A Novel Germ Line-specific Gene of the Phosducin-like Protein (PhLP) Family

A MEIOTIC FUNCTION CONSERVED FROM YEAST TO MICE*

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We identified a new member of the phosducin-like (PhLP) protein family that is predominantly, if not exclusively, expressed in male and female germ cells. In situ analysis on testis sections and analysis of purified spermatogenic cell fractions evidenced a stage-specific expression with high levels of RNA and protein in pachytenic spermatocytes and round spermatids. Three mRNA species were detected, which correspond to different polyadenylation sites and vary in abundance during spermatogenesis. Only low levels of RNA were detected in whole ovary extracts, but expression of the protein was found to complement the defect of a yeast plp2 mutant, suggesting an essential role in germ cell maturation. The murine gene complements the defect of a yeast plp2 mutant with the yeast phosducin-like protein (PhLP) protein family that is predominantly, if not exclusively, expressed ubiquitously (5). Whether tissue-specific homologues exist remains an open question. In the course of screening a mouse testis cDNA library, we have identified a novel germ cell-specific phosducin-like protein, designated MgcPhLP (for “mouse germ cell-specific phosducin-like protein”), which exhibits significant similarities to both the mouse phosducin and phosducin-like proteins. Expression is strictly restricted to the germ cells, suggesting a role for MgcPhLP in regulating cell adhesion during the embryo and the postnatal period.©2003 by The American Society for Biochemistry and Molecular Biology, Inc.

WITHDRAWN

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This article has been withdrawn by Pascal Lopez, Luis A. Lopez-Fernandez, François Cuzin, and Minoo Rassoulzadegan. Ruken Yaman, Frédérique Vidal, Daniel Puel, and Philippe Clertant could not be reached. The Hss26 Northern blots from Fig. 3 (A and D) were previously published in Lopez et al. (2002) Mol. Cell. Biol. 22, 3488-3496, with some data representing different experimental conditions. The withdrawing authors state that the same nitrocellulose filter was reused for two different probes and so the control is the same for both publications. The image in Fig. 6B was inappropriately manipulated. The withdrawing authors state that after 20 years, they cannot provide the original data.

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The abbreviations used are: PhLP, phosducin-like protein; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; EST, expressed sequence tag.

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some 5 (GenBank™ accession number AF146793), which also includes the Clock locus and has been designated Pdc2 (GenBank™ accession number AA300564). The existence of two potential proteins, named protein B and PDCL2 (corresponding, respectively, to amino acids 3–204 and 3–195 of MgcPhLP) had been predicted from the genomic DNA sequence. The complete nucleotide sequence derived from the initial cDNA clone and the 5′- and 3′-RACE products now allows us to determine the correct position of the initiating methionine and thus the complete amino acid sequence.

An analysis of mouse EST libraries showing identities with Mgcphlp sequences (Unigene cluster Mm.143764 plus additional clones) strongly suggested that the gene was at least preferentially expressed in the testis. All identities were found in testis-derived library sources and none among clones derived from other tissues, with the best match found in a purified spermatocyte library (BG100925).

Expression of Mgcphlp in the Testis—Northern blot analysis on a limited series of tissues detected expression only in the testis (Fig. 3C). The RNA was not detected in any of the somatic organs (Fig. 3A). The existence of two potential proteins, named protein B and PDC2 (corresponding, respectively, to amino acids 3–204 and 3–195 of MgcPhLP) had been predicted from the genomic DNA sequence. The complete nucleotide sequence derived from the initial cDNA clone and the 5′- and 3′-RACE products now allows us to determine the correct position of the initiating methionine and thus the complete amino acid sequence.

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Expression of Mgcphlp in the Testis—Northern blot analysis on a limited series of tissues detected expression only in the testis (Fig. 3C). The RNA was not detected in any of the somatic organs (Fig. 3A). A larger series of tests performed by dot blot analysis on a membrane spotted with polya(t)+ RNA from 22 mouse organs (Fig. 3B) again showed expression only in the testis. Mgcphlp RNA was not detected in any of the somatic organs tested. The same conclusion was reached using the most sensitive RT-PCR assay, which could not detect Mgcphlp RNA sequences in two somatic tissues (Fig. 3C). The RNA was present in purified germ cells (see below and Fig. 5) but not in either Sertoli cells, since primary cultures were negative (Fig. 3D), or in cells of the Sertoli line 15P-1 (not shown). Although
expression of the gene cannot be excluded either in yet another tissue, or in a minor fraction of cells, possibly under specific physiological conditions, it would appear to occur mostly in testicular germ cells. In fact, as shown in a subsequent section, Mgcphlp is also expressed in female germ cells during a limited period of meiotic maturation.

In whole testis extracts, three mRNA species were detected. Analysis of cDNA ends by 5'- and 3'-RACE revealed that these RNAs share the same 5'-extremity but differ by their 3'-ends. Three major polyadenylation sites were identified, corresponding to three canonical polyadenylation signals in the genomic sequence (Fig. 4). As shown in Fig. 3D, distribution of the three isoforms significantly differed between the pachytene spermatocyte, the round spermatid and the elongated spermatid fractions prepared by elutriation centrifugation. During post-natal development, RNA was first detected at 20 days post-partum, and the maximum level of expression was reached at day 30. The midsize mRNA was the only one identified at day 20, whereas the other two isoforms appeared only at the later time points.

Stage-specific expression in the testis was confirmed by in situ hybridization. The probe consisted of the entire coding sequence of Mgcphlp and thus detected all three mRNA isoforms. As shown in Fig. 5, expression was detected in pachytene to round spermatids at all spermatogenesis stages. Expression of MgcPhLP was examined at the protein level by using a rabbit antiserum raised against a GST fusion protein (see “Experimental Procedures”). Immunofluorescence staining of purified fractions of male germ cells was clearly positive from the meiotic to late haploid stages of spermatogenesis, and the protein was also present in the mature spermatozoa of epididymal sperm. It was not detectable by Western blotting at 10 days post-partum but was clearly present in the testis at day 18 (Fig. 5, B–F).

Expression in Ovary—Despite the facts that no EST sequences corresponding to Mgcphlp were present in ovarian libraries from various species and that RNA was not detected by dot blot hybridization (Fig. 3B), low levels of expression were evidenced by RT-PCR analysis in total extracts from adult ovary (Fig. 6A). Western blot analysis (Fig. 6B) failed to detect expression of the protein in extracts from either ovary or unfertilized eggs. It was, however, detected in fertilized eggs (Fig. 6C). Taking into account the smaller proportion of germ cells in the female gonad, these results did not exclude the possibility of stage-specific expression during meiotic maturation. This
was shown to be the case by Western blot analysis following injection of human chorionic gonadotropin as part of a superovulation regime (6) (Fig. 6D). The protein was detected as early as 3 h after hormone injection, a time corresponding to the nuclear breakdown step of preovulatory meiotic maturation.

**Association with 14-3-3 Protein(s)—** The 14-3-3 family includes a series of closely related proteins that bind phosphorylated components of signal transduction pathways and modulate their interactions (reviewed in Ref. 14). Several of them, prominently the 14-3-3 θ protein, are expressed in a stage-dependent manner in the spermatogenic differentiation pathway (15). As shown in Fig. 7, a complex of the MgçPhLP protein with 14-3-3 protein(s) was evidenced by immunoprecipitation of testicular protein extracts with polyvalent anti-14-3-3 antibodies followed by Western blot analysis of the precipitated complexes with anti-MgçPhLP antiserum. Further experiments are in progress to identify more precisely the protein(s) present in these complexes.

**Evolutionary Conservation—** The Mgçphlp coding sequence appears to have been relatively well conserved throughout...
evolution. Coding regions of the murine gene are 87–92% identical at the nucleotide level to human EST sequences contained in Unigene cluster Hs.223712, derived exclusively from germ cells or testes. The cluster sequences are located in human chromosome 4q11 region, again in close proximity to the human homologue of the mouse Clock gene. In the S. cerevisiae genomic sequence, two genes, PLP1 and PLP2, encode proteins with a clear similarity to the mammalian phosducins and phosducin-like sequences (4). Alignments of phosducin-like sequences (Fig. 2) show that the murine MgcPhLP protein is more closely related to Plp2. One may note that, in the amino-terminal part of the sequences, an 11-amino acid region implicated in Gβγ binding (16) is completely conserved between the phosducin proteins and Phlp1 but present neither in Plp2 nor in MgcPhLP. That would distinguish two groups of phosducin-like proteins: on one hand, the mammalian phosducins with the region that interacts with Gβγ proteins, and on the other, the MgcPhLP and PLP2 proteins, with a distinct pattern of conserved amino acids. Other discrete patches of pairwise similarities could also be observed in the central and carboxyl-terminal parts of the sequences.

Complementation of a Yeast plp2 Mutant by the Murine Mgcphlp Gene—A yeast strain bearing mutations in the TRP1 and URA3 nutritional markers and in which one PLP2 allele has been replaced by URA3 (plp2::URA3,plp2Δ) was established previously (4). The deletion of PLP2 prevented the recovery of mutated haploid clones upon induction of sporulation in diploid heterozygotes. The mutated strain was transformed with an expression vector for Mgcphlp and TRP1 (see “Experimental Procedures”), and the resulting colonies were induced to sporulate. Isolated spores were tested in duplicate in media selective for either URA3 (plp2Δ) or TRP1.
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(Hmgcplp). Haploid colonies were then revealed by replica plating with yeast strains of either the a or a mating types carrying a mutation in the nutritional HIS gene and selection in histidine-free medium. Haploid derivatives identified as carrying both the URA3 (plp2D) allele and TRP1 (Mgcplp) were grown. Analysis by PCR amplification confirmed the expected absence of the wild type PLP2 and the presence of the Mgcplp allele (Fig. 8A). These complemented haploid clones were successfully grown for successive generations. As expected from the published data, transfer of the empty vector did not result in the production of viable haploid cells.

A Meiotic Function Conserved from Yeast to Mammals?—It was initially reported (4) that haploid derivatives could not be grown from the mixture of meiotic products generated by the diploid heterozygous genotype (plp2::URA3,plp2Δ). One could not on this basis distinguish between a function of the gene during meiosis (or at an early stage of spore formation) and a general requirement for cellular growth. In view of the results shown in Fig. 8B, we may now conclude that the gene is not required for growth. Genotyping of haploid clones that had been maintained in culture for 30–40 generations after their isolation from the complemented parent strain showed that the mouse gene was eventually lost. Growth properties of the clones in the absence of a functional PLP2 gene remained, however, unaffected. It is therefore most likely that the protein, not necessary for growth, was required for the establishment of the haploid state, a conclusion that is consistent with the expression of the mouse gene being restricted to the meiotic and early post-meiotic stages.

DISCUSSION

Phosducins are regulators of G protein activity in the retina, and the phosducin-like proteins are considered to be potential ubiquitous regulators of Gβγ signaling. We describe in this report a novel phosducin-like mRNA specific of the meiotic and post-meiotic germ cells, which shows significant amino acid sequence similarities to the phosducin and phosducin-like proteins of various species. Searching existing EST clones from both mouse and human suggested germ cell specificity, because related sequences were found in EST libraries from testes but not from other tissues. In the mouse, expression of Mgcplp was at least predominantly observed in male and female germ cells, in both sexes at the meiotic and post-meiotic stages. It must be taken into account however, that a detailed in situ analysis could not be performed on every possible tissue, and therefore we cannot entirely exclude the possibility that the gene might be expressed in other tissues in addition and/or only during a limited physiological or developmental period (as is in fact the case). In any case, three Mgcplp RNA isoforms were identified in various stages of differentiation.

In Figure 7, 14-3-3 binding to MgcPhLP was confirmed by Western blot analysis for the sequence described that the position elements mediating polyadenylation of messages during the oocyte meiotic block at ovulation and prior to the oocyte prophase I (17, 18). The presence of stage-specific isoforms of the Mgcplp message may reflect a translational control during meiotic and post-meiotic maturation. The conclusions of RNA analysis were confirmed by direct determination of the protein by polyclonal antibodies specific for the mouse protein. This was especially informative in the ovary, in which expression is normally limited to the small number of oocytes undergoing meiosis but

![Figure 7](image1.png) 14-3-3 binding to MgcPhLP was confirmed by Western blot analysis for the sequence described that the position elements mediating polyadenylation of messages during the oocyte meiotic block at ovulation and prior to the oocyte prophase I (17, 18). The presence of stage-specific isoforms of the Mgcplp message may reflect a translational control during meiotic and post-meiotic maturation. The conclusions of RNA analysis were confirmed by direct determination of the protein by polyclonal antibodies specific for the mouse protein. This was especially informative in the ovary, in which expression is normally limited to the small number of oocytes undergoing meiosis but
could readily be evidenced after hormonal stimulation leading to superovulation.

As is the case for the other phosducin-related proteins, the function of the protein at the molecular level remains largely to be established. A possibly significant feature in this respect is its association with at least one of the proteins of the 14-3-3 family. Binding may be mediated by the RSSVP motif (amino acids 119–123, Fig. 1), which resembles the sites of interaction identified in other 14-3-3-binding proteins (14). 14-3-3 binds phosphorylated serine residues in a number of proteins active in signal transduction. In retinal photoreceptors, 14-3-3 is considered as regulating the binding of phosducin to Gβγ by sequestering the phosphorylated phosducin molecules and blocking their binding. 14-3-3 was also recently shown to interact in the brain with a phosducin-like protein (19, 20). The specificity of 14-3-3 binding and its relationship with the phosphorylation of serine residues in MgcPhLP are currently being studied.

Two phosducin-related genes were recently described in yeast, PLP1 and PLP2 (4). MgcPhLP displays a greater amino acid similarity with PLP2 than with PLP1. The inability of a plp2Δ mutant to generate viable haploid products was successfully complemented by transfer of the mouse gene. Regarding the function of the yeast gene, published data (4) have left two possibilities open; the Plp2 protein could either be necessary for growth in general or specifically required for the generation of haploid products, either during or after meiosis. The observation that haploid clones bearing the plp2Δ mutation could not be grown from a sporulating culture is compatible with both interpretations, thus making it impossible to evaluate unequivocally the phenotype of the diploid homozygous mutant. Our observations rather favor the hypothesis of a meiotic function of the MgcPhlp gene. Indeed, on long term growth of several of the complemented haploid strains, a loss of the murine gene that did not impair their growth ability. A meiotic function of the mouse protein in germ cell differentiation will be established. A possibly significant feature in this respect is the phosphorylation of serine residues in a number of proteins active in signal transduction. In retinal photoreceptors, 14-3-3 is considered as regulating the binding of phosducin to Gβγ by sequestering the phosphorylated phosducin molecules and blocking their binding. 14-3-3 was also recently shown to interact in the brain with a phosducin-like protein (19, 20). The specificity of 14-3-3 binding and its relationship with the phosphorylation of serine residues in MgcPhLP are currently being studied.

Mgcphlp is included in theClock locus (GenBankTM accession number AF146795). Taking into account that phosducin is expressed abundantly in the retina, has been conserved during evolution, and is involved in phototransduction cascades, one could speculate that Mgcphlp expression could be part of the signaling cascade initiated by dark/light stimuli. In the mouse, the ovulation cycle is known to be dependent on light periodicity, and circadian periods have been reported for the ovarian melatonin and rhythm of cAMP accumulation. Increase in Mgcphlp expression within hours after induction of superovulation by human chorionic gonadotropin injection clearly points to hormonal regulation. It is clear, however, that beyond such speculations, the function of the mouse protein in germ cell differentiation will require the use of site-directed and/or temporally controlled mutagenesis technologies.

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