specific anti-21-23kDa bovine brain protein antiserum, allowed the detection of the protein in bovine liver as well as in soluble extracts of rat brain and human platelets[1]. Later on, Roussel et al.[4] revealed by immunocytochemical studies that the anti-21-23kDa protein antiserum produced an intense straining of oligodendrocytes in the central nervous system of developing rats. The labelling was distributed over the entire cytoplasm and was more intense along the membranes of the rough endoplasmic reticulum and the plasma membrane. However, the biological function of the 21-23kDa protein still remained unknown. With the aim of elucidating it, we obtained information from sequence and three-dimensional structure data banks. Comparison with sequence data banks revealed slight homologies with several dozen proteins. By using hydrophobic cluster analysis (HCA), the best similarity was obtained with the N-terminal part of phosphoglycerate kinase (PGK). We built a molecular model of the bovine 21-23kDa protein from the crystallographic data of yeast PGK[5]. The model revealed that the protein possesses a potential nucleotide binding site, and by affinity chromatography the 21-23kDa protein was demonstrated to bind nucleotides, suggesting its involvement in phosphorylation or modulation mechanisms[5].

When Grandy et al.[6] tried to find an opiate receptor, they purified a 23kDa protein from rat brain by morphine affinity chromatography. Although not an opioid receptor itself, the rat 23kDa protein may be associated with such a receptor. The authors screened a rat brain cDNA library and established the sequence of a full-length cDNA coding for the 23kDa protein. The sequence deduced shared 85% identity with the 21-23kDa protein from bovine brain, showing that the rat 23kDa protein was the counterpart of the 21-23kDa protein was the counterpart of the 21-23kDa protein from bovine brain, the observed amino acid substitutions being due to species difference. The presence of 21-23kDa protein family members at cytoplasmic and membrane levels suggested their possible implication in transport or signal mechanisms between membranes and cytoplasm of the cells[7]. Furthermore, a 23kDa protein called neuropolypeptide

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h3 was purified from human brain by Bollengier and Malher[8]. In 1988, by immunochemical experiments, these two authors indicated the presence of h3 in different tissues from human, bovine, rat and chicken sources. Recently, we established the complete amino acid sequence of the human brain h3[9]: 94% sequence identity was observed with the bovine brain 21-23kDa protein, revealing that h3 was the exact counterpart of the 21-23kDa protein from bovine brain and of the 23kDa protein from rat brain. Thus, the three mammalian proteins appeared to be members of the same 21-23kDa protein family. Moreover, multiple Northern analysis revealed the presence of a single mRNA in the different tissues of each species. Indeed, a single band of 1.8 kb, 1.45 kb and 1.2 kb was observed in human, mouse and rat tissues, respectively. The expression rate of the mRNA encoding the 21-23kDa protein appeared to be variable according to the tissue considered. Strikingly, the mRNA was revealed to be particularly highly expressed in rat and mouse testis, but it was not detectable in human testis by Northern blot analysis. This result prompted us to study the location of the 21-23kDa protein in rat and mouse testis. This paper deals with the results obtained by immunostaining of normal and mutant rodent testis and describes some experiments carried out on monkey testis. Although the exact biological role of this protein is not yet elucidated, it is named testicular phosphatidylethanolamine binding protein (tPBP) in reference to the first member of this protein family, namely bovine brain 21-23kDa protein, which was demonstrated to bind hydrophobic ligands[2]. The results obtained, corroborated by protein analysis of isolated testicular cells, revealed that tPBP is mainly encountered in elongated spermatids. In addition, we describe the characterization of the mRNA encoding tPBP in rat and human testis.

Materials and methods

Multiple tissue Northern blots. The membranes were purchased from Clontech Laboratoris. After prehybridization using salmon sperm DNA to decrease non-specific binding, the filters were hybridized with a 380bp cDNA rat liver insert labeled by random priming (1×10^9 cpm/mg) in $2 \times$ SSC buffer (17.5 g of NaCl, 8.8 g of sodium citrate in 800 ml of H_2O, pH 7), 50% formamide, 1% SDS and $5 \times$ Denhardt's reagent (containing 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA) at 42 °C for 72h[9]. Then the membranes were washed in $0.1 \times$ SSC, 0.1% SDS at 50 °C for rat tissues, and in $2 \times$ SSC, 0.05% SDS at 50 °C for mouse tissues. Autoradiography was performed using Amersham Hyperfilm MP with an intensifying screen at −70 °C, and the spot intensity was quantified by densitometry.

Rat testis cDNA library screening. A cDNA library prepared from rat testis by *Eco*RI linker ligation and cloning into *Eco*RI-cleaved lgt11 bacteriophage was purchased from Clontech (Palo Alto, CA). Twenty thousand phages were plated at a density of 10,000 plaques/plate. Phage DNA was then transferred to nylon filters (Hybond N^+, Amersham). Filters were incubated for 3 h at 42 °C in $5 \times$ SSC, 0.1% lauroylsarcosine, 0.02% SDS, 5% block reagent (Boehringer), 50% formamide. Then they were hybridized overnight at 42 °C in the same solution containing the labelled probe (a rat liver 380 bp cDNA labelled by random priming incorporation of dUTP bound to digoxigenin according to the Boehringer protocol). Filters were washed twice in $2 \times$ SSC, 0.1% SDS for 15 min at 20 °C, three times in $0.1 \times$ SSC, 0.1% SDS for 10 min at 65 °C and finally revealed by phosphatase alkaline reaction. Several positive colonies were obtained, replated and tested by hybridization two additional times, then were amplified by the Polymerase Chain Reaction (PCR) by using forward and reverse λgt11 primers. PCR conditions were: a 0.2 mM concentration of each primer, 1 unit of *Taq* polymerase (Perkin-Elmer Cetus, St-Quentin, France), amplification performed for 30 cycles with a hybridization temperature of 37 °C for 1 min, extension at 72 °C for 1 min and denaturation at 95 °C for 1 min. The fragments obtained were submitted to gel electrophoresis and transferred to a nylon filter (Hybond N^+, Amersham). Then, the fragments on the filter were hybridized with the labelled rat liver cDNA probe at 42 °C and the washed at 55 °C in $0.1 \times$ SSC, 0.1% SDS. The positive clones were subcloned in bacteriophage M13 mp19 predigested by *Sma*I enzyme, and the cDNA sequencing was carried out according to Sanger et al.[10].

Polymerase Chain Reaction (PCR) amplification of human testis cDNA. Recently, we determined the sequence of the human brain 21-23kDa protein[9]. From the cDNA sequence encoding the 21-23kDa protein, we designed three human-specific oligonucleotide probes useful for PCR; the three probes were synthesized by GENSET (Paris) using an automated DNA synthesizer. Human testis mRNA purchased from Clontech was submitted to reverse transcriptase, and 1 ng of the obtained cDNA was used for PCR. PCR conditions were: a 0.2 mM concentration of each primer, 1 unit of *Taq* polymerase (Perkin-Elmer Cetus, St-Quentin, France), amplification performed for 30 cycles with a hybridization temperature of 55 °C for 1 min, extension at 72 °C for 1 min and denaturation at 95 °C for 1 min. The fragment obtained hybridized with the rat liver cDNA probe and was submitted to sequencing using a thermoresistant polymerase (Hot tub polymerase, Amersham), according to the Amersham protocol.

Animals used for immunohistochemical studies. Adult male Sprague-Dawley rats (5 months old) and adult male C 57BL mice (3 months old) were killed under chloroform anesthesia. Pubertal male Sprague Dawley

rats were used on day 8, 18, 26 and 35 post-partum to study the onset of tPBP expression in the developing testis. Mutant mice (Oligotriche and Ebouriffée), presenting an arrested spermatogenesis, were also tested; they were obtained from the Pasteur Institute (Paris, France). Mice homozygous for the Oligotriche mutation (*olt/olt*) are characterized by a defect in spermatozoa tail development[11] and by a low production of spermatids and spermatozoa. The other mutant mice, homozygous for the Ebouriffée mutation (*ebo/ebo*) are characterized by spermatoza with an abnormal head shape and a disorganized mid-piece in the tail (unpublished data). Adult marmoset monkey (*Callithrix jacchus*) testis used for this study was kindly provided by Dr. J. M. Gasc (Laboratoire de Médecine expérimentale, Collège de France, Paris).

Preparation of the tissue specimens. Testes from mature monkey, adult rats and mice, pubertal rats and mutant sterile mice were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, or in Bouin's fluid, for 12 to 24 hrs. The tissues were dehydrated in ethanol and embedded in paraffin. 7 mm thick sections of testes were cut and submitted to immunohistochemical analysis.

Immunohistochemistry. The polyclonal antibody against the bovine brain 21-23kDa protein was raised by administration of purified protein aliquots to rabbits[1] at multiple intradermal sites. The immunohistochemical tools and methods employed were described previously[12]. Immunoperoxidase staining was performed using the Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, California) and diaminobenzidine was used for the detection step. All incubations were performed at room temperature.

Normal rabbit serum and anti-21-23kDa antibody previously incubated with the purified bovine protein, were used for negative controls. In addition, the polyclonal antibody raised against the peptide 28 of the Angiotensin Converting Enzyme[13] was used as a control for the late stages of spermatogenesis.

Primary testicular cell cultures. Sertoli and peritubular cells were prepared from testes of 20-day-old Sprague-Dawley rats (Elevage Janvier, Le Genest Saint Isle, France), as previously described[14].

Sertoli cell suspensions were seeded at a density of approximately 1×10^6 cells/ml in 75 cm^2 tissue culture flasks (NUNC, Copenhagen, Denmark). The cells were then incubated at 32 °C in a humidified atmosphere of 5% CO_2 and 95% air (day 0 of culture) in Ham's F12/DMEM (v/v) (Gibco, Life Biotechnologies, France) supplemented with insulin (10 mg/ml), human transferrin (5 mg/ml) and gentamycin (10 mg/ml). Culture media were replenished daily until the end of the experiment. On day 2 of the culture, Sertoli cells were exposed to a hypotonic treatment[15] to remove about 98% of the contaminating germ cells.

Peritubular cells were seeded at a density of approximately 200,000 cells/ml in 75 cm^2 tissue culture flasks (NUNC, Copenhagen, Denmark). The cells were then cultured for 5 days at 32 °C in a humidified atmosphere of 5% CO_2 and 95% air (day 0 of culture) in Ham's F12/DMEM (v/v) (Gibco, Life Biotechnologies, France) supplemented with insulin (10 mg/ml), human transferrin (5 mg/ml) and gentamycin (10 mg/ml) and 10% fetal calf serum. When confluence was reached, cells were removed by treatment with phosphate buffered saline pH 7.4 (PBS) containing 0.05% trypsin and 0.5 mM EDTA, washed and seeded at 1/4 density in 175 cm^2 flasks. The subcultured cells were allowed to grow to confluence for another 4 to 5 days in the presence of 10% fetal calf serum. Finally, before collection, the cells were washed (3×2 hours) with fresh serum-free medium.

Preparation of germ cells. Germ cells (GC) were prepared from adult Sprague-Dawley rats (Elevage Depré, Saint Doulchard, France) as previously described[16], except that the usual trypsin digestion was replaced by a mechanical dispersion of seminiferous tubules. Enriched populations of pachytene spermatocytes (SPC) and early spermatids (SPT) were prepared by centrifugal elutriation with a purity greater than 90%[17]. GC, SPC and SPT were incubated individually at 32 °C for 16 hours in Ham's F12/DMEM (v/v) (Gibco, Life Biotechnologies, France) supplemented with 2mM sodium pyruvate and 6mM sodium DL-lactate, at a density of 2.5×10^6 cells/ml in 175 cm^2 tissue culture flasks (NUNC, Copenhagen, Denmark). After 16 hours, cells and cellular debris were removed by successive centrifugations at 4 °C (2×200 g for 10 minutes followed by 10,000 g for 1 hour). Viability of the cells was >95% at that time, as judged by the trypan blue dye exclusion test.

Preparation of intracellular protein extracts. Sertoli and peritubular cell confluent monolayers were washed twice with phosphate buffered saline (PBS) and then scraped off the dishes with a rubber policeman after addition of 3 ml Tris buffer (50 mM, pH 7.5), supplemented with 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 mM PMSF and 10 mg/ml leupeptin. The cells were homogenized using a pellet pestle and centrifuged at 15,000 g for 30 min at 4 °C. Supernatants were centrifuged at 100,000 g for 1 hour at 4 °C. Freshly prepared GC, SPC and SPT were also treated as described above. All 100,000 g supernatants were snap frozen in liquid nitrogen and stored at −80 °C until used.

Western blot analysis. Lysates of cells isolated from rat testis were submitted to 12.5% SDS-PAGE[18] and the proteins were transferrd to nitrocellulose membranes[19]. The tPBP was detected using a rabbit antiserum directed against the bovine brain 21-23kDa protein. For immunodetection, the antiserum was diluted at 1/5000 and an anti-rabbit IgG alkaline phosphatase conjugate pur-

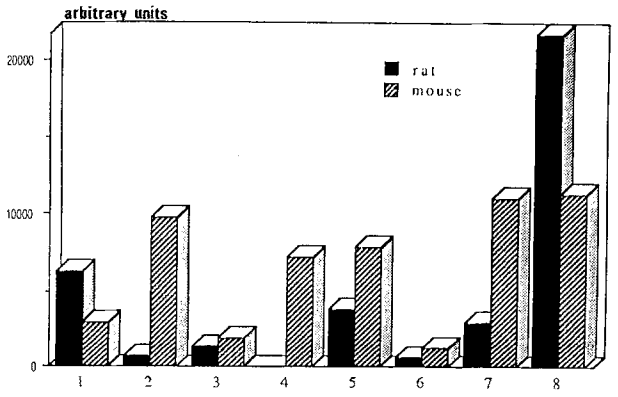

Figure 1. Tissue distribution of mRNA encoding the 21-23kDa protein in mouse and rat species. The blotted membranes were purchased from Clontech laboratories and probed with a labelled 380bp rat liver cDNA (1×10^9 cpm/mg). The radiolabelling of the spots was measured by using a densitometer. Tissues: heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), testis (lane 8).

chased from the Promega Corporation was used as the secondary antibody.

General methods. Protein estimation on 100,000 g supernatants was performed by the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Spermiogenesis and spermatogenesis terminology. The association of the germ cell types (spermatogonia, spermatocytes and spermatids) within the seminiferous tubules defined the spermatogenesis stages: 12 stages were identified in mouse[20] and 14 in rat[21].

Results

Distribution of mRNA encoding the 21-23kDa protein in rat and mouse tissues. Northern blot analysis revealed that the mRNA encoding the 21-23kDa protein was present in all tissues considered from rat and mouse; it was more highly expressed in testis when compared with

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Human          Pro Val Asp Leu Ser Lys Trp Ser Gly Pro Leu Ser Leu Gln Glu Val Asp Glu Gln      19
Rat       Met Ala Ala Asp Ile Ser Gln Trp Ala Gly Pro Leu Ser Leu Gln Glu Val Asp Glu Pro      20
Rat       ATG GCC GCC GAC ATC AGC CAG TGG GCC GGG CCG CTG TCA TTA CAG GAG GTG GAT GAG CCG      60

Human     Pro Gln His Pro Leu His Val Thr Tyr Ala Gly Ala Ala Val Asp Glu Leu Gly Lys Val      39
Rat       Pro Gln His Ala Leu Arg Val Asp Tyr Gly Gly Val Thr Val Asp Glu Leu Gly Lys Val      40
Rat       CCC CAG CAC GCC CTG AGG GTC GAC TAC GGC GGA GTA ACG GTG GAC GAG CTG GGC AAA GTG     120

Human     Leu Thr Pro Thr Gln Val Lys Asn Arg Pro Thr Ser Ile Ser Trp Asp Gly Leu Asp Ser      59
Rat       Leu Thr Pro Thr Gln Val Met Asn Arg Pro Ser Ser Ile Ser Trp Asp Gly Leu Asp Pro      60
Rat       CTG ACG CCC ACC CAG GTC ATG AAT AGA CCA AGT AGC ATT TCA TGG GAT GGC CTT GAT CCT     180
Human                                 AAT AGA CCC ACC AGC ATT TCG TGG GAT GGT CTT GAT TCA      39
                                      ------------ A ---------->

Human     Gly Lys Leu Tyr Thr Leu Val Leu Thr Asp Pro Asp Ala Pro Ser Arg Lys Asp Pro Lys      79
Rat       Gly Lys Leu Tyr Thr Leu Val Leu Thr Asp Pro Asp Ala Pro Ser Arg Lys Asp Pro Lys      80
Rat       GGG AAG CTC TAC ACC CTG GTC CTC ACA GAC CCC GAT GCT CCC AGC AGG AAG GAC CCC AAA     240
Human     GGG AAG CTC TAC ACC TTG GTC CTG ACA GAC CCG GAT GCT CCC AGC AGG AAG GAT CCC AAA      99

Human     Tyr Arg Glu Trp His His Phe Leu Val Val Asn Met Lys Gly Asn Asp Ile Ser Ser Gly      99
Rat       Phe Arg Glu Trp His His Phe Leu Val Val Asn Met Lys Gly Asn Asp Ile Ser Ser Gly     100
Rat       TTC AGG GAG TGG CAC CAC TTC CTG GTG GTC AAC ATG AAG GGC AAC GAC ATT AGC AGT GGC     300
Human     TAC AGA GAA TGG CAT CAT TTC CTG GTG GTC AAC ATG AAG GGC AAT GAC ATC AGC AGT GGC     159

Human     Thr Val Leu Ser Asp Tyr Val Gly Ser Gly Pro Pro Lys Gly Thr Gly Leu His Arg Tyr     119
Rat       Thr Val Leu Ser Glu Tyr Val Gly Ser Gly Pro Pro Lys Asp Thr Gly Leu His Arg Tyr     120
Rat       ACT GTC CTC TCC GAA TAC GTG GGC TCC GGA CCT CCC AAA GAC ACA GGT CTG CAC CGC TAC     360
Human     ACA GTC CTC TCC GAT TAT GTG GGC TCG GCG CCT CCC AAG GGC ACA GGC CTC CAC CGC TAT     219

Human     Val Trp Leu Val Tyr Glu Gln Asp Arg Pro Leu Lys Cys Asp Glu Pro Ile Leu Ser Asn     139
Rat       Val Trp Leu Val Tyr Glu Gln Glu Gln Pro Leu Asn Cys Asp Glu Pro Ile Leu Ser Asn     140
Rat       GTC TGG CTG GTG TAT GAG CAG GAG CAG CCT CTG AAC TGT GAC GAG CCC ATC CTC AGC AAC     420
Human     GTC TGG CTG GTG TAT GAG CAG GAC AGG CCG CTA AAG TGT GAC GAG CCC ATC CTC AGC AAC     279

Human     Arg Ser Gly Asp His Arg Gly Lys Phe Lys Val Ala Ser Phe Arg Lys Lys Tyr Glu Leu     159
Rat       Lys Ser Gly Asp Asn Arg Gly Lys Phe Lys Val Glu Ser Phe Arg Lys Lys Tyr His Leu     160
Rat       AAG TCT GGA GAC AAC CGC GGC AAG TTC AAG GTG GAG TCC TTC CGC AAG AAG TAC CAC CTG     480
Human     CGA TCT GGA GAC CAC CGT GGC AAA TTC AAG GTG GCG TCC TTC CGT AAA AAG TAT GAG CTC     339
                                      <---------- B ----------

Human     Arg Ala Pro Val Ala Gly Thr Cys Tyr Gln Ala Glu Trp Asp Asp Tyr Val Pro Lys Leu     179
Rat       Gly Ala Pro Val Ala Gly Thr Cys Phe Gln Ala Glu Trp Asp Asp Ser Val Pro Lys Leu     180
Rat       GGA GCC CCG GTG GCC GGC ACG TGC TTC CAG GCA GAG TGG GAT GAC TCT GTG CCC AAG CTG     540
Human     AGG GCC CCG GTG GCT GGC ACG TGT TAC CAG GCC GAG TGG GAT GAC TAT GTG CCC AAA CTG     399
                                                <---------- C ----------

Human     Tyr Glu Gln Leu Ser Gly Lys                                                       186
Rat       His Asp Gln Leu Ala Gly LYS                                                       187
Rat       CAC GAT CAG CTG GCT GGG AAG                                                       561
Human     TAC GAG CAG CTG TCT GGG AAG                                                       420
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Figure 2. Protein and cDNA sequences of rat[6] and human[9] brain 21-23kDa protein. The underlined sequences correspond to the sequenced cDNA fragments from human and rat testis: they are identical to the corresponding parts of human and rat brain, respectively. The three dashed arrows indicate the probes specific for the human sequence used for PCR amplification of human testis cDNA.

other organs (fig. 1). In particular, the level of mRNA encoding the rat tPBP is 30 times and 5 times higher than that of the mRNA encoding the rat brain and liver proteins, respectively (fig. 1, lanes 2, 5 and 8).

Identification of the mRNA encoding the rat and human tPBP. Recently, by PCR carried out on rat liver mRNA, we obtained a 380 bp cDNA which was subcloned into the pUC 19 mp 18 vector[9]. This probe was used to screen a human brain cDNA library and to analyze the expression of mRNA encloding the 21-23kDa protein in various tissues and species. Both by cDNA and protein sequencing, we determined the complete amino acid sequence of human brain 21-23kDa protein. Northern blot analysis revealed that the mRNA encoding the 21-23kDa protein was very highly expressed in mouse and rat testis[9].

By using the rat liver cDNA probe (380 bp) subcloned into the pUC 19 mp18 vector, we screened a rat testis cDNA library: ten positive clones were identified and purified. Five of these clones were amplified by PCR and tested by gel elecrophoresis, and the longer one was submitted to sequencing. The sequence obtained consisted of 177 bp and was identical to the corresponding rat brain cDNA sequence (bases 125-301) previously described by Grandy et al.[6] (see also fig. 2), except for position 153 where thymidine is substituted by cytidine; however, the codon codes for the same amino acid residue, namely serine.

The mRNA encoding the tPBP in human testis was not detectable by Northern analysis[9], but when the human testis total mRNA was subjected to PCR amplification using three oligonucleotide probes (fig. 2) the transcript was revealed to be present. Moreover, the sequence determination of the cDNA obtained by PCR (393 bp) showed complete identity with the corresponding region of the human brain cDNA sequence previously described by Seddiqi et al.[9] (see fig. 2).

Cellular localization of rat and mouse tPBP. The histochemical localization of tPBP in rat and mouse testis was carried out using a polyclonal rabbit antiserum directed against the bovine brain 21-23kDa protein[1].

1. Adult rat testis. A strong immunoreactivity was observed in the apical compartment of the seminiferous tubules of adult rat testis which contained the elongated spermatids (fig. 3A). Before stage V of spermatogenesis, the elongated spermatid cytoplasm appeared to be unstained (fig. 3B). At stages V and VI, the cytoplasm of elongated spermatids inserted between round spermatids was highly stained (fig. 3C). At stage VII, the cytoplasm of old spermatids was strongly immunoreactive and later, at stages VIII, IX, and X, only the residual bodies presented strong staining (fig. 3D). The early elongated spermatids of stages XI–XIV showed no immunoreactivity (fig. 3E).

2. Pubertal rat testis. To study the onset of tPBP expression during puberty, rats were killed at days 8, 18, 25 and 35 post-partum. Before day 35 post-partum, the seminiferous tubules which contained spermatogonia, spermatocytes and early spermatids showed no reactivity (see fig. 3F for day 18 post-partum). When the spermatids started to elongate, a faint immunoreactivity could be detected in the most advanced germ cells (fig. 3G).

3. Adult mouse. The immunostaining pattern of adult mouse seminiferous tubules was similar to the labelling observed in rats; however, it displayed a lower intensity (fig. 4A). No immunoreaction was observed before stage V; at stages V and VI, the immunostaining was located on the cytoplasm of the elongated spermatids deeply inserted in the seminiferous epithelium. At stage VII a strong reactivity was seen on late spermatids (fig. 4B) and finally, at stages IX and X, only the remaining cytoplasm of spermatids, namely the residual bodies, appeared strongly stained (fig. 4C). As for the observation made on rat sections, the cytoplasmic droplets attached to the flagellum were immunoreactive (data not shown).

4. Mutant mouse testis. The sterile mutant Oligotriche (*olt/olt*) presents an arrest of spermatogenesis during spermatid elongation and an aborted flagellum formation. In this mutant, faint staining was observed in the cytoplasm of some elongating spermatids. Moreover, strong immunoreactivity was seen in degenerating cells and residual bodies located in the lumen of the seminiferous tubules (fig. 4D) and within the area of the seminiferous epithelium where they had been phagocytozed by Sertoli cells.

In the sterile mutant Ebouriffée (*ebo/ebo*), spermatogenesis is mostly stopped at the end of the spermatid elongation process so that spermatozoa have abnormal heads and a disorganized flagellum. In these animals, the tPBP immunostaining showed no labelling of late spermatids; however, a faint staining was observed in some degenerating late spermatids (fig. 4E).

5. Primates. No immunostaining was observed in the Marmoset monkey (*Callithrix jacchus*) seminiferous epithelium. In particular, the elongated spermatids at late stages of spermatogenesis were not stained (fig. 4F).

6. Positive and negative controls. We tested the labelling of the late spermatids and residual bodies by using a monoclonal antibody raised against peptide A-28 of the angiotensin converting enzyme, which is known to label the late steps of rodent spermatogenesis[13]. The staining observed with this antibody was similar to the labelling obtained with the antibody directed against the bovine brain 21-23kDa protein: only the late, nearly mature spermatids at stage VII and residual bodies were stained (fig. 4G). The sections treated with preimmune IgG, or with the anti-21-23kDa antiserum previously depleted by incubation with the purified bovine brain 21-23kDa protein, showed no reactivity at all.

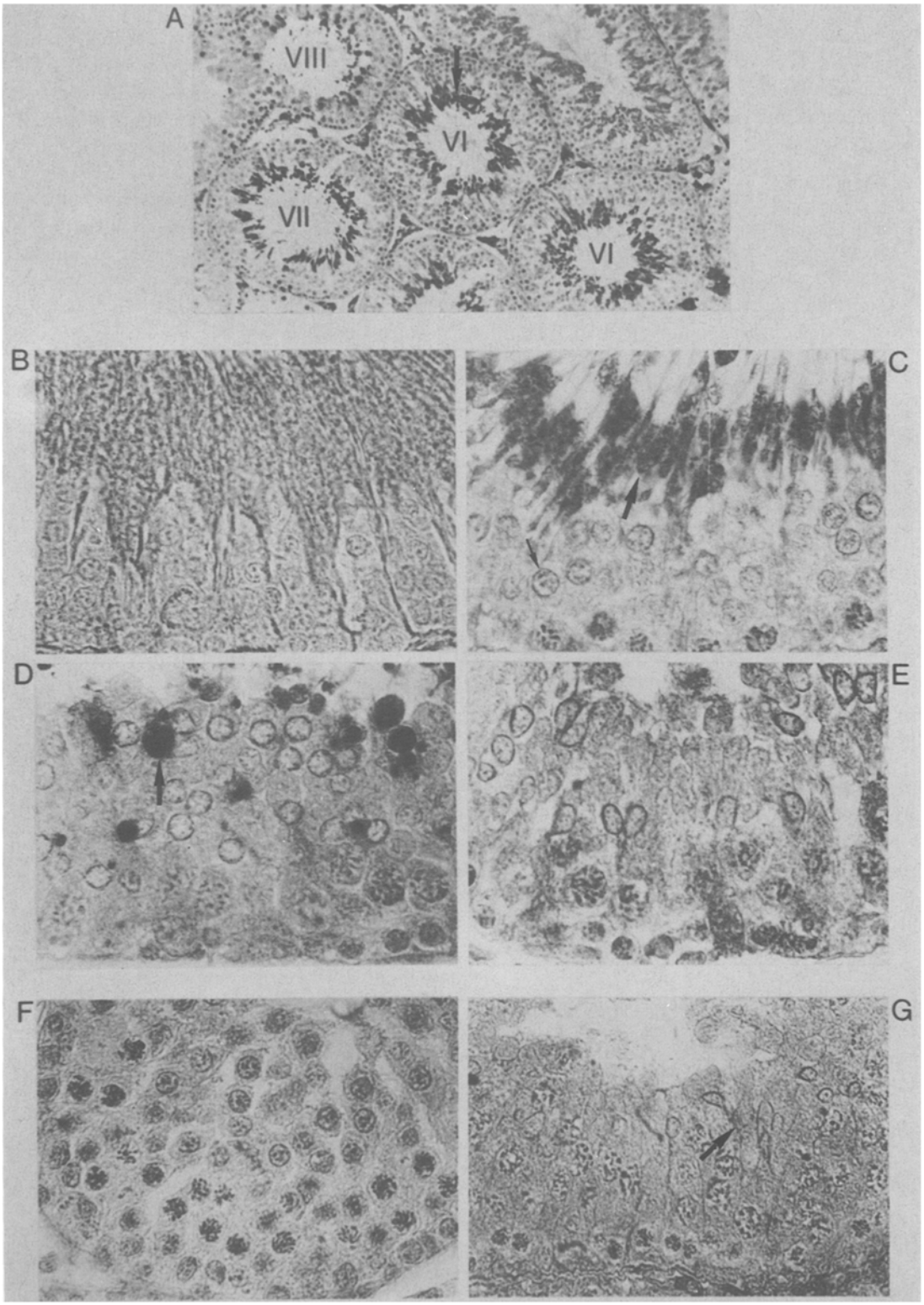

Figure 3. Immunolocalization of tPBP in rat testis. (*A*) Spermatogenesis stages are indicated. Magnification × 100. Only the apical compartment of the seminiferous tubules containing the later spermatids is stained (indicated by the arrow). (*B*), (*C*), (*D*), (*E*) Spermatogenesis at stages III, VI, VII–IX and X–XI, respectively. Magnification × 640. (*B*) There is no staining before stage V. (*C*) The elongated spermatids are strongly stained (large arrow) while the round spermatids are not stained (small arrow). (*D*) At stages VIII–IX, only residual bodies are stained (indicated by the arrow). (*E*) The early elongated spermatids are unstained. (*F*) Day 18 post-partum; spermatogonia, spermatocytes and early spermatids show no immunostaining. (*G*) Day 35 post-partum. A slight immunoreactivity could be detected in the most advanced germ cells (arrow).

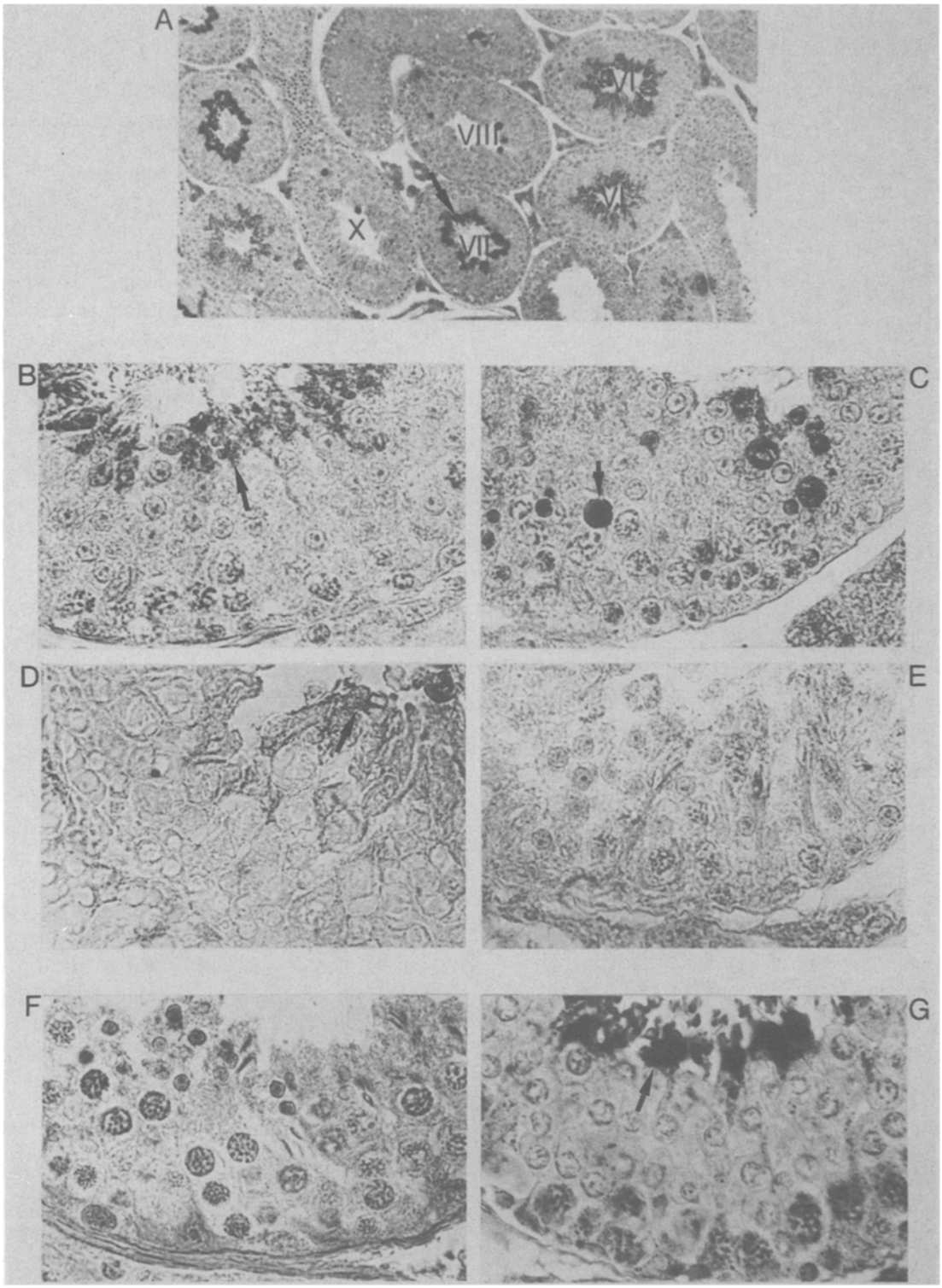

Figure 4. Immunolocalization of the tPBP in mouse and monkey testis and positive control of late steps in rodent spermatogenesis. (*A*) Normal mouse, spermatogenesis stages are indicated. Magnification × 100. Only the apical compartment of the seminiferous tubules containing the late spermatids is stained (indicated by the arrow). (*B*), (*C*) Normal mouse, spermatogenesis at stages VI and IX–X, respectively. Magnification × 640. No immunoreactivity was observed before stage V, at stage VI a strong reactivity was seen on late spermatids (arrow); at stages IX and X, only the residual bodies appeared strongly stained (arrow). (*D*) Mutant mouse *olt/olt* at stages X–XI. Immunoreactivity was seen in degenerating cells and residual bodies located in the lumen of the seminiferous tubules (arrow). (*E*) Mutant mouse *ebo/ebo* at stages IV–V. No labelling of the late spermatids. (*F*) Adult monkey (*Callithrix jacchus*) testis at stage I. The elongated spermatids at late stages of spermatogenesis were not stained. (*G*) Positive control carried out on rat testis at stage VII: labelling obtained by using a monoclonal antibody raised against peptide A-28, a region of the angiotensin converting enzyme, specific for the late steps in rodent spermiogenesis[13].

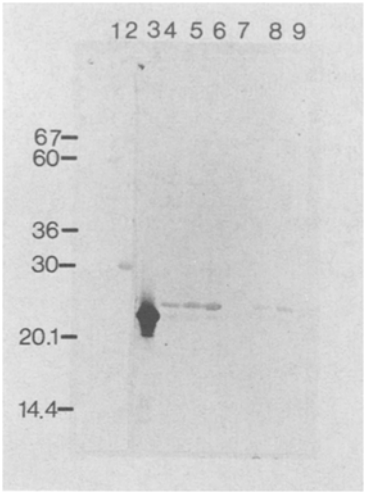

Figure 5. Immunodetection of the tPBP in cellular lysates. Cells were obtained from rat testis by elutriation and were submitted to alkaline lysis. The lysate proteins were separated on SDS/PAGE and electrotransferred onto nitrocellulose membranes. The tPBP was detected using an immunodetection kit purchased from Promega and the antibody previously raised in rabbits and directed against the bovine brain protein. High M_r standards (lane 1), low M_r standards (lane 2), purified bovine brain 21-23kDa protein (lane 3), pachytene spermatocytes (lane 4), round spermatids (lane 5), residual bodies (lane 6), peritubular cells (lane 7), Sertoli cells (lane 8), total germ cells (lane 9).

Western analysis of cell lysates. Isolated testicular cells were submitted to alkaline lysis. After separation on SDS/PAGE followed by electrotransfer on nitrocellulose membrane, tPBP was immunodetected. The testicular protein appeared to migrate with an higher apparent M_r (about 25kDa) protein from bovine brain (fig. 5). Labelling was observed in residual bodies, early spermatids and pachytene spermatocyte-enriched fractions. However, it could be also detected in Sertoli cell fractions (fig. 5), though to a much lesser extent.

Discussion

Although the 21-23kDa protein was initially characterized in brain from human[8], bovine[3] and rat[6] sources, its major site of expression was found to be the rodent testis. However, in this organ the protein displayed a $M_r \approx 25$kDa: thus, we call it testicular phosphatidylethanolamine binding protein (tPBP), with reference to the initial properties shown for the bovine brain 21-23kDa protein[2] and to the evidence that the testicular protein is able to bind phosphatidylethanolamine *in vitro*[22]. In testis, the mRNA amounts seemed to be highly variable, depending on the species considered: it is highly expressed in rat and to a lesser extent in mouse, but it was not detectable in humans[9]. Immunochemical studies carried out on monkey tissue (fig. 4F) indicated that, as in humans, no staining of the tPBP was observed in the testis. The results so far obtained after PCR amplification show that, although it is not detectable, the mRNA is present in human testis. Moreover, a 393 bp-long sequence of the cDNA encoding the human tPBP presents 100% identity with the corresponding sequence of the human brain cDNA previously described[9] (fig. 2). In the rat testis, the level of mRNA encoding tPBP is 30 times higher than the mRNA encoding the rat brain 23kDa protein (fig. 1). The partial sequence established from the rat testis cDNA (177 bp) is identical to the corresponding part of the cDNA encoding the 23kDa protein in rat brain[6]. These results, together with the presence of a single mRNA of the same size in various organs, indicate that the same mRNA is probably expressed in all the tissues of a given species. However, when compared to the 21-23kDa protein isolated from bovine brain, the testicular protein migrated with a higher apparent M_r (fig. 5). At the present time, there is no explanation for this discrepancy: it might be due to differences in sequence, post-translational events, 3D structure modifications, or bound ligands.

The immunohistochemical studies carried out on normal adult and pubertal animals revealed that the expression of tPBP is much higher in late spermatids than in any other cell type of the seminiferous epithelium. This observation was corroborated both by histochemical studies on mouse mutants and by immunostaining of tPBP from isolated testicular cells. Indeed, the protein is slightly expressed in mutant *olt/olt*, which presents a degeneration of the flagellum without any other effects on cell organelles. Moreover, tPBP is not expressed in the *ebo/ebo* mutant which combines several defects at the end of the maturation process in late spermatids. Thus, the use of male mouse mutants presenting an arrest at a late stage of spermatogenesis confirmed that the tPBP is essentially expressed at the end of the elongation process. This result was further substantiated by experiments performed on isolated testicular cells: the electrotransferred tPBP appeared to be weakly expressed in fractions enriched in early spermatids, pachytene spematocytes and Sertoli cells.

When comparing the immunohistochemical labeling of the tPBP with the staining of the Angiotensin Converting Enzyme (ACE) obtained by using the antibodies directed against the peptide A-28, the ACE appeared at the same stages of spermatogenesis as the tPBP. However, ACE passes through the Golgi apparatus[13] before being localized in the cytoplasm, whereas tPBP is encountered only in the cytoplasm of the cells.

In all mammals, transition between spermatids and sperm cells is characterized by the condensation of the nucleus and the delimitation of the greater part of the cytoplasm as a residual body. The latter includes most of the organelles and proteins that are useless for the remaining life-span of the sperm cell; it is finally phagocytozed by Sertoli cells. Furthermore, in many species, a

small portion of the cytoplasm, together with poorly characterized membrane structures, remains in early sperm cells as a cytoplasmic droplet located at the head-tail junction[23]. The cytoplasmic droplet then possibly migrates along the flagellar midpiece during the epididymal transit and is eliminated at this site. Our studies showed the labeling of residual bodies, cytoplasmic droplets and to a lesser extent of Sertoli cells and thus suggest that tPBP is weakly expressed in round spermatids, highly expressed in elongated spermatids, concentrated in residual bodies and cytoplasmic droplets, and finally resorbed by Sertoli cells.

Previously, several authors observed, in different parts of the rat male reproductive organs, a protein which appears to be similar to tPBP. Indeed, Jones and Hall[22] have purified a 23kDa protein identified as a major component of rat epididymal secretions and sperm plasma membranes. Two tryptic peptides (corresponding to 30 residues) were sequenced: they revealed that the testicular protein was probably the counterpart of the bovine brain 21-23kDa protein. According to these authors the 23kDa protein encountered in rat testis was identical to a 22kDa protein previously described[24]. By using antibodies directed against the 22kDa protein, Brooks observed its presence in cytosolic extracts of other tissues from both male and female rats. However, since the protein could not be detected in blood, peritoneal fluid, saliva, milk, uterine fluid, seminal vesicle secretion, coagulating gland secretion or prostatic secretion, it seems that the testis and epididymis may be unique in containing the protein in a soluble form in their luminal secretions. In addition to the occurrence of the 22kDa protein as a soluble moiety in rat testicular and epididymal fluids, the protein was also located on sperm plasma membranes where its distribution was restricted to the surface of the flagellum. Among sperm surface proteins, the 22kDa protein was the major protein containing SH groups and one of the major entities containing disulphide bonds. According to Brooks[24], these properties may be of importance in the maintenance of sperm viability.

More recently, Turner et al.[25] have identified the tPBP as a prominent protein in the rat testicular seminiferous tubules. Furthermore, in studying the sequential interactions of epididymal secretory proteins with spermatozoa during epididymal transit, a protein of 25kDa was observed which remained associated with spermatozoa in substantial amounts during epididymal transit[26]. It appeared to be synthesized, secreted and bound to spermatozoa in the proximal epididymis, substantial amounts of the protein remaining bound to cauda spermatozoa after seven days. Further studies showed that the 25kDa protein was present in the luminal fluid of the epididymis that had been separated from the testis prior to the onset of spermatogenesis, demonstrating that the 25kDa protein is present independently in the testis and the epididymis[27]. Moreover, a partial sequence analysis demonstrated that the testicular 25kDa protein has high sequence homology with the 21-23kDa protein from bovine brain[27].

Despite several suggestions, the role of the 21-23kDa protein and of tPBP remains obscure. Recently, Onoda and Djakiew[28] have identified a tPBP-like molecules in media/lysates of germ cells and in lysates from peritubular and Sertoli cells prepared from rat restis. The authors suggested that the tPBP-like molecule could be a germ cell product responsible for pacracrine regulation of Sertoli cell function. This hypothesis seems premature in view of the ubiquitous localization of the protein in seminiferous tubular cells, and considering that the immunoprecipitation of the round spermatid tPBP-like protein did not have much effect on the down-regulation of Sertoli cell secretion. On the contrary, the observations made in testis, compared with the results in rat brain where oligodendrocytes are particularly stained in developing rats, suggest that the 21-23kDa protein and tPBP may be implicated in cell growth, more especially in cell elongation. During this process, the protein could be associated directly or indirectly with the modulation or modification of membrane structures. Indeed, in brain, the main function of oligodendrocytes is to elaborate myelin sheaths and to maintain their integrity. Moreover, myelin contains a high amount of lipids and more particularly of ethanolamine phosphoglycerides. In testis, it is known that during maturation of spermatozoa in the spididymis there is considerable remodelling of the plasma membrane, especially in its content of ethanolamine phosphoglycerides, which decreases approximately three-fold. The 21-23kDa protein in brain and its counterpart in epididymal plasma could have a role as a lipid carrier protein participating in membrane constitution and preventing formation of lysophosphatides, which are known to perturb membrane structure[22]. Furthermore, it appears that the tPBP pattern of expression is compatible with a functional role in the regulation of sperm maturation, motility and/or fertilization.

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