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Novel group V phospholipase A2 involved in arachidonic acid mobilization in murine P388D1 macrophages.

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Four related genes encode four different secretory phospholipase A₂ (sPLA₂) enzymes in mammals, namely the well described Group I and IIA enzymes and the more recently described Groups IIC and V. A large body of research has putatively demonstrated that the Group IIA sPLA₂ is involved in diverse pathologic processes, such as rheumatoid arthritis, septic shock, intestinal neoplasia, and epidermal hyperplasia, as well as cellular signaling by regulating the formation of arachidionate-derived lipid messengers. However, we demonstrate herein the involvement of another sPLA₂, i.e. the Group V sPLA₂, in arachidonic acid release and prostaglandin production in the mouse macrophage-like cell line P388D₁. Abundant message for Group V sPLA₂ was detected in both resting and activated cells. In contrast, Group IIA sPLA₂ message was undetectable as analyzed by Northern blot and reverse transcriptase-polymerase chain reaction. Moreover, blockage of Group V sPLA₂ gene expression by antisense RNA oligonucleotides resulted in inhibition of prostaglandin E₂ production as well as reduction of the amount of sPLA₂ protein at the cellular surface. Collectively, these results uncover Group V sPLA₂ as a novel effector involved in arachidonic acid-mediated signal transduction.

The phospholipase A₂ (PLA₂) superfamily comprises a number of heterogeneous enzymes whose common feature is to hydrolyze the fatty acid esterified at the sn-2 position of glycerophospholipids (1). Prominent members of this family are the secretory phospholipase A₂ (sPLA₂), proteins of relatively low molecular mass (about 14 kDa), highly enriched in disulfide bonds, and requiring millimolar levels of Ca²⁺ for activity. Four different sPLA₂ enzymes exist in mammalian cells, which include the well described Groups I (pancreatic type) and IIA (synovial type) (1) and the more recently described Groups IIC and V (2–4). Group IIC lacks the “elapid loop” characteristic of Group I enzymes nor the C-terminal extension characteristic of Group II enzymes and contains twelve Cys (2).² Group IIA sPLA₂ (in some literature referred to as Group II) has attracted considerable interest due to its apparent involvement in a number of pathologic conditions, ranging from systemic and acute inflammatory conditions to cancer (5–8). Group II IA sPLA₂ is expressed by most cells and tissues, immunoneflammatory cells (except lymphocytes) being particularly rich sources of this enzyme. Recent work by many laboratories, including ours, has demonstrated that sPLA₂ plays a role in receptor-coupled arachidonate (AA) release and lipid messenger production in many cells. In fact, in P388D₁ macrophages stimulated with lipopolysaccharide (LPS) and platelet-activating factor (PAF), sPLA₂ appears to be the major effector involved (9–11). Mainly due to its ubiquitous distribution among AA-releasing cells as well as its role in inflammatory reactions, it has been generally believed that this sPLA₂ is a Group II enzyme. Moreover, we have demonstrated that another sPLA₂, i.e. the Group V enzyme, is actively involved in AA signaling in macrophages. While the current results do not completely rule out a role for Group IIA sPLA₂ in AA mobilization in P388D₁ macrophages and other cellular systems, they do stress the involvement of Group V sPLA₂ in the response. Thus Group V sPLA₂ emerges as a novel effector involved in AA-mediated signal transduction.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Labeling Conditions**—P388D₁ cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO₂ in Iscove’s modified Dulbecco’s medium (Whittaker Bioproducts, Walkersville, MD; endotoxin content <0.05 ng/ml) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids (Irvine Scientific, Santa Ana, CA). Cells were plated at 10⁶ cells/well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove’s modified Dulbecco’s medium. When required, radiolabeling of the cells with [5,6,8,9,11,12,14,15-³H]arachidonic acid (DuPont NEN; specific activity 100 Ci/mmol; [³H]AA) was achieved by including 0.5 μCi/ml [³H]AA during the overnight adherence period (20 h). Labeled AA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 5 mg/ml albumin.

**Stimulation of P388D₁ cells**—The standard regimen for activating cells with LPS and PAF has been described previously (9–11). Briefly, P388D₁ cells were placed in serum-free medium for 30–60 min before the addition of 200 ng/ml LPS Re595 (Sigma) for 1 h. After the LPS incubation, cells were overlaid with serum-free medium for 5–30 min, after which they were challenged with 100 nM PAF (Sigma) for the time indicated.

**RT-PCR**—One μg of total RNA was used for reverse transcription using Moloney Leukemia Reverse Transcriptase (U. S. Biochemical Corp.). 0.2 μg of cDNA was then subjected to PCR reaction using 2 units

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‡ Dennis, E. A., *Trends Biochem. Sci.*, in press.

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The abbreviations used are: PLA₂, phospholipase A₂; sPLA₂, secretory PLA₂; AA, arachidonic acid; PGE₂, prostaglandin E₂; PAF, platelet-activating factor; LPS, bacterial lipopolysaccharide; RT, reverse transcriptase; PCR, polymerase chain reaction.
of AmpliTaq DNA polymerase (Perkin-Elmer). Reaction conditions were as follows: 95°C, 20 s; 60°C, 30 s; 95°C, 10 s for 30 cycles. Group IIA PLA2 primers used were EF-15, CAG TTT GGG GAA ATG ATT CGG C, and EF-43, GAA ACA TTC AGC GGC GGC TTT A. Group V PLA2 primers used were NcM10e2, CAG GGG GCT TGC TAG AAC CGG C, and EF-43, GAA ACA TTC AGC GGC GGC TTT A. Group IIC PLA2 primers were M8-Ex1, GCC ATT TTC ATC TTC CCT GTC T, and M8-Ex3, TAA GCT GTT AGC AGC AGT C. Ten μl of the reaction was then separated in a 2% agarose gel and transferred to a Nytran nylon membrane using the Turboblotter system (Schleicher & Schuell). Hybridization was carried out for 1 h using ExpressHyb hybridization solution (Clontech).

sPLA2 mRNA Detection by Northern Blot—Total RNA was isolated according to the acid guanidinium thiocyanate/phenol/chloroform method (12). 10–20 μg of RNA was separated in a 0.22 μm formamide gel. Probes used for hybridization were the fragments generated in the RT-PCR reactions above.

sPLA2 Protein Detection on the Surface of P388D1 Cells by Flow Cytometry—Flow cytometry analyses were performed on a Coulter Elite cytometer. A detailed description of the procedure will be published elsewhere. In short, the cells (10⁶) were incubated with a 1:300 dilution of rabbit anti-human synovial sPLA2 antiserum BQY-113A (generously provided by Drs. J. L. Bobbitt and R. M. Kramer, Eli Lilly Co., Indianapolis, IN), followed by washing and labeling with fluorescein isothiocyanate-tagged swine anti-rabbit F(ab)2 Ab (Dako, Carpinteria, CA). P388D1 cell Fe receptors were blocked with a 1:10 dilution of swine serum in phosphate buffer prior to incubating with BQY-113A antiserum.

Western Blot Analysis of sPLA2—The cells were washed twice with serum-free medium and homogenized by 25 strokes in a Dounce homogenizer in a buffer consisting of 20 mM Tris-HCl, 2 mM EDTA, 10 mM sodium-freon medium and homogenized by 25 strokes in a Dounce homogenizer. Homogenate samples (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 18 h. Membranes were then incubated with anti-sPLA2 antiserum for 30 min and then treated with horseradish peroxidase-conjugated protein A (Amersham Corp.). Bands were detected by enhanced chemiluminescence (Amersham).

Antisense Inhibition of Group V PLA2 Expression in P388D1 Cells—Transient transfection of P388D1 cells, with antisense oligonucleotides ASGII-rat, ASGII-mouse, ASGV, ASGV-2, or SGV-2 plus lipofectamine was carried out as described (9, 10). Briefly, P388D1 cells were transfected with oligonucleotide (250 nm) in the presence of 5 μg/ml Lipofectamine (Life Technologies, Inc.) under serum-free conditions for 8–9 h prior to cell activation. When [3H]HIAA-labeled cells were used, the [3H]HIAA was added at the beginning of the transfection (9, 10). Antisense oligonucleotide ASGII-rat (sequence 5′-GAU CCA CUG CCA CCC ACA CC-3′) is complementary to nucleotides 148–168 of the rat Group IIA PLA2 gene; it is 80% homologous to ASGII-mouse (5′-GAU CCA CUG CCA CCC ACA CC-3′) and 55% homologous to mouse ASGV (5′-GUC CGG GGA CGG CCC CAG CC-3′). Antisense oligonucleotide ASGV-2 (5′-GUA UAC UUG CUA CAA AGG CCA C-3′) is complementary to nucleotides 64–84 of mouse Group V PLA2 gene. SGV-2 (5′-GUG CUC GUA CAA GAG UCC-3′) is the sense complement of ASGV-2.

Measurement of PGE2 Production and of Extracellular [3H]HIAA Release—For PGE2 production, LPS-treated cells were incubated with 100 nM PAF for 10 min, after which the supernatants were removed and cleared of detached cells by centrifugation and PGE2 was quantitated using a specific radioimmunoassay (Perspective Systems, Framingham, MA). For [3H]HIAA release experiments, the cells, labeled with [3H]HIAA as described above, were stimulated with 100 nm PAF for 10 min in the presence of 1 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Data Presentation—Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS AND DISCUSSION

When total RNA from resting P388D1 macrophages was extracted and analyzed by Northern blot using Group IIA PLA2

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4 Mouse Group IIC sPLA2, GenBank™ accession number is U18119.
than Group IIA mRNA in P388D1 cells are likely to reflect the normal pattern of gene expression in macrophages rather than a compensatory expression of one gene due to an inactivating mutation in the second gene.

Distinct lines of evidence, such as heparin treatments or use of nonpermeable sPLA2 inhibitors, have suggested that the relevant sPLA2 pool involved in AA signaling is localized at the outer surface of the cells (9–11, 14–17). This is, in addition, consistent with the millimolar Ca2⁺ requirement of the enzyme. It should be noted however, that direct, unambiguous evidence for such an autocrine role of sPLA2 has not yet been provided. In fact, it has also been speculated that the sPLA2 involved in the generation of inflammatory mediators might be acting intracellularly (18). Thus, we aimed at detecting expression of sPLA2 on the surface of the P388D1 cells by flow cytometry. In doing these experiments, we took advantage of the high structural homology existing among sPLA2 proteins, regardless of source or group type. Thus, using an antibody raised against human synovial PLA2 (a Group IIA enzyme), we were able to detect sPLA2 expression at the outer surface of the P388D1 cells. Because these cells lack Group IIA sPLA2 mRNA and perhaps Group IIA sPLA2 protein, the polyclonal antibody used may be cross-reacting with another sPLA2, most likely the Group V enzyme.

Resting cells constitutively expressed rather high levels of sPLA2 protein; depending on cell batch, between 25–35% of the cells are positive for sPLA2 as judged by flow cytometry. Interestingly, the fraction of cells expressing sPLA2 protein at the cell surface was increased after LPS/PAF treatment (Fig. 2). Median intensity fluorescence was 160 and 218 for resting and activated cells, respectively (arbitrary units). Analysis of total cellular sPLA2 by Western blot and subsequent densitometric quantitation of the visualized bands revealed a 50% protein increase in homogenates from LPS/PAF activated cells with respect to homogenates from resting unstimulated cells (1,031 and 1,430 for resting and activated cells, respectively; arbitrary units). These data are consistent with the notion that increased sPLA2 expression at the surface of activated cells is not due to exocytosis of preformed protein but to gene induction and de novo protein synthesis.

We aimed at blocking Group V PLA2 gene expression by using antisense RNA oligonucleotides. This technique was previously used in our laboratory to uncover a role for sPLA2 in receptor-coupled AA release and prostaglandin production in activated P388D1 macrophages (9, 10). At the time these previous experiments were performed, Group V PLA2 had not been discovered and the mouse Group IIA PLA2 sequence had not yet been elucidated. Thus the Ca2⁺-binding loop zone from the
the surface of the P388D₁ cells.

In summary, the current results demonstrate involvement of the novel Group V sPLA₂ in arachidonate signaling in P388D₁ macrophages. It is very important to note however, that our data have not completely ruled out a similar role for Group IIA sPLA₂, if actually present in these cells. Although our inability to detect mRNA for Group IIA PLA₂, using both Northern blots and the highly sensitive RT-PCR technique (3) in the P388D₁ cells even after cell activation, makes it difficult to envision a role for Group IIA PLA₂ in AA metabolism in P388D₁ cells, it is possible that some Group IIA sPLA₂ protein exists that accounts for part of the AA mobilized upon cell activation.

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