Human ADP-ribosylation Factor-activated Phosphatidylcholine-specific Phospholipase D Defines a New and Highly Conserved Gene Family*

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EXPERIMENTAL PROCEDURES

Molecular Isolation of hPLD1—The full-length hPLD1 cDNA was obtained from a x Zap II HeLa cDNA library (Stratagene) as described in the text. Of the nine hPLD1 cDNAs sequenced (Sequenase version 2.0, U.S. Biochemical Corp.; a final sequence was determined from both strands), four represented mRNAs that terminated prematurely and five extended past the putative amino terminus by 48–96 nucleotides. The presumed initiator methionine conforms to the eukaryotic consensus sequence and is the first in-frame methionine in the 5'-untranslated region. The coding region is additionally thought to be full length since a recog-
PLD1, a PC-specific Phospholipase D Gene Activated by ARF

RESULTS

Cloning of a Human PC-specific PLD cDNA—To identify cDNAs encoding PC-specific PLD activity, we took advantage of a screen that had uncovered a yeast PC-specific PLD gene (SPO14 (15)), which in turn identified a GenBank human-expressed sequence tag (EST) encoding a significantly similar peptide sequence. A hybridization probe was generated from random-primed HeLa cDNA encoding polymerase chain reaction and primers specific to the EST and used to screen a HeLa cDNAlibrary. Sequence analysis of hybridizing clones revealed a member of a novel but highly conserved gene family (Fig. 1).
PLD, a PC-specific Phospholipase D Gene Activated by ARF

enzyme (castor bean (18)); the remainder constitutes ESTs or hypothetical open reading frames. A comparison of the related sequences reveals the location of several blocks of highly conserved amino acids, one or more of which might constitute critical portions of the catalytic or cofactor binding sites. Two regions in particular (amino acids 455–490 and 892–926) are highly conserved in all of the PLD1-related genes and contain an invariant charged motif, HXXXXXXD. PLD is thought to be intracellular and membrane-associated (not an integral membrane protein). Consistent with this prediction, PLD encodes neither a signal peptide nor a hydrophobic transmembrane sequence.

hPLD1 Activity—To investigate its catalytic properties and activation requirements, hPLD1 was expressed in Sf9 insect cells using baculovirus and in COS-7 cells. A recombinant protein of approximately 120 kDa was observed (Fig. 2A and data not shown), matching closely the theoretical size of 124 kDa and suggesting that little if any post-translational processing occurs. By definition, PLD catalyzes the hydrolysis of PC to PA and choline. To determine the activity encoded by recombinant hPLD1, control and hPLD1-encoding baculovirus-infected Sf9 cells were assessed using a standard headgroup release assay that measures the amount of tritiated headgroup (e.g. [3H]choline) liberated by hydrolysis of the labeled substrate [3H]PC (Fig. 2B). In lanes 1 and 3, modest levels of endogenous PLD activity are observed in control Sf9 cells (either uninfected or data not shown) infected with an irrelevant construct, the majority of which (94%) is membrane-associated. In contrast, Sf9 cells infected with hPLD1-encoding baculovirus exhibit substantial cytosolic (32-fold above control) and membrane-associated (15-fold above control) PLD activity (lanes 2 and 4). To confirm that hPLD1 encodes a PLD activity, cation-exchange HPLC was used to analyze the water-soluble product(s) which co-eluted with a labeled choline standard (Fig. 2C), and thin layer chromatography was used to demonstrate concurrent production of PA (Fig. 2D, lane 1). Both membrane-associated and cytosolic PLD activities have been described in mammalian tissues and cell lines (2); it has been suggested that the membrane-associated and cytosolic PLD activities have distinct biochemical properties and thus might derive from different gene products. In contrast, we demonstrate here that a recombinant PLD activity derived from a single gene product is located both in the cytoplasm and in association with the membrane. This observation suggests instead that hPLD1 can exist as a stable soluble protein and that controlled interaction with substrate-containing phospholipid surfaces may be a physiologically important mode of regulation for this enzyme, as has been shown for phospholipase A2 and PLC (19).

hPLD1 Selectivity—In addition to PC, PLD activities capable of hydrolyzing both PI and PE have been reported, and the issue of whether a single gene product mediates one or multiple activities has remained unresolved. To assess hPLD1’s substrate selectivity, the standard assay was carried out using [32P]PE and [32P]PI (Fig. 2D, lanes 3 and 5). The results revealed that hPLD1 is unable to hydrolyze PE or PI. All PLD activities described to date also function as transphosphatidylases in the presence of primary alcohols, catalyzing the transfer of the phosphatidyl group from an appropriate substrate to the alcohol and thus generating phosphatidyl alcohol (20). To determine whether hPLD1 was capable of transphosphatidylation activity, EtOH was added to the reaction mixture and the products analyzed by TLC (Fig. 2D, lane 2). The results demonstrated that hPLD1 catalyzes the formation of [32P]phosphatidylethanol when presented with [32P]PC. Since PC-specific PLD is the only enzyme capable of catalyzing this particular reaction, we conclude that hPLD1 must be a PC-specific PLD.

Requirements for hPLD1 Activation—Two distinct rat brain PLD activities have been previously reported: one that is activated by PIP2 and ARF but inhibited by oleate and a second that is insensitive to PIP2 and ARF but requires oleate (6). Analysis of hPLD1 demonstrates that it is activated by PIP2 and strongly inhibited by oleate (Fig. 3, A and B). The stimulation by PIP2 (11-fold) is comparable in both magnitude and concentration dependence with effects reported previously for endogenous PLD activities (6, 9, 21). Similarly, the inhibition by oleate is comparable with that previously reported for a rat brain ARF-stimulated PLD activity (6).

Numerous reports have implicated monomeric G-proteins as
reason that related but different properties have been reported. The existence of at least two human genes suggests that at least one another part of the reported human activity. Moreover, the data not shown), presenting an immediate possibility for some of the other PLD activities that have been described are present in cell ex- properties that clearly implicate it as being responsible for at least part of the PLD activity previously observed in cell ex.

FIG. 3. Effects of PIP<sub>2</sub>, olate, and ARF on hPLD1 activity. PLD activity associated with membranes from Sf9 cells infected with the hPLD1 baculovirus was determined under standard assay conditions except that (A) the PIP<sub>2</sub> content of the vesicles was varied or (B) varying amounts of sodium olate or (C) varying amounts of ARF<sub>1</sub> were added to the incubations. 50 μM GTP-γ-S was included in all assays containing exogenously added ARF<sub>1</sub>. The data shown are means ± S.E. of triplicate determinations. Standard errors fell within the symbols for some data points. ~0.1 μg of membrane protein was present in each sample. hARF-1, human ARF1. D, ARF1 activation of hPLD1 expressed in mammalian cells. COS-7 cells were transfected using Lipofectamine (Life Technologies, Inc.) with 3 μg of LacZ or hPLD1 driven by a cytomegalovirus promoter and assayed after 48 h for PLD activity in the presence or absence or exogenously supplied ARF1 and GTP-γ-S.

regulators of PLD activities in a variety of mammalian cells and tissues (2, 4–6). We found that recombinant human ARF1 strongly activates hPLD1 expressed in both baculovirus or mammalian cells (Fig. 3, C and D) and does so with a magnitude comparable with that previously reported.

FIG. 3.

DISCUSSION

PLD activities have been detected in essentially all organisms (2). Mammalian PLD activities in many tissues and cell lines have been studied in detail, although no clear biochemical classification of their properties has emerged. Moreover, attempts to devise such a classification have been complicated by reports of activities that differ in their subcellular localization, metal ion dependence, phospholipid and detergent require- ments in exogenous substrate assays, and activation by various G-proteins and unidentified accessory proteins (2). Failure to isolate any of the enzymes in sufficient purity has left this matter unresolved. The PLD enzyme we have identified has properties that clearly implicate it as being responsible for at least part of the PLD activity previously observed in cell extracts and partially purified preparations. It is possible that some of the other PLD activities that have been described are mediated by additional members of the hPLD1 gene family. We have identified a second mammalian PLD gene (~60% identi- cal; data not shown), presenting an immediate possibility for another part of the reported human activity. Moreover, the existence of at least two human genes suggests that at least one reason that related but different properties have been reported for PLD has been that the activity obtained from different cell lines or tissues or using different purification techniques is actually composed of different mixtures of at least two distinct gene products that may have different biochemical properties and/or requirements for activation.

ARF-activated PLD is present in Golgi vesicles (22). The demonstration that hPLD1 is activated by ARF<sub>1</sub> lends support to the hypothesis that PLD and specifically hPLD1 are involved in intravesicular membrane trafficking. Previous reports using partially purified PLD had raised the possibility that PLD and ARF interact directly (6, 9). Our results extend previous efforts by using a single recombinant, purified protein as a PLD source, as well as recombinant, purified ARF as the activator. The data strengthen the hypothesis that interaction between ARF and hPLD1 is direct, although rigorous proof will require further experiments. Preliminary results suggest that at least some members of the RhoRac family of small G-proteins acti- vate hPLD1 as well (data not shown). It will be important to determine which of the numerous small G-proteins reported to activate endogenous PLD actually activate hPLD1 to a significant extent.

Preliminary in vivo expression studies of PLD1 (the second mammalian gene) in mouse embryos indicate that expression is detected at high levels in different selected regions of the brain and spinal cord (data not shown). These results raise the possibility that the PLD genes may also play a role in selected signal transduction events. In addition, there may be other members of the mammalian PLD gene family that are as of yet unidentified. The work presented in this paper paves the way for a molecular definition of the PLD enzymes. Ultimately, this advance should provide essential information for future studies designed to reveal the cellular and physiological function of these enzymes and their products.

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