Cloning and Initial Characterization of a Human Phospholipase D2 (hPLD2)

ADP-RIBOSYLATION FACTOR REGULATES hPLD2*

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Phospholipase D (PLD) has been implicated in a variety of cellular processes including vesicular transport, the respiratory burst, and mitogenesis. PLD1, first cloned from human, is activated by small GTPases such as ADP-ribosylation factor (ARF) and RhoA. Rodent PLD2, which is approximately 50% identical to PLD1 has recently been cloned from mouse embryo (Colley, W., Sung, T., Roll, R., Jenco, J., Hammond, S., Altshuller, Y., Bar-Sagi, D., Morris, A., and Frohman, M. (1997) Curr. Biol. 7, 191–201) and rat brain (Kodaki, T., and Yamashita, S. (1997) J. Biol. Chem. 272, 11408–11413). We describe herein the cloning from a B cell library and expression of human PLD2 (hPLD2). The open reading frame is predicted to encode a 933-amino acid protein (M, of 105,995); this corresponds to the size of the protein expressed in insect cells using recombinant baculovirus. The deduced amino acid sequence shows 53 and 90% identity to hPLD1 and rodent PLD2, respectively. The mRNA for PLD2 was widely distributed in various tissues including peripheral blood leukocytes, and the distribution was distinctly different from that of hPLD1. PLD1 and hPLD2 both showed a requirement for phosphatidylinositol 4,5-bisphosphate. Both isoforms showed optimal activity at 10–20 mol % phosphatidylcholine in a mixed lipid vesicle system and showed comparable basal activities in the presence of phosphatidylinositol 4,5-bisphosphate. Unexpectedly, ARF-1 stimulated the activity of hPLD2 expressed in insect cells about 2-fold, compared with a 20-fold stimulation of hPLD1 activity. Thus, not only PLD1 but also hPLD2 activity can be positively regulated by both phosphatidylinositol 4,5-bisphosphate and ARF.

Phospholipase D (PLD) has been implicated in a wide range of physiological processes and diseases including inflammation, secretion, mitogenesis, neuronal and cardiac stimulation, diabetes, and the respiratory burst in neutrophils (1). PLD catalyzes the hydrolysis of phospholipids, usually phosphatidylcholine, to generate phosphatidic acid plus the head group. Phosphatidic acid may act directly as a signaling molecule (2–5) or can be further metabolized to form diacylglycerol by phosphatidic acid phosphohydrolase (6). The latter may function as an activator of protein kinase C isoforms and possibly other diacylglycerol-dependent enzymes. PLD can be activated in cells by a variety of extracellular agonists including those that bind to G protein-coupled receptors (e.g. the fMet-Leu-Phe receptor in neutrophils) and those that stimulate receptor tyrosine kinases (3, 7, 8). Phorbol esters are also potent activators of PLD in a variety of cell systems, implicating protein kinase C in PLD regulation.

PLD activities from mammalian cells were initially studied in broken or permeabilized cell preparations. ADP-ribosylation factor (ARF) was shown to be a potent activator in rat brain preparations (9) and in permeabilized HL-60 cells (10). PLD activity found in the cytosolic fraction obtained from HL-60 was also activated by ARF (5). A role for Rho family small GTPases such as RhoA, Rac, and Cdc42 was established based on inhibition by RhoGDI and by reconstitution studies (11–13). PLD activity in plasma membranes from rat liver was stimulated by RhoA but not ARF (13). In some systems, RhoA and ARF synergize to increase PLD activity (5, 14–16). The actions of RhoA and ARF can be further enhanced by cytosolic factors (12, 16–19) as well as by protein kinase C (5, 15, 16, 20). Protein kinase C activates PLD in a phosphorylation-dependent (21) or phosphorylation-independent (16, 22) manner, depending on the system. Solubilized preparations required PIP$_2$ for activity (9). These and other differences (e.g. subcellular localization, Ca$^{2+}$ requirement, activation or inhibition by oleate, etc.) implied that several isoforms of PLD exist (3, 23).

The first cloned mammalian homologue of a plant PLD, human PLD1 (hPLD1), is regulated by PIP$_2$, ARF, RhoA, and protein kinase C (15, 24) and almost certainly represents the same isoform that had been studied by Brown and colleagues in rat brain (9). A rat PLD1 (rPLD1) has also recently been cloned and characterized (25) and shows similar properties to hPLD1. Two splice variants of PLD1 have been identified (15), but no significant differences in activity or in vitro regulation of the two forms have been observed. A second isoform of PLD, PLD2, was recently cloned from mouse (mPLD2) (26) and rat (rPLD2) (27). Both of the rodent PLD2s require PIP$_2$ for activity and, unlike hPLD1, are constitutively active in the absence of other activating factors when PIP$_2$ is present. Neither ARF nor other protein factors have been reported to further activate rodent PLD2. In the present studies, we have cloned, expressed, and partially characterized a PLD2 isoform from human cells, and

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1 The abbreviations used are: PLD, phospholipase D; hPLD1 and hPLD2, human phospholipase D1 and D2, respectively; rPLD1 and rPLD2, rat phospholipase D1 and D2, respectively; mPLD2, mouse phospholipase D2; kb, kilobase pair(s); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag; HPLC, high performance liquid chromatography; C, choline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC, dipalmityl phosphatidylcholine; PIP, phosphatidylinositol 4,5-bisphosphate; ARF, ADP-ribosylation factor.
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we have compared its properties with those of hPLD1. Unexpectedly, hPLD2 is activated not only by PI(3,4,5)P3 but also by ARF-1. Although the magnitude of the activation of hPLD2 by ARF is smaller than that of hPLD2, these data suggest that hPLD2 contains an ARF binding/regulatory site and that ARF along with other unknown factors may activate this isoform.

**EXPERIMENTAL PROCEDURES**

**Library Screening**—A computer search of the human GenBank expressed sequence tag (EST) data base was conducted using regions sharing homology among PLDs from *Ricinus communis*, cabbage, and *Solanum tuberosum* in order to identify cDNA clones related to PLDs. Two EST clones, yq12f08.r1 and yq16g08.r1, showed a high degree of homology and contained putative open reading frames. Two hybridization probes (350 base pairs each) were generated from these EST clones using polymerase chain reaction (PCR) and primers specific to the open reading frame of these EST clones, yq12f08.r1 (5'-GATGATATCACTTGGTCCACCT-3' and 5'-GATCCAAATCGTG- GCCGGTATT-3') and yq16g08.r1 (5'-GGCCTTACTCCCTGGTCCT-3' and 5'-ATGGTTCGGTCCTGTCCTG-3'). These cDNAs were radio labeled by the random priming method (28) and were used to screen an oligo(dT)-primed HSC-93 B cell plasmid (pREP4) library (containing 3 x 10^10 individual clones), obtained from Dr. Manuel Buchwald (University of Toronto). The average insert size of this library was approximately 1 kb; however, cDNA inserts of sizes of 4.6, 3.2, and 2.3 kb have been recovered in this library (29). Two million clones were screened by Southern analysis starting with 20 plasmid DNA samples, each representing 100,000 individual cDNA clones. The plasmid DNA samples were electrophoresed on a 0.8% agarose gel, transferred onto nylon filters, and then hybridized overnight with the random-primed (Prime-It; Stratagene) 32P-labeled DNA probes at 42 °C in 50% formamide, 3 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 x Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, and 1% SDS. Filters were washed twice at low (42 °C for 10 min with 1 x SSC, 0.1% SDS) stringency and exposed overnight to film. Two positive pools of cDNAs were identified, which were then plated at 50,000 clones/plate; nylon filter lifts from these plates were hybridized overnight with random-primed (Prime-It; Stratagene) [32P]dCTP-labeled probes at 42 °C in 50% formamide, 6 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5 x Denhardt's solution, 100 mg/ml sheared salmon sperm DNA, and 1% SDS. Filters were washed twice under low stringency conditions (42 °C for 10 min with 1 x SSC, 0.1% SDS), and positive clones were identified and purified. Restriction enzyme analysis using Bancroft revealed that all of the clones contained 1.3- or 2.1-kb inserts. The cloned cDNAs were sequenced using the Taq dye terminator method at the University of Georgia Molecular Genetics Facility, Department of Genetics, using Applied Biosystems 373 and 377 automatic sequencers.

5- and 3'-Rapid Amplification of cDNA Ends (RACE) — The full-length hPLD2 cDNA was generated by 5- and 3'-RACE using the Marathon cDNA amplification kit (CLONTECH). The cDNA was reverse transcribed from poly(A)+ mRNA obtained from the B-cell line, HSC-93 (a kind gift from Dr. Manuel Buchwald), using the cDNA synthesis primer provided with the kit. The PCR amplification was carried out using an adaptor primer (AP1; 5'-CATCCATATAGCTC- CATAAGGCGCC-3') provided with the kit and gene-specific forward, 5'-TGCACATGACACCTGCTTGAAGA-3' (3'-RACE) or reverse primers, 5'-GGAGAAT GGCGGGCTGTAGGAGA-3' (5'-RAC- E). The first round PCR was performed according to the recommendations of the manufacturer of 94 °C for 1 min, five cycles at 94 °C for 5 s and 72 °C for 4 min, another five cycles at 94 °C for 5 s and 70 °C for 4 min, followed by 25 cycles at 94 °C for 5 s and 68 °C for 4 min. The second round of PCR was performed under the same conditions using a 1.50 dilution of the first round PCR amplification mixture as the template, adaptor primer 2 (AP2; 5'-ACTACTATAGGGCGCTCGAGG- GC-3') and a nested gene-specific primer, 5'-ATGGCCGGCTGCTGTGA- GACCTGGGAA-3' (5'-RACE). A Southern blot analysis using an end-labeled internal oligonucleotide (5'-CCATCGATCCCTCTCCACATTTG- CCC-3') from the PCR primers confirmed that the PCR product contained the expected sequence. The PCR product was subcloned into the PCRII TA cloning vector (Invitrogen) according to the instructions of the manufacturer. Positive clones were identified by hybridizing filter lifts with the same labeled oligonucleotide that was described for the Southern blots. Clones containing the expected size DNA fragment were then sequenced along both strands.

A single 1.8-kb DNA fragment was obtained after first round 3'-RACE. A Southern blot analysis of this band showed hybridization to an end-labeled oligonucleotide (5'-CCATCGATCCCTCTCCACATTTG- CCC-3'). The fragment was subcloned into PCR2.1, and positive clones were identified by hybridization to the same oligonucleotide probe and analyzed by restriction digests to identify clones containing inserts greater than 1.6 kb. Those clones containing the expected size DNA fragment were sequenced and the sequence determined. The sequence was obtained by ligating both the 5'- and 3'-cDNA fragments at the BstRI restriction site in the overlapping region and subcloned into pBluescript KS+ (Stratagene).

**Northern Blots**—Human multiple-tissue Northern blots (CLONTECH) containing approximately 2 μg of poly(A)+ RNA were probed with full-length cDNA of hPLD1, hPLD2, or β-actin according to the manufacturer's instructions. The blots were exposed to Hyperfilm MP x-ray film (Amersham Pharma Biotech) at −80 °C with two intensifying screens for 4–24 h before developing the film. Blots exposed to multiple probes were stripped of the first probe as suggested by the manufacturer's protocols.

**Expression of hPLD2 in Insect Cells**—The hPLD2 cDNA was inserted into the unique EcoRI and Kpn1 sites of the pAcHIL-T-A baculovirus transfer vector (Pharmingen). Plasmids used for co-transfections were purified using Qiagen kits. SF9 insect cells used for co-transfection and amplification were grown in IPL-41 insect medium (Life Technologies, Inc.), supplemented with 10% fetal calf serum (Atlanta Biologicals), 4 g/liter yeastolate (Life Technologies, Inc.), 2.6 g/liter tryptose phosphate (Life Technologies, Inc.), and 10 μg/ml gentamicin (Life Technologies, Inc.). Monolayers of exponentially growing SF9 insect cells (3 x 10^6 cells in a 60-mm plate) were transfected with a mixture of the transfer vector pAcHIL-T-A or hPLD2 containing transfer vector at 5 μg/ml and 1 μg/ml of BaculoGold virus DNA (0.5 μg) (Pharmingen) according to the manufacturer's recommendations. The virus was amplified by infecting a monolayer culture of SF9 cells (8 x 10^5 cells in a 75-cm^2 flask, approximately 80% confluent) for 5 days at 27 °C. The high titer virus stocks were stored at 4 °C.

High five (Hi5) cells (Invitrogen) were used for expression of hPLD2. Monolayers of exponentially growing cells were cultured in ExCell 401 serum-free insect culture medium (JRH) in a 27°C incubator. Insect cells (8 x 10^5 cells in a 75-cm^2 flask) were infected with recombinant baculovirus obtained from the co-transfection. The infected cells were grown for 3 days in ExCell 401 medium. At the end of the infection period, the cells were detached, centrifuged at 500 x g for 5 min to pellet, washed once with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4), and resuspended in cold lysis buffer (50 mM Tris, pH 7.5, 20 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 1 mM aprotinin, and 1 mM pepstatin). The cells were then disrupted by N2 cavitation for 20 min at 500 psi and 4°C and centrifuged at 500 x g to remove intact cells. The cell homogenate was then centrifuged at 540,960 x g for 20 min to obtain membrane and cytosolic fractions.

**Immunoblotting**—Membrane and cytosolic fractions subjected to 8% SDS-polyacrylamide gel electrophoresis and were electrophotically transferred to nitrocellulose at room temperature for 1 h at 5 V using a semidyed transfer cell (Bio-Rad). PLD2 was detected by Western blot analysis using a primary antibody to either a peptide (DRPFEDFIDRETT) specific to PLD2 or an anti-tetra-His antibody (Qiagen) and alkaline phosphatase-conjugated secondary antibodies (IGG). A rabbit polyclonal antiserum was raised against the N-terminus of the first domain of hPLD2 (H. Brown, unpublished). The IgG fraction was purified using a primary antibody to either a peptide (DRPFEDFIDRETT) specific to PLD2 or an anti-tetra-His antibody (Qiagen) and alkaline phosphatase-conjugated secondary antibodies (IGG). A rabbit polyclonal antiserum was raised against the N-terminus of the first domain of hPLD2 (H. Brown, unpublished).
RESULTS

Cloning of hPLD2 cDNA—To isolate a novel human PLD2 cDNA, a human B-cell library was screened with probes made from two partial cDNA clones obtained from the GenBank™ EST database as described under “Experimental Procedures.” These EST sequences encode a portion of human PLD1. A clone was isolated by low stringency hybridization, and an open reading frame of 250 base pairs was identified. Primers specific to regions of the coding sequence were developed, and the full-length 3433 base pairs cDNA clone was generated by 5'- and 3'-RACE. Sequence analysis revealed a large open reading frame encoding a 933-amino acid protein with a calculated molecular mass of 106,000 (Fig. 1A). This region contained a 5'-translation initiation codon ATG, an in-frame stop codon, and a poly(A) tail, suggesting that the entire 3'-untranslated region was obtained. The hPLD2 mRNA transcript was found to be approximately 3.5 kb in size by Northern blot analysis of poly(A)+ mRNA obtained from the HSC-93 B-cell line using the open reading frame of the 1.8-kb 5'-RACE product as a probe (data not shown), indicating that the cDNA sequence reported is likely to be full-length. The sequence showed 53% amino acid sequence identity with hPLD1. hPLD2 also contained the conserved regions I–IV reported to be conserved among the eukaryotic PLDs (41), which include regions thought to be required for catalysis (1 and IV) (Fig. 1). Analysis of the predicted amino acid sequence for known protein motifs did not reveal the presence of Src homology 2 or 3 domains. Sequence comparison demonstrated that hPLD2 was 90% identical at the amino acid level with both mPLD2 and rPLD2 isoforms. The dendrogram in Fig. 1 shows that hPLD2 clearly groups with the PLD2 isoforms (mPLD2 and rPLD2) and is more distantly related to the PLD1 proteins.

Tissue Distribution of hPLD2 mRNA—The expression of both hPLD1 and hPLD2 in various human tissues was determined by hybridizing human multiple tissue Northern blots with random primed full-length cDNA as described under “Experimental Procedures.” As shown in Fig. 2, a ~3.5-kb message corresponding to hPLD2 is expressed in a variety of tissues including heart, placenta, pancreas, spleen, thymus, prostate, uterus, brain, lung, kidney, small intestine, colon, and peripheral blood leukocytes. Liver and skeletal muscle expressed relatively low amounts of hPLD2 mRNA. Peripheral blood leukocytes contained an additional transcript of slightly larger size, suggesting the possibility of an alternatively spliced form of hPLD2 mRNA in this tissue. In contrast, hPLD1 mRNA (~6-kb message) is expressed in high levels in brain placenta, spleen, uterus, and small intestine and in greatest abundance in pancreas and heart, and no signal was seen in peripheral blood leukocytes.

Expression of hPLD2 in Insect Cells—hPLD2 was expressed in Hi5 insect cells using a baculovirus expression system. Three days after infection of Hi5 cells with both hPLD2-expressing baculovirus, cells were disrupted, and samples of the lysates were analyzed for the expression of the hPLD2 protein by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining and immunoblot analysis. Coomassie staining revealed a distinct protein band of approximately 105 kDa in samples of cell lysates from infected Hi5 cells, but very low PLD activity was seen in uninfected cells (Fig. 3A). To further verify that the protein expressed was His-tagged hPLD2, Western blot analysis was performed utilizing anti-tetra-His (anti-His4) antibody as well as an antipeptide antibody to hPLD2 described under “Experimental Procedures.” Both antibodies detected a protein corresponding in size to the expressed protein seen by protein staining. Membrane and cytosol samples were also obtained from infected Hi5 cells, and the majority of the PLD2 was found in the membrane fraction (data not shown), although small quantities of the enzyme were also seen in the cytosol. PLD activity was seen in samples of cell lysates from infected Hi5 cells, but very low PLD activity was seen in uninfected cells (Fig. 3C). Lysates from Hi5 cells infected with native baculovirus (no PLD2) yielded PLD activity levels similar to uninfected cells (data not shown).

Regulation of hPLD2 Activity by Lipids—The catalytic properties of hPLD2 were examined using membrane fractions from control Hi5 insect cells or from the same cells infected with recombinant baculovirus encoding hPLD2. The optimal phosphatidylcholine concentration was determined by varying the mol % phosphatidylcholine in phospholipid dispersions, adjusting the final total lipid concentration with PE while maintain-
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FIG. 2. Tissue distribution of hPLD1 and hPLD2 mRNA. Multiple human tissue Northern blots were analyzed as described under “Experimental Procedures.” Each lane contains approximately 2 μg of poly(A)+ RNA. Blots were hybridized with the indicated full-length cDNA or a β-actin probe. The hybridized membrane was exposed to x-ray film for 24 h.

Panel A, proteins (40 μg) obtained from cell lysates of uninfected Hi5 cells (U, lane 2) or cells infected for 3 days with hPLD2 expressing baculovirus (lane 3) were separated by SDS-polyacrylamide gel electrophoresis on an 8% gel and stained with Coomassie Blue. The position of molecular weight markers are shown (lane 1). The 105-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown). Panel B, Western blot analysis of cell lysates, using anti-His4 antibody and an hPLD2-specific antipeptide antiserum. Antibodies were both used in dilutions of 1:100. Panel C, cell lysates (5 μg of protein) were acquired from uninfected Hi5 cell or cells infected with nonrecombinant baculovirus alone (not shown). The 116-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown). The 105-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown). The 105-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown). The 105-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown). The 105-kDa band observed in the hPLD2 lane (arrow) is not present in uninfected Hi5 cells (U) or cells infected with nonrecombinant baculovirus alone (not shown).

Adjusting for this difference, hPLD1 and hPLD2 had comparable basal activities in the presence of optimal PIP2, unexpectedly, its activity was further increased (about 2-fold) by ARF-1 (Fig. 6, right panel). This stimulation showed an EC50 of about 4 μM ARF-1. Activation by ARF-1 required the guanine nucleotide (data not shown), consistent with the function of PLD2 as an effector of ARF-1. Thus, although the response obtained with hPLD2 in the presence of ARF is not as robust as that obtained with hPLD1, the pattern of activation is similar.

To compare the absolute activities of hPLD2 and hPLD1, we expressed both proteins in Hi5 insect cells. Using Coomassie protein staining of the PLD, hPLD2 was expressed at an approximately 1.7-fold higher amount than Myc-tagged hPLD1. Adjusting for this difference, hPLD1 and hPLD2 had comparable basal activities in the presence of optimal PIP2 and PC (hPLD2 basal activity was actually 65% of hPLD1 basal activity, using 0.75 and 1.25 μg of protein, respectively, in the activity assay). Under these conditions, the ARF-stimulated hPLD1 activity was 8-fold higher than the basal hPLD2 activity. We also expressed the mouse PLD2, which has been reported to have a high basal activity (26). Equal amounts of hPLD2 and mPLD2 under these conditions gave similar basal activities.

We also tested RhoA, Rac1, and protein kinase C for their abilities to stimulate PLD activity in insect cell membranes under a variety of assay conditions (e.g., with or without ATP-Mg2+). While these factors all significantly stimulated hPLD1 activity, they had no effect on hPLD2 activity (data not shown).

DISCUSSION

Sequence Analysis—The hPLD2 cDNA cloned from a human B-cell (HSC-93) library shows 90% predicted amino acid sequence identity to rPLD2 and mPLD2, but only 53% identity with hPLD1, identifying it as the human form of PLD2 (see dendrogram in Fig. 1). PLD2s are shorter than PLD1s, and hPLD2 lacks a 116-amino acid region that is present in the middle of hPLD1, although the functional significance of this
difference is unclear. Sequence comparisons of hPLD2 to known PLD isoforms demonstrate that regions I, II, III, and IV, which are conserved across plant, yeast, and mammalian species, are also present in hPLD2 (Fig. 1A). Previous studies have shown that residues within regions I (HHXXKXXVXXDLXXGRXXDXLHXXL) and IV (YVHXXKXXVXXDDXXIGSAXLXXGRA) are conserved across plant and animal PLDs but also in other phosphatidate transferase (cardiolipin synthase and phosphatidylserine synthase), nucleases, and an outer envelope protein from pox viruses (24, 32, 33) that has recently been described as having phospholipase C and A activities (34). Mutation of any of these three residues in either region renders mPLD2 inactive (35), indicating that both regions are essential for PLD activity and probably constitute a single active site. No other domains of interest were obvious.

Tissue Distribution—Northern blots of hPLD2 mRNA reveal
the widespread expression of a 3.5-kb transcript (Fig. 2) in all tissues examined except liver, skeletal muscle, and perhaps testes. The tissue distribution for hPLD2 is similar to that reported for rPLD2 (27) and mPLD2 (26), except that PLD2 message is expressed at significantly higher levels in spleen and thymus in humans compared with rodents. hPLD1 has a much more restricted distribution than hPLD2, with a 6-kb transcript readily detectable in heart, brain, placenta, pancreas, spleen, small intestine, and uterus. Interestingly, the mRNA for hPLD2 but not PLD1 was observed in peripheral blood leukocytes. A PLD activity has been studied extensively in plasma membranes from peripheral blood leukocytes, and while it showed a modest activation by ARF (17), many of its properties differ greatly from those of PLD1. For example, in contrast to PLD1, its major in vitro activators include RhoA and an unidentified 50-kDa cytosolic factor (12, 17).

Our results indicate that in leukocytes, the message for hPLD2 is expressed at a much higher level than that for hPLD1. However, the in vitro properties of the neutrophil enzyme also differ in several important respects from those of recombinant hPLD2. For example, we see no effect on hPLD2 activity of added neutrophil cytosol (which contains both RhoA and the 50-kDa cytosolic factor).2 Thus, it is unclear whether hPLD2 is the isoform responsible for the previously reported PLD activity in this cell type. Of potential interest in this regard, peripheral blood leukocytes express a 3.8-kb transcript in addition to the 3.5-kb transcript that is expressed more widely. This could indicate either more extensive polyadenylation or an alternatively spliced isoform of hPLD2, as was recently described for hPLD1 (15) and rPLD1 (25). It is also interesting to note that significant levels of hPLD1 and hPLD2 mRNAs are coexpressed in many tissues (e.g. heart, brain, placenta, spleen, liver, lung, and intestine). The complex range of activators and regulators of PLD activity that have been seen in some of these tissues such as brain, liver, and lung (36–39) may be due in part to the expression of two or more PLD isoforms in these tissues.

**Basal Activity of the Expressed Protein**—PLD2 from mouse has been previously described as having a high specific activity, 5 μmol/min/mg protein (26), corresponding to a turnover number of 500 min⁻¹. Although hPLD2 expressed and assayed in insect cell membranes demonstrated readily observable basal activity, this activity was relatively low compared with that of a similar quantity of hPLD1 when the latter is activated with ARF-1. mPLD2 activity was reported using enzyme that had been solubilized from the membrane and isolated by immunochromatographic methods. In the present studies, membranes from Hi5 insect cells expressing hPLD2 were used. These membranes have essentially zero background PLD activity and have a potential advantage in that endogenous unknown protein or lipid factors in the membrane may contribute to modulating PLD activity and may therefore be important for observing the effects of regulatory factors. The difference in basal activities does not appear to be a result of species differences, since we have expressed mPLD2 in Hi5 cells and find its basal activity in membranes from these cells similar to that of hPLD2 when activity is corrected for minor differences in expression levels. The activity of rat PLD2 has also been reported to be relatively low (27). Thus, differences in basal activity between the mouse and the human enzymes are likely to be due to removal of inhibitory factors from the mouse enzyme upon isolation. Consistent with this interpretation, uncharacterized inhibitors of mPLD2 have been described (26), and fodrin (40), clathrin assembly protein 3 (41), and an unidentified 30-kDa protein (42) and synaptotagmin (43) reportedly inhibit PLD1. (The latter appears to inhibit PLD activity by hydrolyzing the phosphates from the cofactor PIP2 (44)).

**ARF Regulation of hPLD2**—The most surprising result from the present studies is that ARF-1 stimulates PLD2 activity. ARF was first described as an activator of cholera toxin-induced ADP-ribosylation of Gα (45, 46) and was subsequently found to play an essential role in membrane trafficking in Golgi related to recruitment of coatamer from the cytosol to budding Golgi membrane (47, 48). Pioneering studies conducted by the groups of Sternweis (9) and Cockcroft (10) showed that PLD activity in brain membranes and permeabilized HL-60 cells was stimulated by ARF. More recently, Hammond et al. (15) showed that ARF activates purified recombinant hPLD1. Phosphatidic acid generated from PLD1 has been implicated as the factor that regulates coatamer assembly and membrane trafficking (49, 50), although diacylglycerol, which can be generated indirectly by hydrolysis of phosphatidic acid, has also been implicated (51). The localization of PLD1 in Golgi membranes (26, 52) is consistent with this proposed function. The widespread cellular distribution of ARF within cells, including plasma membrane, mitochondria, and endoplasmic reticulum⁴ implies that ARF may have effector functions in membranes other than Golgi. In this regard, we have shown that ARF stimulates PLD activity in purified neutrophil plasma membranes cooperatively with RhoA and an unidentified 50-kDa cytosolic factor (17). It may be significant that transfected mPLD2 (Myc-tagged) localizes to the plasma membrane in REF-52 cells (26).

The present studies report for the first time that ARF activates PLD2. Using the hPLD2 expressed in insect cell membranes, ARF-1 was able to increase the activity of hPLD2 expressed in insect cells about 2-fold over basal activity. This compares with at least a 20-fold effect of ARF on hPLD1 expressed in the same membranes and assayed under the same conditions. This relatively smaller effect of ARF on PLD2 may imply that additional regulatory factors or processes are needed for optimal activation of hPLD2, and these may include both inhibitory and stimulatory factors. Nevertheless, an ARF effect on hPLD2 activity may imply the presence of an ARF effector site on PLD2. Studies aimed at identifying an ARF binding site on hPLD2 and hPLD1 are in progress. Until such factors are identified and reconstituted in a cell-free system, the mechanism of ARF activation cannot be addressed directly for PLD2.

In conclusion, we have cloned and expressed the human PLD2 enzyme and demonstrated its regulation by ARF. This PLD isoform was found to be widely expressed in a variety of human tissues. The cloning of hPLD2 will allow further characterization of the regulation and enhance the understanding of the role these enzymes play in cellular signaling pathways.

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**REFERENCES**


² I. Lopez, R. S. Arnold, and J. D. Lambeth, unpublished observations.

³ R. A. Kahn, personal communication.
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