Cloning and expression analysis of murine phospholipase D1

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Activation of phosphatidylcholine-specific phospholipase D (PLD) occurs as part of the complex signal-transduction cascade initiated by agonist stimulation of tyrosine kinase and G-protein-coupled receptors. A variety of mammalian PLD activities have been described, and cDNAs for two PLDs recently reported (human PLD1 and murine PLD2). We describe here the cloning and chromosomal localization of murine PLD1. Northern-blot hybridization and RNase protection analyses were used to examine the expression of murine PLD1 and PLD2 in a variety

of cell lines and tissues. PLD1 and PLD2 were expressed in all RNA samples examined, although the absolute expression of each isoform varied, as well as the ratio of PLD1 to PLD2. Moreover, *in situ* hybridization of adult brain and murine embryo sections revealed high levels of expression of individual PLDs in some cell types and no detectable expression in others. Thus the two PLDs probably carry out distinct roles in restricted subsets of cells rather than ubiquitous roles in all cells.

INTRODUCTION

A commonly observed event in signal transduction is the modification of the cell membrane by phospholipases that utilize membrane phospholipids as substrates to generate intracellular second messengers [1]. Recently, increasing attention has been focused on the role of phosphatidylcholine-specific phospholipase D (PLD) enzymes, which catalyse the hydrolysis of phosphatidylcholine to yield choline and phosphatidic acid (reviewed in [2]).

Phosphatidic acid has been shown to act directly as a signalling molecule [3–5]. It can serve as a substrate for other phospholipases [6] and can be converted into diacylglycerol [7] or lysophosphatidic acid [8,9]. Localized phosphatidic acid concentration has been proposed to promote coatomer binding or membrane curvature [10–12]. Finally, phosphatidic acid generation correlates with actin polymerization [13]. In the context of these specific roles, PLD has been proposed to function in regulated secretion, cytoskeletal reorganization, transcriptional regulation and cell-cycle control (reviewed in [14,15]).

PLD activity is present in a wide variety of cell types including blood platelets, hepatocytes, lymphocytes, fibroblasts, neuronal cells, muscle cells and endothelial cells. PLD activation has been correlated with metabolic regulation, hormone responses, mitogenesis, cardiac and brain function, immune response, senescence and neoplasia (reviewed in [2]). Activation of PLD within cells occurs as a consequence of the stimulation of both tyrosine kinase and G-protein-coupled receptors (reviewed in [2]), and at least one isoform of the enzyme has been shown to be activated directly by protein kinase C (PKC) and members of the ADP-ribosylation factor (ARF) and Rho small G-protein families [16].

Direct evidence on the diverse cellular roles suggested for PLD by biochemical studies has proven difficult to generate in the absence of molecular reagents, and, until recently, no phos-

phatidylcholine-specific PLD genes were identified. A plant PLD cDNA was reported in 1994 [17], for which GenBank searches indicated that homologous sequences existed in a variety of species including bacteria, yeast and mammals [18]. It was not initially clear though that the human homologues of plant PLD would encode proteins with the long-studied mammalian PLD activities, since, on a biochemical level, plant PLD and human PLD are quite distinct (reviewed in [19]). Plant PLD is constitutively active and does not require lipid cofactors, such as phosphatidylinositol 4,5-bisphosphate (PIP₂) [17,20]. In contrast, the best-studied partially purified mammalian PLD activities displayed an absolute requirement for PIP₂ and were quiescent until activated by protein cofactors such as ARF, Rho and PKC (reviewed in [19]).

Nonetheless, human PLD1 (hPLD1), the first identified mammalian homologue of the plant PLD, was found to exhibit dependence on ARF, Rho, PKC and PIP₂ [18]. A second mammalian gene, murine PLD2 (mPLD2), has also been identified [21]. mPLD2 is also PIP₂-dependent but differs from hPLD1 in that it exhibits constitutive activity *in vitro* and *in vivo*. In addition, hPLD1 and mPLD2 are found in discrete subcellular locations, suggesting that they undertake distinct cellular functions. Biological roles for PLD1 and PLD2 are currently under investigation. In yeast, only one PLD homologue exists, known as SPO14, and yeast deficient in SPO14 exhibit a block in meiosis [22].

To undertake cellular and animal studies, it will be necessary to use PLD1 and PLD2 genes from the same species. Here we report the identification and cloning of mPLD1, the mouse homologue of the human PLD1 gene. mPLD1 protein is 92% identical with hPLD1, but only 60% identical with mPLD2. Analysis of mPLD1 and mPLD2 RNA distribution reveals that they are both expressed in many of the tissues previously reported

Abbreviations used: PLD, phospholipase D; hPLD1, human PLD1; mPLD1, mouse PLD1; mPLD2, mouse PLD2; PIP₂, phosphatidylinositol 4,5-bisphosphate; ARF, ADP-ribosylation factor; PKC, protein kinase C; UTR, untranslated region; RFLP, restriction fragment length polymorphism.

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The amino acid and nucleotide sequences for murine phospholipase D1 have been deposited in GenBank under the accession number U87868.

to display PLD activity. Thus it is possible that together PLD1 and PLD2 account for a major part of the long-studied mammalian phosphatidylcholine-specific PLD activities.

EXPERIMENTAL

Molecular isolation of mPLD1

Partial mPLD1 cDNAs were obtained from λ ZAP II E10.5 mouse embryo and neonatal brain cDNA libraries (Stratagene) as described in the Results section. The reduced stringency conditions used to search for cognate genes consisted of hybridization overnight at 65 °C in solution consisting of 6 $\times$ SSC, 10% Denhardt's, 0.1% SDS and 0.1% sodium pyrophosphate (where 1 $\times$ SSC is 0.15 M NaCl + 0.015 M sodium citrate and Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), and then washing three times for 30 min each at 50 °C in 2 $\times$ SSC/1% SDS. The six overlapping mPLD1 cDNA clones sequenced (Sequenase Version 2.0, United States Biochemicals; a final sequence was determined from both strands) encoded approx. 2.4 kb of coding and 3' untranslated region (UTR) sequence but lacked 5' coding and UTR sequences. The 5' sequence was obtained using degenerate PCR to amplify nts 300–1200 (approximately) employing primers based on conserved peptides present in the N-termini of hPLD1 and a *Caenorhabditis elegans* PLD1 homologue found in the GenBank database (U55854). The remainder of the coding sequence and the 5'UTR were generated using rapid amplification of cDNA ends PCR [23]. The mPLD1 amino acid and nucleotide sequences have been deposited in GenBank under the accession number U87868.

Cell culture

HL60, NIH3T3 and HeLa cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C.

RNA extraction and Northern-blot and *in situ* hybridizations

Total RNA was isolated from tissue culture cell lines and murine tissues using Trizol (Gibco-BRL). Northern-blot and *in situ* hybridizations were carried out as described previously [24–26] using a 740 nt cDNA probe for mPLD1 (nt 414–1155) and a 850 nt cDNA probe for mPLD2 (nt 308–1155).

RNase protection assay

A 398 nt hPLD1 *Acc65I*–*BglII* cDNA fragment (nt 384–781) was subcloned in pBluescript-SK (Stratagene), and a 357 nt hPLD2 fragment (nt 79–435 of the existing composite GenBank expressed sequence tags) was subcloned into pGEM7Zf (Promega) into the *Bam*HI and *Xba*I sites. A 358 nt mPLD1 cDNA fragment (nt 2384–2741) and a 330 nt mPLD2 fragment (nt 2060–2390) were subcloned in pBluescript-SK into *Eco*RI and *Xho*I sites. Linearized plasmids were transcribed *in vitro* using RNA polymerases in the presence of [³²P]UTP to generate labelled antisense RNAs, which were gel isolated, and employed to detect PLD RNA using a commercial RNase protection kit (Ambion). Total RNA samples were prepared as described above.

Interspecific mouse backcross mapping

Interspecific backcross progeny were generated by mating (C57BL/6J $\times$ *Mus spretus*)F₁ females and C57BL/6J males as described [27]. A total of 205 N₂ mice were used to map the

PLD1 and PLD2 loci (see the Results section for details). DNA isolation, restriction-enzyme digestion, agarose-gel electrophoresis, Southern-blot transfer and hybridization were performed essentially as described [28]. All blots were prepared with Hybond N⁺ nylon membrane (Amersham). The PLD1 probe, an approx. 660 bp *Eco*RI–*Hind*III cDNA coding fragment, was labelled with [α -³²P]dCTP using a random-primed labelling kit (Stratagene); washing was performed to a final stringency of 1 $\times$ SSC/0.1% SDS, 65 °C. Fragments of 5.9, 4.0 and 1.7 kb were detected in *Pst*I-digested C57BL/6J DNA and fragments of 6.4, 2.5 and 1.7 kb were detected in *Pst*I-digested *M. spretus* DNA. The PLD2 probe, an approx. 1.8 kb *Eco*RI cDNA coding fragment, detected 10.0 and 6.0 kb *Xba*I fragments in C57BL/6J DNA and a 17.5 kb *Xba*I fragment in *M. spretus* DNA. The presence or absence of the *M. spretus*-specific fragments was followed in backcross mice.

The probes and restriction fragment length polymorphisms (RFLPs) for the loci linked to PLD1 including *Il7*, *Crh* and *Evi1* have been described previously [29,30]; those linked to PLD2 include *Myhsf1*, *Trp53* and *Nf1* [31]. Recombination distances were calculated as described [32] using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Isolation of a cDNA encoding mPLD1

We had previously reported the isolation of hPLD1 from a HeLa cell library [18]. To search for related genes in a more complex set of tissues, we used the entire coding region of hPLD1 as a probe to screen mouse embryonic and neonatal brain cDNA libraries under conditions of reduced stringency as described in the Experimental section. Two sets of plasmid clones representing distinct PLD genes were isolated. Six overlapping cDNA clones encoded a sequence that was highly similar to hPLD1. The extensive similarity suggested that this cDNA is the cognate to hPLD1, and the cDNA was therefore designated mPLD1. In addition, 20 separate overlapping cDNAs were isolated that encoded a related but novel cDNA, mPLD2. mPLD2 is 60% identical on an amino acid level with hPLD1 and it encodes a PLD enzyme that differs from hPLD1 in its biochemical regulation and subcellular distribution [21]. The mPLD1 cDNA was completed using a combination of degenerate PCR and rapid amplification of cDNA ends PCR to obtain the 5' coding regions and UTR.

The mPLD1 cDNA is 3656 nt in length (Figure 1). The presumed initiator methionine (nt 1–3) conforms to the eukaryotic consensus sequence and is the first in-frame methionine in the 5' UTR. The coding region is in addition thought to be full-length, since an in-frame stop codon is located 60 nt upstream of the presumed start codon. Moreover, the presumed coding sequence is similar to hPLD1 at both the N- and C-termini [18]. A recognizable polyadenylation signal sequence was not found at the 3' end of the composite cDNA, suggesting that the entire 3' UTR was not obtained. The mPLD1 mRNA transcript was found to be approx. 4.5 kb in size by Northern-blot analysis (see Figure 3), suggesting that the cDNA sequence reported here lacks approx. 700 nt in total from the 5' and 3' UTRs, assuming a polyadenylated tail length of approx. 150 nt.

mPLD1 encodes a 1036-amino-acid protein that is 92% identical with hPLD1 (Figure 2). Two alternatively spliced forms of hPLD1 have been identified, differing in the inclusion (hPLD1a) or exclusion (hPLD1b) of a 38-amino-acid exon [16]. mPLD2 lacks both the amino acids corresponding to this exon

The presumed initiator codon is shown in bold typeface. Boxes indicate the locations of four conserved regions found in all known PLD homologues. Underlined amino acids indicate the duplicated motif that is presumed to form the active site required for catalysis. The filled circle indicates the site at which an alternative splice site is located (see the text and Figure 2 for details).

Figure 3 Northern-blot analysis of mPLD1 and mPLD2

RNA samples were electrophoresed and visualized using ethidium bromide to determine the integrity and amounts loaded for each sample. The RNA was transferred to a nylon membrane and sequentially hybridized with mPLD1, stripped, and hybridized with mPLD2. The hybridized membrane was exposed to X-ray film for 2 weeks for each probe. Stripping and rehybridization can diminish subsequent signals, so it is possible that PLD may be more abundant than the result shown here would suggest.

Figure 4 RNase protection of mPLD1 and mPLD2

[32 P]UTP-labelled RNA human and mouse PLD1 and PLD2 probes prepared as described in the Experimental section were hybridized to unlabelled RNA samples containing yeast RNA, sense control RNAs or RNAs prepared from cell lines and tissues. A 20 μ g amount of sample RNA was used for all hybridizations except for HL60, for which 2 μ g of RNA was used for the PLD1 protection and 50 μ g was used for PLD2. Top left, protection of the labelled probes by control sense RNAs (positive control) demonstrates the expected size products after a 2 h exposure to X-ray film. Top middle, lack of protection by yeast RNA (negative control) after a 2-day exposure to X-ray film demonstrates that the protection of the fragments is specific. Top right, RNAs prepared from NIH3T3, HL60 and HeLa cells all protect both probes, as shown by a 2-day exposure. Relatively similar amounts of PLD1 and PLD2 are present in NIH3T3 and HeLa cells. HL60 contains much more PLD1 than PLD2, as evidenced by the stronger signal in the PLD1 lane despite the fact that the PLD2 sample contained 25-fold more RNA. Bottom, protection by RNA prepared from several tissues demonstrates that PLD1 and PLD2 are both present in all samples but differ in relative abundance (2-day exposure). All samples were assayed in multiple (at least three) experiments and representative results are shown.

G-proteins and PKC. The other group is oleate-dependent and does not require PIP_2 , small G-proteins or PKC. Within each group, numerous activities have been described that vary slightly in their specific properties, cofactor requirements, responsiveness to activators or predicted molecular size (reviewed in [14]). Undetermined at present is how many PLD genes or gene families exist. Accordingly, we sought to determine the pattern of expression of PLD1 and PLD2 in cell lines and multiple murine tissues. The finding of extensive PLD1 and PLD2 expression would suggest that these genes play widespread roles. In contrast, the finding of restricted expression in a subset of the tissues in which PLD activity can be demonstrated would suggest that additional unrecognized cognate genes or PLD families should

exist. Analysis of mPLD1 and mPLD2 RNA distribution was performed using several approaches.

Northern-blot analysis

Total RNA was prepared from a variety of murine tissues, electrophoresed in a formaldehyde/agarose gel, and hybridized to mPLD1 and mPLD2 (Figure 3). The PLD1 and PLD2 RNAs were detected in all tissues sampled, although the absolute levels of expression varied by 10–100-fold. PLD1 was found to be expressed at relatively high levels in kidney and lung, and PLD2 was expressed most strongly in brain and lung. These results are consistent with previous reports. Kidney, lung and brain have all been described as rich sources from which PLD can be prepared using biochemical means [2]. More striking, however, was the finding that the relative amounts of PLD1 and PLD2 in each tissue vary dramatically. For example, levels of expression of PLD1 RNA are found to be much higher than PLD2 in the kidney, and the opposite is observed in the brain. This result suggests that PLD1 and PLD2 may carry out specific cellular roles in restricted tissues, rather than a ubiquitous role in every tissue. Since the levels of PLD RNA detected roughly correlate with the tissues in which high levels of PLD activity have previously been reported, the result does not lend support to the hypothesis that additional PLD genes or families are likely to exist.

RNase protection analysis

RNase protection analyses were carried out to confirm and extend the results of the Northern-blot analyses. Probes and hybridization conditions were established for both the human and murine PLD1 and PLD2 genes (Figure 4). As shown, protected bands of approx. 360–390 nt (mPLD1, hPLD1) and 300 nt (mPLD2, hPLD2) are observed with a 2 h exposure at room temperature when control sense RNAs are present in the reaction mixture. In contrast, protected bands are not observed with a 2-day exposure when yeast RNA is used as a sample template. Three cell lines, NIH3T3 (murine), HL60 (human) and HeLa (human), were examined for PLD expression. NIH3T3 and HeLa cells expressed equivalent levels of PLD1 and PLD2. In contrast, HL60, a pre-leukaemic cell line used in many reports to study the role of PLD in the regulation of secretion, expresses much higher levels of PLD1 than PLD2.

The results on RNA samples prepared from individual tissues are consistent with the Northern-blot analyses. All tissues tested express both PLD genes, and the relative amounts of the two genes in kidney and brain mirror the reciprocal relationships observed using Northern-blot analysis.

In situ hybridization

Northern-blot and RNase-protection assays permit the determination of levels of expression in specific tissues as averaged out for all cells. To examine expression of the PLD genes by individual cells, we carried out *in situ* hybridization on sections of murine embryos and adult brain (Figure 5). Both PLD1 and PLD2 are expressed in a restricted manner. PLD1 is expressed most strikingly in selected ventricular cells lining the spinal cord and brain (Figures 5A, 5B, 5L and 5J). The level of expression decreases dramatically as the cells differentiate into neurons and migrate to the outer layer of the spinal cord and brain. Expression is also seen in the retina (Figure 5C), and in multiple regions in the adult brain. In the dentate gyrus (a subregion in the hippocampus; Figure 5N), PLD1 expression is observed in large

Figure 5 *In situ* hybridization of mPLD1 and mPLD2

PLD1 and PLD2 RNA antisense probes were used to detect PLD gene expression in adult brain (**N, O**) and in embryonic (day E11.5) tissues (**A–M**). **A, E, G, I, L, N, O**, Bright field; **B, C, D, F, H, J, K, M**, dark field; **B, C, J, N**, PLD1; **D, F, H, K, M, O**, PLD2. Panels **N** and **O** were generated using non-radioactive *in situ* hybridization; the remainder of the sections were hybridized with ^{33}P -labelled probes. **A, B, E, F, G, H, L, M**, transverse sections through the forebrain and hindbrain; **C, D**, retina and optic nerve tract; **G, H**, higher-power magnification of **E, F**; **I, J, K**, transverse sections of the spinal cord; **N, O**, dentate gyrus of the adult brain. Expression of PLD1 is observed in ventricular cells in the lateral wall of the third ventricle (**B**), in the retina (**C**), in ventral ventricular cells in the spinal cord (**J**), and in neuronal-appearing cells in the adult brain (**N**). PLD2 expression is observed in the optic nerve tract (**D**), in the hippocampus anlage (**F, H**), in ventricular (proliferating) and motor (differentiating) neurons in the spinal cord (**K**), in a cranial nucleus in the hindbrain (**M**), and in cells in the adult brain consistent in appearance with glia (**O**). Expression in the hippocampal anlage is observed only on one side of the brain in (**F**) because the embryo was sectioned at an angle and the region on the right side does not include the hippocampal anlage. Abbreviations: dg, dentate gyrus; d, dorsal; hb, hindbrain; hc, hippocampus; lv, lateral ventricle; sc, spinal cord; v, ventral.

cells consistent in appearance with neurons. Finally, PLD1 expression is observed during development in a restricted region of the nasal neuroepithelium (not shown).

PLD2 is expressed in a partially overlapping pattern at higher levels. Most strikingly, PLD2 is expressed at high levels in the hippocampus at the earliest time at which it is defined as a structure (Figures 5E, 5F, 5G and 5H). In addition, PLD2 expression is observed in ventricular neural cells (Figures 5I and 5K) as well as in differentiating neurons outside of the ventricular region (Figures 5I, 5K, 5L and 5M). PLD2 is also expressed in multiple regions of the adult brain. In the dentate gyrus, in contrast with PLD1, PLD2 is expressed in smaller cells (Figure 5O) consistent in appearance with glia (astrocytes and oligodendrocytes). Finally, PLD2 is expressed during development at

lower levels in mesenchymal cells derived from the neural crest that are destined to form bones of the skull (not shown).

The functional significance of the restricted expression is unknown, since the cellular roles proposed for PLD1 and PLD2 both cover a wide range of biological processes and are unproven. However, the finding does illustrate that PLD1 and PLD2 are not expressed ubiquitously and therefore may be required for specific regulated cell processes (reviewed in [15]) instead of 'housekeeping' activities.

Chromosomal localization of mPLD1 and mPLD2

The mouse chromosomal locations of PLD1 and PLD2 were determined by interspecific backcross analysis using progeny

Figure 6 Murine chromosomal locations of PLD1 and PLD2

The segregation patterns of PLD1 and PLD2 and their flanking genes in 99 and 130 backcross animals respectively that were typed for all loci are shown at the top. For individual pairs of loci, additional animals were typed (see the text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J $\times$ *M. spretus*) F_1 parent. Solid boxes represent the presence of a C57BL/6J allele and open boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. Partial chromosome 3 and 11 linkage maps showing the location of PLD1 and PLD2 in relation to linked genes are shown at the bottom of the Figure. Recombination distances between loci in cM are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD, U.S.A.).

derived from matings of (C57BL/6J $\times$ *M. spretus*) F_1 $\times$ C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 2300 loci that are well distributed among all the autosomes as well as the X chromosome [27]. C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analysed by Southern-blot hybridization for informative RFLPs using mouse cDNA coding region probes. The 6.4 and 2.5 kb *Pst*I *M. spretus* RFLPs (see the Experimental section) were used to follow the segregation of the PLD1 locus in backcross mice. The mapping results indicated that the two fragments co-segregated, and PLD1 is located in the proximal region of mouse chromosome 3 linked to *Il7*, *Crh* and *Evi1*. Although 99 mice were analysed for every marker and are shown in the segregation analysis (Figure 6), up to 145 mice were typed for some pairs of markers. Each locus was analysed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere-*Il7*-2/143-*Crh*-3/135-*Pld1*-5/145-*Evi1*. The recombination frequencies [expressed as genetic distances in centimorgans (cM) $\pm$ the standard error] are: *Il7*-1.4 $\pm$ 1.0-*Crh*-2.2 $\pm$ 1.3-*Pld1*-3.5 $\pm$ 1.5-*Evi1*.

A 17.5 kb *Xba*I *M. spretus* RFLP (see the Experimental section) was used to follow the segregation of the PLD2 locus in

backcross mice. PLD2 mapped to the central region of mouse chromosome 11 linked to *Myhsf1*, *Trp53* and *Nf1*. In this case, 130 mice were analysed for every marker and are shown in the segregation analysis (Figure 6). Up to 144 mice were typed for some pairs of markers. Again, each locus was analysed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere-*Myhsf1*-4/132-*Pld2*-0/130-*Trp53*-4/144-*Nf1*. The recombination frequencies (expressed as genetic distances in cM $\pm$ the standard error) are *Myhsf1*-3.0 $\pm$ 1.5-(*Pld2*, *Trp53*)-2.8 $\pm$ 1.4-*Nf1*. No recombinants were detected between PLD2 and *Trp53* in 130 animals typed in common, suggesting that the two loci are within 2.3 cM of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosomes 3 and 11 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided by Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME, U.S.A.). PLD1 mapped in a region of the composite map containing one mouse mutation potentially attributable to PLD1 misregulation, known as *coa*. *coa* is associated with a coat colour dilution and white spotting,

hence the name cocoa [35]. More relevant to PLD1, *coa* also causes a platelet-storage-pool deficiency characterized by decreased levels in serotonin and dense granules, suggestive of a potential problem in a secretory pathway.

PLD2 mapped in a region of the composite map in which there were several interesting mouse mutations within 10 cM, including vibrator (*vb*), insulin-dependent diabetes locus 4 (*idd4*) and juvenile cystic kidney disease (*jck*). *vb* is characterized by degeneration of selected neurons in the central nervous system and features dilated cisternae of the endoplasmic reticulum in cell bodies, axons and dendrites with eventual severe intracellular vacuolization and some cell death [36]. The nature of the *idd4* phenotype is not known, but suggests an abnormality in a signal-transduction pathway [37]. The *jck* mutation is also not well understood, but may involve problems with vesicular trafficking in kidney cells [38].

The proximal region of mouse chromosome 3 shares regions of homology with human chromosomes 8q and 3q (Figure 6). Our placement of mPLD1 in this interval suggested that hPLD1 should map to one of these two regions. Mapping of hPLD1 was carried out using a National Institute of General Medical Science somatic cell hybrid mapping panel. A human specific RFLP was found for panel no. 2 from human chromosome 3, and thus the inferred human location is 3q24-q28 (results not shown). Coincident chromosomal localizations of mPLD1 and hPLD1 in syntenic regions confirms that mPLD1 and hPLD1 are cognate genes. The central region of mouse chromosome 11 shares a region of homology with human chromosome 17. The tight linkage in mouse between PLD2 and *Trp53* suggests that the human homologue of PLD2 will reside on 17p, since human *Trp53* has been mapped to chromosome 17p13.1.

A number of human diseases map within regions that overlap the locations of the PLD1 and PLD2 loci. Lacking insight into expected phenotypes for misregulated expression of the PLD genes, however, it is difficult to predict whether the PLD genes represent likely candidates for these diseases. Information gained from the study of PLD1 and PLD2 and from gene-inactivation experiments should eventually shed light on the possibility that the PLD genes are involved in a specific disease or syndrome.

DISCUSSION

The cloning of mPLD1 revealed that it is highly conserved with hPLD1. A comparison of the mouse and human proteins showed no deletions or insertions and only 4% non-conservative substitutions. The proteins encoded are very likely to be indistinguishable on a functional level, in contrast with PLD2, which exhibits numerous differences, including its regulation and sub-cellular localization.

The expression analyses presented here demonstrate that the study of PLD1 and PLD2 is likely to be germane to the numerous reports of PLD activity in a wide variety of tissues and cell lines. Thus far, PLD1 and PLD2 expression has been observed in at least seven separate cell lines (this report and unpublished work). No cell line has yet been found that fails to express both genes, although as shown above, the relative levels of expression can vary dramatically. Levels of expression can also change significantly with differentiation (W. B. Bollag, unpublished work). Similarly, PLD1 and PLD2 expression has been found in all adult tissues examined (including several additional ones not shown here) on a gross level. However, *in situ* hybridization results suggest that, within a given tissue, the PLDs may be expressed at high levels in some cells and undetectable levels in

others. Taken together, these results suggest two possibilities. First, it may be that PLD1 and/or PLD2 are not required by all cells. As discussed in a recent review, PLD1 and PLD2 may carry out specific roles related to regulated secretion or specific signal-transduction responses, and these types of behaviour may be restricted to certain subgroups of cells [15]. Alternatively, we cannot exclude the possibility that there may be additional PLD genes that carry out these functions in cells that do not express significant levels of PLD1 and PLD2, although at present there exists no evidence to support this hypothesis.

PLD activities have been reported in numerous tissues [2] and have been partially purified from discrete subcellular fractions [39–41]. It will be important to determine if PLD1 and PLD2 account for all of the reported (PIP₂-dependent) activities (as well as which activities derive from PLD1 and which from PLD2) or whether evidence suggests that additional genes should be sought.

We thank Yue Zhang, Tsung-Chang Sung, JoAnne Engebrecht and Andrew Morris for critical comments on the manuscript, and Mary Barnstead for excellent technical assistance. We also thank Forrest Spencer for mapping the human PLD1 gene as part of the XREF project. This research was supported by a grant from the NIH (HD29758) and from Onyx (Richmond, CA, U.S.A.) to M.A.F., and in part, by the National Cancer Institute, DHHS, under contract with ABL. S.E.T. was supported by a grant from the NIH (NS35843). C.K.S.-L. was supported by a summer student award from the American Heart Association-Georgia Affiliate to W.B.B.

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