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Novel Group V Phospholipase A_2 Involved in Arachidonic Acid Mobilization in Murine $P388D_1$ Macrophages*

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Four related genes encode four different secretory phospholipase A_2 ($sPLA_2$) 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 $sPLA_2$ is involved in diverse pathologic processes, such as rheumatoid arthritis, septic shock, intestinal neoplasia, and epidermal hyperplasia, as well as in cellular signaling by regulating the formation of arachidonate-derived lipid messengers. However, we demonstrate herein the involvement of another $sPLA_2$, *i.e.* the Group V $sPLA_2$, in arachidonic acid release and prostaglandin production in the mouse macrophage-like cell line $P388D_1$. Abundant message for Group V $sPLA_2$ was detected in both resting and activated cells. In contrast, Group IIA $sPLA_2$ message was undetectable as analyzed by Northern blot and reverse transcriptase-polymerase chain reaction. Moreover, blockage of Group V $sPLA_2$ gene expression by antisense RNA oligonucleotides resulted in inhibition of prostaglandin E_2 production as well as reduction of the amount of $sPLA_2$ protein at the cellular surface. Collectively, these results uncover Group V $sPLA_2$ as a novel effector involved in arachidonic acid-mediated signal transduction.

The phospholipase A_2 (PLA_2)[1] 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 A_2s ($sPLA_2$), proteins of relatively low molecular mass (about 14 kDa), highly enriched in disulfide bonds, and requiring millimolar levels of Ca^{2+} for activity. Four different $sPLA_2$ enzymes exist in mammalian cells, which include the well described Groups I (pancreatic type) and IIA (synovial type) (1)[2] and the more recently described Groups IIC and V (2–4). Group IIC lacks the "elapid loop" characteristic of Group I PLA_2s but possesses the C-terminal amino acid extension characteristic of Group II enzymes and contains sixteen Cys. Group V $sPLA_2$ contains neither the elapid loop characteristic of Group I enzymes nor the C-terminal extension characteristic of Group II $sPLA_2$s and contains twelve Cys (2).[2]

Group IIA $sPLA_2$ (in some literature referred to as Group II) has attracted considerable interest due to its apparent involvement in a number of pathological conditions, ranging from systemic and acute inflammatory conditions to cancer (5–8). Group IIA PLA_2 is expressed by most cells and tissues, immunoinflammatory cells (except lymphocytes) being particularly rich sources of this enzyme. Recent work by many laboratories, including ours, has demonstrated that $sPLA_2$ plays a role in receptor-coupled arachidonate (AA) release and lipid messenger production in many cells. In fact, in $P388D_1$ macrophages stimulated with lipopolysaccharide (LPS) and platelet-activating factor (PAF), $sPLA_2$ 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 $sPLA_2$ is a Group IIA enzyme. In the current work, however, we demonstrate that another $sPLA_2$, *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 $sPLA_2$ in AA mobilization in $P388D_1$ macrophages and other cellular systems, they do stress the involvement of Group V $sPLA_2$ in the response. Thus Group V $sPLA_2$ emerges as a novel effector involved in AA-mediated signal transduction.

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling Conditions—$P388D_1$ cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 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 10^6 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-^{3}H]arachidonic acid (DuPont NEN; specific activity 100 Ci/mmol) ([^{3}H]AA) was achieved by including 0.5 μCi/ml [^{3}H]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 $P388D_1$ cells—The standard regimen for activating cells with LPS and PAF has been described previously (9–11). Briefly, $P388D_1$ 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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[1] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; AA, arachidonic acid; PGE_2, prostaglandin E_2; PAF, platelet-activating factor; LPS, bacterial lipopolysaccharide; RT, reverse transcriptase; PCR, polymerase chain reaction.

[2] Dennis, E. A., *Trends Biochem. Sci.,* in press.

of AmpliTaq DNA polymerase (Perkin-Elmer). Reaction conditions were as follows: 95 °C, 20 s; 60 °C, 30 s; 72 °C, 30 s for 30 cycles. Group IIA PLA_2 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 PLA_2 primers used were NcM10e2, CAG GGG GCT TGC TAG AAC TCA A, and M10Ex4R, AAG AGG GTT GTA AGT CCA GAG G. Group IIC PLA_2 primers were M8-Ex1, GGC ATT GCC ATC TTC CTT GTC T, and M8-Ex3.1, TAA GCT TGT GGT 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).

$sPLA_2$ 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 M formaldehyde gel. Probes used for hybridization were the fragments generated in the RT-PCR reactions above.

$sPLA_2$ Protein Detection on the Surface of $P388D_1$ Cells by Flow Cytometry—Flow cytometry analyses were performed on a Coulter Elite cytofluorimeter. A detailed description of the procedure will be published elsewhere.[3] In short, the cells (10^6) were incubated with a 1:300 dilution of rabbit anti-human synovial $sPLA_2$ antiserum BQY-113A (generously provided by Drs. J. L. Bobbitt and R. M. Kramer, Ely Lilly Co., Indianapolis, IN), followed by washing and labeling with fluorescein isothiocyanate-tagged swine anti-rabbit $F(ab')_2$ (Dako, Carpintería, CA). $P388D_1$ cell Fc 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 $sPLA_2$—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 EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, 0.1% 2-mercaptoethanol, pH 7.5. 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-$sPLA_2$ 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 PLA_2 Expression in $P388D_1$ Cells—Transient transfection of $P388D_1$ cells with antisense oligonucleotides ASGII-rat, ASGII-mouse, ASGV, ASGV-2, or SGV-2 plus lipofectamine was carried out as described (9, 10). Briefly, $P388D_1$ 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 [^{3}H]AA-labeled cells were used, the [^{3}H]AA was added at the beginning of the transfection (9, 10). Antisense oligonucleotide ASGII-rat (sequence 5′-GAU CCU CUG CCA CCC ACA CC-3′) is complementary to nucleotides 148–168 of the rat Group IIA PLA_2 gene; it is 80% homologous to ASGII-mouse (5′-GAU CCU UUG CCA CCC AGG CC-3′) and 55% homologous to mouse ASGV (5′-GUC CCG GGA CCG CCC CAG CC-3′). Antisense oligonucleotide ASGV-2 (5′-GGA CUU GAG UUC UAG CAA GCC-3′) is complementary to nucleotides 64–84 of mouse Group V PLA_2 gene. SGV-2 (5′-GGC UUG CUA GAA CUC AAG UCC-3′) is the sense complement of ASGV-2.

Measurement of PGE_2 Production and of Extracellular [^{3}H]AA Release—For PGE_2 production, LPS-treated cells were stimulated with 100 nM PAF for 10 min, after which the supernatants were removed and cleared of detached cells by centrifugation and PGE_2 was quantitated using a specific radioimmunoassay (Perspective Systems, Framingham, MA). For [^{3}H]AA release experiments, the cells, labeled with [^{3}H]AA 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 $P388D_1$ macrophages was extracted and analyzed by Northern blot using Group IIA PLA_2 probes, no signal was detected (Fig. 1*A*). Positive signal for Group IIA PLA_2 was found, however, when RNA from DBA/2J mouse intestine was analyzed. A clearly positive signal was detected when probes based on the Group V PLA_2 were used for hybridization with RNA from resting $P388D_1$ macrophages (Fig. 1*B*). Group IIC $sPLA_2$ mRNA was also undetectable by the same technique, whereas positive controls using C57Bl/6J and C3H/HeJ mouse testis RNA gave a strong hybridization signal (data not shown).[4]

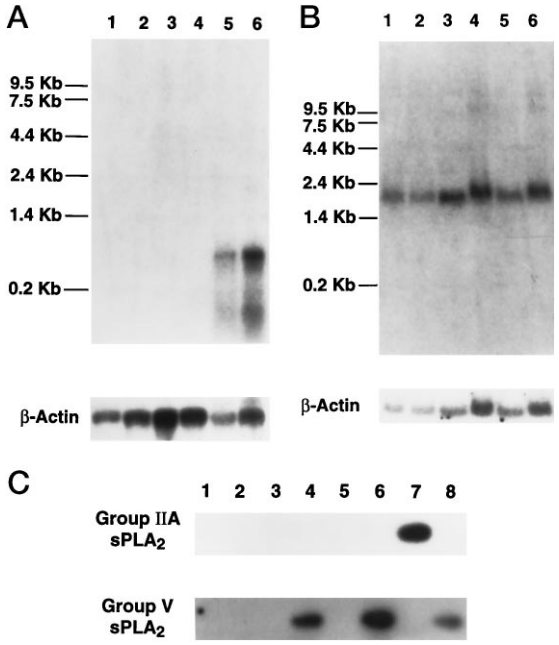

FIG. 1. *A*, Northern blot of Group IIA PLA_2 isolated from $P388D_1$ total RNA. The Group IIA PLA_2 probe used for hybridization was the fragment generated in the RT-PCR reaction below. *Lanes 1* and *3* contain $P388D_1$ total RNA; *lanes 2* and *4*, activated $P388D_1$ total RNA; *lanes 5* and *6*, total RNA isolated from DBA/2J mouse intestine. The β-actin probe was CRTL-BAIM, TCT GCG CAA GTT AGG TTT TGT CAA AG. *B*, Northern blot of Group V PLA_2 isolated from $P388D_1$ total RNA. *Lanes 1, 3* and *5*, $P388D_1$ total RNA; *lanes 2, 4* and *6*, activated $P388D_1$ RNA. The Group V PLA_2 probe used for hybridization was the fragment generated in the RT-PCR reaction below. *C*, RT-PCR of Group IIA and Group V PLA_2 from total RNA. Probes used were EF-23, GTG GGA GTA CTT GTA GGT CAG T (Group IIA PLA_2), and CTV410, GGT AAG AAT GGC ACA GTC TTT (Group V PLA_2). Group II PLA_2 (*top*): *lane 1* contains no cDNA, *lane 3* contains $P388D_1$ cDNA, *lane 5* contains activated $P388D_1$ cDNA, and *lane* 7 contains DBA/2J intestine cDNA. No sample was loaded on *lanes 2*, *4*, *6*, and *8*. Group V PLA_2 (*bottom*): *lane 2* contains no cDNA, *lane 4* contains $P388D_1$ cDNA, *lane 6* contains activated $P388D_1$ cDNA, *lane 8* contains DBA/2J intestine cDNA. No sample was loaded on *lanes 1*, *3*, *5*, and *7*.

Remarkably, when RNA from cells activated with LPS plus PAF were used (9, 10), the same results as noted above were obtained, *i.e.* no signal for Groups IIA and IIC PLA_2 (Fig. 1*A*; data not shown), and clearly a positive signal for Group V PLA_2 (Fig. 1*B*). Independent confirmatory evidence of the above data was obtained by RT-PCR. Again, mRNA for Group V PLA_2 was detected in both resting and activated cells, with Groups IIA and IIC being undetectable (Fig. 1*C*; data not shown).

Our inability to detect any Group IIA mRNA in the $P388D_1$ cells led us to sequence the gene (*Pla2g2a*) encoding that enzyme in these cells. We found that it did not contain the T insertion frame-shift mutation at position 166 that was recently reported to produce only barely detectable but inactive Group IIA mRNA (7, 8). DBA/2 mice, from which $P388D_1$ cells are derived (13), were also shown not to have this mutation (7, 8). Thus, the relatively high levels of Group V mRNA rather

[3] J. Balsinde, M. A. Balboa, and E. A. Dennis, manuscript in preparation.

[4] Mouse Group IIC $sPLA_2$ GenBank™ accession number is U18119.

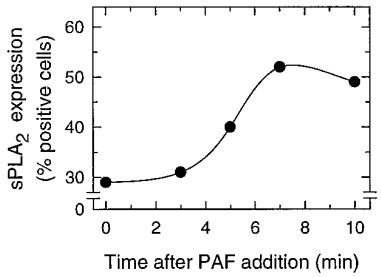

FIG. 2. **$sPLA_2$ detection on the surface of $P388D_1$ cells by flow cytometry.** $sPLA_2$ expression in LPS-primed cells, treated with PAF for the times indicated, was analyzed by using the BQY-113A anti-$sPLA_2$ antiserum. Background fluorescence was established by incubating the cells without the BQY-113A antiserum.

than Group IIA mRNA in $P388D_1$ 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 $sPLA_2$ inhibitors, have suggested that the relevant $sPLA_2$ 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 Ca^{2+} requirement of the enzyme. It should be noted however, that direct, unambiguous evidence for such an autocrine role of $sPLA_2$ has not yet been provided. In fact, it has also been speculated that the $sPLA_2$ involved in the generation of inflammatory mediators might be acting intracellularly (18). Thus, we aimed at detecting expression of $sPLA_2$ on the surface of the $P388D_1$ cells by flow cytometry. In doing these experiments, we took advantage of the high structural homology existing among $sPLA_2$ proteins, regardless of source or group type. Thus, using an antibody raised against human synovial PLA_2 (a Group IIA enzyme), we were able to detect $sPLA_2$ expression at the outer surface of the $P388D_1$ cells. Because these cells lack Group IIA $sPLA_2$ mRNA and perhaps Group IIA $sPLA_2$ protein, the polyclonal antibody used may be cross-reacting with another $sPLA_2$, most likely the Group V enzyme.

Resting cells constitutively expressed rather high levels of $sPLA_2$ protein; depending on cell batch, between 25–35% of the cells are positive for $sPLA_2$ as judged by flow cytometry. Interestingly, the fraction of cells expressing $sPLA_2$ protein at the cell surface was increased after LPS/PAF treatment (Fig. 2).[5] Mean intensity fluorescence was 160 and 218 for resting and activated cells, respectively (arbitrary units). Analysis of total cellular $sPLA_2$ 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 $sPLA_2$ 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 PLA_2 gene expression by using antisense RNA oligonucleotides. This technique was previously used in our laboratory to uncover a role for $sPLA_2$ in receptor-coupled AA release and prostaglandin production in activated $P388D_1$ macrophages (9, 10). At the time these previous experiments were performed, Group V PLA_2 had not been discovered and the mouse Group IIA PLA_2 sequence had not yet been elucidated. Thus the Ca^{2+}-binding loop zone from the rat Group IIA PLA_2 sequence was utilized for the design of antisense oligonucleotides in those previous studies. This is a very highly conserved zone among $sPLA_2$s. In fact, the antisense oligonucleotide that we used previously (9, 10), based on the rat Group IIA sequence (referred to as ASGII-rat), inhibited both $sPLA_2$ protein expression and PGE_2 production just as well as antisense oligonucleotides directed against the equivalent sequences in the mouse Group IIA and V PLA_2 genes (referred to as ASGII-mouse and ASGV, respectively) (Fig. 3). It is therefore likely that the antisense oligonucleotide used in our previous studies (9, 10) was affecting Group V $sPLA_2$ in addition to any effect on Group IIA $sPLA_2$. We have now designed new antisense oligonucleotides based on exon II of the mouse Group V $sPLA_2$ sequence. After treating the cells with the oligonucleotides, the cells were activated with LPS plus PAF, and subsequently $sPLA_2$ protein and PGE_2 levels were quantitated by flow cytometry and radioimmunoassay, respectively. Antisense oligonucleotide ASGV-2 blocked $sPLA_2$ expression by about 60–70% (Fig. 3*A*) and inhibited PGE_2 production to the same extent (Fig. 3*B*). As a control for the transfection, we used the sense complement of ASGV-2 (referred to as SGV-2), which had no effect on either of the two parameters measured. These results further underscore the involvement of Group V $sPLA_2$ in generating lipid mediators at

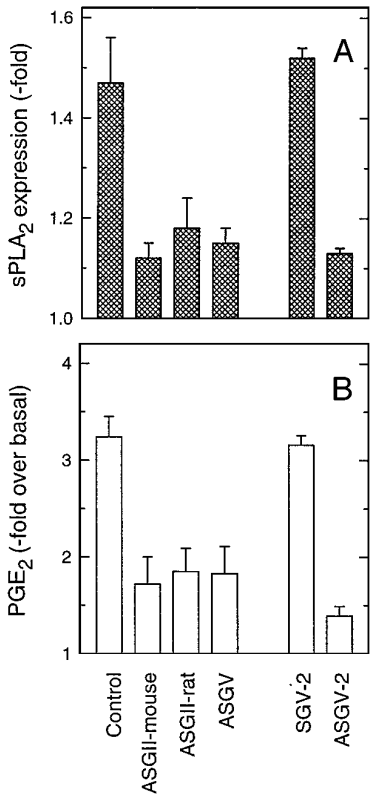

FIG. 3. **Inhibition of $sPLA_2$ protein expression and lipid mediator production by antisense RNA technology.** $P388D_1$ cells were treated with antisense oligonucleotides ASGII-rat, ASGII-mouse, ASGV, ASGV-2, or SGV-2 plus LipofectAMINE. Subsequently, the cells were treated with 200 ng/ml LPS for 1 h and then with 100 nM PAF for 10 min. Afterwards, the cell supernatants were removed and PGE_2 levels were quantitated by radioimmunoassay (*panel B*). To quantitate $sPLA_2$ expression after the different treatments, the cells were processed for flow cytometry analysis as described in the legend to Fig. 2 (*panel A*). Data are expressed relative to the response observed in unstimulated cells. Actual values were as follows. For unstimulated cells, 31 ± 2% cells expressed $sPLA_2$, and 2.7 ± 0.9 ng PGE_2 was produced per 10^6 cells. For stimulated cells lacking antisense (referred to in the figure as *Control*), 44 ± 2% of cells expressed $sPLA_2$ and 9.2 ± 1.4 ng PGE_2 was produced per 10^6 cells.

[5] Although LPS priming is required for the cells to expose new $sPLA_2$ protein after stimulation with PAF, LPS alone does not significantly increase the level of $sPLA_2$ at the outer surface.

the surface of the $P388D_1$ cells.

In summary, the current results demonstrate involvement of the novel Group V $sPLA_2$ in arachidonate signaling in $P388D_1$ macrophages. It is very important to note however, that our data have not completely ruled out a similar role for Group IIA $sPLA_2$, if actually present in these cells. Although our inability to detect mRNA for Group IIA PLA_2, using both Northern blots and the highly sensitive RT-PCR technique (3) in the $P388D_1$ cells even after cell activation, makes it difficult to envision a role for Group IIA PLA_2 in AA metabolism in $P388D_1$ cells, it is possible that some Group IIA $sPLA_2$ protein exists that accounts for part of the AA mobilized upon cell activation.

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