Molecular Cloning, Splice Variants, Expression, and Purification of Phospholipase C-δ4*

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Complementary DNAs encoding a previously unidentified phosphoinositide-specific phospholipase C (PLC) isozyme were cloned from a rat brain cDNA library by the polymerase chain reaction with degenerate oligonucleotide primers based on sequences common to three known δ-type PLC isozymes. The encoded polypeptide contains 772 amino acids (calculated molecular mass, 88,966 daltons) and is similar in primary structure to δ-type PLC isozymes, with overall sequence identities of 45% to PLC-δ1, 72% to PLC-δ2, and 47% to PLC-δ3. Thus, the new PLC isozyme was named PLC-δ4. Recombinant PLC-δ4 was purified from extracts of HeLa cells that had been infected with vaccinia virus containing the corresponding cDNA. The purified protein exhibited an apparent molecular mass of 90 kDa on SDS-polyacrylamide gels. The specific activity of PLC-δ4 and its dependence on Ca^2+ were similar to those of PLC-δ1. The distribution of PLC-δ4 in 16 different rat tissues was studied by immunoblot analysis with PLC-δ4-specific antibodies of fractions obtained after an enzyme-enrichment procedure. The 90-kDa immunoreactive protein was detected unambiguously in only eight tissues and was present at concentrations that were low compared to those of other major PLC isozymes. A 93-kDa immunoreactive protein was also prominent in testis but was not detected in the other seven positive tissues. The 93-kDa enzyme appears to be derived from a splice variant of the mRNA that encodes the 90-kDa PLC-δ4 and contains an additional 32 amino acids between the X and Y catalytic domains. Splice variants have not previously been detected for δ-type PLC isozymes.

Phosphatidylinositol-specific phospholipase C (PLC)¹ plays an important role in receptor-mediated signal transduction by generating two second messenger molecules, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, from phosphatidylinositol 4,5-bisphosphate (PIP₂) (1). PLC actually comprises a diverse family of enzymes that differ in structure and tissue distribution (2–4). Nine mammalian enzymes, two Drosophila enzymes, one Dictyostelium enzyme, and one yeast enzyme have to date been characterized at the cDNA level; all are single polypeptides and can be divided into three types (β, γ, and δ) on the basis of size and amino acid sequence (3, 4). The β type includes four mammalian enzymes (PLC-β1, PLC-β2, PLC-β3, and PLC-β4) and two Drosophila enzymes (PLC-νorpA and PLC-p21), the γ type includes two mammalian enzymes (PLC-γ1 and PLC-γ2), and the δ type includes three mammalian enzymes (PLC-δ1, PLC-δ2, and PLC-δ3) and the Dictyostelium and yeast enzymes (3, 4).

The amino acid sequences of PLC isozymes are relatively variable with the exception of two well conserved regions, identified as the X (~170 amino acid residues) and Y (~260 residues) domains, that appear to constitute the catalytic site. The amino acid sequence similarity in the X and Y domains is ~60 and 40%, respectively, among the nine mammalian enzymes (3, 4); the similarity is greater when members of the same type of PLC are compared. The sequence between the X and Y domains is short (40–110 residues) in the β- and δ-type isozymes. However, in γ-type isozymes, this region is much longer (~400 residues) and contains two Src homology 2 (SH2) domains, which bind phosphorylated tyrosine-containing sequences in other proteins, and one SH3 domain, which interacts with proline-rich sequences in cytoskeletal proteins (5, 6). Furthermore, unlike γ- and δ-type enzymes, β type isozymes have a long carboxyl-terminal sequence (~450 residues) downstream of the Y domain.

All mammalian and Drosophila PLCs possess an amino-terminal region of ~300 residues that precedes the X domain and contains a pleckstrin homology (PH) domain. The PH domain is a loosely conserved protein module of ~100 amino acids and targets various proteins to the membrane surface by interacting with either the βγ subunits of G proteins or PIP₂ (7, 8).

The various PLC isoforms appear to be activated by different receptors through different mechanisms. Activation of γ-type enzymes is achieved by phosphorylation by autophosphorylated tyrosine kinases as a result of binding to these kinases via the SH2 domains. Isozymes of the β type are activated as a result of binding either to the α subunits of Gα class G proteins, via the long carboxyl-terminal region, or to Gβγ subunits, probably through the PH domain (4, 9). Regulation of δ-type enzymes is not yet understood. Despite the presence of PH domains in γ- and δ-type enzymes, there is no evidence that these isoforms are modulated by Gβγ subunits.

As part of our continuing effort to detect previously unidentified PLC isoforms and gather clues pointing to a regulatory mechanism for δ-type PLC enzymes, we screened a rat brain cDNA library by the polymerase chain reaction (PCR) with oligonucleotide primers based on the amino acid sequences conserved in the X and Y domains of δ-type isozymes. Here, we now describe the molecular cloning of a cDNA corresponding to a new δ-type PLC, named PLC-δ4.

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¹ The abbreviations used are: PLC, phospholipase C; PI, phosphatidylinositol; IP₃, inositol 1,4,5-trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; SH, Src homology; PH, pleckstrin homology; PCR, polymerase chain reaction; DTT, dithiothreitol; HPLC, high performance liquid chromatography; RT, reverse transcription; nt, nucleotide; kb, kilobase(s).
PCR Amplification—Alignment of the amino acid sequences of all known PLC isozymes reveals several regions of highly conserved sequences within the X and Y domains (2, 3). Three blocks of conserved amino acids, SSHNTLY (E/K/M)P(I/V/F)T/(Y/H)TG, and (I/V)LNL/IV/L/I(K/N/H/I)KL, in the X domain were chosen for the synthesis of forward oligonucleotide primers. Two reverse primers were synthesized on the basis of conserved sequences, G(W/L/T/Q)AIGAGT/TC(G/C)TT, in the Y domain. The sequences of the primers (named after the first two amino acid residues of the corresponding conserved sequence) were as follows: primer SS, CGGAATCT(C/T)IGCA(T/C)GACT(T/G); primer VA, CGGAATCT(C/T)IGCA(T/C)GACT(T/G); primer TL, CGGAATCT(C/T)IGCA(T/C)GACT(T/G); primer GW, TCGA(G/A)CCI(A/G)TI(A/G)T(T/C)TA(C/T)GG; primer IL, TCGA(G/A)CCI(A/G)TI(A/G)T(T/C)TA(C/T)GG; and primer VA, CGGAATCT(C/T)IGCA(T/C)GACT(T/G); primer VA, CGGAATCT(C/T)IGCA(T/C)GACT(T/G).

The underlined sequences at the 5’-end of each oligonucleotide indicated restriction enzyme cleavage sites (EcoRI and SphI sites for the forward and reverse primers, respectively) and two nucleotides (CG) were added to facilitate cloning of PCR products. Primers SS and VA were used to amplify a 2.7-kb insert that contained a full-length open reading frame for a PLC-like enzyme, a potential translational termination codon, and a poly(A) tail.

Expression and Purification of PLC-δ4—DNA encoding the entire rat PLC-δ4 sequence was subcloned into the pTM1 vaccinia virus expression vector (11), with the PLC-δ4 coding sequence downstream of the bacteriophage T7 promoter in the resulting PTM1-PLC-δ4 vector. Recombinant vaccinia viruses encoding PLC-δ4 were generated by transfecting PTM1-PLC-δ4 into CV-1 cells, which had been infected with wild type vaccinia virus (12), and were finally selected by propagation in human TK- cells.

For the purification of PLC-δ4, HeLa cells were grown at 37°C to a density of 1 × 10^9 cells/ml in DMEM supplemented with 5% horse serum, infected at a ratio of 10 virus particles per cell with the recombinant virus and a VTF-7-3 recombinant vaccinia virus that contained the bacterial T7 RNA polymerase gene, and harvested 2 days after infection (12). Cell pellets (80 ml) were washed three times with phosphate-buffered saline, suspended in 2 volumes of homogenization buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml)), and disrupted by sonication. The homogenate was centrifuged at 100,000 × g for 1 h, and the resulting supernatant (200 ml) was collected and divided into five 40-ml portions, each of which was applied to a preparative TSKgel DEAE-SPW HPLC column (21.5 × 150 mm) that had been equilibrated with 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 0.1 mM DTT. The columns were eluted at a flow rate of 5 ml/min with linear gradients of 0–0.3 M NaCl for 35 min and 0.3–1 M NaCl for 5 min. Fractions (5 ml) were collected and assayed for PLC as described (13). The fractions (49–51 min) corresponding to the peak of phosphatidylinositol-hydrolyzing activity from each column were pooled and concentrated. The subsequent three chromatography steps on a preparative TSKgel phenyl-SPW column, a TSKgel heparin-SPW column, and a Mono Q column were performed according to procedures similar to those previously described (13).

Preparation of Antibodies to PLC-δ4—Peptides corresponding to PLC-δ4 amino acid residues 454–464 (KDEGSDLDPAS) and residues 759–772 (VYTCMQEDLDMDEP) were synthesized and conjugated to keyhole limpet hemocyanin with glutaraldehyde. Antisera to the peptides were generated separately in rabbits, and specific antibodies were purified by immunoaffinity chromatography with purified PLC-δ4 protein.

**Tissue Distribution of PLC-δ4**—Frozen rat tissues that had been harvested in liquid nitrogen were purchased from Pel-Freeze BiologicaLs. Rats were previously hepatectomized Sprague-Dawley rats 48 h after removal of approximately two-thirds of the liver mass (14). Each tissue (5–10 g) was thawed in 50 ml of homogenization buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and calpain inhibitors I and II (each at 4 μg/ml)) and homogenized in a tissue homogenizer (15) with Teflon pestle (10 strokes). The homogenate was centrifuged at 1000 × g for 10 min. The supernatant was adjusted to 2 μl of heparin-Sepharose CL-6B (Phar- macia Biotech Inc.) column (20 ml of gel packed in a 1.5 × 15 cm Econo column) that had been equilibrated with 20 ml of Hepes (pH 7.0) containing 1 mM EGTA and 0.1 mM DTT. Bound proteins were eluted at a flow rate of 4 ml/min with the equilibration buffer containing 1.2 M NaCl. Fractions (16 ml) were collected and assayed for PLC activity. Essentially no detectable PLC activity eluted in six fractions, which were pooled and concentrated in a stirred ultrafiltration cell fitted with a YM 30 membrane (Amicon). After the final NaCl concentration was adjusted to 50 mM, the concentrate was centrifuged at 100,000 ×g for 10 min. Proteins (20 mg) from the supernatant were injected into a TSKgel heparin-SPW HPLC column (7.5 × 75 mm) and fractionated as described previously (13). Fraction (61–64 min) was collected and used for PLC activity. For immunoblot analysis, fractions were concentrated with Centricron-30 (Amicon), and one-fourth of the pooled peak fractions (61–64 min) from each tissue was resolved by SDS-polyacrylamide gel electrophoresis on a 7% gel. Proteins were transferred to nitrocellulose and incubated with purified antibodies to PLC-δ4, and immune complex was visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG.

**RT-PCR**—Sprague-Dawley rats were sacrificed by cervical dislocation, and various tissues were collected in liquid nitrogen. Total RNA was isolated
was isolated from these frozen tissues by the guanidinium isothiocyanate method, and poly(A)^+ RNA was purified from total RNA with Oligotex-dT (Qiagen). RT-PCR was carried out with a StrataScript kit (Stratagene). First-strand cDNAs were synthesized with 100 ng of poly(A)^+ RNA from each tissue and 300 ng of oligo(dT) primer in a 50-μl reverse transcriptase reaction mixture. The first-strand products were amplified with two sets of PLC-δ4-specific primers (first set, nucleotides (nt) 1108–1130 and 1640–1662; second set, nt 1305–1325 and 1516–1535 (nucleotides were numbered beginning with the first residue of the ATG initiation codon; those on the 5'-side of residue 1 are indicated by negative numbers)) by two consecutive rounds of PCR. The first amplification was performed for 30 cycles of 15 s at 94 °C, 15 s at 60 °C, and 30 s at 72 °C with 3 μl of the cDNA products and the first set of primers; the first extension step was increased to 5 min. The second-round amplification was achieved by 20 cycles with the second set of primers and 0.5 μl of the first-round PCR products as template. The final products were separated on a 1.8% agarose gel. The expected 230-bp PLC-δ4 cDNA product was obtained after amplification of the RNA samples by amplifying the first-strand products with rat β-actin primers (Clontech); 30 cycles of PCR were performed under the same conditions as described above, and a 764-bp fragment was amplified.

Cloning and Sequencing of Two Alternatively Spliced Forms of PLC-δ4—PCR of rat testis poly(A)^+ RNA yielded 330- and 270-bp bands in addition to the 230-bp band. These two additional fragments were subcloned directly into the pGEM-T plasmid vector (Promega). Restriction enzyme analysis and sequencing of these two fragments revealed that an additional 96 and 42 nt, respectively, were inserted immediately before the Y region of the PLC-δ4 open reading frame. To determine whether these two additional fragments were amplified from alternatively spliced forms of PLC-δ4 mRNA in testis, the entire coding region of PLC-δ4 cDNA was amplified with two sets of primers (first set, nt –115–95 and 2421–2444; second set, nt –58–35 and 2477–2497), all of which corresponded to the 5'- or 3'-untranslated regions of the cDNA. Two rounds of PCR were performed as described above but with a 1-min extension step. The second-round PCR products were digested with Stul and EcoRI for restriction enzyme analysis. PLC-δ4 cDNA contains only one site for each of these two restriction enzymes; thus, digestion of the second-round PCR products should yield three distinct fragments of 1493, 597, and 411 bp. The presence of additional two forms of PLC-δ4 cDNA in testis would be expected to result in the generation of additional 507- and 453-bp fragments.

RESULTS

PCR Amplification, Cloning, and Sequencing of PLC-δ4 cDNA—The existence of previously unidentified PLC-δ isozymes was investigated by subjecting a rat brain cDNA library to three sequential PCR amplifications. Five blocks of conserved amino acid sequences, three (SS, EP, and IL) from the X domain and two (GW and VA) from the Y domain, were chosen for the synthesis of degenerate oligonucleotide primers. The outermost pair of primers (SS and VA) was used for the first PCR amplification and was based on sequences common to all three types of PLC isozymes, whereas the other three primers (EP, IL, and GW), used for the second (EP and GW) and third (IL and GW) PCR amplifications, were specific to δ-type isoforms. The first- and second-round PCR products were treated with restriction enzymes known to cleave sequences corresponding to various PLC isozymes before serving as templates for the next round of PCR. The link between the X and Y domains of PLC isozymes varies both in length and sequence. Thus, whereas the presence of conserved residues in the X and Y domains identifies a PCR product as a member of the PLC family, a comparison of the linker sequence with known sequences further reveals whether it represents a newly discovered PLC-δ.

The third PCR amplification yielded a 426-bp fragment that revealed a PLC-δ-like sequence distinct from those of known PLC isozymes. Removal of the sequences corresponding to the X and Y domains yielded a 312-bp fragment that was used as a probe to screen the rat brain library. Two clones with a 2.7-kb insert were obtained. Complete sequencing of these two clones revealed an open reading frame of 2,316 bp flanked by a 142-bp 5'-untranslated region and a 255-bp 3'-untranslated region including a poly(A) tail. The translational initiation site (ATG) was assigned to the first methionine codon at nt 143 because of the presence of an in-frame stop codon upstream of this methionine and flanking sequences that fulfill the Kozak criteria for initiation (15). An in-frame translational termination codon (TGA) was present after codon 772. Therefore, we concluded that this new PLC contained 772 amino acids, with a calculated molecular mass of 88,966 daltons (Fig. 1A).

A comparison of the deduced amino acid sequence with known PLC sequences revealed that the predicted protein was similar in primary structure and overall structural organization to PLC-δ type isozymes, with overall sequence identities of 45, 72, and 47% to PLC-δ1, PLC-δ2, and PLC-δ3, respectively. Thus, the protein encoded by the cloned cDNA was named PLC-δ4 (Fig. 1B).

Purification and Characterization of PLC-δ4—To confirm that the isolated PLC-δ4 cDNA encodes a PLC enzyme, we subcloned the entire coding region of the cDNA into a vaccinia virus vector and expressed the encoded protein in HeLa cells. The expressed PLC-δ4 protein was purified by four successive column chromatographies (Fig. 2). The first chromatographic step, performed with a DEAE-HPLC column, separated PLC-δ4 from several endogenous PLC enzymes; immunoblot analysis with isozyme-specific antibodies revealed that the first activity peak (Fig. 2A) contained PLC-δ1, PLC-δ1, PLC-δ1, and PLC-δ3 from HeLa cells (data not shown), whereas PLC-δ4 was detected in the second peak. HeLa cells infected with a control vaccinia virus vector not containing the PLC-δ4 cDNA yielded the first activity peak but not the second. The marked retention of PLC-δ4 by the heparin column (third step) facilitated its purification (Fig. 2C). SDS-polyacrylamide gel electrophoresis analysis of the peak fractions pooled from the last step on a Mono Q column revealed an apparently homogeneous (>90%) preparation of a 90-kDa protein (Fig. 2D), in good agreement with the calculated molecular mass of PLC-δ4.

Because of the low level of expression, only 200 μg of purified PLC-δ4 could be obtained from 20 liters of cultured HeLa cells, despite good recovery yield (overall 25% relative to the DEAE column preparation). In contrast, ~1 mg of purified PLC-δ1 was obtained from 8 liters of cultured HeLa cells that had been transfected with the same virus vector harboring PLC-δ1 cDNA.2

The catalytic activities of PLC-δ1 and PLC-δ4 expressed in HeLa cells were measured with [3H]PtdInsP2 at various concentrations of free Ca2+; both enzymes exhibited similar specific activities and dependence on Ca2+ (data not shown).

Tissue Distribution of PLC-δ4 as Determined by Immunoblot Analysis—The concentration of PLC-δ4 in most rat tissues is low, and it was not possible to detect the isozyme in crude homogenates by immunoblot analysis. It was thus necessary to prepare samples enriched in PLC-δ4 before such analysis. Tissue homogenates were extracted with 2 M KCl to solubilize PLC isozymes associated with particulate fractions. After dialysis, the extracts were fractionated on a conventional heparin column (mainly to remove turbid material) and then on a heparin HPLC column. Under the experimental conditions described in Fig. 2C, PLC-δ4 bound tightly to the HPLC column and eluted at 62 min, whereas most other isozymes eluted earlier: γ2 at 32 min, γ1 at 37 min, δ1 at 42 min, β1 and β3 at 51 min, β4 at 54 min, δ2 at 66 min, and δ3 at 69 min (data not shown). The resulting PLC-δ4-enriched fractions were subjected to

2 S. B. Lee and S. G. Rhee, unpublished data.
Fig. 1. **Amino acid alignment of PLC-δ1, PLC-δ2, PLC-δ3, and PLC-δ4.** A, comparison of the amino acid sequence of PLC-δ4 deduced from a rat brain cDNA sequence with those of rat PLC-δ1 (16), bovine PLC-δ2 (17), and human PLC-δ3 (R.W. Kriz, D. Park, S.G. Rhee, and J. Knopf, unpublished results). Dashes represent identities with the PLC-δ4 sequence, and dots indicate gaps introduced into the sequences to optimize the alignment. Amino acid residue numbers are shown at the right of each line. The position where 32 and 14 amino acids are inserted in the putative splice variants, ALT-I and ALT-II, respectively, is shown by . Double underlines indicate amino acid sequences used for the production of anti-peptide antibodies (Ab-454 and Ab-759) specific to PLC-δ4. Demarcation of the X and Y domains is indicated.

**B** PLC-δ type

| Rat PLC-δ4 | 112 118 286 439 757 | 729 772 |
|------------|----------------------|--------|
| 84%        | 84%                  | 33%    | 76% | 33% |

| Bovine PLC-δ2 | 112 128 286 441 757 | 764 |
|---------------|----------------------|-----|
| 84%           | 84%                  | 33% | 76% | 33% |

| Human PLC-δ3 | 38% 128 286 439 757 | 729 779 |
|--------------|----------------------|--------|
| 38%          | 38%                  | 28%    | 55% | 28% |

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immunoblot analysis with affinity-purified monospecific antibodies to PLC-δ4 residues 454–464 (Ab-454) or 759–772 (Ab-759) (Fig. 3). The intensity of the 90-kDa band was strongest with testis and decreased in the order of brain > skeletal muscle > thyroid gland > stomach > thymus > aorta > heart. 

No band was detected unambiguously with normal or regenerating liver, kidney, prostate, adrenal gland, intestine, pancreas, or lung. In addition to the 90-kDa band, testis showed a strong band of 93 kDa and a weak band of 86 kDa. Only the 86-kDa band was detected in spleen.

By comparing the immunoblot intensities of the samples with those of purified PLC-δ4, the amount of PLC-δ4 in various tissues was measured quantitatively. The complex formed between PLC-δ4 and antibodies was visualized with 125I-labeled protein A, and the amount of radioactivity was determined with a PhosphorImager. From the amounts of protein loaded on the two heparin columns and the SDS gel, it was deduced that the amount of PLC-δ4 expressed in nanograms per milligram of protein in the KCl extracts was 7.2 for brain, 20 for testis (combining 90- and 93-kDa forms), 6.4 for skeletal muscle, and 5.6 for thyroid.

Tissue Distribution of PLC-δ4 mRNA as Determined by RTPCR—We also examined the distribution of PLC-δ4 mRNA in rat tissues by RTPCR with two pairs of PLC-δ4-specific primers. The relative intensities of the expected 230-bp band (Fig. 4A) match qualitatively those of the PLC-δ4 protein band on immunoblot analysis (Fig. 3) when the relative efficiency of PCR amplification is taken into account.

Alternatively Spliced Forms of PLC-δ4 mRNA—In addition to a 230-bp band of weak intensity, the RTPCR products from testis poly(A)+ RNA contained two larger (330 and 270 bp) bands of stronger intensity. These larger bands were also present in the products from thyroid gland, normal liver, prostate, intestine, and pancreas (Fig. 4A). Cloning and sequencing analysis of the two larger RTPCR products revealed 96- and 42-nt inserts positioned in-frame between nt 1461 and 1462 of the PLC-δ4 cDNA (Figs. 1 and 4). These inserts add 32 and 14 amino acid residues and would result in protein action mixtures derived from brain and skeletal muscle...
DISCUSSION

Our results indicate that PLC-δ4 is expressed at low concentrations in a limited number of rat tissues. The 90-kDa form was unambiguously detected by immunoblot analysis in only 8 of 16 tissues examined, even after enrichment, and the 93-kDa form was detected only in testis. Brain is one of the rat tissues relatively rich in PLC-δ4, but the enzyme concentration of 7.2 ng per milligram of crude extract protein is significantly lower than those of PLC-δ1 (70 ng/mg), PLC-γ1 (140 ng/mg), and PLC-δ3 (180 ng/mg). Faint RT-PCR bands derived from PLC-δ4 mRNA were visible in a greater number of tissues, probably because of the greater sensitivity of this procedure.

Regenerating liver was examined for PLC-δ4 expression because a PLC-δ-like enzyme that is not recognized by antibodies to either PLC-δ1 or PLC-δ2 was shown to be expressed specifically during rat liver regeneration (14) and because our immunoblot analysis showed that PLC-δ3 is not the PLC isozyme specific to regenerating liver. The present study suggests that PLC-δ4 also is not expressed in regenerating liver.

Like the other three δ-type PLCs, PLC-δ4 contains a PH domain sequence at the amino terminus. However, none of the four PLC-δ enzymes is significantly activated by Gβγ subunits. The PH domain of PLC-δ1 contains a 14-residue sequence (KVKSRRFERFYK) enriched in basic amino acids (net positive charge of 5) that was shown to form the core of the binding site for the negatively charged IP3 and PI(4,5)P2 and was
suggested to tether the enzyme to membrane surfaces containing PIP2 (18). The basic amino acid-rich sequence is well conserved in PLC-δ4 (KVRTKSWKKLRYFR); indeed, the δ4 sequence has a net positive charge of 7 and thus would be concentrated by solid lines above or below each schematic. Numbers above each schematic represent amino acid positions at boundaries of the splice-variant domains, and those on the right indicate total number of amino acids.

Two splice variants of PLC-δ4 mRNA, ALT-I and ALT-II mRNAs, were detected by PCR. ALT-I mRNA encodes the 93-kDa enzyme that contains an additional 32 amino acids located between the X and Y domains of PLC-δ4. It does not appear that ALT-II mRNA, which would produce an enzyme with an additional 14 amino acids in the same region between the X and Y domains, is translated in sufficient quantities to be detected by immunoblot analysis. PLC-δ4 is thus the first example of a δ-type PLC that exists in splice variants. Previously identified splice variants correspond to β- and γ-type PLC isozymes including rat PLC-β1 (19), bovine PLC-β2 (20), Drosophila PLC-p21 (21), and Drosophila PLC-norpA (22). Examination of the splicing differences in these PLC isozymes, including rat PLC-δ4, reveals that all occur outside of the X and Y domains (Fig. 6). Rat PLC-β1 and Drosophila PLC-p21 are alternatively spliced in the carboxy-terminal region following the Y domain, where the Gβγ-binding site is located (12, 23). Splicing differences in Drosophila PLC-norpA variants occur in the PH domain, to which Gβγ subunits and PIP2 might bind. It is thus possible that the differences among splice variants result in differences in the ability to interact with signaling components.

Splice variants of rat PLC-δ4, like those of bovine PLC-β4 (20), differ in the region separating the X and Y domains. This separating region of γ-type PLC isozymes plays important roles by interacting with various signaling components through its SH2, SH3, and PH domains. Whether the region linking the X and Y domains of β- and δ-type PLC enzymes also interacts with signaling molecules is not known. However, this region of all mammalian β- and δ-type isozymes is rich in acidic amino acids; 20 of 70, 26 of 76, 29 of 137, and 24 of 100 residues are acidic in β1, β2, β3, and δ4 isoforms, respectively, and 11 of 50, 19 of 53, 17 of 44, and 17 of 66 residues are acidic in δ1, δ2, δ3, and δ4 isoforms, respectively. Unlike the other three δ-type PLC isozymes, PLC-δ4 contains a high density (12 of 66 residues) of serine and threonine in the region separating the X and Y domains. All mammalian β-type isoforms with the exception of PLC-β4 also contain a high density of serine and threonine residues in this region. Furthermore, as with other splice variants (22), PLC-δ4 and ALT-I exhibit distinct tissue distributions. Together, these observations suggest that the 90- and 93-kDa PLC-δ4 enzymes might be regulated differentially.

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Molecular Cloning, Splice Variants, Expression, and Purification of Phospholipase C-4
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