Identity between the Ca\(^{2+}\)-independent Phospholipase A\(_2\) Enzymes from P388D\(_1\) Macrophages and Chinese Hamster Ovary Cells*  

(Received for publication, October 16, 1996, and in revised form, January 15, 1997)

María A. Balboa‡, Jesús Balsinde‡, Simon S. Jones§, and Edward A. Dennis‡¶

From the ‡Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093-0601 and the §Genetics Institute, Cambridge, Massachusetts 02140

A novel Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)) has recently been purified and characterized from P388D\(_1\), macrophages (Ackermann, E. J., Kempner, E. S., and Dennis, E. A. (1994) J. Biol. Chem. 269, 9227–9233). This enzyme appears to play a key role in regulating basal phospholipid remodeling reactions. Also an iPLA\(_2\) from Chinese hamster ovary (CHO) cells has been purified, molecularly cloned, and expressed (Tang, J., Kriz, R., Wolfman, N., Shaffer, M., Seehra, J., and Jones, S. S. (1997) J. Biol. Chem. 272, 8567–8575). We report herein that the cloned CHO iPLA\(_2\) is equivalent to the mouse enzyme purified from P388D\(_1\) cells. Polymerase chain reaction amplification of cDNA fragments from P388D\(_1\) cells using primers based on the CHO iPLA\(_2\) sequence, revealed a high degree of homology between the mouse and hamster enzymes at both the nucleotide and amino acid levels (92 and 95%, respectively). Identity between the two proteins was further demonstrated by using immunochemical, pharmacological, and biochemical approaches. Thus, an antisera generated against the CHO enzyme recognized the P388D\(_1\) cell enzyme and gave similar molecular masses (about 83 kDa) for the two enzymes under the same experimental conditions. Further, the CHO enzyme has exactly the same sensitivity to inhibition by a variety of compounds previously shown to inhibit the P388D\(_1\) enzyme, including bromoethyl lactone, palmitoyl trifluoromethyl ketone, and methyl arachidonyl fluorophosphonate. Additionally, covalent modification of the CHO enzyme by \(^{3}H\)bromoethyl lactone is dependent on active enzyme as is the P388D\(_1\), iPLA\(_2\). Finally, both enzymes have the same specific activities under identical experimental conditions.

Phospholipase A\(_2\) (PLA\(_2\)) consists of a superfamily of enzymes that regulate phospholipid metabolism and generate bioactive lipid mediators such as the eicosanoids and platelet-activating factor (for review see Ref. 1). Since the PLA\(_{2}\)s have been implicated in a number of tissue dysfunctions ranging from inflammation to ischemia, much attention has been devoted to the study of the mechanism of action and biochemical characteristics of these enzymes. Depending on their site of action, the PLA\(_{2}\)s can be subdivided into two types: the extracellular, secreted enzymes and the intracellular, cytoplasmic enzymes (1). Among the latter, two groups of enzymes can be considered, namely the group IV, Ca\(^{2+}\)-dependent cytosolic PLA\(_2\), and the Ca\(^{2+}\)-independent PLA\(_2\) (iPLA\(_2\)).

In contrast to the group I–IV PLA\(_{2}\)s, the Ca\(^{2+}\)-independent PLA\(_{2}\)s (iPLA\(_{2}\)) have been poorly studied, since most of them are labile, constitute only a minor fraction of the total cellular protein, and have lower specific activities. The iPLA\(_{2}\)s have been grouped into three main categories based on their biochemical and localization characteristics: lysosomal, brush-border membranes, and intracellular (for review, see Ref. 2). The lysosomal and brush-border membrane iPLA\(_{2}\)s appear to be conserved among the species where they have been identified. However, the intracellular iPLA\(_{2}\) enzymes appear to represent a much more diverse and broad group of enzymes, whose relationship is not so evident. Only four intracellular iPLA\(_{2}\)s have been purified to homogeneity, namely a 40-kDa enzyme from myocardium (3), a 39-kDa enzyme from bovine brain (4), an 80-kDa enzyme from P388D\(_1\), macrophages (5), and very recently a 28-kDa enzyme from rabbit kidney (6). The enzymes from myocardium and P388D\(_1\) cells are both modulated by ATP and form oligomeric complexes of about 400 kDa. However, besides their very distinct molecular mass, they also differ significantly in substrate preference and detergent sensitivity (3, 5).

The first molecular cloning of an iPLA\(_{2}\) from CHO cells is reported in the accompanying manuscript (7). Due to the role of iPLA\(_{2}\) in P388D\(_1\) cell metabolism (8), it was essential to determine whether or not the enzyme present in these cells was the same as that cloned by Tang et al. (7). Using a variety of techniques, we demonstrate herein that the enzyme from P388D\(_1\) cells and the cloned enzyme from CHO cells is the same molecular entity expressed in different species. Thus, the availability of the macrophage iPLA\(_{2}\) sequence should ensure rapid progress in understanding its physiological function.

**EXPERIMENTAL PROCEDURES**

Materials—1-Palmitoyl-2-\(^{14}\)Cpalmitoyl-sn-glycero-3-phosphocholine was obtained from Amersham Corp. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine was purchased from Avanti (Birmingham, AL). \((E)\)-6-(bromomethylene)-tetrahydro-3-(4-[\(^{3}H\)naphthalenyl]-2H-pyran-2-one (\([\(^{3}H\) ]BEFL) was a generous gift of Drs. Randy H. Weiss and Philip Needleman (Monsanto Co., St. Louis, MO). Palmitoyl trifluoromethyl ketone (PACOCF\(_{3}\)) and BEL were synthesized in our laboratory as described previously (9). Methyl arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical Co. (Ann Arbor, MI).

**iPLA\(_{2}\)** Assay—The iPLA\(_{2}\) activity has been previously described (5). Briefly, 10–50 ng of purified iPLA\(_{2}\) was assayed in a buffer consisting of 100 mM Heps, 400 µM Triton X-100, 100 µM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1-palmitoyl-2-\(^{14}\)Cpalmitoyl-sn-glycero-3-phosphocholine (200,000 cpm), 5 mM EDTA, and 0.1 mM ATP (pH 7.5). The
mixture was incubated at 40 °C for 30 min with shaking, the reaction was stopped by adding 2.5 ml of Dole reagent (2-propanol, heptane, 0.5 M H2SO4; 400:100:20 (v/v/v)), and products were processed by a modified Dole extraction procedure (10). When the irreversible inhibitor BEL, MAFP, or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was used in the assays, the enzyme was preincubated with the inhibitor for 5 min at 40 °C. The remainder of enzyme activity was assayed by adding the substrate mixture. When the reversible inhibitor PACOCF3 was used, it was added directly to the assay mixture.

Immunoblot—Purified proteins were analyzed by a 10% SDS-polyacrylamide gel electrophoresis (Novex), transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp.). Nonspecific binding was blocked by incubating the membranes with a buffer consisting of 5% nonfat milk, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100 for 60 min. Membranes were incubated with the antiserum generated against the CHO iPLA2 (1:200 into the blocking buffer) for 30 min at 40 °C. After washing out the excess of [3H]BEL, proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% gel.
acetic acid and incubated for 30 min with a fluorographic reagent (Amplify, Amersham). Bands were visualized by autoradiography (9). When MAFP was used to prevent [3H]BEL binding, purified enzyme was preincubated with 20 μM MAFP for 15 min at 40 °C prior to the addition of [3H]BEL.

![Fig. 6](image)

**Comparison between the nucleotide (A) and amino acid (B) sequences of mouse and hamster iPLA2.** A colon denotes nucleotides or amino acids that are identical; a dot denotes a conservative amino acid change.

---

FIG. 6 Comparison between the nucleotide (A) and amino acid (B) sequences of mouse and hamster iPLA2. A colon denotes nucleotides or amino acids that are identical; a dot denotes a conservative amino acid change.
were based on the CHO iPLA2 sequence (Ref. 7; GenBankTM accession number I15470). The 5'-end (primer region), we performed a standard rapid amplification of cDNA ends-PCR (9). Briefly, cDNA was amplified by PCR using a dT(18)-primer used (AGG ATG CAG TTC TTC GGA GGA A, which is equivalent to nucleotides 205–226 in the hamster sequence). After attaching a poly(A) tail to the 5'-end of the PCR product obtained, amplification of this product was accomplished by PCR using a dT(18)-primer and a mouse reverse primer, ACT CCA GTT GGA AGA GCC GGA A, which is equivalent to nucleotides 598–619 in the hamster sequence. This is the same molecular size as that obtained for the CHO iPLA2 enzyme, since the inhibitory agent is generated in situ from BEL by the hydrolytic action of the enzyme on the lactone ring (10). Indeed, in previous studies with the P388D1 iPLA2, DTNB completely inactivated the CHO iPLA2 (Fig. 4) and prevented labeling of the enzyme with [3H]BEL (10). Although DTNB also inhibits the CHO iPLA2 (Fig. 4), we have employed herein the irreversible inhibitor MAFP (11), because it is a much more specific reagent than DTNB. MAFP (20 μM) completely inactivated the CHO iPLA2, as shown by immunoblot (Fig. 1). Since BEL is an irreversible inhibitor, it was possible to label the macrophage iPLA2 by incubation with [3H]BEL (10). However, labeling with BEL required the presence of active enzyme, since the inhibitory agent is generated in situ from BEL by the hydrolytic action of the enzyme on the lactone ring (10). Indeed, in previous studies with the P388D1, iPLA2, DTNB prevented labeling of the enzyme with [3H]BEL (10). Although DTNB also inhibits the CHO iPLA2 (Fig. 4) and prevented labeling of the enzyme with [3H]BEL (Fig. 5). Moreover, in the absence of MAFP treatment, autoradiographic analysis of [3H]BEL-treated enzyme revealed a single spot at about 85 kDa. This is the same molecular size as that obtained for the P388D1 iPLA2 utilizing a similar strategy (10) as well as that found by immunoblot (Fig. 1).

The nucleotide sequence of the P388D1 iPLA2, as analyzed by poly(A) tail to the 5'-end of the PCR product obtained, amplification of this product was accomplished by PCR using a dT(18)-primer and a mouse reverse primer, ACT CCA GTT GGA AGA GCC GGA A, which is equivalent to nucleotides 205–226 in the hamster sequence. A final round of amplification was performed using the mouse reverse primer TCT CCA GAG AGC CCT GAT GCA CCT G, which is equivalent to nucleotides 598–619 in the hamster sequence. After spraying a poly(A) tail to the 5'-end of the PCR product obtained, amplification of this product was accomplished by PCR using a dT(18)-primer and a mouse reverse primer, ACT CCA GTT GGA AGA GCC GGA A, which is equivalent to nucleotides 205–226 in the hamster sequence. A final round of amplification was performed using the mouse reverse primer TTT CTC CCG GAC ACG TTC ACT T (equivalent to nucleotides 101–123 in the hamster sequence). PCR conditions were as follows: denaturing, 95 °C for 30 s; annealing, 55 °C for 45 s; extension, 72 °C for 120 s; 40 cycles. A last extension step lasted 10 min. PCR products were purified by agarose gel electrophoresis and sequenced by automatic DNA cycle sequencing (Applied Biosystems 373 automated DNA sequencer, Perkin-Elmer).

RESULTS AND DISCUSSION

The intracellular iPLA2 from P388D1 cells was purified and characterized in our laboratory as an approximately 80-kDa protein (5). The iPLA2 purified by Tang et al. (7) from CHO cells and the protein expressed by the cDNA have a molecular size of 84.5 kDa. Besides this similarity, the purification strategy used by Tang et al. (7) contains some analogous steps to those used for the macrophage iPLA2 (5), and the enzymatic profile of the CHO enzyme is comparable. Therefore, we hypothesized that both proteins would share a high degree of similarity if not identity. We began to address this hypothesis by taking advantage of the polyclonal antibody generated by Tang et al. (7) against the C-terminal portion of the CHO protein. The antiserum recognized pure iPLA2 from P388D1 cells, as shown by immunoblot (Fig. 1, lane 2). Purified CHO iPLA2 was run in parallel as a control and showed identical mobility, focusing both proteins in the 80–85-kDa molecular mass range (Fig. 1, lane 1).

To further establish a similarity between the two proteins, a series of inhibition studies were conducted. We have previously shown that the iPLA2, from P388D1 cells is potently and irreversibly inhibited by bromoeno lactone (BEL). After a preincubation time of 5 min at 40 °C, BEL inhibits pure iPLA2 with an IC50 of about 60 nM (10). Likewise, iPLA2 from CHO cells was inhibited in a dose-dependent manner by BEL with an IC50 of 120 nM (Fig. 2A). This inhibition was irreversible even after exhaustive dialysis of treated enzyme compared with control (Fig. 2B). Also, P388D1 iPLA2 is reversibly inhibited by PACOCF3 (IC50 = 4 μM), and again CHO iPLA2 was inhibited by the same reagent with an IC50 of 3 μM (Fig. 3).

Since BEL is an irreversible inhibitor, it was possible to label the macrophage iPLA2 by incubation with [3H]BEL (10). However, labeling with BEL required the presence of active enzyme, since the inhibitory agent is generated in situ from BEL by the hydrolytic action of the enzyme on the lactone ring (10). Indeed, in previous studies with the P388D1, iPLA2, DTNB prevented labeling of the enzyme with [3H]BEL (10). Although DTNB also inhibits the CHO iPLA2 (Fig. 4), we have employed herein the irreversible inhibitor MAFP (11), because it is a much more specific reagent than DTNB. MAFP (20 μM) completely inactivated the CHO iPLA2, as shown by immunoblot (Fig. 1).
pects of cell physiology, such as new membrane synthesis that allows cell proliferation, or fatty acid exchange within phospholipids that allows adaptive homeostatic changes. Although the discovery by Gross and co-workers (14) that some iPLA_2_s are potently and selectively inhibited by BEL has accelerated research on this enzyme, study of the iPLA_2 is hampered by the fact that it is extremely difficult to obtain sufficient amounts of pure protein for biochemical and sequence analysis.

We have presented evidence herein that the enzyme purified and cloned by Tang et al. (7) is the equivalent in hamster to the enzyme we identified (15), purified (5), characterized (5, 10, 11), and studied at the cellular level (8, 12) in mouse P388D_1 macrophages. By using a wide variety of approaches, we have found biochemical, immunological, pharmacological, and genetic similarities between the two proteins, strongly suggesting that the same molecular entity is expressed in different species. There is, however, an apparent difference between the two enzymes concerning ATP sensitivity. Tang et al. (7) failed to observe any ATP effect on a partially purified preparation of CHO iPLA_2. We have confirmed this finding using the assay system employed for the P388D_1 iPLA_2. We have previously reported on the apparent activation of P388D_1 iPLA_2 by ATP (5). The effect was shown to depend on the presence of Triton X-100 in the assay system. This, along with the fact that several other nucleotides manifested the same effect (i.e., ADP, UTP, GTP) raised the possibility that it might not be relevant as a regulatory mechanism. We have now data with P388D_1 iPLA_2 showing that, rather than stimulating the iPLA_2, the ATP stabilizes the enzyme and protects it from denaturation; hence, higher activity is found in the presence than in the absence of ATP. Considering the fact that the two proteins have slightly different amino acid sequences and purification schemes, it is possible that the CHO enzyme lacks the region with which ATP interacts. Alternatively, it is possible that the CHO enzyme is more resistant to denaturation than the P388D_1 enzyme.

The availability of the iPLA_2 cDNA and protein sequence of the 80-kDa iPLA_2, which is now classified as a Group VI PLA_2 (16), will allow new experimental avenues to be explored in defining the role(s) of iPLA_2 in phospholipid metabolism and cellular function.

Acknowledgments—We thank Richard Loo (University of California at San Diego) and Jin Tang (Genetics Institute) for providing pure iPLA_2 from P388D_1 macrophages and CHO cells, respectively.

REFERENCES
1. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
2. Ackermann, E. J., and Dennis, E. A. (1995) Biochim. Biophys. Acta 1259, 125–136
3. Hazen, S. L., and Gross, R. W. (1991) Biochem. J. 280, 581–587
4. Hirashima, Y., Farooqui, A. A., Mills, J. S., and Horrocks, L. A. (1992) J. Neurochem. 59, 708–714
5. Ackermann, E. J., Kempner, E. S., and Dennis, E. A. (1994) J. Biol. Chem. 269, 9227–9233
6. Portilla, D., and Dai, G. (1996) J. Biol. Chem. 271, 15451–15457
7. Tang, J., Kriz, R., Wolfman, N., Shaffer, M., Seehra, J., and Jones, S. S. (1997) J. Biol. Chem. 272, 8567–8575
8. Balsinde, J., Bianco I., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8527–8531
9. Frohman, M. A., Dush, M. K., and Martin, G. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8998–9002
10. Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) J. Biol. Chem. 270, 445–450
11. Lio, Y-C., Reynolds, L. J., Balsinde, J., and Dennis, E. A. (1996) Biochim. Biophys. Acta 1302, 55–69
12. Balsinde, J., and Dennis, E. A. (1996) J. Biol. Chem. 271, 6758–6765
13. Ma, Z., Ramanathad, S., Corbett J. A., Bohrer, A., Gross R. W., McDaniel, M. L., and Turk J. (1996) J. Biol. Chem. 271, 1029–1042
14. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) J. Biol. Chem. 266, 7227–7232
15. Ross, M. L., Deems, R. A., Jesaitis, A. J., Dennis, E. A., and Ulevitch, R. J. (1985) Arch. Biochem. Biophys. 238, 247–258
16. Dennis, E. A. (1997) Trends Biochem. Sci. 22, 1–2

---

2 Y. C. Lio and E. A. Dennis, manuscript in preparation.