Vol. 183, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1992 Pages 121-127

CYTOSOLIC PHOSPHOLIPASE A_2 FROM U937 CELLS : SIZE OF THE FUNCTIONAL ENZYME BY RADIATION INACTIVATION

Nathalie M. Tremblay[‡], Donald Nicholson[‡], Michel Potier[§] and Philip K. Weech[‡*]

[‡]Merck Frosst Centre for Therapeutic Research, Merck Frosst Canada Inc., PO Box 1005, Pointe-Claire - Dorval, Québec, Canada H2W 4P8

[§]Section de Génétique Médicale, Hôpital Sainte-Justine, 3175 Chemin Sainte Catherine, Montréal, Canada H3T 1C5

Received December 26, 1991

We have studied the cytosolic phospholipase A_2 ($cPLA_2$) of human U937 cells by radiation inactivation in order to characterize the functional form of the native enzyme by a method that was independent of the discrepancies observed by SDS-PAGE and cDNA cloning. The Radiation Inactivation Size of $cPLA_2$ was reproducible and gave a value of 76,800-80,100 daltons. We eluted the active enzyme from polyacrylamide-gradient gel electrophoresis at a molecular weight of 77,000, confirming the irradiation result. We conclude that $cPLA_2$ is active as the monomeric enzyme and is composed of a single major functional domain that is sensitive to irradiation. © 1992 Academic Press, Inc.

Interest in cytosolic phospholipase A_2 ($cPLA_2$) stems from the search for an enzyme that shows selectivity for releasing arachidonic acid in preference to other fatty acids in the cell. The $cPLA_2$ from cultured human U937 cells selectively hydrolyses phospholipids containing an arachidonyl ester and, thus, this enzyme could be responsible for the release of arachidonic acid from cell membrane phospholipids in vivo. In resting cells this is the rate-limiting step in the biosynthesis of eicosanoids important in inflammatory disorders [1]. The partial characterization of $cPLA_2$ isolated from cultured human U937 cells was described by three groups [2-4] and two of these groups

Abbreviations:

RIS: Radiation Inactivation Size; D_{37}: the radiation dose, in MRads, that decreases the enzyme activity to 37% of the control activity; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PAGGE: polyacrylamide gradient gel electrophoresis.

*To whom correspondence should be addressed.

0006-291X/92 $1.50
Copyright © 1992 by Academic Press, Inc.
All rights of reproduction in any form reserved.

subsequently reported cloning its cDNA and expressing a high molecular weight enzyme [5,6].

In the first reports there was ambiguity in the exact molecular weight of this PLA_2, with estimates of 56,000 [2], 100,000 [4] 110,000 [3] by SDS-PAGE, and 68,000 [2] 70,000 [4] and 100,000 [3] by gel filtration . The molecular cloning and expression of the $cPLA_2$ cDNA showed that this enzyme has a greater molecular weight than the secreted human PLA_2 (Mr 14,000) [7-9] and bears no apparent sequence homology to the latter. Nevertheless, there is no published evidence explaining the discrepancy between the molecular weight inferred from the cDNA sequence i.e. Mr 85,200, and the earlier reports from the same groups of Mr 110,000 and 100,000 [3,4]. Some secreted PLA_2 have been reported to be active as dimers [10-12]. We have determined the Radiation Inactivation Size (RIS) [13] of partially purified $cPLA_2$ in order to obtain a direct and independent estimate of the size of the functional unit of this enzyme. We confirmed the molecular size by eluting active enzyme from polyacrylamide-gradient gel electrophoresis.

MATERIALS AND METHODS

Cells and Isolation of PLA_2

U937 cells [14] were obtained from the American Type Culture Collection (Rockville, Maryland) and grown in suspension using RPMI 1640 medium (0.03% w/v L-glutamine, 0.2% w/v $NaHCO_3$) supplemented with 10% fetal calf serum (not heat-inactivated), Penicillin 50 U/ml, Streptomycin 50 µg/ml, and 1.3% dimethyl sulfoxide (v/v) (J. T. Baker, Phillipsburg, NJ). Cells were seeded at 1-1.2 x 10^5 cells/ml into Bellco stirred vessels containing 8 l of medium and allowed to grow for a minimum of 4 days to 6-8 x 10^5 cells/ml. Sixty four litres of culture were used to isolate each preparation of PLA_2. Cells were pelleted by centrifugation (600 x g, 15 min), resuspended to 10^8 cells/ml in phosphate buffered saline pH 7.4 containing 2 mM EDTA and 1 mM PMSF, and lysed by Dounce homogenization and nitrogen cavitation (800 psi, 15 min). The cytosol was isolated as the supernatant of sequential centrifugations at 1,000 x g for 15 min, 10,000 x g for 20 min, and 100,000 x g for 60 min.

PLA_2 was partially purified from the cytosol by sequential chromatography as follows. Two volumes of 30% $(NH_4)SO_4$ was added to the cytosol, stirred overnight at 4°C, and centrifuged at 330 x g for 20 min. The supernatant was incubated for about 3h at 4°C with Phenyl-Sepharose (Pharmacia, 250 ml) equilibrated with 10 mM Tris-HCl, pH 8.0, 10% glycerol, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 0.02 % w/v NaN_3 (buffer A) containing 20% $(NH_4)SO_4$. The gel was washed with the equilibration buffer

and then PLA_2 was eluted with buffer A containing 30% ethylene glycol. The active fractions were pooled, adjusted to 100 mM NaCl, pH 7.4 and loaded onto a DEAE-Sepharose column (Pharmacia, 5 x 2 cm) equilibrated with buffer A containing 100 mM NaCl, pH 7.4 and then washed with the latter buffer. PLA_2 was eluted with the same buffer containing 1 M NaCl. This fraction was applied to a column of Sepharose 6B (Pharmacia, 2.6 x 100 cm) equilibrated with buffer A containing 20 mM Tris and 100 mM KCl in place of NaCl. The active fractions were pooled and applied to two columns in series: Heparin-Sepharose (Pharmacia, 1 x 10 cm) and Polypore-DEAE (2.1 x 100 mm, Applied Biosystems Inc., Foster City, CA) equilibrated with buffer A containing 20 mM Tris and 100 mM KCl in place of NaCl. The Heparin-Sepharose column was disconnected and the Polypore-DEAE column was eluted with a linear gradient from 100 mM to 1 M KCl in its equilibration buffer. Pooled active fractions were used for the experiments below.

PLA_2 Assay

A substrate containing equimolar concentrations (1.5 μM) of phosphatidylethanolamine and phosphatidylcholine was prepared as follows. 1 μCi 1-hexadecyl-2-[^{3}H]-arachidonyl-phosphatidylcholine (10 μl, NET-981, New England Nuclear), and 1 μCi 1-palmitoyl-2-[^{14}C]-arachidonyl-phosphatidylethanolamine (20 μl, NEC-783, New England Nuclear), was mixed with 14.4 μg egg yolk phosphatidylcholine (1 mg/ml ethanol, Sigma) or 14.4 μg 1-palmitoyl-2-arachidonyl-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL). This mixture was diluted with 2.5 ml 50 mM sodium borate pH 9.0, 5 mM $CaCl_2$ (buffer B) and vortexed or sonicated (1-palmitoyl-2-arachidonyl-phosphatidylcholine). The incubation mixture contained 50 μl substrate, with enzyme and buffer B to 250 μl. The reaction was initiated by the addition of substrate and incubated at 37°C for 15 min. The reaction was stopped and fatty acid extracted [15]. 750 μl of the heptane phase was passed through a column of 300 mg BioSil A silicic acid (BioRad) in disposable columns (Quick-Sep, ISO-LAB, Akron, OH) equilibrated with heptane, and eluted with 1 ml diethyl ether. The heptane and ether eluates were combined and radioactivity was measured by scintillation counting with 7.5 ml Econofluor-2 (DuPont New England Nuclear).

Radiation Inactivation Analysis

PLA_2 fractions were desalted through NAP-5 columns (Pharmacia) equilibrated with 100 mM NH_4HCO_3, 1 mM EDTA, 0.02% NaN_3, 0.05% bovine serum albumin w/v. A 25 fold weight excess of trehalose over PLA_2 fraction was added and the mixture was dried in aliquots in Eppendorf tubes in a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). Triplicate samples were subjected to various doses of irradiation from ^{60}Co in a GammaCell model 220 (Atomic Energy of Canada Ltd., Ottawa, Canada) at about 36.5°C, with non-irradiated samples serving as controls, [10]. Each sample was redissolved in assay buffer B and was assayed in duplicate for remaining PLA_2 activity, giving six measurements of PLA_2 activity at each radiation dose. Using least squares regression of log % remaining activity on radiation exposure (MRad) we calculated the dose (D_{37}) required to reduce PLA_2 activity to 37% of the control value. Using this value we calculated the Radiation Inactivation size (RIS) of PLA_2 using the relation [16,10]

$$RIS = \frac{6.4 \times 10^5}{D_{37}} \qquad \text{[Equation 1]}$$

$$\log RIS = 5.89 - \log D_{37,t} - 0.0028t \qquad \text{[Equation 2]}$$

Electrophoresis

Nondenaturing polyacrylamide-gradient gel electrophoresis was performed with gels of 8-30% acrylamide (Jule Inc. Biotechnology Division, New Haven, CT) with 90 mM boric acid, 2.6 mM EDTA pH 8.3 as buffer. Gels were pre electrophoresed for 20 mins at 70V, samples were applied and electrophoresis continued for 20 mins. at 70V then 16 hours at 130V. Part of the gel was stained with silver [17] and a part was cut into 3 mm slices to elute proteins. Slices were allowed to stand in 200 µl electrophoresis buffer containing 5mM dithiothreitol, 150 mM $CaCl_2$ and 1mg/ml gamma-globulin for 3 hours at 4° C, and the supematant was assayed for PLA_2 activity. Protein was measured by the method of Lowry *et al.* [18].

RESULTS AND DISCUSSION

Essentially the entire peak of PLA_2 activity was pooled at each stage of chromatography, in order to obtain a representative pool of $cPLA_2$. PLA_2 activity was enriched 401-fold in the pooled Polypore-DEAE fraction, compared with the cytosol, to a specific activity of 3.0 nmol/min/mg. Similar activity was observed against both 2-arachidonyl-phosphatidyl choline and 2-arachidonyl-phosphatidyl ethanolamine when both were simultaneously present at equimolar concentration. The activity was entirely inhibited by excess EDTA.

The Polypore-DEAE fraction was heterogeneous on silver-stained non-denaturing gradient-gel electrophoresis, but PLA_2 activity was eluted as a single peak at Mr 77,000 (slice 14, Fig.1). A similar result was obtained in a second experiment. Furthermore, this active band reacted with rabbit antisera raised against synthetic polypeptides corresponding to $cPLA_2$ residues 54-66 and 731-749 (results not shown) [5,6].

Following drying and redissolving control, non-irradiated aliquots of the Polypore-DEAE fraction we determined the range over which PLA_2 activity was linear with respect to enzyme concentration (Fig. 2). Then we assayed the control and irradiated samples using an amount equal to the maximum amount of control sample in the linear range.

Looking at the three irradiation experiments together (Fig. 3A,B,C) we found that PLA_2 activity was lost from the samples as a simple function of radiation dose, within the limits of precision of the assay, down to 26% of the control value with correlation coefficients of -0.987, -0.993, -0.986 (Fig. 3A,B,C). We calculated the size of the PLA_2 using Equation 1 and the interpolated value for D_{37}. Two separately pooled fractions from the same preparation gave values of 80,800 (± 6,600 standard error of the estimate) and 78,500 (± 5,800) daltons, and a separate preparation gave a value of 81,000 (± 2,400)

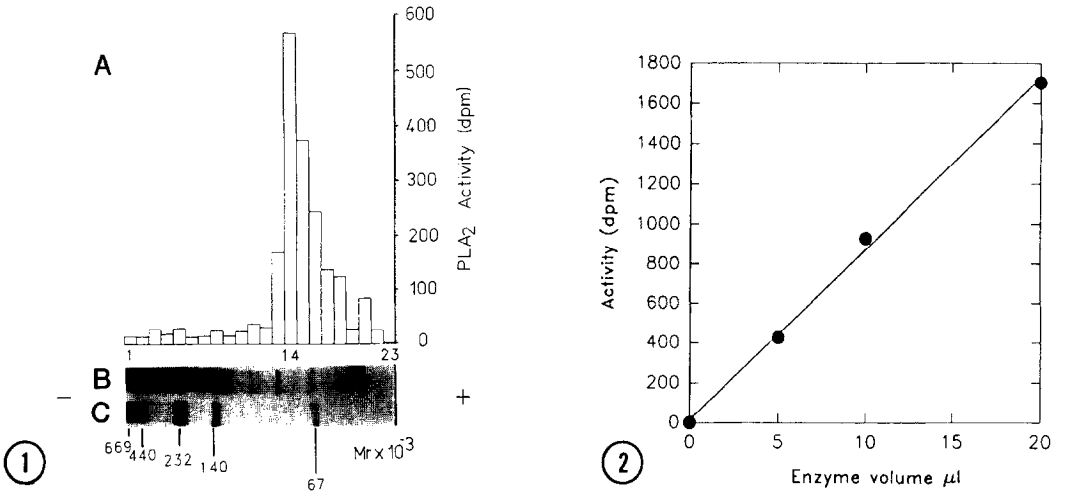

Figure 1

Nondenaturing polyacrylamide-gradient gel electrophoresis of partially purified cPLA$_2$. A: PLA$_2$ activity eluted from gel slices, slices are numbered from the top of the gel, B: Silver stained electrophoresis of the same Polypore-DEAE fraction, C: Standard proteins (Pharmacia).

Figure 2

Phospholipase A$_2$ activity against 2-arachidonyl-phosphatidylethanolamine as a function of amount of enzyme in control nonirradiated samples incubated with substrate for 15 min.

daltons, Figs. 3A,B,C respectively. The standard error of the estimate (in daltons) was calculated by projection of the limits of the standard error of the estimate of activity (at 37% control) onto the activity vs irradiation dose curve to estimate the limits of accuracy of D_{37}. The mean value for the Radiation Inactivation Size of U937 PLA$_2$ from our three experiments was 80,100 daltons. Calculation using Equation 2 and a temperature of

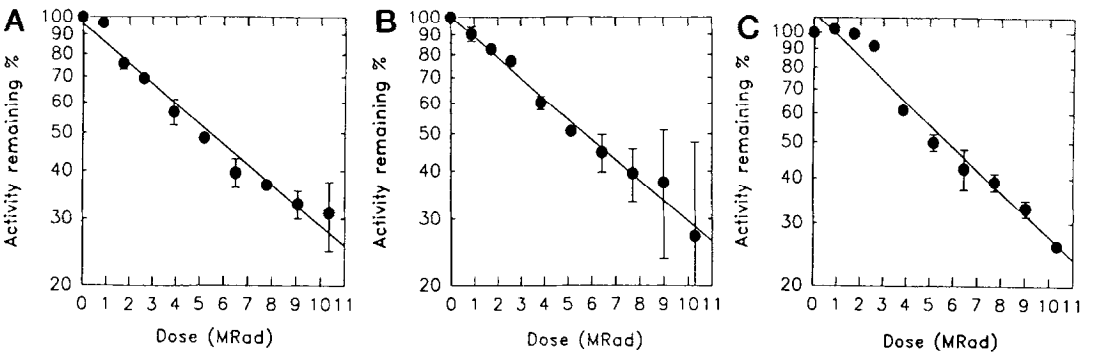

Figure 3

Phospholipase A$_2$ activity against 2-arachidonyl-phosphatidylethanolamine following irradiation.

36.5°C gave values for the RIS of 77,400, 75,200, 77,600, mean 76,800. We do not believe that these values are significantly different from the mean RIS 80,100 (equation 1) given i) the scatter of values and reported precision (1 in 28) of the temperature coefficient of RIS [19,10] and ii) the comment of Beauregard *et al.* [10] that the temperature correction is insignificant with irradiation at 1 MRad/h.

Given that the $cPLA_2$ cDNA appears to encode a protein of Mr 85,200 [5,6] our results from the radiation inactivation of $cPLA_2$ (Mr 80,100) conform to a model in which the active form of this enzyme is a monomer composed of a single functional domain containing the entire potential translated sequence. This is the simplest type of result of radiation inactivation [10,20] and is in contrast to the observation of independent inactivation of the several domains within a multidomain protein, as was found with the ligand-binding and guanylate cyclase activities of the Atrial Natriuretic Peptide receptor [21]. We conclude that $cPLA_2$ is functional as a monomer. Jain *et al.* [22 made the same conclusion for several low molecular weight secreted PLA_2 from kinetic studies although others had previously concluded that these PLA_2 may be active as dimers [10-12].

ACKNOWLEDGMENTS

We thank Nicole Sawyer, France Laliberté and Louise Charlton for help in preparation of cytosol, development of the PLA_2 assay and secretarial assistance, respectively, and Dr. Ian Street for helpful discussion.

REFERENCES

[1] Samuelsson, B. (1983) *Science 220*, 568-575.
[2] Diez, E. & Mong, S. (1990) *J. Biol. Chem. 265*, 14654-14661.
[3] Clark, J.D., Lin, L. L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A. Y., Milona, N. & Knopf, J.L. (l99l) Cell 65, 1043-1051.
[4] Kramer, R. M., Roberts, E. F., Manetta, J. & Putnam, J. E. (1991) *J. Biol. Chem. 266*, 5268-5272.
[5] Clark, J. D., Lin, L. L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. & Knopf, J. L. (1991) *Cell 65*, 1043-1051.
[6] Sharp, J.D., White, D.L., Chiou X.G., Goodson, T., Gamboa, G.C., McClure, D., Burgett, S., Hoshins, J., Skatrud, P.L., Sportsman, J.R., Becker, G.W., Kang, L.H., Roberts, E.F., Kramer, R.M. (1991) J.Biol. Chem. 266, 14850-14853.
[7] Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizard, R. & Pepinsky, R. B. (1989) *J. Biol. Chem. 264*, 5768- 5775.
[8] Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J. & Johnson, L. K. (1989) *J. Biol. Chem. 264*, 5335-5338.

[9] Verheij, H. M., Westerman, J., Sternby, B. & De Haas, G. H. (1983) *Biochim. Biophys. Acta 747*, 93-99.
[10] Menashe, M., Romero, G., Biltonen, R. L. & Lichtenberg, D. (1985) *J. Biol. Chem. 261*, 5328-5333.
[11] Romero, G., Thompson, K. & Biltonen, R. L. (1987) *J. Biol. Chem. 262*, 13476-13482.
[12] Tomasselli, A. G., Hui, J., Fisher, J., Zürcher-Neeley, H., Reardon, I. L., Oriaku, E., Kézdy, F. J. & Heinrikson, R. L. (1989) *J. Biol. Chem. 264*, 10041-10047.
[13] Beauregard, G., Maret, A., Salvayre, R. & Potier, M. (1987) *Methods Biochem. Anal. 32*, 313-343.
[14] Sundstrom, C. & Nilsson, K. (1976) *Int. J. Cancer 17*, 565-577.
[15] Dole, V. P. & Meinertz, H. (1960) *J. Biol. Chem. 235*, 2595-2599.
[16] Kepner, G.R. & Macey, R.I. (1968) *Biochim. Biophys. Acta 163*, 188-203.
[17] Weech, P. K., Camato, R., Milne, R. W. & Marcel, Y. L. (1986) *J. Biol. Chem. 261*, 7941-7951.
[18] Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
[19] Kempner, E.S. & Haigler, H.T. (1982) *J. Biol. Chem. 257*, 13297-13299.
[20] Kempner, E. S. & Fleischer, S. (1989) *Methods in Enzymology 172*, 410- 439.
[21] Tremblay, J., Huot, C., Koch, C. & Potier, M. (1991) *J. Biol. Chem. 266*, 8171-8175.
[22] Jain, M.R., Ranadive, G., Yu, B-Z., Verdij, H.M. (1991) Biochemistry, 30, 7330-7340.