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Involvement of Epigenetic Mechanisms in the Regulation of Secreted Phospholipase A_2 Expressions in Jurkat Leukemia Cells

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Abstract

Epigenetic changes provide a frequent mechanism for transcriptional silencing of genes in cancer cells. We previously established that epigenetic mechanisms are important for control of group IIA phospholipase A_2 (*PLA2G2A*) gene transcription in human DU-145 prostate cells. In this study, we analyzed the involvement of such mechanisms in the regulation of five $sPLA_2$ isozymes and the M-type receptor of $sPLA_2$ ($sPLA_2$-R) in human leukemic Jurkat cells. These cells constitutively expressed $sPLA_2$-IB, $sPLA_2$-III, $sPLA_2$-X, and $sPLA_2$-R but not $sPLA_2$-IIA and $sPLA_2$-V. Transcription of $sPLA_2$-IIA and $sPLA_2$-V was, however, detected after exposure of cells to the DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC). Expression of $sPLA_2$-IIA was further enhanced by additional exposure to interferon-γ and blocked by inhibitors of specificity protein 1, nuclear factor κB, and Janus kinase/signal transducer and activator of transcription–dependent pathways. Sequence analysis and methylation-specific polymerase chain reaction of bisulfite-modified genomic DNA revealed two 5′-CpG sites (−111 and −82) in the $sPLA_2$-IIA proximal promoter that were demethylated after 5-aza-dC treatment. These sites may be involved in the DNA binding of specificity protein 1 and other transcription factors. Similar findings after treatment of human U937 leukemia cells with 5-aza-dC indicate that this mechanism of *PLA2G2A* gene silencing is not restricted to Jurkat and DU-145 cells. These data establish that regulation of $sPLA_2$-IIA and $sPLA_2$-V in Jurkat and other cells involves epigenetic silencing by DNA hypermethylation.

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Introduction

Secreted phospholipases A_2 ($sPLA_2$, phosphatide *sn*-2-acylhydrolases, EC 3.1.1.4) belong to the superfamily of phospholipases that not only catalyze production of bioactive lipid mediators of cell signaling pathways but also function as receptor ligands, independently of any enzymatic activity [1–5]. By way of these functions, the various $sPLA_2$ isozymes play crucial roles in several physiological and pathophysiological processes involving alterations in cellular proliferation, growth, differentiation, and apoptosis. The most thoroughly characterized isozyme, $sPLA_2$-IIA, is constitutively expressed in a variety of cell types including chondrocytes, synoviocytes, mesangial cells, astrocytes, and smooth muscle cells (for review [4]).

Recognition that expression of $sPLA_2$-IIA is increased in numerous cancer cells has formed the basis of the $sPLA_2$-IIA pro-oncogenic activity concept [6–12]. The precise role of the enzyme in tumorigenesis, nevertheless, remains unclear. In human pancreatic cancer cells and gastric adenocarcinoma, expression of $sPLA_2$-IIA is associated with prolonged survival and reduced tendency to metastasis, suggesting antioncogenic properties [13,14]. Moreover, $sPLA_2$-IIA

Abbreviations: 5-aza-dC, 5-aza-2′-deoxycytidine; C/EBP-β, CAAT-enhancer–binding protein β; CAPE, caffeic acid phenethyl ester; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon γ; γ-IRE, γ-interferon response element; LPS, lipopolysaccharide; IL-1β, interleukin 1β; IL-6, interleukin 6; Jak, Janus kinase; NF-κB, nuclear factor κB; NF-Y, nuclear factor Y; PKA, protein kinase A; PMA, phorbol-12-myristate 13-acetate; RT-PCR, reverse transcription–polymerase chain reaction; Sp1, specificity protein 1; $sPLA_2$-IB, secreted phospholipase A_2 of group IB; $sPLA_2$-IIA, secreted phospholipase A_2 of group IIA; $sPLA_2$-V, secreted phospholipase A_2 of group V; $sPLA_2$-X, secreted phospholipase A_2 of group X; STAT1, signal transducer and activator of transcription 1; TNF-α, tumor necrosis factor α

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is not appreciably expressed in a number of cancer cell lines including human leukemia Jurkat T cells, monocytic U937 cells [15–17], fibrosarcoma HeLa cells [18], and prostate cancer DU-145 cells [9,19].

Recently, we established that lack of $sPLA_2$-IIA expression and failure of cytokines to induce $sPLA_2$-IIA expression in malignant DU-145 prostate cells can be explained by an epigenetic silencing regulatory mechanism and not by *PLA2G2A* gene mutations connected with a failed expression of intact $sPLA_2$-IIA protein [19]. Such mechanisms involve aberrant methylation of cytosines belonging to 5′-CpG islands, short stretches of DNA rich in 5′-CpG dinucleotides, and often associated with gene promoters (for review [20,21]). In cancer cells, the methylation may retard expression of genes critical to cell proliferation and apoptosis [21].

With the above considerations in mind, we proposed that epigenetic mechanisms are involved in the silencing of $sPLA_2$-IIA in a number of so-called "$sPLA_2$-IIA–negative" cells. To address this hypothesis, we analyzed the expression of $sPLA_2$-IIA and four other $sPLA_2$ isozymes (IB, III, V, and X) in connection with the M-type receptor of $sPLA_2$ ($sPLA_2$-R) in cytokine-primed Jurkat cell lines before and after treatment with demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC). We also determined the methylation status of several distinct 5′-CpG sites in the proximal $sPLA_2$-IIA promoter region.

Materials and Methods

Materials

Recombinant human interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were purchased from Roche Diagnostics Applied Science (Mannheim, Germany). PD98059, Janus kinase (Jak) inhibitor I, phorbol-12-myristate 13-acetate (PMA), SP-600125, and H-1152 were obtained from Calbiochem (San Diego, CA). Forskolin, 5-aza-dC, mithramycin A, and caffeic acid phenethyl ester (CAPE) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Phorbol-12-myristate 13-acetate, PD98059, forskolin, SP-600125, mithramycin A, Jak inhibitor I, and CAPE were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of solvents were 0.3% or less. Controls using DMSO alone were run in all cases.

Cell Culture and Incubation

Jurkat (human T lymphocyte acute leukemia) and U937 (human hystiocytic lymphoma) cell lines were purchased from German Collection of Microorganisms and Cell Cultures (Berlin, Germany). They were cultured in a standard cell medium RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For all experiments, exponentially growing subconfluent cells were used at passages 5 to 8.

For 5-aza-dC treatment, cells were cultured in RPMI 1640 cell medium with 10% fetal calf serum and 5-aza-dC added to the final concentration of 1 to 10 μM from a freshly prepared 10-mM stock solution. After 3 days, during which the culture medium containing 5-aza-dC was renewed once each day, the cells were harvested and analyzed for $sPLA_2$-IIA protein and mRNA levels.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis of $sPLA_2$ Enzyme Expressions

RNA was isolated after lysis of Jurkat T cells in TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. Isolated RNA was converted to cDNA using the GeneAmp RNA-PCR Kit (PerkinElmer LAS GmbH, Jügesheim, Germany). Portions of the reverse-transcribed reaction products were then amplified by polymerase chain reaction (PCR) for the identification of $sPLA_2$-IB, -IIA, -III, -V, -X, the M-type of the $sPLA_2$ receptor ($sPLA_2$-R), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. The applied primer pairs are summarized in the Table 1.

The oligonucleotides used for analysis of mRNA were synthesized according to published nucleotide sequences of human *$sPLA_2$-IB*, *$sPLA_2$-IIA*, *$sPLA_2$-III*, *$sPLA_2$-V*, *$sPLA_2$-X*, *$sPLA_2$-R*, and *GAPDH* genes (http://www.ensembl.org/index.html). Primer pairs for PCR were applied in a final concentration of 0.8 μM. The conditions for amplification were as follows: 40 cycles at 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 1 minute. Buffers and reagents were from GeneAmp Kit (PerkinElmer LAS GmbH). Amplified products were analyzed by electrophoresis on agarose gels.

Quantitative Determination of $sPLA_2$-IIA by Enzyme-Linked Immunosorbent Assay

Release of $sPLA_2$-IIA protein into the culture medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cayman Chemical, MI). Culture medium was removed, centrifuged for 10 minutes at 400*g* to remove cell debris, and used for $sPLA_2$-IIA protein determinations. Total cell protein was determined using a bicinchoninic acid assay kit (Sigma-Aldrich).

Searching for 5′-CpG Islands and Potential Binding Sites for Transcription Factors in Proximal Promoter Regions of $sPLA_2$ Isozymes and M-type Receptor of $sPLA_2$

To address the question of whether 5′-CpG islands are present in the promoters of the $sPLA_2$ isozymes and $sPLA_2$-R, we used MethPrimer software (http://www.urogene.org/methprimer) to analyze the promoter regions −1000 to +200 relative to the transcription starting sites of each gene [22]. We also used TFSEARCH software [23,24] and an object-oriented transcription factors search (ooTFD) software [25] to assess whether the proximal promoter of *PLA2G2A* contains binding sites for transcription factors known to be crucial for the $sPLA_2$-IIA expression.

DNA Methylation Analysis of Selected 5′-CpG Sites in the Proximal $sPLA_2$-IIA Promoter Region

Genomic DNA was extracted from control and 5-aza-dC–treated Jurkat cells using DNazol (Invitrogen Life Technologies, Karlsruhe,

Table 1. Sequences of the Applied Forward (f) and Reverse (r) Primers.

Genes	Sequences	Size (bp)
PLA2G1B	5′-AAA TGA TCA AGT GCG TGA TCC-3′ (f) 5′-TTG CTG CTA CAG GTG ATT GC-3′ (r)	243
PLA2G2A	5′-GTG ATC ATG ATC TTT GGC CTA CTG CA-3′ (f) 5′-TCT CCC TCG TGG GGA GCA ACG ACT-3′ (r)	411
PLA2G3	5′-ACAACTCTTCTATGCCTGG-3′ (f)′ 5′-TGTGACATCCCTAACTTCC-3′ (r)	256
PLA2G5	5′-GGG CTG CAA CAT TCG CAC AC-3′ (f) 5′-CCT CTC TCA GGA ACC AGG CAG-3′ (r)	278
PLA2G10	5′-CCA TCG CCT ATA TGA AAT ATG G-3′ (f) 5′-TAG GAA CTG GGG GTA GAA GAG-3′ (r)	295
PLA2G1R	5′-CAG AAG AAA GGC AGT TCT GGA TTG-3′ (f) 5′-AAA GCC ACA TCT CTG GCT CTG ATT-3′ (r)	496
GAPDH	5′-CGG AGT CAA CGG ATT TGG TCG TAT TG-3′ (f) 5′-GCA GGA GGC ATT GCT GAT GAT CTT G-3′ (r)	439

Germany) following the manufacturer's instructions. Sodium bisulfite treatment of the genomic DNA was performed using the MethylDetector kit from Active Motif (Carlsbad, CA) [26–28].

To amplify the bisulfite-modified DNA, a seminested PCR was performed with primers covering regions within the proximal promoter region of the *sPLA$_2$-IIA* gene from −351 to +152 relative to the transcription start site [29]. As extrinsic primers, 5′-CTC ATA CAT ATC AAA TCA T-3′ and 5′-GTA ATT GGT AGT TTT TTT G-3′, and as intrinsic primers, 5′-CTC ATA CAT ATC AAA TCA T-3′ and 5′-GTA AAT GAG TTT ATA GTT TG-3′, were applied in a final concentration of 0.8 μM. The conditions of PCR were as follows: 20 cycles at 95°C for 45 seconds, 44°C for 60 seconds and 72°C for 45 seconds using the extrinsic primers, and 35 cycles at 95°C for 30 seconds, 45°C for 45 seconds, and 72°C for 30 seconds using the intrinsic primers. The buffers and reagents were from GeneAmp Kit (PerkinElmer LAS GmbH). The PCR products were purified using a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany). DNA sequences of both strands were analyzed with the ABI PRISM 310 Genetic Analyzer (PerkinElmer Applied Biosystems, Foster City, CA). To verify the efficiency of bisulfite treatment, the sequence of bisulfite-treated genomic DNA was compared with that of untreated genomic DNA. As a further control, Jurkat DNA treated *in vitro* with *Sss* I methyltransferase and purchased from New England BioLabs Inc. (Ipswich, MA) was used.

In addition to sequence analysis of bisulfite-modified genomic DNA, methylation-specific PCR was performed. For this purpose, the following forward (f) and reverse (r) primers were designed and applied:

5′-TGG TAT TAG TTA TTG ATA CGT-3′, f for methylated 5′-CpG (−186);
5′-CGG TAT TAG TTA TTG ATA TGT-3′, f for unmethylated 5′-CpG (−186);
5′-ATT ATT TAG GGG TAT GGG CGA-3′, f for methylated 5′-CpG (−111);
5′-ATT ATT TAG GGG TAT GGG TGA-3′, f for unmethylated 5′-CpG (−111);
5′-TTT TGA GTT TAT TAA TTG ATT ACG T-3′, f for methylated 5′-CpG (−82);
5′-TTT TGA GTT TAT TAA TTG ATT ATG T-3′, f for unmethylated 5′-CpG (−82);
5′-CTC CAA AAT TAT ATC CCC AAA-3′, r primer.

The conditions of amplification for methylation analysis were as follows: 40 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds in the case of the primer pairs to analyze the 5′-CpG sites at positions −186 and −82. In case of the 5′-CpG site at position −111, a touchdown PCR with an annealing temperature between 45 and 50°C was performed. Finally, PCR products were analyzed by electrophoresis on agarose gels.

Data Analysis

Data were analyzed by one-way analysis of variance coupled with Dunnett's *post hoc* test to compare each experimental group with a nominated control group using SPSS 14.0 software. Differences were considered significant at $P < .05$.

Results

Basal and Cytokine-Induced Expression of Secreted Phospholipases A_2 and Phospholipase A_2 Receptor in Human Jurkat Cells

According to our reverse transcription–PCR (RT-PCR) data, Jurkat cells constitutively expressed sPLA$_2$-IB, sPLA$_2$-III, and sPLA$_2$-X isozymes, whereas no transcripts of sPLA$_2$-IIA and sPLA$_2$-V were detectable (Figure 1). A moderate stimulation of sPLA$_2$-IIA and sPLA$_2$-V transcription occurred after exposure of cells to IFN-γ. The sPLA$_2$-R was constitutively expressed and stimulated by all cytokines that were examined (Figure 1). Of note, the level of *sPLA$_2$-X* mRNA decreased considerably after treatment of Jurkat cells with TNF-α.

Preincubation of Jurkat cells with the methyltransferase inhibitor, 5-aza-dC, induced the transcription of sPLA$_2$-IIA, increased existing expression of sPLA$_2$-R, but did not significantly affect expression of sPLA$_2$-IB, sPLA$_2$-III, sPLA$_2$-V, or sPLA$_2$-X (Figure 1). The failed strong effect of 5-aza-dC treatment on sPLA$_2$-IB, sPLA$_2$-III, and sPLA$_2$-X expression was reproducible using RT-PCR with lower amounts of amplification cycles (30 instead of 40 cycles to exclude a possible end point amplification; data not shown). The additional presence of proinflammatory cytokines, IFN-γ, TNF-α, IL-1β, or IL-6, was required to induce sPLA$_2$-V by 5-aza-dC treatment (Figure 1).

Similar results to the above at the protein level for sPLA$_2$-IIA were obtained as determined using a commercially available ELISA kit. After exposure of Jurkat cells to IFN-γ for 72 hours, the level of sPLA$_2$-IIA secreted into the culture medium increased significantly from 8.1 ± 2.9 to 75.1 ± 45.6 pg/mg cell protein (Figure 2). A strong up-regulation of sPLA$_2$-IIA resulted after simultaneous treatment of cells with cytokines and 5-aza-dC (Figure 2). The sPLA$_2$-IIA protein level released into culture medium increased from 708.4 ±

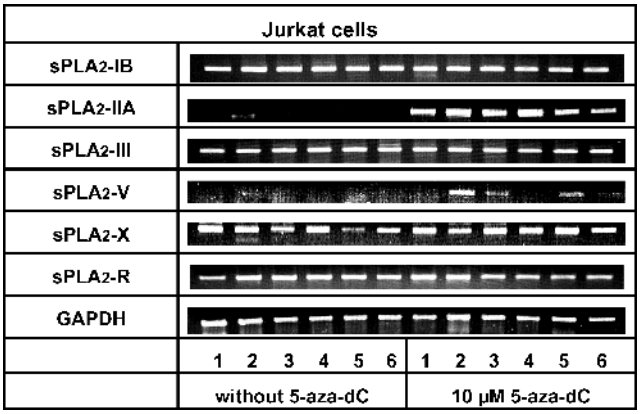

Figure 1. Agarose gel electrophoresis of RT-PCR amplificates of different secreted phospholipase A_2 isozymes (sPLA$_2$-IB, sPLA$_2$-IIA, sPLA$_2$-III, sPLA$_2$-V, and sPLA$_2$-X) and the M-type receptor of sPLA$_2$ (sPLA$_2$-R) in comparison to *GAPDH* mRNA in control and cytokine-primed Jurkat cells, with and without exposure to the DNA demethylating agent, 5-aza-dC. Cells were incubated for 72 hours with vehicle, 10 μM 5-aza-dC, or 5-aza-dC in combination with different proinflammatory cytokines at final concentrations of 25 ng/ml. Lane 1 in both 5-aza-dC–treated and untreated cells represents controls (without addition of cytokines) for cytokine-treated cells. Lanes 2 to 6 represent cells treated with IFN-γ, IL-1β, IL-1β + TNF-α (both at 12.5 ng/ml), TNF-α, and IL-6, respectively. Data are representative of at least three independent experiments giving similar results.

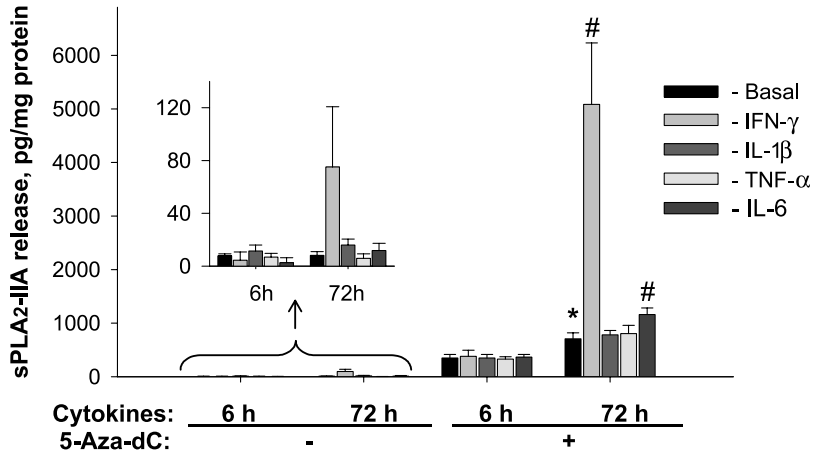

Figure 2. Bar graphs showing effects of the demethylating agent, 5-aza-dC, alone and in combination with proinflammatory cytokines on $sPLA_2$-IIA protein levels in conditioned culture medium of Jurkat cells. Cytokines were added at a final concentration of 25 ng/ml. Cells were exposed to 10 μM 5-aza-dC or vehicle (DMSO) for 6 and 72 hours. The insert shows at higher resolution the results for $sPLA_2$-IIA protein release in cells that were not exposed to 5-aza-dC. Results are means ± SDs of three separate analysis in quadruplicates (n = 12). *P < .05 *versus* cells not exposed to 5-aza-dC; #P < .05 *versus* cells exposed to 5-aza-dC but not to cytokines.

112.0 pg/mg cell protein in cells treated with 5-aza-dC for 72 hours to 5084.4 ± 1149.0 pg/mg cell protein when cells were exposed to IFN-γ and 5-aza-dC during the same period.

A comparison of $sPLA_2$-IIA mRNA expression and protein release in cells treated with 5-aza-dC combined with IFN-γ indicates stronger effects on $sPLA_2$-IIA protein release (Figure 2) than on mRNA level (Figure 1). A possible reason for this difference is that after 72 hours of incubation when the protein release and $sPLA_2$-IIA mRNA levels were determined, the peak level of mRNA was earlier and transient, whereas the amount of $sPLA_2$-IIA protein in the culture medium reflects the accumulation of the released enzyme during the incubation period of 72 hours. This conclusion is consistent with findings of Vial et al. [30], who observed a peak level of $sPLA_2$-IIA mRNA at 16 hours and its decline at 32 hours, whereas the $sPLA_2$-IIA protein level remained high until the end of incubation. Analysis of the active concentration range for IFN-γ indicated that IFN-γ–mediated up-regulation of $sPLA_2$-IIA expression in Jurkat cells was dose-dependent, with a half-maximal effect at ~6 ng/ml of IFN-γ (Figure 3*A*).

Regulation of $sPLA_2$-IIA Expression by the Activity of Signaling Pathways

A marked increase of $sPLA_2$-IIA protein release occurred when 5-aza-dC– and IFN-γ–treated cells were incubated with the mitogen-

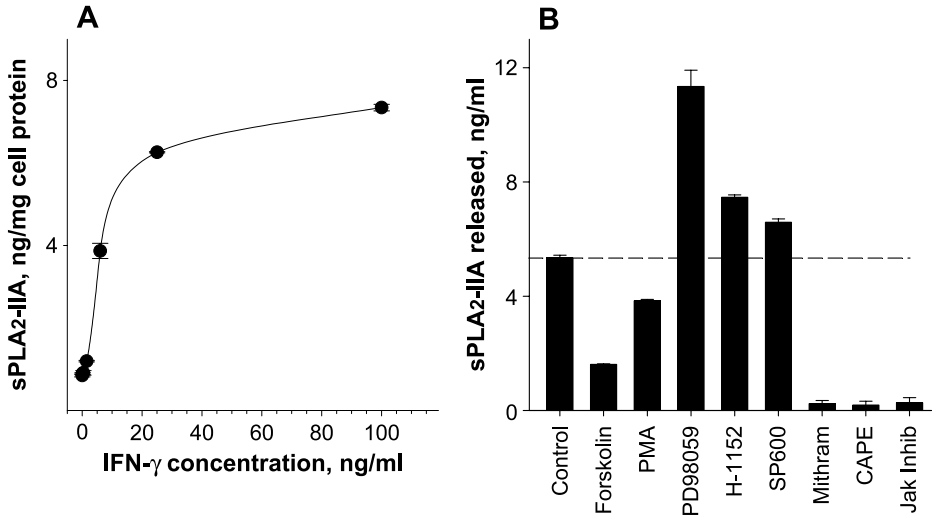

Figure 3. Dose-dependent effects of IFN-γ (A) and effects of pharmacological manipulations of cell signaling pathways (B) on $sPLA_2$-IIA protein release from Jurkat cells treated with 5-aza-dC. Concentrations of $sPLA_2$-IIA protein in cell incubates determined after a 72-hour exposure of cells to 10 μM 5-aza-dC and increasing concentrations of IFN-γ are shown in panel A. Effects of cell signaling activators and inhibitors on the levels of $sPLA_2$-IIA after a 72-hour incubation with 25 ng/ml IFN-γ and 10 μM 5-aza-dC are shown in panel B. Forskolin (10 μM), PMA (30 ng/ml), H-1152 (10 μM), PD98059 (PD98; 50 μM), indirubin (100 nM), SP-600125 (SP600; 5 μM), mithramycin A (Mithram; 250 nM), CAPE (25 μM), or Jak inhibitor I (Jak Inhib; 1 μM) were added simultaneously with IFN-γ and 5-aza-dC. The data shown are the means ± SDs of analysis in triplicate and are representative of three independent experiments.

activated/extracellular response protein kinase/extracellular signal–regulated kinase 1/2 inhibitor, PD-98059 (Figure 3*B*). Expression was also increased, albeit to a lesser extent, after incubation of 5-aza-dC– and IFN-γ–treated cells with H-1152, a Rho-kinase inhibitor, and SP-600125, a Jun N-terminal kinase inhibitor.

An inhibition of $sPLA_2$-IIA protein release was found when Jurkat cells treated with 5-aza-dC and IFN-γ were exposed simultaneously to forskolin (Figure 3*B*). A similar inhibitory effect was seen by PMA as an activator of protein kinase C. Finally, mithramycin A, an inhibitor of specificity protein 1 (Sp1) binding to DNA, CAPE, an inhibitor of NF-κB, and Janus kinase inhibitor I completely abolished the reexpression of $sPLA_2$-IIA induced by 5-aza-dC and IFN-γ in Jurkat cells (Figure 3*B*).

Effects of 5-aza-dC Treatment on the Methylation Status of Selected 5′-CpG Sites in the Proximal $sPLA_2$-IIA Promoter

The analyses using MethPrimer demonstrated that distinct 5′-CpG islands are present only in *PLA2G10* and *PLA2-R* genes. No 5′-CpG islands were detected in the promoter regions of *PLA2G2A* (Figure 4*A*), *PLA2G1B*, *PLA2G3*, and *PLA2G5* genes (data not shown). Furthermore, using TFSEARCH and ooTFD software, we found that the $sPLA_2$-IIA promoter region from −260 to +20 contained a number of potential binding sites, including those for signal transducer and activator of transcription (STAT) [−255, −246], NF-κB [−251, −241], CAAT-enhancer–binding protein β (C/EBP-β) [−177, −169], NF-Y [−114, −97], Sp1 [−86, −76], and γ-IRE [−197, −189] (Figure 4*A*).

In Figure 4*B*, data of sequence analyses of 5′-CpG sites −186, −111, −82, and +8 in the reverse strand are shown. A complete conversion of guanine into adenine demonstrated that the bisulfite modification was effective. Similar results were obtained by analysis of the forward strand (data not shown). As a positive control, enzymatically hypermethylated genomic DNA from Jurkat cells was used. Here, the residual unmethylated portions of 5′-CpG sites −111, −82, and +8 disappeared completely as a sign of total methylation of these sites (Figure 4*B*).

Finally, sequence analyses of genomic DNA from Jurkat cells demonstrated that the 5′-CpG sites −186, −111, −82, and +8 were methylated to ~100%, ~83%, ~79%, and nearly 100%, respectively. After 5-aza-dC treatment for 72 hours, the methylated portions of 5′-CpG sites −111, −82, and +8 decreased to ~20%, 0%, and ~83%, respectively. The methylation status of the 5′-CpG site −186 did not changed significantly after exposure of cells to 5-aza-dC (Figure 4*B*).

The data obtained by sequence analyses were supported by methylation-specific PCR, showing that the 5′-CpG −82 site was strongly demethylated and the −111 site partially demethylated by 5-aza-dC; in contrast, no demethylation of the 5′-CpG −186 site was evident (Figure 4*C*).

Reexpression of $sPLA_2$-IIA in Human U937 Leukemia Cells

As shown in Figure 5, treatment of U937 leukemia cells with 5-aza-dC restored $sPLA_2$-IIA expression, and this was further markedly facilitated by simultaneous addition of IFN-γ. The levels of $sPLA_2$-IIA released into the medium of these cells were distinctly lower compared with those released into the medium by Jurkat cells. The pattern of reexpression was, however, overall qualitatively similar in both leukemic cell lines, except that IL-6 increased $sPLA_2$-IIA release in Jurkat cells, but not in U937 cells, and TNF-α increased $sPLA_2$-IIA release in U937 cells, but not in Jurkat cells (Figures 2 and 5).

Discussion

In this study of five secreted phospholipases A_2 (groups IB, IIA, III, V, and X) in T-lymphoblastic Jurkat cells, we show that epigenetic mechanisms regulate the expression of two isozymes, $sPLA_2$-IIA and $sPLA_2$-V. Scanning of 5′-flanking promoter regions (−1000; +200) of corresponding *PLA2G2A* and *PLA2G5* genes indicated a relatively low number of 5′-CpG sites and no 5′-CpG islands. In contrast, *PLA2G10* and *PLA2-R* genes are rich in 5′-CpG islands (data not shown). However, the constitutive expression of *PLA2G10* and *PLA2-R* and failed strong effects of 5-aza-dC on the expression of both genes argue against a significant involvement of epigenetic mechanism such as hypermethylation in the regulation of these genes in Jurkat cells.

Nevertheless, that the *PLA2G2A* and *PLA2G5* genes have no 5′-CpG islands and the presence of clusters of methylated 5′-CpG sites or even a single site—instead of 5′-CpG islands within the binding regions of crucial transcription factors—can be sufficient for gene silencing as shown for other genes [31–33]. Such sites may also be responsible for silencing of *PLA2G2A* and *PLA2G5*.

By using TFSEARCH [23,24] and ooTFD [25] software, we confirmed a number of specific cognate binding sites in the proximal promoter region from −260 to +20 of the *PLA2G2A* gene for transcription factors known to be important for the *PLA2G2A* promoter activity, including Sp1, NF-κB, STAT, and C/EBP-β [4,18,19,29]. Two additional binding sites were found for NF-Y, also called CCAAT box-binding factor [34], and γ-interferon response element (γ-IRE) in the same region (Figure 4*A*). The former transcription factor, NF-Y, is considered to be important for the *PLA2G2A* promoter activity [18]. How far the latter transcription factor, γ-IRE, in addition to previously examined transcription factors, is actually essential for promoter activity, however, requires further investigation.

The data from sequence analyses and methylation-specific PCR demonstrated that four 5′-CpG sites (−186, −111, −82, and +8) at this $sPLA_2$-IIA promoter region were almost completely methylated. After 5-aza-dC treatment, two of the sites (−82 and −111) became quantitatively demethylated, whereas some demethylation occurred at the +8 site and negligible demethylation at the −186 site.

The demethylation of 5′-CpG sites at −82 and −111 of the $sPLA_2$-IIA proximal promoter regions by 5-aza-dC may be of special importance. As shown in Figure 4*A*, site −82 is within the potential Sp1 binding domain, 5′-GACCACGCC-3′, near the TATA box of the $sPLA_2$-IIA promoter [18,35], whereas site −111 is part of the DNA binding region for NF-Y/CCAAT box-binding factor. Recently, it was demonstrated that binding of both transcription factors is crucial for full induction of the promoter activity of the human *PHGDH* gene in HeLa cells [36]. Possibly, therefore, hypermethylation of 5′-CpG sites at −82 and −111 of the proximal *PLA2G2A* promoter may result in failure of both transcription factors to bind at putative Sp1 and NF-Y binding sites, with subsequent absence of $sPLA_2$-IIA expression. The importance of Sp1 binding in the regulation of $sPLA_2$-IIA expression is supported by our findings that blockade of Sp1 binding sites by mithramycin A completely abolished the induction of $sPLA_2$-IIA by 5-aza-dC and IFN-γ. Mithramycin A acts as an inhibitor of Sp1. It binds to 5′-CpG sites and 5′-CpG–rich tracts as a dimer that forms in the presence of magnesium [37]. The importance

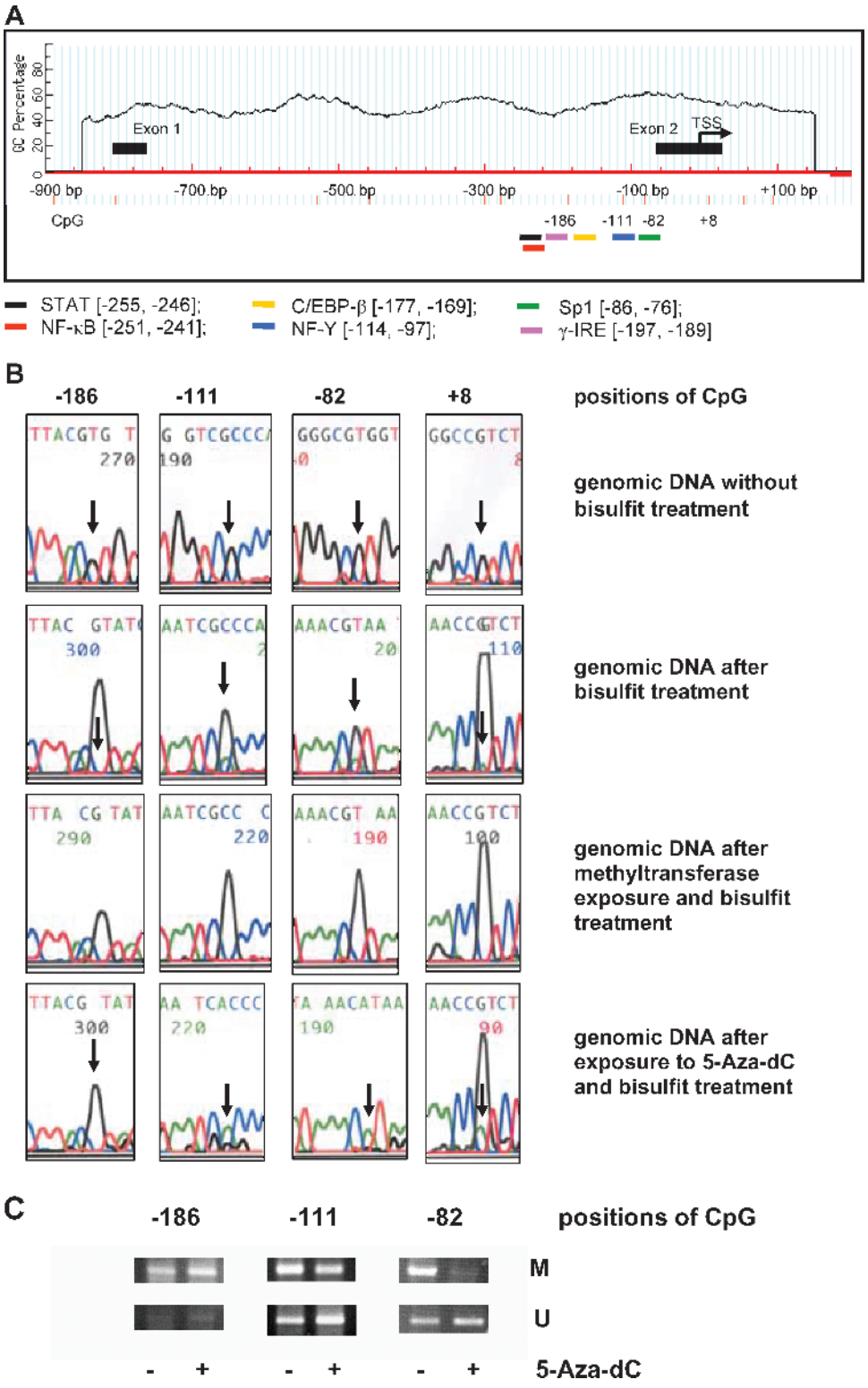

Figure 4. Methylation of the proximal promoter of the *PLA2G2A* gene. (A) Location of 5′-CpG sites and potential binding regions for transcription factors in the proximal promoter of *PLA2G2A* gene was assessed using MethPrimer and TFSEARCH software as described in the Materials and Methods section. The gene sequence is numbered [−900, +200 bp] relative to the transcription start site [29] and the proximal promoter region [−260, +20] of *PLA2G2A*. Positions of potential binding sites for transcription factors, STAT, NF-κB, C/EBP-β, NF-Y, Sp1, and γ-IRE, are illustrated. (B) Methylation status of selected 5′-CpG sites in the proximal *PLA2G2A* promoter of Jurkat genomic DNA before and after exposure to 10 μM 5-aza-dC as described in the Materials and Methods section. Representative results of sequence analyses of the bisulfite-treated genomic reverse DNA strands are shown. Arrows indicate the conversion of reverse 5′-CpG sites, −186, −111, −82, and +8, in dependence on the methylated status. (C) Methylation-specific PCR analyses of genomic DNA before and after exposure of Jurkat cells to 5-aza-dC. M or U indicate the presence of methylated or unmethylated 5′-CpG sites.

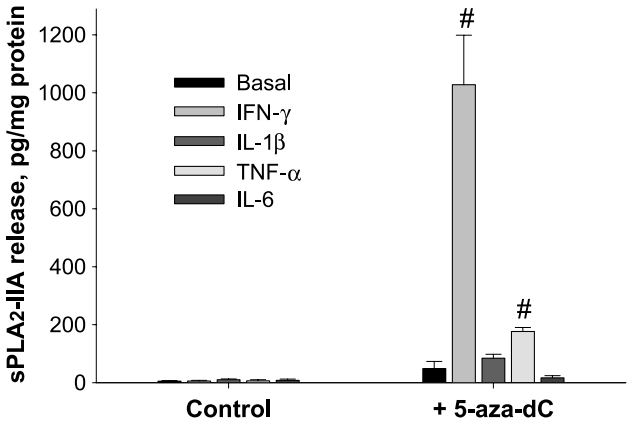

Figure 5. Effects of 5-aza-dC alone and in combination with proinflammatory cytokines on $sPLA_2$-IIA protein levels released by U937 leukemia cells. Cytokines were added at a final concentration of 25 ng/ml, and cells were exposed to 10 μM 5-aza-dC or vehicle (DMSO) for 72 hours, respectively. Results are the means ± SDs of three separate analysis in quadruplicates ($n = 12$). #$P < .05$ *versus* cells exposed to 5-aza-dC but not to cytokines.

of NF-Y for $sPLA_2$-IIA promoter activity, however, still has to be analyzed more thoroughly.

Our findings that the Jak inhibitor I and CAPE, an inhibitor of NF-κB, completely blocked the 5-aza-dC– and IFN-γ–mediated induction of $sPLA_2$-IIA (Figure 3) suggest that for an optimal $sPLA_2$-IIA expression, in addition to Sp1, the activities of NF-κB and STAT are necessary. The activities of these transcription factors are modulated by upstream cell signaling pathways. As demonstrated in a variety of cell types—from human aortic smooth muscle cells, HepG2 hepatoma cells, and prostate epithelial cells to human malignant prostate cell lines (PC-3 and LNCaP)—all of these pathways have negative regulatory influences on $sPLA_2$-IIA expression [4,19]. Our data indicate the presence of similar negative regulatory influences on the expression of $sPLA_2$-IIA in Jurkat cells.

Our data, however, also indicated different effects of cAMP/protein kinase A (PKA) signaling pathway on the $sPLA_2$-IIA expression in Jurkat cells compared with other cell types [38,39]. In normal prostate epithelial cells and tumor prostate PC-3 cells, for example, activation of cAMP/PKA by forskolin in the presence of IFN-γ results in a synergistic up-regulation of $sPLA_2$-IIA [19]. In contrast, an inhibitory effect of forskolin has been observed in malignant prostate LNCaP cells [19] and lipopolysaccharide (LPS)–stimulated alveolar macrophages [40], a response similar to the effect observed in the present study in Jurkat cells. The reason for the divergent effects of the cAMP/PKA signaling pathway among different cell types presently remains unclear. Interestingly, trichostatin A, an inhibitor of histone deacetylase activity, decreased the LPS-stimulated $sPLA_2$-IIA expression in alveolar macrophages [40]. It is known that histone deacetylase activity is inhibited by PKA-dependent phosphorylation [41]. This suggests that histone deacetylation as a further epigenetic mechanism may be involved in the forskolin-mediated effect on the LPS-stimulated $sPLA_2$-IIA expression in alveolar macrophages. Whether a similar mechanism is also acting in Jurkat cells remains unknown.

The importance of diverse cell signaling cascades in the regulation of $sPLA_2$-IIA expression underscores the opportunity that, in addition to the direct demethylation of 5′-CpG sites in the *PLA2G2A* promoter itself, indirect mechanisms may also be responsible for the reexpression of $sPLA_2$-IIA mediated by 5-aza-dC. It is conceivable that this may involve demethylation of 5′-CpG sites in the promoters of corresponding upstream signaling components or transcription factors. Indeed, our search of 5′-CpG islands confirmed the presence of distinct islands in gene promoters of relevant regulators of $sPLA_2$-IIA expression, including Sp1, NF-κB p65, Jak/STAT, Rho-kinase, and C/EBP-β (data not shown).

Interferon response elements have also been identified to exhibit 5′-CpG islands [42]. Of note, the $sPLA_2$-IIA promoter at positions [−197, −189] has a potential binding site for the γ-IRE (Figure 4*A*). It is interesting that, in leukemic cells, such regulatory elements were down-regulated due to hypermethylation of 5′-CpG motifs in their promoter regions [42]. Therefore, it is possible that IREs are also involved in the restoration of $sPLA_2$-IIA expression by 5-aza-dC.

In general, the regulation of the *PLA2G2A* promoter is complex and includes species- and cell type–specific mechanisms of induction by proinflammatory mediators (for review [3,4,29]). TNF-α and IL-1β effectively up-regulate the expression of $sPLA_2$-IIA in numerous cell types; moreover, a strong up-regulation of $sPLA_2$-IIA was found solely by IFN-γ [4] including cell lines with reexpressed *PLA2G2A* after 5-aza-dC treatment such as malignant prostate DU-145 cells [19] or Jurkat and U-937 cells as shown here.

The observation that the inhibition of DNA methylation can restore the cytokine-mediated effects on $sPLA_2$-IIA and $sPLA_2$-V expression may have important implications in the development of novel strategies to treat leukemia. Cytokines are known to be crucial in a variety of cellular and molecular events [43,44]. For example, IFN-γ stimulates antiproliferative pathways in tumor cell lines [45]. Expression data in immortalized fibroblasts revealed that among genes with methylation-dependent silencing, approximately 50% were regulated through interferon-dependent growth-suppressive signaling pathways [46]. Furthermore, the efficiency of cytokine-mediated effects can be amplified in cells treated with 5-aza-dC. For example, 5-aza-dC and IFN-γ at relatively low individual concentrations acted in a proapoptotic and synergistic manner in resistant neuroblastoma, medulloblastoma [47], and metastatic uveal melanoma cells [48].

Finally, our current data demonstrate that epigenetic silencing of *PLA2G2A* and *PLA2G5* is not restricted to Jurkat cells but also occurs in leukemic U937 cells. The pathophysiological relevance of *PLA2G2A* and *PLA2G5* silencing in malignant cells remains unclear. Nevertheless, the finding of the present study support the growing body of evidence that the antioncogenic and proapoptotic properties of $sPLA_2$ isozymes may have importance for novel strategies targeting defective apoptosis pathways in tumors [49–53]. It should be noted, however, that 5-aza-dC treatment induces not only some well-established antioncogenic genes but also a set of prometastatic genes [54]. Therefore, establishing in detail which genes are up-regulated by 5-aza-dC treatment is important to understanding the overall prooncogenic or antioncogenic effects of such a treatment.

In conclusion, the data of this study indicate that DNA methylation plays a crucial role in the regulation of $sPLA_2$-IIA and $sPLA_2$-V in Jurkat and U937 cells. Whether a direct hypermethylation of single 5′-CpG sites of the proximal promoter may explain $sPLA_2$-IIA silencing in Jurkat and other cells or whether the hypermethylation of 5′-CpG sites in the promoter of corresponding upstream acting transcription factors or signaling components are responsible requires further investigation.

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