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Mini Review

Multiple roles of phosphoinositide-specific phospholipase C isozymes

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Phosphoinositide-specific phospholipase C is an effector molecule in the signal transduction process. It generates two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate. Currently, thirteen mammal PLC isozymes have been identified, and they are divided into six groups: PLC-β, -γ, -δ, -ε, -ζ and -η. Sequence analysis studies demonstrated that each isozyme has more than one alternative splicing variant. PLC isozymes contain the X and Y domains that are responsible for catalytic activity. Several other domains including the PH domain, the C2 domain and EF hand motifs are involved in various biological functions of PLC isozymes as signaling proteins. The distribution of PLC isozymes is tissue and organ specific. Recent studies on isolated cells and knockout mice depleted of PLC isozymes have revealed their distinct phenotypes. Given the specificity in distribution and cellular localization, it is clear that each PLC isozyme bears a unique function in the modulation of physiological responses. In this review, we discuss the structural organization, enzymatic properties and molecular diversity of PLC splicing variants and study functional and physiological roles of each isozyme. [BMB reports 2008; 41(6): 415-434]

INTRODUCTION

Phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG and IP_3 initiate further signal transduction pathways through activation of protein kinase C (PKC) and intracellular calcium release (1-3; Fig. 1a).

The first evidence of PLC activity was suggested by Hokin *et al*. in 1953 who reported specific hydrolysis of phospholipids in pigeon's pancreas slices after cholinergic stimulation (4). The authors showed that the enhanced turnover of phosphorylinositol groups of phosphatidylinositol occurred in cells as a response to a variety of stimuli. In 1983, Streb *et al*. demonstrated that IP_3 generated from PIP_2 hydrolysis is responsible for mobilization of intracellular calcium in the pancreatic acinar cells (5). Takenawa *et al*. purified the first PLC with a molecular weight of 68 kDa in 1981 (6). Subsequently, a number of PLCs of different molecular masses, isoelectric points and calcium dependency have been identified in several tissues. In the late 80 s, three PLC isozymes, namely PLC-γ, -β, and -δ, were isolated and their cDNA sequences were obtained (7). Since then, multiple types of PLC were found from various tissues using either an RT-PCR method by specifically designed primers or a screening method using low stringency hybridization with probes made from the conserved domain X or Y. So far, 13 PLC isozymes have been identified in different mammalian tissues and they belong to six different subtypes.

Identification of PLC isozymes, PLC-ε and PLC-η, has changed our understanding of the relations between receptor tyrosine kinase (RTK)-PLC-γ and G protein-coupled receptor (GPCR)- PLC-β (Fig. 1b). Little is known about functions, relations and hierarchy of PLC isozymes. In this review, we discuss the questions of structural organization, enzymatic properties and molecular diversity of splicing variants of PLC isozymes in relation to their function and physiological significance.

Structure of PLCs

Catalytic X and Y domains

Two highly conserved amino acid regions in PLC isozymes are the X and Y domains, which are located between EF-hand motifs and C2 domain. The domains are composed of alternating α-helices and β-strands and resemble incomplete triose phosphate isomerase (TIM) α/β-barrel (8). Crystallographic analysis

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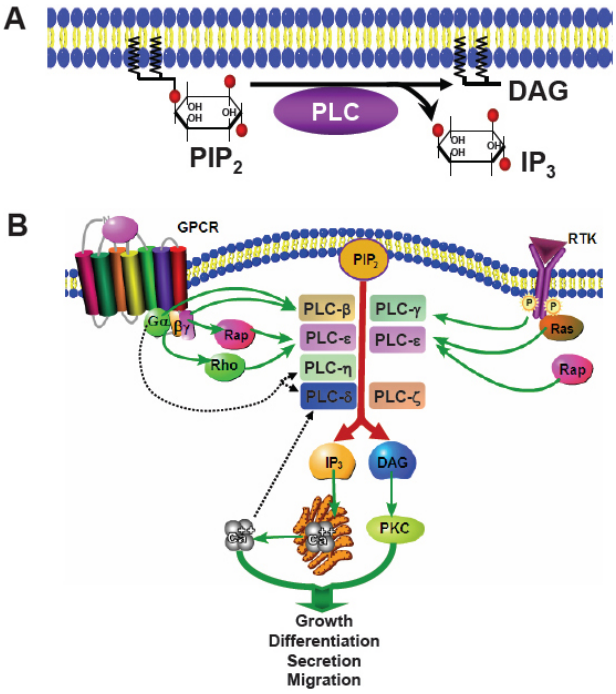

Fig. 1. Activation and function of various PLC isozymes. A) Phospholipase-dependent PIP_2 hydrolysis. PLC catalyzes the hydrolysis of PIP_2, a member of membrane phospholipids. Two products, diacylglycerol (DAG) and IP_3 induce the activation of PKC and intracellular Ca^{2+} release, respectively. B) PLC related signaling pathway. Various extracellular signals stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphoinositide-specific phospholipase C (PLC). PLC hydrolyzes PIP_2 to two important second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). These two second messengers lead to the activation of several protein kinase C (PKC) isoforms and the release of calcium from intracellular stores, respectively. PLC-β subtypes are activated by GPCR through several mechanisms. Pertusis toxin (PTX)-insensitive heterotrimeric G_q family proteins (G_q, G_{11}, G_{14}, G_{15} and G_{16}) activate PLC-β subtypes via GTP-loaded Gα subunits. PLC-β subtypes are also activated by Gβγ subunits liberated from PTX-sensitive G_i family proteins. Various growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) can activate PLC-γ isozymes. Upon growth factor stimulation, PLC-γ is recruited to activated growth factor receptors via SH2 domain-phosphotyrosine interaction and then subjected to phosphorylation by receptor tyrosine kinases. PLC-ε can be activated by both GPCR and RTK activation with distinct activation mechanism. The activation mechanism for PLC-δ, η, ζ remains to be revealed.

revealed that histidine residue functions as a general base leading to formation of 1,2-cyclic inositol 4,5-bisphosphate (9). Ca^{2+} ions are required for lowering the pKa and promoting the nucleophilic attack on 1-phosphate. The following stabilization of the cyclic phosphate intermediate at the active site produces acyclic product (10). Catalytic residues are highly conserved in all eukaryotic PLC isozymes. Based on structural analysis of PLC-δ1, it has been suggested that Lys^{438}, Lys^{440}, Ser^{522} and Arg^{549} residues are present at the active site which are implicated in interactions with 4- or 5-phosphate of the substrate headgroup (9, 11). Preferential hydrolysis of PIP_2 has been attributed to a positive charge at 549 position of PLC-δ1, which is conserved in the eukaryotic PLC catalytic core, This finding is supported by the fact that replacement of a charged residue causes suppression of PIP_2 hydrolysis but not of PI (12).

PH domain

It has been shown that the PH domain in PLC-δ1 binds PIP_2 and advances the access of PLC-δ1 onto the membrane surface (13). The PH domain of PLC-β2 and PLC-β3 binds specifically to the heterotrimeric G protein subunit, Gβγ (14). The domain also mediates interactions with PIP_3, which is required for PI3K-dependent PLC-γ1 translocation and activation (15). PLC-γ1 and γ2 contain an additional PH domain which is split by two tandem Src homology 2 (SH2) and a Src homology 3 (SH3) domains. The C-terminal half of the PLC-γ split PH domain has been implicated to interact directly with the TRPC3 calcium channel, thereby providing a direct coupling mechanism between PLC-γ and agonist-induced calcium entry (16).

C2 domain and EF-hands

The C2 domain of PLC-δ1 possesses three to four Ca^{2+} binding sites (8). Calcium ions bind to the C2 domain and enhance the enzymatic activity of PLC-δ1 by forming an enzyme-phosphatidylserine-Ca^{2+} ternary complex. EF-hand motifs are helix-turn-helix structural domains which bind Ca^{2+} ions. It was shown that the deletion of EF-hand motifs of PLC-δ1 resulted in a decrease in PLC activity in a Ca^{2+}-independent manner (17, 18). Although the EF-hand motif of PLC isozyme may have an important regulatory function, currently there is no strong evidence to support the notion that EF-hand motifs bind to metal ions.

Tissue distribution of PLCs

Thirteen mammalian PLC isozymes have been identified, so far in various tissues and cell types. Analysis of expressed sequence tags (EST) allows a systematic search of gene expression. We used EST database in NCBI Unigene, (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) to study the tissue distribution of PLC isozymes and to analyze their mRNA expression. We found that PLC-β1 is highly expressed in the cerebral cortex and hippocampus (19) compare to limited expression of PLC-β2 in hematopoietic cells (20, 21); PLC-β3 is found in the brain, liver, and parotid gland (22); PLC-β4 is present at the highest level in the cerebellum and retina (23-25). Our results showed that the highest EST score for PLC-β isozymes was found in the brain (~ 20-44% of analyzed EST sequences) (Table 1). EST sequences of PLC-β2 have greater value in blood and the bone marrow compare to other tissues.

Two mammalian subtypes of PLC-γ isozymes have been identified. PLC-γ1 mRNA is widely detected in various tissues. It is abundantly expressed in embryonal cortical structures, neurons, oligodendrocytes and astrocytes (26) Unlike PLC-γ1,

Table 1. Tissue-specific expression of PLCs as deduced from human UniGene database

PLC isozymes	PLC-β1	PLC-β2	PLC-β3	PLC-β4	PLC-γ1	PLC-γ2	PLC-δ1	PLC-δ3	PLC-δ4	PLC-ε	PLC-ζ	PLC-η1	PLC-η2
GENE ID	23236	5330	5331	5332	5335	5336	5333	113026	84812	51196	89869	23007	9651
UniGene No.	Hs.431173	Hs.355888	Hs.591953	Hs.472101	Hs.268177	Hs.413111	Hs.80776	Hs.380094	Hs.632528	Hs.655033	Hs.97542	Hs.567423	Hs.170156
No. of Total EST	142	198	115	280	360	238	390	196	82	175	69	83	67
No. of Other (mixture)	20	50	11	47	43	67	52	31	15	33	15	17	7
No. EST counted	122 (100%)	148 (100%)	104 (100%)	233 (100%)	317 (100%)	171 (100%)	338 (100%)	165 (100%)	67 (100%)	142 (100%)	54 (100%)	66 (100%)	60 (100%)
Tissues													
embryo tissue	2 (1.6%)	1 (0.7%)	12 (11.5%)	5 (2.1%)	33 (10.4%)	2 (1.2%)	9 (2.7%)	4 (2.4%)	2 (3.0%)	1 (0.7%)	-	9 (13.6%)	1 (1.7%)
brain	44 (36.1%)	36 (24.3%)	20 (19.2%)	44 (18.9%)	68 (21.5%)	16 (9.4%)	81 (24.0%)	52 (31.5%)	16 (23.9%)	30 (21.1%)	1 (1.9%)	25 (37.9%)	16 (26.7%)
eye	7 (5.7%)	2 (1.4%)	3 (2.9%)	16 (6.9%)	14 (4.4%)	1 (0.6%)	9 (2.7%)	9 (5.5%)	8 (11.9%)	10 (7.0%)	-	6 (9.1%)	12 (20.0%)
ear	2 (1.6%)	-	-	2 (0.9%)	-	-	-	-	-	1 (0.7%)	-	-	-
mouth	1 (0.8%)	-	-	2 (0.9%)	4 (1.5%)	1 (0.6%)	8 (2.4%)	-	3 (4.5%)	2 (1.4%)	-	-	-
thymus	1 (0.8%)	5 (3.4%)	-	2 (0.9%)	16 (5.0%)	1 (0.6%)	24 (7.1%)	-	1 (1.5%)	1 (0.7%)	-	3 (4.5%)	-
thyroid	-	-	1 (1.0%)	5 (2.1%)	12 (3.8%)	3 (1.8%)	1 (0.3%)	3 (1.8%)	2 (3.0%)	1 (0.7%)	-	-	-
heart	-	-	-	4 (1.7%)	2 (0.6%)	1 (0.6%)	3 (0.9%)	2 (1.2%)	1 (1.5%)	1 (0.7%)	-	-	-
kidney	5 (4.1%)	3 (2.0%)	3 (2.9%)	6 (2.6%)	8 (2.5%)	14 (8.2%)	10 (3.0%)	4 (2.4%)	4 (6.0%)	2 (1.4%)	-	-	2 (3.3%)
liver	5 (4.1%)	1 (0.7%)	5 (4.8%)	4 (1.7%)	1 (0.3%)	5 (2.9%)	2 (0.6%)	5 (3.0%)	1 (1.5%)	1 (0.7%)	-	-	-
lung	3 (2.5%)	6 (4.1%)	-	31 (13.3%)	19 (6.0%)	5 (2.9%)	14 (4.1%)	17 (10.3%)	8 (11.9%)	8 (5.6%)	-	1 (1.5%)	8 (13.3%)
stomach	-	-	4 (3.8%)	10 (4.3%)	6 (1.9%)	6 (3.5%)	1 (0.3%)	3 (1.8%)	2 (3.0%)	-	-	-	-
spleen	-	6 (4.1%)	-	-	-	1 (0.6%)	9 (2.7%)	-	-	1 (0.7%)	-	-	-
intestine	7 (5.7%)	5 (3.4%)	13 (12.5%)	15 (6.4%)	24 (7.6%)	3 (1.8%)	12 (3.6%)	7 (4.2%)	3 (4.5%)	13 (9.2%)	-	3 (4.5%)	3 (5.0%)
pancreas	3 (2.5%)	1 (0.7%)	2 (1.9%)	17 (7.3%)	1 (0.3%)	2 (1.2%)	1 (0.3%)	13 (7.9%)	-	5 (3.5%)	-	-	11 (18.3%)
testis, prostate	15 (12.3%)	4 (2.7%)	4 (3.8%)	13 (5.6%)	24 (7.6%)	7 (4.1%)	76 (22.5%)	12 (7.3%)	7 (10.4%)	6 (4.2%)	53 (98.1%)	16 (24.2%)	-
ovary, placenta,uterus	16 (13.1%)	16 (10.8%)	15 (14.4%)	6 (2.6%)	31 (9.8%)	11 (6.4%)	30 (8.9%)	16 (9.7%)	2 (3.0%)	11 (7.7%)	-	2 (3.0%)	-
lymph node	-	11 (7.4%)	1 (1.0%)	-	9 (2.8%)	73 (42.7%)	-	2 (1.2%)	-	-	-	-	4 (6.7%)
parathyroid	4 (3.3%)	-	-	-	-	-	-	-	-	-	-	-	-
mammary gland	-	-	2 (1.9%)	18 (7.7%)	11 (3.5%)	4 (2.3%)	5 (1.5%)	2 (1.2%)	1 (1.5%)	-	-	-	2 (3.3%)
ganglia	-	1 (0.7%)	-	1 (0.4%)	1 (0.3%)	1 (0.6%)	1 (0.3%)	-	-	-	-	-	-
skin	-	2 (1.4%)	14 (13.5%)	12 (5.2%)	11 (3.5%)	2 (1.2%)	7 (2.1%)	7 (4.2%)	-	1 (0.7%)	-	-	1 (1.7%)
muscle	2 (1.6%)	2 (1.4%)	-	9 (3.9%)	7 (2.2%)	1 (0.6%)	3 (0.9%)	2 (1.2%)	5 (7.5%)	4 (2.8%)	-	1 (1.5%)	-
bone	-	1 (0.7%)	-	-	2 (0.6%)	2 (1.2%)	2 (0.6%)	-	-	1 (0.7%)	-	-	-
bone marrow	-	14 (9.5%)	-	-	2 (0.6%)	1 (0.6%)	1 (0.3%)	-	-	-	-	-	-
blood	-	27 (18.2%)	-	2 (0.9%)	5 (1.6%)	5 (2.9%)	8 (2.4%)	3 (1.8%)	-	1 (0.7%)	-	-	-
adipo tissue	1 (0.8%)	-	1 (1.0%)	-	1 (0.3%)	1 (0.6%)	-	-	-	-	-	-	-
nerve	1 (0.8%)	-	-	1 (0.4%)	2 (0.6%)	-	1 (0.3%)	-	-	-	-	-	-
connective tissue	3 (2,5%)	4 (2.7%)	4 (3.4%)	8 (3.4%)	3 (0.9%)	2 (1.2%)	20 (5.9%)	2 (1.2%)	1 (1.5%)	41 (28.9%)	-	-	-

The NCBI UniGene database represents the total number of EST clones for each PLC isotypes from a variety of adults and fetal tissues, including identified and unidentified sequences (e.g. pooled sequences from mixture tissues or unknown). For systemic presentation of tissue distribution the EST clone, unidentified sequences were subtracted from the total amount of sequences. The remaining number of sequences is set to 100% and the tissue-specific sequences are calculated in percent of total sequences.

PLC-γ2 mRNA is expressed in the limited areas of anterior pituitary and cerebellar Purkinje and granule cells (27). The expression of PLC-γ2 is primarily limited to cells of haematopoietic lineage. Our data search results revealed that the PLC-γ1 EST number is high in the brain and embryonic tissues, while the highest PLC-γ2 EST number is observed in lymph nodes. The observed expression patterns can serve as an explanation to the predominant role of PLC-γ1 in embryonic cell development and the role of PLC-γ2 in immune response (28-30).

PLC-δ1 is present in high abundancy in the brain, heart, lung, skeletal muscle and testis (31). EST sequences analysis showed that PLC-δ1 isozyme is found at high levels in the brain, lung, reproductive organs, thymus and connective tissue (Table 1). PLC-δ3 is detected abundantly in brain, skeletal muscle and heart (32). EST sequences for PLC-δ3 isozyme are mainly represented in brain, lung, pancreas, and reproductive organ (Table 1). PLC-δ4 mRNA is expressed in various tissues with the highest levels detected selectively in the brain, skeletal muscle, testis and kidney (33).

PLC-ε mRNA expression has been detected in various tissues, including brain, lung, and colon, with the highest expression detected in heart (34). Two splicing variants of PLC-ε have been reported, i.e., PLC-ε1a and PLC-ε1b. They have different sequences at the N-terminus that precedes the CDC25 domain. PLC-ε1a transcripts are expressed in various tissues except in peripheral blood leukocytes. PLC-ε1b mRNA has a limited expression in the placenta, lung and spleen. EST sequences of PLC-ε are dominant in connective tissues and brain.

Northern blot analysis of mouse tissues and EST sequences analysis using human UniGene database showed that PLC-ζ is only found in the testis (35). However, PLC-ζ transcripts are detected in spermatid cDNA but not in testis cDNA in the absence of spermatid, suggesting that the PLC-ζ expression within the testis is sperm-specific.

Two PLC-η isozymes, PLC-η1 and PLC-η2, were identified in human and mouse. The highest level of PLC-η1 mRNA is observed in the brain and kidney and smaller levels are detected in the lung, spleen, intestine, thymus and pancreas (36).

PLC-η2 mRNA is detected in the brain and intestine (37, 38).

Diverse splicing variants of PLCs

Most genes express a limited number of mRNA isoforms. But there are a number of genes that use alternative splicing to generate several numbers of isoforms. Alternative splicing variants have been previously reported for several of PLC isozymes including rat PLC-β1 (39), human PLC-β2 (40), rat PLC-β4 (41), rat PLC-δ4 (33), and human PLC-ε (42). Here, we analyzed and identified additional splicing variants for PLC isozyme using BLAST server in mouse species. Subsequently additional splicing variants for PLC isozyme were verified in exon-intron sequences from genomic sequences using UCSC Genome Bioinformatics web server (http://genome.ucsc.edu/cgi-bin/hgBlat).

Two splicing variants of the PLC-β1 isozyme derived from a single gene have been identified in human and rat that differ in their C-terminal sequences (39, 43). The mouse PLC-β1 gene possesses two alternative splicing variants. PLC-β1a contains putative NLS (nuclear localization sequences) and NES (nuclear export sequences) regions. Two variants of PLC-β isozyme differ in their cellular localization, suggesting that this region regulates the transit in and out of the nucleus (39). Two splicing variants of human PLC-β2 were reported; PLC-β2a and PLC-β2b. They differ in 15 amino acid residues at the C-terminal region as was shown in haematopoietic cells (21, 40). In the case of mouse PLC-β2 gene, we have found a splicing variant, which has truncated the entire C-terminal region. The alternative splicing variant of the mouse PLC-β3 gene showed

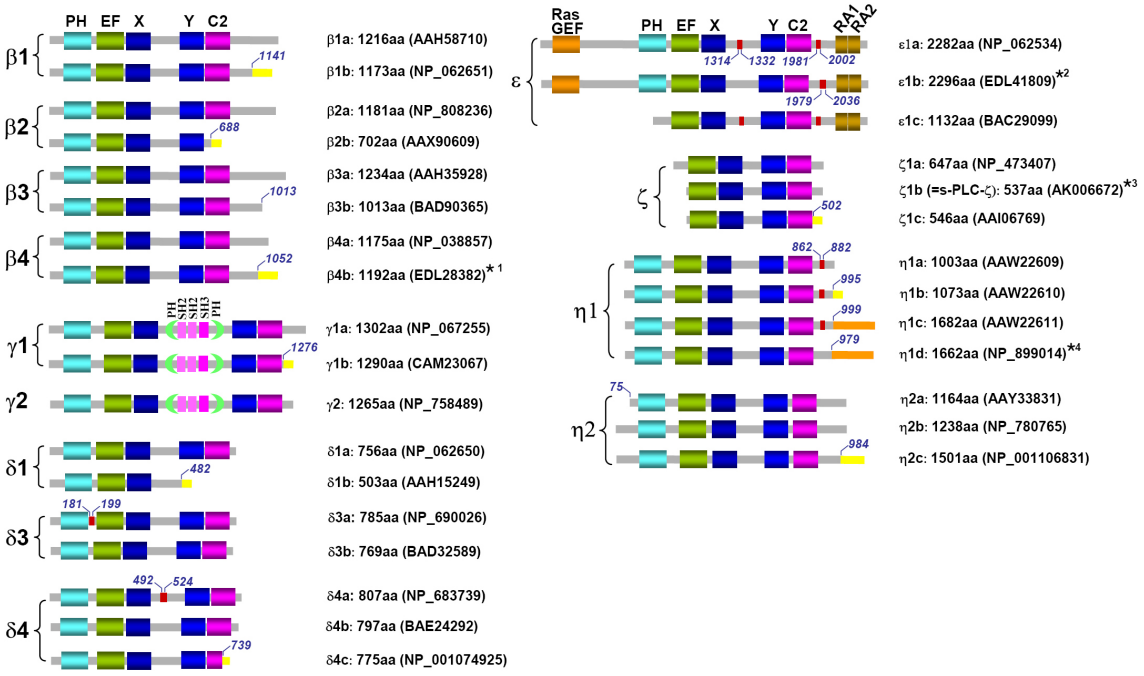

Fig. 2. Schematic diagram of alternative splicing variants for PLC isozymes in mouse. Schematic representing shows the PH domain, EF-hand motifs, catalytic X and Y domain, and C2 domain for the functional domains in all PLC isozymes. The total numbers of amino acid residues and GeneBank accession numbers for each splicing variant are written next to the diagram. The region of alternatively splicing variants for PLC isozyme, which differ in their amoino acid residues, is indicated by a square with yellow and organge color. Insertion of addtional exon is indicated by red color. The position of alternatively splicing residue and insertion of additional residue at each PLC isozyme are labelled with arabic numerals in blue color. All of PLC-β transcripts are found to have two alternative splicing variants, only differing in C-terminal sequences. Interestingly, in the mouse PLC-β2 gene, we found an alternative splicing variant form, containing the truncated C-terminal region. Like alternative splicing variants of PLC-β, two different transcripts in C-terminal regions of PLC-γ1 were identified in mouse. There are more than one additional transcripts found in PLC-δ isozyme in mouse. Surprisingly, mouse PLC-δ1b differs from PLC-δ1a by truncation of catalytic Y domain and C2 domain. Two and three alternative splicing variants of PLC-δ3 and -δ4 exist in mouse, respectively. Mouse PLC-ε isozyme has two novel splicing variants at different region. Mouse PLC-ε1c lacks Ras-GEF domain and partial PH domain at N-terminus. In sperm-specific PLC-ζ, deduced amino acid sequences at C-terminal region differ from that of PLC-ζ in mouse. We found a novel splicing variant of mouse PLC-η1, excluding 20 amino acid residues in C-terminal region (we designed it as PLC-η1d). In addition, we found the three different splicing variants of PLC-η2 in mouse. In PLC-γ isozymes, two SH2, SH3 and split PH domain are indicated by green, pink and purple color, respectively. Protein GeneBank accession number of alternative splicing variant for PLC is inserted in a bracket (*1: 28aa missing from methionine start site; *2: Nucleotide GeneBank accession number; *3: Exon1 and Exon2 missing; *4: 21aa missing from methionine start site).

that it contains the internal stop codon between 3039 and 3040 open reading frame in the wild PLC-β3 gene. Several alterative splicing variants of PLC-β4 have been reported. We found and identified two alternative splicing variants from rat and bovine brain (24, 41, 44). The third splicing variant of rat PLC-β4 has an additional 37 nucleotide exon at the C-terminal region (23). All PLC-β genes have two alternative splicing variants, which differ in their C-terminal sequences (Fig. 2).

Two alternative splicing variants of PLC-γ1 were found to differ in their C-terminal sequences. The distinct function of the two alternative splicing variants still remains unknown. Only one transcript of the PLC-γ2 gene was identified in the mouse (Fig. 2).

Alternative splicing variants of PLC-δ isozymes represent several different patterns of splicing variants unlike those of PLC-β isozymes. Mouse PLC-δ1b differs from PLC-δ1a by 274 amino acid residues that extend from the catalytic Y domain to the stop sequence, which are replaced with 21 distinct amino acid residues. Mouse PLC-δ1b has a truncated catalytic Y domain, which implies that this variant may have no enzymatic activity. PRIP-1 (PLC-related inactive protein-1) has a high sequence similarity to PLC-δ1 but has no PIP_2-hydrolyzing activity. PRIP-1 regulates IP_3-mediated Ca^{2+} signaling by binding of IP_3 (45). It would be interesting to investigate whether PLC-δ1b can also have such a regulatory function in PLC signaling. The second splicing variant of PLC-δ3 demonstrates the deletion of 51 nucleotides between the PH domain and EF-hand motifs. There are three alternative splicing variants of mouse PLC-δ4 (Fig. 2). PLC-δ4a contains additional 32 amino acid residues in the linker region between the catalytic X and Y domains. The earlier study reported that all of the above three splice variants have the catalytic X domain and/or the linker region for X and Y domains (46). We found an additional splicing variant of PLC-δ4b that is different from the earlier reported PLC-δ4 isoforms at the C-terminal region.

Two splicing variants of human PLC-ε with a different N-terminal region have been reported (42), but mouse PLC-ε isozyme was found to have two novel splicing variants in a different region. Surprisingly, mouse PLC-ε1c lacks the Ras-GEF domain and partial PH domain at the N-terminus. Additionally, we searched the mouse EST database and identified one EST clone (EST clone; CJ197436) that included partial sequences for N-terminus of PLC-ε1c.

s-PLC-ζ, an alternative splicing variant for PLC-ζ has been reported (47). It contains two internal stop codons at the N-terminus and lacks one and a half of EF-hand motifs. It was reported that this splicing variant of PLC-ζ does not affect Ca^{2+} oscillations. Moreover, we found an additional splicing variant of PLC-ζ, which has the C-terminal sequence different from that of s-PLC-ζ.

Three splicing variants of PLC-η1 have been reported in human and mouse (36). We found a novel splicing variant of mouse PLC-η1. It excludes 20 amino acid residues in the C-terminal region (designated as PLC-η1d; Fig. 2). Five alternative splicing variants of PLC-η2 exist in human (37), and three splicing variants of PLC-η2 have been found in mouse. PLC-η2b and PLC-η2c contain additional 74 amino acid residues at N-terminal region. Moreover, splicing variant of PLC-η2c differ from the other PLC-η2 subtypes at the C-terminal region.

Molecular function and regulation of PLCs

PLC-β isozymes

Regulation of PLC-β subtype activity: PLC-β is a member of a large family of PLC enzymes and includes PLC-β1∼4. PLC-β isozymes share many of the structural features present in other members of the PLC family, including conserved catalytic X and Y domains as well as two membrane-phospholipid binding regions, the PH and C2 domains. PLC-β isozymes, however, are distinguished by the presence of an elongated C-terminus consisting of about ∼450 residues, which contains many of the determinants for interaction with Gq as well as for other functions such as membrane binding and nuclear localization (3, 48-50).

Four PLC-β isotypes and additional splicing variants have been identified in mammals. These isoforms are regulated by heterotrimeric GTP-binding proteins, and have a high GTPase stimulating (GAP) activity. Mammalian PLC-β isozymes are differentially distributed in tissues, with the PLC-β1 being most widely expressed, especially in specific regions of the brain. PLC-β1 exists as alternative splicing variants PLC-β1a and -β1b, which differ in their C-terminal residues (39). The primary transcript of PLC-β4 gene is also alternatively spliced in the region corresponding to the C-terminal region of the protein downstream of the C2 domain (41). In the cytoplasm, PLC-β functions as effector enzymes for receptors belonging to the rhodopsin superfamily of transmembrane proteins that contain seven transmembrane spanning segments. They are activated by a variety of stimuli and require special combinations of Gα and Gβ/γ subunits to couple to the effector (3). Activation could be also induced by phosphatidic acid (PA) in that recent studies suggest a novel role for PA in the regulation of GPCR signal transduction that is mediated through PLC β1 homodimerization (51). With the exception of PLC-β4, PLC-β isozymes are also activated by Gβγ dimmers (52-55). The relative sensitivity of PLC-β isozymes to Gβγ subunits differs from that to Gqα subunits; PLC-β1 is the least sensitive to Gβγ (52, 53). Direct binding measurements indicate that, although the Gβγ dimer interacts with PLC-β1, -β2, and -β3, it exhibits a high affinity only for PLC-β2 (56).

It has been shown that a variety of physiological stimuli that activate PLC-β isozymes in normal platelets were not able to activate these isozymes in platelets derived from αq knockout mice; αq is the only member of the Gqα subfamily expressed in normal platelets (57). Thus, Gαq appears essential for PLC-β activation and cannot be replaced by Gβγ in this regard. The region of PLC β that interacts with Gqα subunits differs from

that responsible for interaction with Gβγ. Thus, C-terminal truncation of PLC-β2 generated enzymes that were activated by Gβγ but not by Gαq (58). The PH domain of PLC-β2 exhibits high affinity for Gβγ subunits bound to membranes (59). Experiments with antisense oligonucleotides directed to mRNAs encoding various G protein subunits suggested that the m1 muscarinic acetylcholine receptor interacts only with G protein complexes composed of the subunits αq, α11, β1, β4, and γ 4 in order to activate PLC in RBL-2H3 cells, despite the fact that the subunits α14, β2, β3, γ2, γ3, γ5, and γ7 are also expressed in these cells (60). G protein–initiated signaling is turned off by hydrolysis of the GTP bound to the Gα subunit, a reaction catalyzed by the intrinsic GTPase activity of the α subunit itself, and the subsequent reassociation of the GDP-bound α subunit with the βγ subunits. This deactivation process was studied in detail by reconstituting the m1 muscarinic acetylcholine receptor, G protein, and PLC-β1 in lipid vesicles (61).

PLC-β1 isozyme present the nucleus is located in nuclear speckles: PLC signaling occurs not only at the plasma membrane but also in the nucleus (62, 63). PLC-β1 is the major PLC isozyme in the nucleus of various cells (64, 65), and the C-terminal region of the protein is required for nuclear localization, even though also PLC-β2, -β3 and -β4 have been shown to be in this organelle (66).

Where is located PLC-β1 located in the nucleus? PLC-β1 has been reported to localize to nuclear speckles, together with PIP kinases, PIP_2, diacylglycerol kinase θ (DGK θ), PLC-δ 4, PI 3-Kinase C2α, phosphatase and tension homologue deleted on chromosome 10 (PTEN) and SH2-domain containing inositol phosphatase 2 (SHIP2) (67-69). It has been demonstrated an association between PLC-β1 and both DGK ζ and PIP Kinase α, in immunoprecipitation experiments with a PLC-β1 specific antibody. With immuno-electron microscopy we also showed that DGK θ, PLC-β1 and PIP_2 are associated to electron-dense particles within the nucleus, that correspond to nuclear speckles as revealed by using the antibody against SC-35 (70).

PLC-β1 in hematological malignancies: The involvement of PLC-β1 in hematopoietic differentiation, i.e. affecting CD24 expression (71) prompted us to investigate the role of this signaling molecule in hematological malignancies, focusing particularly on patients affected by myelodysplastic syndromes (MDS) at higher risk of evolution into acute myeloid leukemia (AML). By using fluorescence *in situ* hybridization (FISH) analysis, our group demonstrated, in a small number of high-risk MDS patients (72), that subjects bearing a mono-allelic cryptic deletion of the PLC-β1 gene had a worse clinical outcome, as compared with patients having both alleles. In a subsequent study, it has also been shown that the expression profile of both PLC-β1a and PLC-β1b mRNAs, which are the two alternative splicing subtype of PLC-β1, is altered in high-risk MDS, as compared to healthy donors. Interestingly, MDS cells always expressed higher levels of PLC-β1b mRNA as compared to PLC-β1a mRNA; this difference may reflect a specific role of PLC-β1 in MDS, given that the PLC-β1a splicing subtype demonstrates both nuclear and cytoplasmatic localization, while PLC-β1b splicing subtype is localized only in the nucleus. Furthermore, our recent studies demonstrated that not only is Akt specifically phosphorylated in high-risk MDS patients, but also mTOR and its downstream targets are activated (73), affecting cell survival and proliferation of MDS cells.

PLC-β isozyme expression and the epigenetic effect of DNA methyltransferase inhibition: Interestingly, it has been shown that a DNA methyltransferase inhibitor currently approved for the treatment of MDS and under experimental evaluation for other hematological malignancies, i.e. azacitidine, also affects PLC-β1 expression (74). Besides showing response rates of 50–80% in MDS, azacitidine has been reported to have a significant impact on the quality of life and progression into AML (75). Nevertheless, the molecular mechanisms underlying this drug are not completely understood. Low-dose regimens with azacitidine have been assumed to act by reversing the epigenetic silencing of target genes involved in the control of cell growth and differentiation. However, although demethylation of a hypermethylated p15/ INK4B gene has been demonstrated in MDS patients treated with demethylating therapy (76), this observation does not necessarily mean that this is the main mechanism of action of the drug *in vivo*. Moreover, since p15 is unlikely to be the only hypermethylated and silenced gene in MDS, it was important to examine the presence of other targets for demethylating treatment. We have shown that in patients diagnosed with MDS who was treated with azacitidine a correlation of PLC-β1 expression with an almost complete remission (77). In particular a patient after reaching a partial hematologic remission, following the International Working Group (IWG) response criteria (78) and temporarily decreased hematological response, subsequently restored, finally reached a complete remission. During the therapy, the patient demonstrated an increase in PLC-β1 expression, as well as a down-regulation in the level of activated Akt, postulating an association between PLC β1 expression and azacitidine effectiveness. All in all these observations hint at the likelihood that nuclear PLC-β1 is a good candidate for both MDS prognosis and epigenetic effect of antileukemic drugs. Indeed the fact that PLC-β1 could at the nucleus in a peculiar way, different from that at the plasma membrane, i.e. as a check point for the G1 phase of the cell cycle, is strengthened by the results showing that nuclear PLC-β1 activity is switched on and off by phosphorylative events and targets cyclin D3 during both cell growth and differentiation (79-81).

Functions of PLC-β isozyme in mice: PLC-β1 null mice showed the epileptic seizures and this led to the sudden death of the mice. This result suggests that PLC-β1 is essential for the normal functioning of inhibitory neuronal circuitry (82). Chemoattractant-mediated PLC activity was impaired in the neutrophills iso-

lated from PLC-β2-deficient mice. This led to the decreased intracellular calcium release, superoxide production and cell surface expression of MAC-1 (83). PLC-β3-null mice demonstrated increased sensitivity to morphine compared with the wild type mice, which indicates that PLC-β3 is a significant negative modulator for opioid response (84). The mice lacking PLC-β4 showed the ataxia and impaired visual processing, which are results of impaired PLC-linked signal transduction (82, 85).

PLC-γ isozymes

Regulation of PLC-γ isozyme activity: Two mammalian PLCγ forms (PLC-γ1 and PLC-γ2) have been identified. As described in previous sections, these enzymes are characterised by the insertion of a highly structured region (PLC-γ-specific array, γSA) comprising a split PH domain flanking two tandem SH2 domains and SH3 domain between the two halves of the TIM-barrel catalytic domain (86, 87). This region has been implicated in activation of PLC-γ isozymes downstream of receptors with intrinsic or associated tyrosine kinase activity. Although not fully understood, regulatory mechanisms are best characterised for PLC-γ1 activated in response to polypeptide growth factors that bind to receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (88, 89). The SH2 domains of PLC-γ1 mediate binding to autophosphorylated tyrosine residues within the intracellular region of the receptor; this association is important for both membrane recruitment and tyrosine phosphorylation of PLC-γ1. Growth factor stimulation, such as stimulation by PDGF, induces PLC-γ1 phosphorylation at three tyrosine residues: Tyr^{771} and Tyr^{783} within the γ-specific region and Tyr^{1254} within the C-terminal tail; however, a recent study has shown that only phosphorylation at Tyr^{783} is essential for lipase activation (90). This has been attributed to the intra-molecular binding of phosphorylated Tyr^{783} to the C-terminal SH2 domain and further speculated that this interaction induces a conformational change required for activation (91).

PLC-γ1 and PLC-γ2 can also be activated downstream of a variety of receptors that lack intrinsic tyrosine kinase activity. For PLC-γ1 this has been reported for receptors including GPCRs such as the angiotensin II and bradykinin receptors, cytokine receptors and immunoreceptors such as the T cell receptor (92-95). PLC-γ2 is activated downstream of immunoglobulin and adhesion receptors on immune cells such as B cells, platelets and mast cells (96-98). In these instances, PLC-γ isozymes are phosphorylated by non-receptor tyrosine kinases in the context of membrane-localised multimolecular signalling complexes. These assemblies have been well characterised in the context of antigen receptor signalling on B and T cells and include kinases of the Src, Syk and Tec families in addition to adaptor molecules such as BLNK and LAT that serve to recruit the different components to the complex (99). In tumour cells and some non-neoplastic cells, PLC-γ1 interacts with another important adaptor protein GIT1 (GPCR kinase-interacting protein-1) (100-102). This interaction facilitates Src kinase-mediated PLCγ1 activation downstream of GPCRs, RTKs and integrins and thereby could integrate signal transduction inputs emanating from these different classes of receptor (100-102).

The multi-domain structure of PLC-γ1 permits interactions with a whole host of additional binding partners. The SH2 domains, SH3 domain and split PH domain have all been implicated in various protein/protein interactions. Some of these interactions, including EF-1α and the inositol 5′-phosphatase, SHIP1, enhance PLC-γ1's activity whereas others such as Cbl and Grb2 serve as negative regulators of the enzyme (103-106). Localization and activation of PLC-γ isozymes at the cell periphery are also governed by direct interactions with F-actin and are therefore subject to changes in the architecture and functioning of the cytoskeleton (107-110).

Function of PLC-γ isozyme in mice: The expression patterns of PLC-γ1 and PLC-γ2 show some degree of overlap; however, even in regions of co-expression, the two isoforms appear to perform independent, non-redundant functions (111). PLC-γ2 is most highly expressed in cells of the haematopoietic system and plays a key role in regulation of the immune response (19, 112). Consistent with this, PLC-γ2 null mice display defects in the functioning of B cells, platelets, mast cells and natural killer cells (29, 30). Loss of PLC-γ2 signalling in human B cells underlies the immunodeficiency syndrome X-linked agammaglobulinaemia (113). Furthermore, a point mutation in the murine PLC-γ2 gene has been linked to inflammatory and autoimmune responses through PLC-γ2 hyperactivation in cells of both the innate and acquired immune system (114).

PLC-γ1, in contrast, is ubiquitously expressed and appears to regulate a multitude of cellular functions in many tissues. PLC-γ1 null mice die by embryonic day 9 highlighting the widespread importance of this enzyme (115). Although PLC-γ1 is classically activated in response to growth factor stimulation, its role in the regulation of cell proliferation remains controversial. Neutralising antibodies and dominant negative PLC-γ1 fragments have been used to demonstrate a role for PLC-γ1 in mitogenic signalling (116, 117). However, observations made using signalling-restricted receptors and PLC-γ1 null fibroblasts support the idea that PLC-γ1 is not required for growth factor-stimulated proliferation (28, 116-118). More definitive roles for PLC-γ1 have been established in the regulation of cellular differentiation and survival programmes (119-121). Recently, several studies implicated PLC-γ1 as a determinant of the directionality of cell movement towards EGF and proposed that it can couple to the actin polymerisation machinery via multiple routes including regulation of coffilin and Rac/Cdc42 GTPases (122). Furthermore, *in vivo* data where PLCγ activity is compromised support on important role for PLC-γ1 in cancer metastasis (123, 124).

Brain function in PLC-γ isozyme: PLC-γ1 also contributes to the functioning of specific cell types. Within the immune system, PLC-γ1 regulates the activation of T cells and mast cells there-

by complementing the role played by PLC-γ2 (125-127). In the brain, PLC-γ1 is activated in response to Nyk, fibroblast growth factor (FGF), and Trk family RTKs and has been implicated in the regulation of neuronal differentiation and neurite outgrowth (128, 129). There is also evidence to suggest that PLC-γ1 forms an integral part of neural networks that underlie disparate features of brain function such as emotion, memory and motor activity (130-132). Consistent with the involvement of PLC-γ1 in the regulation of mood, polymorphisms in the human PLC-γ1 gene have been linked to the pathogenesis of bipolar disorder (133).

Some of the roles of PLC-γ1 in the brain may be linked to regulation of ion channel function, as many ion channels are acutely sensitive to the levels of membrane PIP_2 (134). PLC-mediated hydrolysis of PIP_2 underlies the rapid suppression of multiple potassium channel currents (135-137). PLC-γ1 has also been shown to regulate agonist-induced calcium entry through plasma membrane channels of the canonical TRP (transient receptor potential) family (138, 139); this effect is independent of PLC-γ1-mediated calcium release from intracellular stores. The second messenger DAG can directly activate several TRPC channels (140, 141); however, studies have shown that this function is independent of PLC-γ1's lipase activity and relies on the direct binding of PLC-γ enzymes and TRPC3 (139). A model has been proposed whereby the C-terminal half of PLC-γ1's split PH domain associates with a region on TRPC3 to form a lipid-binding intermolecular PH domain although subsequent structural data suggest that the split PH domain of PLC-γ1 folds together to form an intact internal PH domain (16, 142).

PLC-γ isozyme is involved in cellular proliferation: Several additional studies have reported on phospholipase-independent functions of PLC-γ1. Early studies focusing on the role of PLC-γ1 in proliferation demonstrated that lipase activity was dispensable for induction of DNA synthesis in quiescent fibroblasts and attributed the mitogenic properties of the catalytic mutant to the SH3 domain (143, 144). Binding sites for many PLC-γ1 interaction partners have been mapped to the SH3 domain (145, 146); in particular, SH3 domain binding of the Ras exchange factor, SOS1, enhances Ras activation providing a potential link to cell cycle regulation (147). More recently, it has been reported that the SH3 domain of PLC-γ1 can act as a guanine nucleotide exchange factor (GEF) for the brain-specific nuclear GTPase PIKE (PI3K enhancer) and dynamin-1 (148, 149). This novel activity of PLCγ1 may underlie several features of PLC-γ1 cellular function including regulation of proliferation and survival (150, 151).

One aspect of study of PLC-γ isozymes recently consolidated and further extended their regulatory and biological roles. In addition to growth factors and immune receptors, integrins have been shown to activate PLC-γ isozymes in specific cell types including osteoclasts, fibroblasts and T cells (152-155). Further, PLC-γ2 activation downstream of integrins in platelets has been implicated in the regulation of platelet adhesion and spreading (156-158) and several studies show that PLC-γ1 may also regulate cell attachment and morphology (152, 155). In keeping with the involvement of PLC-γ isozymes in influencing the dynamics of cell shape, PLC-γ1 plays a role in the regulation of cell migration not only in response to growth factors but also downstream of integrin engagement, as observed in several cell types including tumour cells, endothelial cells and fibroblasts (101, 159).

PLC-δ isozymes

Regulation of PLC-δ isozyme activity: PLC-δ is considered the most basic isozyme because its structure is very simple, comprising a PH domain, EF hand motif, X and Y domains, and C2 domain. In addition, comparison of DNA sequences suggests an evolutionary relation in which PLC-δ appeared in primitive eukaryotes. PLC-δ isozyme is composed of three isozyme, PLC-δ1, -δ3, and -δ4 (160), and similarities in amino acid of each domain between these isozymes are very high.

PLC is a basically soluble protein that is localized mainly in the cytosol, and it is translocated to the plasma membrane, where it functions to hydrolyze PIP_2, in response to cell activation. Thus, targeting of PLC to the plasma membrane is a critical event for generating signals. Lemmon *et al*. proposed the "tether and fix" theory, by which binding to plasma membrane and activation of PLC-δ1 were described (161). First, PLC-δ1 is tethered to the plasma membrane through interactions of PH domain and PIP_2. This is still inactive form. Additional other actions with the membrane mediated by the C2 domain and catalytic domains expose the active site. This fixed (active) form hydrolyzes PIP_2 efficiently. This process may be triggered by a small increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Although all PLC isozymes require calcium for activity, PLCδ-isozyme is one of the most sensitive to calcium, suggesting activity of PLCδ-isozyme is regulated by $[Ca^{2+}]_i$ (162, 163). It is also worth noting that Gα (h). (tissue transglutaminase II (TGII)) associates directly and activates PLC-δ1 *in vitro* (164). TGII has transglutaminase activity as well as GTPase activity. More recent report indicated that expression of TGII mutants lacking the interaction with α-adrenoreceptor (αAR) or PLC-δ1 reduced the increase in $[Ca^{2+}]_i$ (165), suggesting the involvement of PLC-δ1 in αAR signaling. Very interestingly, Rho GTPase-activating protein (RhoGAP), p122, was also reported to bind and activate PLC-δ 1 directly (166). Microinjection of the GTPase-activating domain of p122 suppressed the formation of stress fibers and focal adhesions induced by lysophosphatidic acid, suggesting a specific GTPase-activating activity of p122 for Rho (167). These results demonstrate that p122 synergistically functions as a RhoGAP and an activator of PLC-δ1 *in vivo* and induces cytoskeletal reorganization.

Functions of PLC-δ isozyme in mice: Loss of function in PLC-δ isotype gene-deficient mice has revealed the critical function

of each PLC isozyme *in vivo*. PLC-δ1 null mice showed marked hair loss associated with abnormal hair follicle structures and epidermal hyperplasia in interfollicle epidermis (168). Hyperthickened epidermis and increased dermal cellularity are often observed in mice with skin inflammation (169). In fact, expression of IL-1β and IL-6, pro-inflammatory cytokines, was increased significantly and infiltration of leukocytes such as macrophages, granulocytes, and T lymphocytes was observed in skin of PLC-δ1 null mice (170). These findings suggest that lack of PLC-δ1 results in induction of skin inflammation. Since these defects were clearly canceled by treatment of PLC-δ1 null mice with potent anti-inflammatory reagents, epidermal hyperplasia of PLC-δ1 null mice may be caused by skin inflammation.

The appearance of a PLC-δ1 null mouse is very similar to that of a nude mouse (171). Hematoxylin and eosin (HE)-staining of skin sections from PLC-δ1 null and nude mice revealed that the hair shafts of both mice are bent and fail to penetrate the epidermis. In nude mice, the gene encoding the transcription factor Foxn1 is spontaneously mutated (172), and this mutation leads to insufficient hair keratin mHa3 expression and abnormal hair shaft structures (173). Expression of mHa3 was decreased remarkably in the skin of PLC-δ1 null mice. In addition, exogenously expressed Foxn1 induced the expression of PLC-δ1, indicating that Foxn1 functions as an upstream molecule of PLC-δ1. Furthermore, *in situ* hybridization analysis revealed that PLC-δ1was abundantly expressed in hair follicles of control mice, whereas only faint expression of PLC-δ1 was observed in hair follicles of nude mice. These results indicate that Foxn1is the upstream regulator of PLC-δ1 expression and that PLC-δ1 may be involved in the pathway from Foxn1 to hair keratin mHa3 expression.

Although PLC-δ3 null mice show no abnormality so far, PLC-δ1/-δ3 double null mice resulted in embryonic lethality at E11.5-E13.5 caused by differential defect of placental development (174). PLC-δ1/-δ3 double null mice exhibited severe disruption of the normal labyrinth architecture in the placenta. The labyrinth layer of placenta contains a large number of maternal and embryonic vessels and is the exchange site of oxygen, nutrient, and waste between mother and embryo. Remarkably, the numbers of these vessels in the labyrinth layer of PLC-δ1/-δ3 double null mice placentas were severely reduced. Furthermore, PLC-δ1/-δ3 double null mice embryos supplied with a normal placenta by the tetraploid aggregation method (175) survived beyond E14.5 and the placenta of rescued PLC-δ1/-δ3 double null mice embryos contained many maternal and embryonic vessels, clearly showing that the embryonic lethality is caused by a defect in trophoblasts. These results indicate that PLC-δ1 and PLC-δ3 are essential in trophoblast for placental development.

PLC-δ4 null mice appeared healthy, however, PLC-δ4 null male mice either produced a few smaller litters or are sterile (176). *In vitro* fertilization studies showed that insemination with PLC-δ4 null mice sperm resulted in significantly fewer eggs becoming activated and that the calcium transients associated with fertilization are absent or delayed. These results suggest that PLC-δ4 in sperm plays an essential role in an early step of fertilization. Histochemical analysis of testes revealed that PLC-δ4 is concentrated in the anterior acrosomal region of sperm. Furthermore, PLC-δ4 null mice sperm were unable to initiate the acrosome reaction, an exocytotic event required for fertilization and induced by interaction with the egg coat, the zona pellucida (ZP). These data demonstrate that PLC-δ4 functions in the ZP-induced acrosome reaction during mammalian fertilization. $[Ca^{2+}]_i$ has a primary role in the execution of the acrosome reaction (177, 178). Wild-type sperm treated with ZP exhibited a continuous $[Ca^{2+}]_i$ increase. In contrast, the addition of ZP induced a minor $[Ca^{2+}]_i$ increase in PLC-δ4 null mice sperm (179). These data indicate that the abnormal acrosome reaction induced by ZP in PLC-δ4 null mice's sperm is due to impaired intracellular $[Ca^{2+}]_i$ mobilization in these sperm and that PLC-δ4 plays a crucial role in the acrosome reaction during natural fertilization.

PLC-δ1 is identified as anti-oncogene: Recently PLC-δ1 was identified as anti-oncogene protein in human. Since chromosome 3p22 was frequently deleted in esophageal squamous cell carcinoma (ESCC), Fu *et al*. searched genes that is located in this region and found that PLC-δ1 is a strong candidate for tumor-suppressor gene (180). Absent expression of PLC-δ1 was frequently detected in both primary ESCCs and ESCC cell lines, which was significantly associated with the promoter hypermethylation. They also reported introduction of PLC-δ1 into ESCC cells suppressed their tumorigenic ability assayed by colony formation in soft agar and tumor formation in nude mice. In addition, down-regulation of PLC-δ1 protein was significantly correlated with ESCC metastasis. Taken together with the observations of hyperprasia and enhanced proliferation in the skin of PLC-δ1 null mice, PLC-δ1 may play an important suppressive role in the development and progression of ESCC.

On the other hand, Yuan *et al*. isolated a new gene frequently deleted in liver cancer, *DLC-1*. Human DLC-1 shares high homology with rat p122 RhoGAP (181), a PLC-δ1-binding protein as described before. Since DLC-1 inhibited human cancer cell growth and the *in vivo* tumorigenicity in nude mice, it may possible that p122 functions as an anti-oncogene by synergic interaction of PLC-δ1 and bying modulating the Rho-mediated actin cytoskeleton.

Nuclear function of PLC-δ1: Yagisawa's group first reported that PLC-δ1 has both nuclear export and import sequences that contribute to its shuttling between the cytoplasm and nucleus (182). Although PLC-δ1 is generally found in the cytoplasm of quiescent cells, it localizes in nuclear structures in the G_1/S boundary of the cell cycle (183). Recently Rebecchi's group demonstrated that suppression of PLC-δ1 by siRNA increases the level of cyclin E, a key regulator of the G_1/S boundary, alters S-phase progression, and inhibits cell proliferation (184). In addition, transient expression of PLC-δ1 suppressed the ex-

pression of cyclin E at the G_1/S boundary. These results suggest that PLC-δ1 plays a role in cell cycle. Taken together with the report that nuclear PIP_2 level was increased to several folds at the G_1/S boundary and at least doubled at G_0, respectively, the change in the PIP_2 level may regulate nuclear functions such as a chromatin remodeling mediated by mammalian SWI/SNF-like BAF complex (185).

PLC-δ1 is involved in osmotic response: To understand transductions of mechanical and osmotic stimuli, Ferrer-Montiel's group used an expression cloning approach to screen a human dorsal root ganglion cDNA library to look for proteins that respond to hypotonicity by raising the $[Ca^{2+}]_i$ (186). They identified GAP43, a membrane-anchored neuronal protein implicated in axonal growth and synaptic plasticity, as an osmosensory protein that induces $[Ca^{2+}]_i$ in response to hypotonicity. Interestingly, hypotonicity promoted the selective association of GAP43 with PLC-δ1, and concomitant increase in IP_3 formation. These findings indicate that PLC-δ1 is involved in the pathway from hypo-osmotic activation of GAP43 to Ca^{2+} increase. Specific extracellular stimuli or receptors that couple to PLC-δ1 have not been well clarified yet, though, these results suggest that the most primitive and evolutionary conserved type of PLC-δ plays a role in the most fundamental situation.

PLC-ε isozyme

Regulation of GPCR-mediated PLC-ε activity: PLC-ε was first identified in *C. elegans* as a novel effector molecule of LET-60, nematode Ras (187). Three independent research groups have identified the mammalian PLC-ε in 2001 (34, 188, 189). It is the largest PLC isozyme identified up to date that has two domains which are not found in other PLC isozymes. RA domains are located at the C-terminus of PLC-ε and mediates GTP-dependent interaction with Ras family small G-proteins such as Ras or Rap (189, 190). CDC25 homology domain is located at the N-terminus of PLC-ε and functions as a guanine nucleotide exchange factor (GFF) for Rap1A, one of Ras family small G-proteins (191). These structural features suggest that PLC-ε has a role in interplay between the Ras-mediated and PLC- dependent signaling pathways.

Various ligands can lead to activation of PLC-ε. Several ligands of $G\alpha_s$-coupled GPCRs such as adrenaline and PGE_1 activate PLC-ε (192, 193). Epac, a cyclic adenosine-3'5-monophosphate (cAMP)-dependent GEF for Rap GTPases, activates Rap2B upon GPCR stimulation. Activated Rap2B then associates with the RA2 domain of PLC-ε and induces PIP_2 hydrolysis. GPCR ligands coupled with $G\alpha_{12}$ and $G\alpha_{13}$ such as LPA, S1P and thrombin, can activate PLC-ε (194-196). Various RhoGEFs are activated by $G\alpha_{12}$ and $G\alpha_{13}$ and they induce the formation of GTP-loaded RhoA. Activated RhoA stimulates PLC-ε activity by direct binding to the specific region of the PLC-ε Y domain. Several GPCR ligands such as LPA, thrombin and endothelin can activate PLC-ε as well as PLC-β. The isozyme-specific function of PLC-β or PLC-ε in GPCR signaling remains to be revealed. However, Kelly *et al*. suggested that activation of these isoforms occurs in a temporally distinct manner whereby PLC-β3 is activated acutely and PLC-ε in a sustained manner (195).

Regulation of growth factor-mediated PLC-ε activity: PLC-ε is activated by stimulation with growth factors such as EGF and PDGF. Upon growth factor stimulation, activated Ras recruits PLC-ε into the plasma membrane through direct association with the RA2 domain (189). PLC-ε also translocates to the perinuclear area to associate with activated Rap1A. The CDC25 domain of PLC-ε functions as a GEF for Rap1A and helps persistent Rap1A-dependent PLC-ε activation (191). RA2 domain also mediates the interaction with ubiquitin E3 ligase Siah, which leads to growth factor-dependent degradation of PLC-ε (197).

The relationship between PLC-γ1 and PLC-ε in growth factor-dependent signaling remains to be revealed. Kataoka *et al* demonstrated that a PDGF receptor mutant deficient in PLC-γ1 docking site retains its ability to activate PLC-ε (198). However, Schmidt *et al*. suggested that PLC-γ1 can be an upstream regulator of PLC-ε and may exert the effect via activation of RasGRP3 and Rap2B (199).

Function of PLC-ε isozyme in mice: Null mice studies revealed that PLC-ε plays a role in heart development and functions. Kataoka *et al*. showed that the ablation of PLC-ε leads to the defects in the development of the aortic and pulmonary valves (200). On the other hand, Smrcka *et al*. reported that mice lacking PLC-ε showed decreased contractile responses to acute isoproterenol administration, which indicates that the loss of PLC-ε can sensitize the heart to the development of hypertrophy in response to chronic cardiac stress (201). PLC-ε is also important for the development of several other organs. Hinkes *et al*. reported that mutations in the catalytic region of PLC-ε resulted in the human renal pathology such as diffuse mesangial sclerosis or focal semental glomerulosclerosis (202). It is suggested that the absence of PLC-ε may block kidney development at the capillary loop stage leading to the renal failure. Epidermal morphogenesis of *C. elegans* requires PLC-ε and its knock-down or mutation caused embryonic lethality (203).

PLC-ε is involved in cell proliferation: Several reports showed that PLC-ε is involved in cell proliferation. Mice lacking PLC-ε activity are less susceptible to carcinogen induced tumor formation (204). It was suggested that PLC-ε-dependent potentiation of skin inflammation is responsible for the promoting tumor formation (205). In addition, it was evidenced that PLC-ε plays a role in agonist-dependent proliferation. PLC-ε induced PDGF-dependent cell growth in BaF3 cells overexpressing PDGF receptor (198) or EGF-dependent cell growth in HEK293 cells or MEF cells (197, 206). A recent report indicates that thrombin-dependent astrocyte proliferation is mediated by PLC-ε (207). In that report, GEF activity of PLC-ε was required for the Erk activation and cell astrocyte proliferation. In addi-

tion, PLC-ε promotes EGF-dependent cell growth by inhibition of EGF receptor downregulation (206). Taken together, PLC-ε seems to promote cell growth contributing to the normal developmental processes or tumor formation.

PLC-ζ and PLC-η isozymes

Regulation of PLC-ζ isozyme activity: PLC-ζ is identified as sperm-specific PLC that is first identified from EST sequences of mouse and human testis. The molecular weight of PLC-ζ is about 70 kDa and is the smallest in size among other mammalian PLC isozymes (35). Interestingly, injection of the recombinant PLC-ζ protein generated Ca^{2+} oscillation in the egg, caused by IP_3 production in fertilizated egg. Primary sequences of PLC-ζ showed that it consists of the EF-hand domain, catalytic X and Y domains, and the C2 domain. Comparative sequence analysis revealed that the catalytic X and Y domains bear about 64% of sequential amino acid similarity between PLC-ζ and PLC-δ1 (208). Also, five catalytic residues - His^{311}, Glu^{341}, Asp^{343}, His^{356}, and Glu^{390} at the catalytic X domain, are highly conserved between PLC-ζ and PLC-δ1, indicating that catalytic activation of PLC-ζ is similar to that of PLC-δ1. The deletion of the EF-hand domain of PLC-ζ abolished catalytic activity *in vitro*, suggesting that the EF-hand motif plays an important role in maintaining PLC activity (209).

Unlike other PLC isozymes, PLC-ζ lacks the PH domain, so it is not clear how PLC-ζ can target its membrane-bound substrate, such as PIP_2. It may be possible through the C2 domain. Deletion of the C2 domain from PLC-ζ reduced Ca^{2+} sensitivity only slightly, whereas PLC-ζ without the C2 domain does not cause Ca^{2+} oscillation in mouse egg. This indicates that the C2 domain of PLC-ζ is essential for its function but dispensable for Ca^{2+} ion binding (210).

Nuclear function of sperm-specific PLC-ζ isozyme: Originally, PLC-ζ protein has been discovered in soluble sperm extracts from mouse, hamsters and pigs. An earlier study showed that PLC-ζ protein was detected in both the soluble cytosolic fractions of sperm extracts, as well as in the pellet of extracts that contained the sperm heads (35). When PLC-ζ is injected into an embryo at a later stage of development, it continues to undergo nuclear sequestration. It suggests that in the case of PLC-ζ nuclear localization is required for egg activation and development. Indeed, PLC-ζ contains a nuclear localization signal, which promotes its accumulation in the pronuclei that form at the completion of meiosis II (208).

Regulation of PLC-η isozyme activity: Recently, we and other groups have independently identified two PLC-η isozymes called PLC-η1 and PLC-η2, in human and mice (36-38). Like other PLC isozymes, primary sequences of PLC-η isozymes showed that the PH domain, four EF-hand motifs, catalytic X and Y domains, and the C2 domain. Primary sequences of PLC-η isozymes contain a long C-terminal region. However, BLAST analysis of the C-terminal extended region of PLC-η1 and PLC-η2 revealed no homology with any other protein. Most PLC isozymes exist in the cytosol and translocate to the plasma membrane upon agonist-induced stimulation (128, 211, 212). Surprisingly, extrinsic expression of PLC-η2 led to its predominant localization in the plasma membrane without extracelluar stimuli (38). When PH domain was deleted from PLC-η2, PLC- η2 was localized mainly in cytosol, but it was not detected in plasma membrane. This may suggest that the PH domain of PLC-η isozymes is essential for membrane localization. Both PLC-η1 and PLC-η2 isozymes exhibited Ca^{2+}-dependent PIP_2 hydrolysis *in vitro* (36, 38). Co-expression of PLC-η2 with Gβγ resulted in enhanced PLC activity. We investigated whether PLC-η isozyme is activated by GPCR stimulation. We demonstrated that LPA- or PACAP can induce intracellular Ca^{2+} release in a dose-dependent manner in Neuro2A cells. However, LPA- or PACAP-induced Ca^{2+}release was dramatically reduced in PLC-η1 knock-down cell line (unpublished data), suggesting that PLC-η isozymes are activated through GPCR stimulation.

Both PLC-η isozymes are highly expressed in neuron-enriched regions in the brain, suggesting that PLC-η isozymes are particularly involved in the regulation of neural or neuroendocrine systems. However, physiological roles and cellular mechanisms of PLC-η isozymes remain to be studied.

Conclusion

Recent identification of novel PLC isozymes and various splicing variants can lead to a better understanding of complex signaling networks. Isozyme-specific coupling to receptors is mediated by intracellular regulators such as G-proteins and adaptor molecules including NHERF2, and Shank 2.

Multiple splicing variants of PLC isozymes seem to contribute to functional diversities. In fact, PLC-β1 splicing led to the differential subcellular localization and functions of each splicing variant. We found a new splicing variant of PLC-ε devoid of CDC25 domain and PLC-δ1 variant devoid of Y domain and C2 domain. PLC-ε CDC25 domain functions as a GEF for Rap1A and helps persistent activation of PLC-ε during growth factor-dependent activation process. The lack of this domain may lead to more transient activation of this splicing variant, which provides another functional diversity. The lack of catalytic Y domain of mouse PLC-δ1b suggests the possibility that this splicing variant may suppress PH domain-dependent activation of PLC-δ1a. Otherwise, PLC-δ1b may have a novel function similar to that of PRIP which regulates IP_3-dependent Ca^{2+}release. The splicing variant specific functions of PLC isozymes need to be investigated to fully understand PLC-dependent signaling pathway.

The functional diversity of PLC is provided by their emerging novel molecular events. The lipase-independent functions of PLC-isozymes have been intensively revealed. PLC-γ1 has GEF activity in its SH3 domain and regulates PIKE or dynamin activity. Also, growth hormone-induced signaling is attenuated

by the complex formation mediated by PLC-γ1 SH2 and SH3 domains. PLC-ε is a so-called dual enzyme since it has GEF activity along with its PIP_2 hydrolyzing activity. Knock-out animal studies revealed the various physiological functions of PLC isozymes. It is necessary to define more precisely the domain functions in the animal model in the future. Knock-in mice for PLC-γ1 tyrosine phosphorylation site mutants are currently being generated and this would unveil the function of PLC-γ1 tyrosine phosphorylation.

Much remains to be done in the investigation of the specific roles of newly identified members of PLC isozyme and their hierarchy in signal transduction networks. In addition, it is indispensable to consider splicing variant specific functions of PLC isozymes. Advanced genetic and proteomic technology would provide us with answers to unsolved questions in this exciting field.

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