Multiple Splice Variants of the Human Calcium-independent Phospholipase A₂ and Their Effect on Enzyme Activity*

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Recently, the cloning of a novel Ca²⁺-independent phospholipase A₂ (iPLA₂) from Chinese hamster ovary cells as well as from mouse and rat sources containing a C-terminal lipase motif and eight N-terminal ankyrin repeats has been described. In this report we describe the cloning of the human iPLA₂ cDNA and its expression in B-cells and show that the iPLA₂ gene undergoes extensive alternative splicing generating multiple isoforms that contribute to a novel mechanism to control iPLA₂ activity. The full-length cDNA clone encodes a 806-amino acid protein with a calculated molecular mass of 88 kDa. The protein contains a lipase motif, GXXSXXG, and ankyrin repeats, as described for the hamster and rodent forms of the enzyme but has an additional 54-amino acid proline-rich insertion in the last of the eight ankyrin repeats (residues 395–449). Furthermore, at least three additional isoforms most likely due to alternative splicing were identified. One that is present as a partial cDNA in the expressed sequence tag database is similar to iPLA₂ but terminates just after the lipase active site, and two other isoforms contain only the iPLA₂ ankyrin repeat sequence (ankyrin-iPLA₂-1 and -2). Ankyrin repeats are involved in protein-protein interactions and because the purified iPLA₂ enzyme exists as a multimeric complex of 270–350 kDa, the expression of just the ankyrin-iPLA₂ sequence suggested that these may also interact with the iPLA₂ oligomeric complexes and perhaps modulate PLA₂ activity. Transfection of the human iPLA₂ cDNA into COS cells resulted in a substantial increase in calcium-independent PLA₂ activity in cell lysate. No activity above background was observed following ankyrin-iPLA₂-1 cDNA transfection. However, co-transfection of the ankyrin-iPLA₂-1 and the iPLA₂ cDNAs resulted in a 2-fold reduction in activity compared with iPLA₂ alone. A similar co-transfection of ankyrin-iPLA₂-1 cDNA with the cPLA₂ cDNA had no effect on PLA₂ activity. These results suggest that the ankyrin-iPLA₂ sequence can function as a negative regulator of iPLA₂ activity and that the alternative splicing of the iPLA₂ gene can have a direct effect on the attenuation of enzyme activity.

Phospholipases A₂ (PLA₂) are a rapidly growing family of diverse enzymes that hydrolyze fatty acids at the sn-2 position of phospholipids (1, 2). PLA₂ enzymes can be subdivided into two classes, extracellular or intracellular, depending on the enzymes localization during catalysis (2). The intracellular PLA₂s can be further categorized into calcium-dependent, best exemplified by the cytosolic phospholipase A₂ (cPLA₂) (3), and calcium-independent forms (iPLA₂), which tend to be quite diverse and have until recently been less characterized at the molecular level. The calcium-independent PLA₂s have a wide tissue distribution (4) and have been purified from human myocardium (5), bovine brain (6), P388D₁, murine macrophages (7), and rabbit kidney (8). They all have distinct molecular masses, indicating the diversity of PLA₂s. Recently, an 85-kDa iPLA₂ was purified and cloned from CHO cells (9), and its sequence was found to be analogous to the 85-kDa iPLA₂ from P388D₁ cells (10) as well as the sequence for iPLA₂ from rat pancreatic islet (11). The amino acid sequence indicated the presence of eight ankyrin repeats and the GXXSXXG conserved catalytic sequence, as found in other lipases. Although there were apparent differences in ATP sensitivity among the enzymes, the biochemical, immunological, and sequence data indicate that these three enzymes are likely to be species variants of the same protein. In both P388D₁ cells and in rat pancreatic islets it is thought that iPLA₂ has a function in membrane phospholipid remodeling (12). It has been postulated that the rat iPLA₂ may be involved in arachidonic acid release leading to activation of β-cell ion channels (11).

Arachidonic acid is also the main precursor for important biological mediators such as leukotrienes (LT) (13). The oxygenation of arachidonic acid catalyzed by 5-lipoxygenase is the first step in the biosynthesis of leukotrienes and leads to the formation of LTA₄, which can be further metabolized to LTC₄ and LTD₄. In our ongoing studies of leukotriene synthesis and phospholipase activity in human B lymphocytes, we have demonstrated conversion of arachidonic acid to LTB₄ and expression of 5-lipoxygenase in human B lymphocytes (14, 15). Although cellular homogenates of B lymphocytes can release arachidonic acid from phospholipids in vitro, exogenous arachidonic acid is a prerequisite for leukotriene synthesis in intact cells (16). To elucidate the expression of PLA₂(s) in human B lymphocytes, we have examined in this report the expression of the different PLA₂s at the transcriptional level and describe the cDNA cloning of the human 85-kDa iPLA₂ and its various isoforms and their effect on enzyme activity.

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The abbreviations used are: PLA₂, phospholipase(s); A₂, cPLA₂; cytosolic PLA₂; iPLA₂, calcium-independent PLA₂; sPLA₂, secretory PLA₂; CHO, Chinese hamster ovary; LT, leukotriene; RT, reverse transcriptase; PCR, polymerase chain reaction; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; bp, base pair(s).
MATERIALS AND METHODS

Chemicals and Reagents—Chemicals were from Sigma or Aldrich. Cell culture medium and fetal bovine serum were from Life Technologies, Inc. Restriction enzymes and Taq DNA polymerase were from Boehringer Mannheim or Pharmacia Biotech Inc. The human B lymphoblastic cell line BL-41 E95A was kindly provided by Dr. Klein (Karolinska Institute), whereas the Raji cell line was obtained from ATCC. All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer.

Cell Culture Conditions—The B-cell lines BL-41 E95A (17) and Raji were cultivated in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and grown in humidified 5% CO2 at 37°C for 1 week before experimentation.

Figure 1. Alignment of CHO iPLA2 cDNA with DNA sequences of human EST clones 30643 and 46450. A comparison of the two EST sequences with the CHO iPLA2 cDNA indicated that these ESTs contained the 3′ end of the human iPLA2 cDNA sequence. Each contained about a third of the full-length iPLA2 sequence as judged from the size of the CHO sequence. However, at the 5′ end of these clones, EST 46450 aligned more closely to the CHO sequence than did EST 30643. Identical sequence is boxed, and the termination codon for the CHO cDNA is double underlined. The human iPLA2 PCR primers 1 and 2, which were based on the EST 46450 sequence, are underlined, and the polyadenylation signal is shown with asterisks. The dashes indicate the presence of gaps in the sequence.
fied atmosphere with 5% CO₂ (16). The cultures were seeded at a cell density of 0.2 x 10⁶ cells/ml and harvested at approximately 1 x 10⁶ cells/ml.

RT-PCR Analysis of B-cell PLA₂ Expression—Total cellular RNA was isolated from 10–20 x 10⁶ cells of the B-cell lines described above using Trizol-Ready (Life Technologies, Inc.) according to the instructions of the manufacturer. Reverse transcription of total RNA (2 μg) was performed using Expand Reverse transcriptase (Boehringer Mannheim) and priming with random hexamers or oligo(dT). The reverse transcription reaction was carried out at 42 °C for 60 min and then terminated by heating at 65 °C for 5 min. PCR was performed in a total volume of 50 μl containing 2 μl of the reverse transcription reaction mixture, 0.2 mM deNTPs, 0.5 μM of each primer, and 2 units of Taq polymerase in PCR buffer (50 mM KCl, 1.5 mM MgCl₂, and 10 μM Tris-HCl, pH 9.0) (Pharmacia). The conditions for PCR reactions, if not indicated otherwise were: an initial denaturation step at 94 °C for 5 min, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. Control reactions including minus reverse transcriptase and amplification of a glyceraldehyde 3-phosphate dehydrogenase fragment were also performed. Primers used for PCR were: cPLA₂ (sense, 5'-CGATGATACCAAAAATGATT-3'); cPLA₂ (antisense, 5'-TGCTAGCTGAGAGGATGTG-3'); sPLA₂(I) (sense, 5'-AACGTACGCTCAGCTCC-3'); sPLA₂(I) (antisense, 5'-GGAATGCCTCGACCGATATCAT-3'); iPLA₂-34 (sense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); iPLA₂-34 (antisense, 5'-GGCCTTCGTTGATGAGG-3'); sPLA₂(II) (sense, 5'-GCGATGAGGAG-3'); sPLA₂(II) (antisense, 5'-ATACTTCTT-3'); lipoprotein-associated PLA₂ (antisense, 5'-GCCCA-3'); lipoprotein-associated PLA₂ (sense, 5'-TAGATATCCTGG CCCCA-3'); lipoprotein-associated PLA₂ (antisense, 5'-GTTGTCATTG AACCAAGAGG-3'). The PCR products were analyzed on 1.5% low melt agarose gels.

Cloning of Human iPLA₂—The CHO cell-derived iPLA₂ amino acid sequence (9) was used to perform a TBLASTN database search of GenBank®. The sequences of two human expressed sequence tag (EST) clones, 46450 (accession number H10676) and 30643 (accession number M18691), were found to show considerable identity to the CHO cell iPLA₂. To obtain a full-length human iPLA₂ cDNA clone, 5'-rapid amplification of cDNA ends (RACE) was used to amplify the sequence from various cDNA sources (see Fig. 4). Amplification was carried out using a human iPLA₂-specific 3' primer (iPLA₂-2), a 5' anchor primer (CLONTECH), and Marathon Ready cDNAs (CLONTECH) as template. The Expand High Fidelity PCR system (Boehringer Mannheim) was used for the 5' RACE using the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The amplified products were analyzed on 0.8% agarose gels, and DNA fragments were recovered (Qiagen). Taq polymerase was used to add a 5' overhang to the fragment, which was then cloned into the pCR2.1 TA cloning vector (Invitrogen). Sequencing of both strands of the cloned 5'-RACE fragment was performed as described above.

A full-length human iPLA₂ clone was obtained by obtaining the 3' end of the iPLA₂ sequence in clone EST 46450 with the 5' sequence obtained by the RACE reaction. Briefly, the 5'-RACE fragment was released from pCR2.1 by an HindIII/HpaI double digest. The released fragment was gel purified, extracted, and sticky/blunt end ligated into HindIII/HpaI-digested Lamin B/EST 46450. Sequencing of the full-length iPLA₂ was performed to verify the integrity of the sequence.

Transient Expression in COS-7 Cells—Plasmid DNA (5 μg) containing iPLA₂, ankyrin-iPLA₂-1, or cPLA₂ cDNAs cloned into the eukaryotic expression vector pcDNA 3.1+ (Invitrogen) was transfected into COS-7 cells using LipofectAMINE (Life Technologies, Inc.). Transfections were in six-well plates in triplicate. Following the transfection, the DNA and LipofectAMINE (60 μl) were each mixed with 800 μl of medium (Opti-MEM) and then combined and incubated for 45 min at 20 °C to allow formation of DNA-liposome complexes. Subsequently, 6.4 ml of medium was added to each tube, and the transfection mixture was transferred to washed COS-7 cells. Transfection was allowed to proceed for 5 h at 37 °C in 80-mm dishes and terminated by replacement of the transfection mixture with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

PLA₂ Enzyme Activity—Enzyme activities were determined using a vesicular based assay containing equal concentrations (5 μM) of 1-palmitoyl-2-[1-14C]arachidonyl phosphatidylethanolamine (55 μCi/mmol) and 1-palmitoyl 2-[1-14C]arachidonyl phosphatidylethanolamine (55 μCi/mmol), NEN Life Science Products). The radioactive phospholipids were dried under nitrogen and resuspended in either 180 μl of calcium-free assay buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mg/ml albumin) or calcium-containing assay buffer (80 mM glycine, pH 9.0, 5 mM CaCl₂, 2 mM dithiothreitol, and 1 mg/ml albumin). Cells were collected 48 h after transfection by scraping, washed twice, and resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM dithiothreitol, and 20% glycerol). Cells were then lysed by sonication (2 x 5 s), the resulting homogenate was centrifuged at 10,000 x g for 20 min at 4 °C, and the supernatant was collected. Calcium-dependent and -independent PLA₂ activity in the supernatant was determined by the addition of 20 μl of supernatant and 80 μl of one of the above assay buffers, and the reaction was allowed to proceed for 20 min at 37 °C before termination with the addition of 400 μl of methanol containing 0.5% acetic acid and 10 μM arachidonic acid. The sample was then applied to a Sep-Pak Vac (Waters) C18 cartridge, and fatty acids were eluted in 500 μl of methanol. The [1-14C]arachidonate content of the eluate was analyzed by high pressure liquid chromatography using a Nova-Pak C18 column (3.9 x 150 mm) at a flow rate of 1 ml/min (the mobile phase was methanol/H₂O/trichloroacetic acid (85:15:0.01 by volume), and radioactivity was detected using a Beckman 171 radiotrace detector coupled on-line to a Waters 996 diode array spectrophotometer. Peak area integration was performed using Mille

RESULTS

PLA₂ Expression in B-cell Lines—Human monoclonal B-cells express both FLAP and 5-lipoxygenase and have the ability to make LTs only after the addition of exogenous arachidonic acid and stimulation with calcium ionophore and a reducing agent (14, 15). The reason for the lack of cell leukotriene biosynthesis in B-cells without these added factors is unclear. Furthermore, stimulation with various agents known to promote arachidonic acid release fails to induce a similar release in B-cells. However, B-cells possess phospholipase activity and sonicates of these cells release arachidonic acid (16). Therefore, to investigate the presence of PLA₂ in B-cells, we have examined the expression of the various PLA₂ enzymes at the tran-
scriptional level using RT-PCR. Total RNA from the human monoclonal B-cell lines Raji and BL-41 E95A was used as template for the RT-PCR reaction, and primers were designed for the amplification of a fragment of the following PLA2 cDNAs: cPLA2 (3), iPLA2, sPLA2 types I, II, and V (2, 18), and lipoprotein-associated PLA2 (19). The sequence used for the design of the iPLA2 primers was obtained by a TBLASTN data base search using the CHO cell-derived amino acid sequence. Two human EST clones, 46450 and 30643, revealed 84 and 65% sequence identity, respectively, to the CHO cell-derived sequence, strongly suggesting that they represented the human form of the enzyme. Sequencing of the two clones revealed that EST 46450 contained a partial cDNA for the human homolog of iPLA2, whereas EST 30643, which also contained part of the human iPLA2 cDNA sequence, differed from EST 46450 at the 5' end (Fig. 1). The difference in sequence at the 5' end of these clones was not due to a misspliced intron because the 3' intron junction consensus sequence was not present. Therefore, it could be either a cDNA library artifact or the iPLA2 sequence is subject to alternative splicing.

The iPLA2 primers 1 and 2 (Fig. 1) along with the primers for the various PLA2s described above were used to amplify B-cell cDNA and revealed that the iPLA2 sequence was easily amplified from these cells. A weak signal for the cPLA2 sequence was detectable, but none of the other PLA2 sequences were detected (data not shown). The weak signal obtained for cPLA2 is in agreement with previous results showing that the cPLA2 mRNA (20, 21) or protein (22) was either not present or detectable at low levels in B-cells. The results from one typical RT-PCR using primers iPLA2-1 and -2 is shown in Fig. 2. At least three distinct amplified products were observed. The most abundant product was the expected 217-bp fragment, whose identity was confirmed by sequencing, as well as two additional fragments with the apparent sizes of 330 and 380 bp, respectively. The 380-bp fragment was sequenced and found to contain the 5' end of EST clone 30643 (Fig. 3A). This iPLA2 EST contains a 168-bp insert between iPLA2 PCR primers 1 and 2, indicating that there are splice variants of iPLA2. The insertion introduces a premature termination codon that would result in the expression of a truncated iPLA2 (Fig. 3B). The nature of the 330-bp fragment is currently under investigation, although it is related to iPLA2 because it hybridizes to an iPLA2 oligonucleotide probe (data not shown).

Cloning of the Human iPLA2 cDNA—The results from the RT-PCR indicated that iPLA2 is at least one of the more significant PLA2 enzymes in B-cells, and therefore we decided to characterize it more extensively. To obtain a full-length iPLA2 cDNA clone, 5' RACE was performed on various cDNAs to amplify the remainder of the sequence. Fig. 4 shows the results from one such reaction and the substantial amplification of a 2.2-kilobase DNA fragment from testis cDNA. This fragment was subcloned, and sequence data were obtained for five different clones. Three clones had identical sequence and contained an open reading frame encoding the human iPLA2 cDNA sequence (Fig. 5). One of the clones had an insertion just before the iPLA2 catalytic domain that produced a frameshift, thus leading to a truncated iPLA2 without the catalytic domain (Fig. 3B). This clone was identified as ankyrin-iPLA2-1 to indicate that it coded for only the ankyrin repeats. Similarly, the insert at the termination codon that is boxed and in bold. B, comparison of iPLA2 with the ankyrin-iPLA2 isoforms showing the various insertions. The iPLA2 PCR primers 33 and 34 are indicated in bold and underlined, and the termination codons for the isoforms are shown in bold and dotted underlined. The lipase active site sequence is boxed and in bold. C, the in-frame 216-bp 5' deletion in ankyrin-iPLA2-2. The dots indicates the presence of gaps in the sequence.
other clone, ankyrin-iPLA₂-2, had the identical 53 bp insertion as the previous isoform but also had an additional 52 bp insertion 80 bp 5' to the ankyrin-iPLA₂-1 insertion, thus generating a different C terminus but again without the catalytic domain (Fig. 3B). In addition, this clone contained a 216 bp in-frame deletion in the 5' region of the cDNA resulting in the removal of amino acids 71–143 (Figs. 3C and 5). To prove that the observed different forms of iPLA₂ were not due to artifacts in the 5'RACE reaction, testis cDNA as well as two different testis cDNA libraries and B-cell cDNA were subjected to PCR using primers iPLA₂-33 and -34 (Fig. 3B), which spanned the region of interest. Two detectable amplified products were obtained, one major product corresponding to iPLA₂ and a minor one corresponding to ankyrin-iPLA₂-1, whereas ankyrin-iPLA₂-2 was not observed (data not shown). The absence of isoform 2 is not too surprising because it could be at levels that would require more rounds of amplification or the use of ankyrin-iPLA₂-2-specific primers.

A full-length iPLA₂ cDNA sequence was obtained by combining the 3' end of EST 46450 with the appropriate 5'RACE fragment (see “Materials and Methods”). The inferred human iPLA₂ amino acid sequence was compared with the recently reported sequences and found to have an overall identity of 90% to hamster, rat, and mouse iPLA₂ sequences (Fig. 5A). The major difference between the human sequence and the other species is the insertion of an additional 54 amino acids that would interrupt the last putative ankyrin repeat as defined in the hamster and rat iPLA₂ sequences (9, 11) (Fig. 5). This sequence is also present in the other human iPLA₂ isoforms. A search of GenBank™ with this additional 54-amino acid sequence did not reveal any significant homologies.

In addition to the human iPLA₂ cDNA there are at least three other iPLA₂ isoforms. The EST 30643 sequence, if it were full-length, could encode an iPLA₂ that contained the ankyrin repeats and lipase active site but with a truncated C terminus (Fig. 5). The other two iPLA₂ isoforms ankyrin-iPLA₂-1 and -2 contain only the ankyrin repeats because they terminate before the lipase active site (Fig. 5). A diagrammatic representation of the iPLA₂ isoforms is illustrated in Fig. 5B. The ankyrin-iPLA₂-1 sequence is identical to the human iPLA₂ up to the last three C-terminal amino acids and terminates about 40 residues before the active site serine. In contrast, although the ankyrin-iPLA₂-2 sequence has the same iPLA₂ ankyrin repeats, it also contains more structural and sequence variation than does ankyrin-iPLA₂-1. First, there is the 72-amino acid deletion between residues 70–143, and second, the additional insertion in the iPLA₂-2 cDNA (Fig. 3C) alters the sequence of the last 50 C-terminal amino acids (Fig. 5, A and B). The presence of these iPLA₂ isoforms indicates that the iPLA₂ sequence is subjected to a significant amount of alternative splicing.

Effect of Ankyrin-iPLA₂ Sequence on iPLA₂ Activity—The ankyrin structural motif appears to have a function in the formation of various types of protein-protein interactions (23). Deletion of the ankyrin repeats of the CHO cell iPLA₂ results in the loss of lipase activity, suggesting that this structure is required for enzyme activity (9). A possible function for the ankyrin repeats in iPLA₂ is to participate in the formation of the large oligomeric structures (270–350 kDa) found for iPLA₂ upon gel filtration (7, 9). If this is the case then the ankyrin-iPLA₂ sequences (iPLA₂-1 and -2) may also participate in the formation of the iPLA₂ oligomeric structures in the cell and have some effect on iPLA₂ activity. To test this, iPLA₂ cDNA was co-transfected into COS cells with either pcDNA 3.1 vector or ankyrin-iPLA₂-1 cDNA, and PLA₂ activity was measured in the cell lysate in the presence and the absence of calcium (Fig. 6, A and B). A control transfection for PLA₂ activity using the cPLA₂ cDNA was also performed. Transfection of iPLA₂ and control vector resulted in a substantial increase in PLA₂ activity in cell lysate over control (vector alone) in the absence (Fig. 6A) or the presence of calcium (Fig. 6B), indicating that the iPLA₂ cDNA sequence codes for an active enzyme. Increased PLA₂ activity was also observed upon transfection of the cPLA₂ cDNA but only in the presence of calcium, as would be expected (Fig. 6B). Transfection of just the ankyrin-iPLA₂ cDNA sequence did not result in any PLA₂ activity over background (data not shown). However, replacement of the vector control in the iPLA₂ cDNA transfection with ankyrin-iPLA₂-1 cDNA caused a 2-fold decrease in PLA₂ activity in the cell lysate in both assays (Fig. 6, A and B). Including the ankyrin-iPLA₂-1 cDNA in the cPLA₂ cDNA transfection had no effect on PLA₂ activity (Fig. 6B). These results would suggest that the ankyrin-iPLA₂ sequences can specifically modulate iPLA₂ activity.

DISCUSSION

The human iPLA₂ sequence presented here contains the ankyrin and the GXSGX lipase motifs described for the hamster and rodent enzymes (9, 11). A major difference between the human enzyme and that of other species is the insertion of a 54-amino acid proline-rich sequence in the eighth ankyrin motif (residues 395–449). A recent report from Tang et al. (9), which describes a partial human iPLA₂ cDNA clone, found at the exact same position in their clone some sequence that was not present in the CHO iPLA₂, which they attributed to potential unspliced intron sequences. The insertion described here for human iPLA₂, which was detected in all sequenced iPLA₂ clones, does not contain any splice junction consensus sequence, nor does it disrupt the reading frame. Whether the human gene contains an additional exon or this form is also present in the other species but has not yet been cloned remains to be determined. The possibility that iPLA₂ expression could give rise to splice variants was suggested by finding two partial iPLA₂ cDNAs in the EST data base with different 5' ends. The difference was found to be due to a 168 bp insertion in EST 30643. Both EST 30643 and iPLA₂ cDNA sequences were easily detected by RT-PCR of B-cell RNA suggesting that both iPLA₂ isoforms are expressed. Presently, we are trying to obtain a full-length EST 30643 clone to confirm that this truncated isoform of iPLA₂ is catalytically active. Two additional iPLA₂ splice variants were also detected, ankyrin-iPLA₂-1 and -2, which contain only the ankyrin repeats and no lipase catalytic site.
FIG. 5. Amino acid sequence and alignment of human iPLA₂ and the various isoforms with the CHO sequence. A, the amino acid sequence of human and CHO cell iPLA₂ as well as the various iPLA₂ isoforms were compared using the Genetics Computer Group program GAP.

B
transfected with the iPLA₂ cDNA alone, the iPLA₂ oligomer is most likely composed entirely of active iPLA₂ monomers. Co-transfection of both iPLA₂ and ankyrin-iPLA₂ cDNAs could result in iPLA₂ oligomers that contain various combinations of both iPLA₂ and ankyrin-iPLA₂-1 monomers. What is not known is the stoichiometry of the active iPLA₂ enzyme complex. Does it contain only iPLA₂ monomers, or can it tolerate formations with ankyrin-iPLA₂? The size of the iPLA₂ complex, based on gel permeation chromatography, ranges from 270 to 350 kDa (7, 9), suggesting that there are at most four iPLA₂ monomers/complex. If this is the case and we assume that there is equal expression of both subunits in the transfected cells and binding affinity is not changed between the different subunits, then iPLA₂-(ankyrin-iPLA₂-1) complexes should be enzymatically active, because enzyme activity was only decreased 2-fold in the co-transfected cells. However, a more thorough investigation using purified subunits will have to be done to confirm this. Although we can show that ankyrin-iPLA₂ can have an effect on iPLA₂ activity, its in vitro role remains to be determined. The level of ankyrin-iPLA₂-1 expression is at least 10-fold lower than that of iPLA₂, suggesting that if it participates in the iPLA₂ enzyme complex, it must have a much more subtle effect on enzyme activity in the cell. Nevertheless, what we have shown is that alternative splicing of the iPLA₂ gene can have a direct effect on iPLA₂ activity.

In fact, alternative splicing of genes containing sequences that encode both an activity domain and some kind of protein binding domain appears to be a common mechanism to control activity. A similar situation to that of iPLA₂ is the IκB inhibition of NF-κB activity. IκB proteins contain six or seven ankyrin-like repeats, which have been shown to be essential for retaining NF-κB in the cytoplasm and inhibit DNA binding by Rel/NF-κB (25, 27). IκB is derived by alternative splicing of the murine p105 gene, which is the precursor for the p50 component of the NF-κB, p50-p65 heterodimer (28, 29). The N-terminal half of p105 contains p50, which is derived by proteolytic cleavage, whereas the C-terminal half has eight ankyrin repeats (29). In addition, there are multiple isomers of IκBα, all derived by alternative splicing of the p105 gene and each with unique IκB activities (30). The two ankyrin-iPLA₂ isoforms described here do have structural and sequence differences, but whether or not they have unique inhibitory activities remains to be determined. Additional examples of alternative splicing having positive and negative effects on activity are two genes involved in programmed cell death: ich-1 (Caspase-2) (31), which encodes a cysteine protease, and bel-x, a bel-2-related regulatory gene (32). Again it is analogous to the iPLA₂ situation described above in that both genes produce a long transcript that codes for a functional product that is inhibited by a truncated version encoded by a shorter alternatively spliced transcript (31–33).

In the two B-cell lines tested, the iPLA₂ cDNA sequence was easily amplified, whereas only a weak signal was obtained for the cPLA₂ cDNA. The sPLA₂ groups I, II, and V and the lipoprotein-associated PLA₂ sequences were not detected. The weak PCR signal obtained for cPLA₂ is consistent with previous observations.

The existence of these truncated forms of iPLA₂ is intriguing because the ankyrin motif has been shown to be involved in various types of protein-protein interactions (23–26). The truncated forms could function as negative regulatory proteins by docking to iPLA₂ binding sites in the cell and thereby prevent docking of the catalytically active enzyme. Alternatively, they may interfere with the formation of the quaternary structure of iPLA₂ and in this way alter enzyme activity. An oligomeric form of the enzyme may indeed be the active state of the enzyme because removal of ankyrin repeats results in loss of enzyme activity (9). The fact that ankyrin-iPLA₂-2 can alter iPLA₂ activity was shown by co-transfection of the iPLA₂ and ankyrin-iPLA₂-1 cDNAs into COS cells. The co-transfection of both constructs results in a 2-fold decrease in PLA₂ enzyme activity compared with that observed for the co-transfection of iPLA₂ cDNA and vector DNA. Co-transfection of ankyrin-iPLA₂-1 cDNA with the cPLA₂ cDNA had no effect on PLA₂ enzyme activity. Thus the interaction of ankyrin-iPLA₂-1 with iPLA₂ results in a decrease in enzyme activity. The most likely explanation for this is a competition between ankyrin-iPLA₂-1 and iPLA₂ monomers to form the oligomeric species. In cells

FIG. 6. Effect of ankyrin-iPLA₂-1 on iPLA₂ activity in transfected COS-7 cells. PLA₂ cDNAs were cloned into the eukaryotic expression vector pcDNA 3.1, and COS-7 cells were then transfected with 5 μg each of the following DNAs: transfection 1, iPLA₂ cDNA and pcDNA 3.1 vector; transfection 2, iPLA₂ and ankyrin-iPLA₂-1 cDNAs; transfection 3, cPLA₂ cDNA and pcDNA 3.1 vector; transfection 4, cPLA₂ and ankyrin-iPLA₂-2 cDNAs; and transfection 5, 10 μg of pcDNA 3.1. Cell lysates were prepared 48 h after transfection and PLA₂ activity determined in the absence (A) and the presence (B) of calcium. Activity is defined as the release of [14C]arachidonic acid (cpm/mg protein). The results are the means of duplicate samples from two different transfections.

The seven ankyrin repeats in the human sequence are underlined (I–VII), and the eighth repeat, which is underlined in the CHO sequence, is interrupted by an insertion in the human sequence. The active site (GTSTG) is indicated in bold, and termination is indicated by an asterisk. The EST 30643 sequence is the partial sequence from the EST clone. Shading indicates identity, and the dots indicate the presence of gaps in the sequence. B, a diagrammatic representation of the iPLA₂ isoforms. The 54-amino acid insertion into the eighth ankyrin repeat of the human sequences and the 72-amino acid deletion in ankyrin-iPLA₂-2 are illustrated. The active site serine is indicated for both the CHO and human sequence. The C-terminal hatched region in ankyrin-iPLA₂-2 denotes nonidentical sequence, and the number of amino acids in each sequence is also shown.
ous findings that showed that cPLA₂ expression in B-cells was either very low or undetectable (20–22). Based on this it would suggest that iPLA₂ may be one of the more significant PLA₂s in B-cells. In fact the partial human iPLA₂ cDNA sequence recently described (9) was cloned from a B-cell line cDNA library. However, no physiological stimuli is at present known to induce leukotriene synthesis in human B lymphocytes; hence they are dependent on exogenous arachidonic acid for leukotriene synthesis. Perhaps, what is required is the correct stimulus to activate the iPLA₂ enzyme. It has been demonstrated that the majority of the 5-lipoxygenase enzyme in nonstimulated B lymphocytes is located at the nucleus (16). Thus, an increase in the intracellular calcium concentration, which renders translocation of 5-lipoxygenase to the nucleus, might not be a prerequisite for leukotriene synthesis in B lymphocytes, because the 5-lipoxygenase already is located at the nucleus. Therefore, it is tempting to speculate that some stimulus that activates the calcium-independent iPLA₂ without increasing the intracellular calcium concentration may be sufficient for the induction of leukotriene synthesis in B lymphocytes. However, in a recent review, Balsinde and Dennis suggest that the major function of iPLA₂ is in membrane remodeling and not in arachidonic acid release, although involvement in the latter cannot be completely ruled out (34). Therefore, the function and the regulation of iPLA₂ in B lymphocytes and its role in leukotriene synthesis remain to be determined.

In conclusion, we describe in this report the cDNA sequence of the human iPLA₂ and its various splice variants. We furthermore present data indicating that a splice variant of the iPLA₂ containing only the ankyrin motifs and not the active site specifically modulates iPLA₂ activity when the proteins are co-expressed in COS-7 cells. These findings suggest that alternative splicing of the iPLA₂ pre-mRNA can result in the production of regulatory subunits that can modify iPLA₂ activity in vivo.

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REFERENCES
1. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
2. Dennis, E. A. (1997) Trends Biochem. Sci. 22, 1–2
3. Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L. L. (1995) J. Lipid Med. Cell. Sign. 12, 83–117
4. Ackermann, E. J., and Dennis, E. A. (1995) Biochim. Biophys. Acta 1259, 125–136
5. Haten, S. L., and Gross, R. W. (1991) Biochem. J. 280, 581–587
6. Hirashima, Y., Farooqi, A. A., Mills, J. S., and Horrocks, L. A. (1992) J. Neurochem. 59, 708–714
7. Ackermann, E. J., Kemper, E. S., and Dennis, E. A. (1994) J. Biol. Chem. 269, 9227–9233
8. Portilla, D., and Dai, G. (1996) J. Biol. Chem. 271, 15451–15457
9. Tang, J., Kriz, R. W., Wolfman, N., Shaffer, M., Sechra, J., and Jones, S. S. (1997) J. Biol. Chem. 272, 8567–8575
10. Balboa, M. A., Balsinde, J., Jones, S. S., and Dennis, E. A. (1997) J. Biol. Chem. 272, 8576–8580
11. Ma, Z., Ramanadham, S., Kempe, K., Chi, X. S., Ladenson, J., and Turk, J. (1997) J. Biol. Chem. 272, 11118–11127
12. Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8527–8531
13. Samuelsson, B. (1983) Science 220, 568–575
14. Jakobsson, P. J., Odlander, B., Steinhilber, D., Rosen, A., and Claesson, H. E. (1991) Biochim. Biophys. Res. Commun. 178, 302–308
15. Jakobsson, P. J., Steinhilber, D., Odlander, B., Radmark, O., Claesson, H. E., and Samuelsson, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3521–3525
16. Jakobsson, P. J., Shaskan, P., Larsson, P., Feltenmark, S., Odlander, B., Agullagamvalloes, M., Ojala, P., Biberfeld, P., and Claesson, H. E. (1995) Eur. J. Biochem. 232, 37–46
17. Avila-Carino, J., Torstenssdottir, S., Ehin-Henriksson, B., Lenoir, G., Klein, G., Klein, E., and Masucci, M. G. (1987) Int. J. Cancer 40, 691–697
18. Chen, J., Engle, S. J., Seilhamer, J. J., and Tischfeld, J. A. (1994) J. Biol. Chem. 269, 2365–2368
19. Tew, D. G., Southan, C., Rice, S. Q., Lawrence, M. P., Li, H., Boyd, H. F., Moors, K., Gliger, I. S., and Macphee, C. H. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 591–599
20. Feltenmark, S., Sunanith, G., Larsson, P., Jakobsson, P. J., Bjorkholm, M., and Claesson, H. E. (1996) Br. J. Haematol. 90, 585–594
21. Jakobsson, P. J., Larsson, P., Feltenmark, S., Odlander, B., Runarsson, G., Bjorkholm, M., and Claesson, H. E. (1995) Adv. Prostaglandin Thromboxane Leukotriene Res. 23, 293–298
22. Gilbert, J. J., Stewart, A., Courtney, C. A., Fleming, M. C., Reid, P., Jackson, C. G., Wise, A., Wakelam, M., and Harnett, M. M. (1996) J. Immunol. 156, 2054–2061
23. Bork, P. (1993) Proteins 17, 363–374
24. Bennett, V., and Gilligan, D. M. (1993) Ann. Rev. Cell. Biol. 9, 27–66
25. Reg, A. A., and Baldwin, S. S. (1993) Genes Dev. 7, 2064–2070
26. Thompson, C. C., Brown, T. A., and McKnight, S. L. (1991) Science 253, 762–768
27. Gilmore, T., and Morn, P. (1993) Trends Genet. 9, 427–433
28. Inoue, J. I., Kerr, L. D., Kataoka, A., Verma, I. M., (1992) Cell 68, 1109–1120
29. Blank, V., Kourilsky, P., and Israel, A. (1992) Trends Biochem. Sci. 17, 135–140
30. Grumont, R. J., and Gerondakis, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4367–4371
31. Wang, L., Miura, M., Bergeron, L., Shu. H., and Yuan, J. (1994) Cell 78, 739–750
32. Boise, L. H., Gonzalez-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, S., Nunez, G., and Thompson, C. B. (1993) Cell 74, 597–608
33. Ottval, Z. N., and Kursmeyer, S. J. (1994) Cell 79, 189–192
34. Balsinde, J., and Dennis, E. A. (1997) J. Biol. Chem. 272, 16069–16072
Multiple Splice Variants of the Human Calcium-independent Phospholipase A₂ and Their Effect on Enzyme Activity

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