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Differential Expression of Secretory Phospholipases A_2 in Normal and Malignant Prostate Cell Lines: Regulation by Cytokines, Cell Signaling Pathways, and Epigenetic Mechanisms

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Abstract

Upregulation of group IIA phospholipase A_2 (sPLA$_2$-IIA) correlates with prostate tumor progression suggesting prooncogenic properties of this protein. Here, we report data on expression of three different sPLA$_2$ isozymes (groups IIA, V, and X) in normal (PrEC) and malignant (DU-145, PC-3, and LNCaP) human prostate cell lines. All studied cell lines constitutively expressed sPLA$_2$-X, whereas sPLA$_2$-V transcripts were identified only in malignant cells. In contrast, no expression of sPLA$_2$-IIA was found in PrEC and DU-145 cells, but it was constitutively expressed in LNCaP and PC-3 cells. Expression of sPLA$_2$-IIA is upregulated in PC-3 and in PrEC cells by IFN-γ in a signal transducer and activator of transcription-1–dependent manner, but not in LNCaP cells. Additional signaling pathways regulating sPLA$_2$-IIA expression include cAMP/protein kinase A, p38 mitogen-activated protein kinase, protein kinase C, Rho-kinase, and mitogen-activated/extracellular response protein kinase / extracellular signal–regulated kinase. No deletions were revealed in the *sPLA$_2$-IIA* gene from DU-145 cells lacking the expression of sPLA$_2$-IIA. Reexpression of sPLA$_2$-IIA was induced by 5-aza-2′-deoxycytidine demonstrating that DNA methylation is implicated in the regulation of sPLA$_2$-IIA. Together, these data suggest that sPLA$_2$-IIA and sPLA$_2$-V, but not sPLA$_2$-X, are differentially expressed in normal and malignant prostate cells under the control of proinflammatory cytokines; epigenetic mechanisms appear involved in the regulation of sPLA$_2$-IIA expression, at least in DU-145 cells.

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Introduction

Secretory phospholipases A_2 (sPLA$_2$, phosphatide *sn*-2-acylhydrolases; EC 3.1.1.4) belong to a growing and structurally heterogenous superfamily of phospholipases that play a critical role in a number of relevant physiological processes including defense mechanisms, production of bioactive lipids, and cell signaling [1–3]. At present, 10 different human sPLA$_2$ isozymes are known. Among these, the group IIA isozyme, sPLA$_2$-IIA, is the best established to contribute to the pathogenesis of inflammatory diseases such as sepsis and septic shock, rheumatoid arthritis, and atherosclerosis [1–3]. Other studies suggest that sPLA$_2$-IIA is also important for the genesis of different cancers [4–11]. For example, increased expression of sPLA$_2$-IIA correlates with tumor grade in human colon cancer and Barrett's adenocarcinoma [12,13].

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; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-6, interleukin-6; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PKC, protein kinase C; PMA, phorbol-12-myristate 13-acetate; RT-PCR, reverse transcription–polymerase chain reaction; SP1, specificity protein 1; sPLA$_2$-IIA, secretory phospholipase A_2 of group IIA; STAT1, signal transducer and activator of transcription-1; TNF-α, tumor necrosis factor-alpha

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Furthermore, increased levels of this enzyme have been found in the extracellular microenvironment of prostate cancer tissues and cell lines [4–6] correlating with poor survival [7] and high aggressiveness [8].

The role of $sPLA_2$-IIA in tumorigenesis, however, remains unresolved. A protective function of $sPLA_2$-IIA has been suggested from data in Min mice, in which mutations of the adenomatous polyposis coli gene lead to the development of multiple adenomas throughout their small and large intestines [14]. However, this function could not be proven in humans where *$sPLA_2$-IIA* gene mutations do not appear to play a major role in the development of colorectal cancers [15,16]. Otherwise, in human pancreatic cancer cells and gastric adenocarcinoma, the expression of $sPLA_2$-IIA was associated with prolonged survival and less frequent metastasis [14,17,18]. In contrast, prooncogenic properties of $sPLA_2$-IIA upregulation have been suggested from studies on different tumor cell lines and xenograft tumor models [6,19,20].

The expression of $sPLA_2$-IIA is regulated through a number of growth peptides, cytokines, and transcription factors in a cell-type–specific manner [1–3,21]. However, the role of proinflammatory cytokines and related signaling pathways in the regulation of $sPLA_2$-IIA in prostate cancer cell lines remains to be determined. This question has special interest because of the well-known relationship between progression of cancers and levels of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-6 (IL-6) [22–26]. It was suggested that transcription factors such as nuclear factor-kappa B (NF-κB) and members of the signal transducer and activator of transcription (STAT) family play pivotal roles in transmitting proinflammatory cytokine signals during tumor development [27]. These transcription factors are necessary at the same time for $sPLA_2$-IIA promoter activity [3].

The potential contribution of proinflammatory cytokines to the expression of $sPLA_2$ in prostate cancer cells provided the premise for the present study. To this end, we analyzed the expression of three $sPLA_2$ isozymes in malignant prostate cell lines and compared the expression pattern with that found in normal prostate epithelial cells.

Materials and Methods

Materials

Recombinant human interleukin-1β (IL-1β), IL-6, TNF-α, and IFN-γ were purchased from Roche Diagnostics Applied Science (Mannheim, Germany). 2′-Amino-3′-methoxyflavone (PD98059), Janus kinase (JAK) inhibitor-I, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1*H*-imidazole (SB-202190), phorbol-12-myristate 13-acetate (PMA), and (*s*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-homopiperazine, 2HCl (H-1152) were obtained from Calbiochem (San Diego, CA). Forskolin, *N*-acetyl-L-cysteine, 5-aza-2′-deoxycytidine (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, SB-202190, 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

Normal human prostate epithelial cells (PrEC; Cambrex Bio Science, Walkersville, MD) were maintained up to a maximum of six passages in prostate epithelial growth medium supplemented with bovine pituitary extract, epidermal growth factor, insulin, transferrin, hydrocortisone, retinoic acid, epinephrine, triiodothyronine, and gentamicin–amphotericin solution on dishes coated with collagen type I (BioCoat; BD Falcon, Heidelberg, Germany). Every 2 to 3 days, the medium was changed, and before reaching confluence, the cells were passaged using trypsin/ethylenediaminetetraacetic acid.

Human prostatic malignant cell lines (PC-3, DU-145, and LNCaP cells) were purchased from the German Collection of Microorganisms and Cell Cultures (Berlin, Germany). They were cultured in a standard cell culture medium RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 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 5-aza-dC treatment, DU-145 cells were cultured in RPMI 1640 cell medium containing 10% FCS and 5-aza-dC added to the final concentration of 1 to 10 μM from a freshly prepared 10-mM stock solution. After 2 to 4 days, the cells were harvested and analyzed for $sPLA_2$-IIA protein and mRNA levels [28].

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis

RNA was isolated after lysing 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). A portion of the reverse transcription (RT) reaction products was then amplified for the identification of $sPLA_2$-IIA, -V, -X, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene using polymerase chain reaction (PCR). The applied primer pairs were 5′-GTG ATC ATG ATC TTT GGC CTA CTG CA-3′ and 5′-TCT CCC TCG TGG GGA GCA ACG ACT-3′ for $sPLA_2$-IIA, giving PCR products with a length of 411 bp, 5′-GGG CTG CAA CAT TCG CAC AC-3′ and 5′-CCT CTC TCA GGA ACC AGG CAG-3′ for $sPLA_2$-V, giving PCR products with a length of 278 bp, 5′-CCA TCG CCT ATA TGA AAT ATG G-3′ and 5′-TAG GAA CTG GGG GTA GAA GAG-3′ for $sPLA_2$-X, giving PCR products with a length of 295 bp, and 5′-CGG AGT CAA CGG ATT TGG TCG TAT TG-3′ and 5′-GCA GGA GGC ATT GCT GAT GAT CTT G-3′ for GAPDH amplifying products with a length of 439 bp. The oligonucleotides for the analysis of mRNA were synthesized according to the published nucleotide sequences of human *$sPLA_2$-IIA*, *$sPLA_2$-V*, *$sPLA_2$-X*, and *GAPDH* genes [21,29,30]. In the PCR, primer pairs were applied to 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. The buffers and reagents used were from GeneAmp Kit (PerkinElmer LAS GmbH). After amplification, products were analyzed by electrophoresis on agarose gels. GAPDH mRNA was determined in every sample as a reference.

Enzyme-Linked Immunosorbent Assays Specific for $sPLA_2$-IIA and Transcription Factors

The $sPLA_2$-IIA protein released into the medium was determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Cayman Chemical, MI). Culture medium was removed, the cell surface–bound $sPLA_2$-IIA was extracted with ice-cold PBS, pH 7.4, containing 1 M NaCl, and the extract was collected with cell culture medium, centrifuged for 10 minutes at 400*g* to remove cell debris, and was used for $sPLA_2$-IIA protein

determination in the ELISA. Total cell protein was determined using a Bicinchoninic Acid assay kit (Sigma-Aldrich).

For the determination of NF-κB, CAAT-enhancer–binding protein-β (C/EBP-β), specificity protein 1 (SP1), and STAT1 DNA binding activities, cells were incubated in six-well plates. After incubation, nuclear extracts were prepared using a nuclear extraction kit (Active Motif, Carlsbad, CA). The DNA binding capacities of NF-κB p65 and C/EBP-β in nuclear extracts were estimated using ELISA-based assays (Active Motif) according to the manufacturer's instructions. STAT1 and SP1 DNA binding activities were studied using STAT1 and SP1 ELISA kits from Panomics, Inc. (Fremont, CA). The phosphorylation of STAT1 was measured using a cell-based ELISA kit (RayBiotech, Inc., Norcross, GA) according to the manufacturer's instructions.

DNA Sequencing

DNA sequences were analyzed with a genetic analyzer (ABI PRISM 310; Perkin-Elmer Applied Biosystems, Foster City, CA).

Data Analysis

Data were analyzed by two-tailed and unpaired Student's *t* test to calculate the indicated *P* values. Differences were considered significant at $P < .05$.

Results

Basal and Cytokine-Induced Expression of Group IIA, V, and X Phospholipases A_2 in Normal and Malignant Prostate Cells

In prostate cancer cells, PC-3 and LNCaP, $sPLA_2$-IIA mRNA was constitutively expressed, whereas in normal PrEC and malignant DU-145 cells, no $sPLA_2$-IIA transcripts were detectable (Figure 1). However, exposure to IFN-γ led to the induction of $sPLA_2$-IIA expression in PrEC and to further upregulation in PC-3 cells. No cytokine-mediated induction of $sPLA_2$-IIA transcripts occurred in DU-145 cells. Similar results were also obtained at the protein level where negligible amounts of $sPLA_2$-IIA were released from DU-145 cells after incubation with cytokines (Figure 2). In LNCaP cells, $sPLA_2$-IIA protein was produced under normal conditions at about 700-fold higher levels compared to PC-3 cells (Figure 2, *A* and *B*). This high basal expression in LNCaP cells was further increased by IL-1β, TNF-α, and IL-6 treatments by 5.0-, 2.0-, and 1.5-fold, respectively, but not by IFN-γ.

Transcripts of $sPLA_2$-V were consistently detected in PC-3 and LNCaP cells, but levels were relatively insensitive to the addition of cytokines (Figure 1). In PrEC cells, no $sPLA_2$-V transcripts were detected, and these could not have been induced by any of the investigated cytokines. Under basal conditions, moderate levels of $sPLA_2$-V mRNA were detected in DU-145 cells; these levels were strongly induced by IFN-γ. No inductive effects on $sPLA_2$-V expression were observed in this cell line with IL-1β, IL-6, and TNF-α (Figure 1). The $sPLA_2$-X isozyme was strongly expressed in all studied cell lines, and no marked response to the added cytokines was observed. In all samples, levels of GAPDH mRNA were comparable (Figure 1).

Analysis of the active concentration range for IFN-γ indicated that IFN-γ–mediated upregulation of $sPLA_2$-IIA in normal PrEC and malignant PC–3 cells was dose-dependent (Figure 2*C*). A half-maximal effect was observed at ~4 ng/ml IFN-γ in PC-3 cells and at ~12 ng/ml IFN-γ in PrEC cells.

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

Because, in DU-145 cells, no basal or cytokine-induced $sPLA_2$-IIA expressions were identifiable, we analyzed signaling components only in PrEC, PC-3, and LNCaP cells. A marked increase in release of $sPLA_2$-IIA protein was observed in PrEC and PC-3 cells when these cells were simultaneously stimulated with both IFN-γ and forskolin, in comparison to cells exposed to IFN-γ alone (Figure 3). In contrast, forskolin attenuated the $sPLA_2$-IIA expression induced by IL-1β in LNCaP cells. Phorbol-12-myristate 13-acetate, an activator of protein kinase C (PKC), inhibited the cytokine-stimulated expression of $sPLA_2$-IIA in all three analyzed cell types. H-1152, an inhibitor of Rho-kinase, and PD-98059, an inhibitor of mitogen-activated/extracellular response protein kinase, increased further the cytokine-induced $sPLA_2$-IIA production in PrEC, PC-3, and LNCaP cells. Conversely, a significant suppression of $sPLA_2$-IIA occurred in prostate cell lines after exposure to SB-202190, mithramycin A, JAK inhibitor-1, and CAPE, which inhibit p38 mitogen-activated protein kinase (MAPK), SP1 binding activity, the JAK/STAT pathway, and NF-κB, respectively. Other NF-κB inhibitors, such as pyrrolidine dithiocarbamate, *N*-acetyl-L-cysteine, and 20S proteasome inhibitor, also decreased the amounts of $sPLA_2$-IIA released into the medium (data not shown).

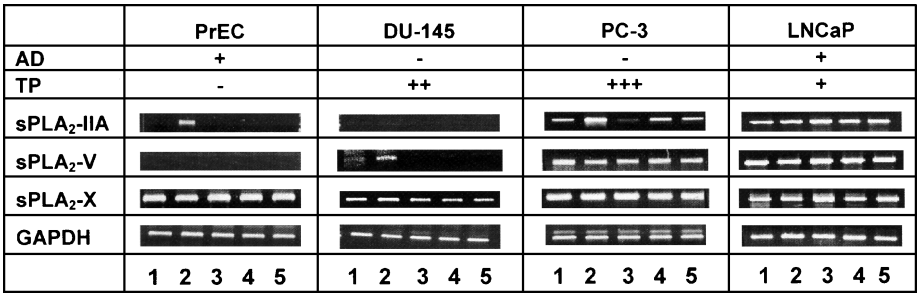

Figure 1. Agarose gel electrophoresis showing amplifiers of different secretory phospholipase A_2 isozymes ($sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X) and GAPDH mRNA after RT-PCR analysis expressed in normal and malignant prostate cell lines under basal conditions and after exposure to proinflammatory cytokines. Cells were incubated for 24 hours in medium containing 10% FCS alone and additions of cytokines at a final concentration of 25 ng/ml. Lanes 1 to 5 are controls (without additions of cytokines), IFN-γ, IL-1β, IL-6, and TNF-α, respectively. Data are representative of at least three independent experiments giving similar results. AD and TP indicate the androgen dependence and tumorigenicity potential according to literature [32,34].

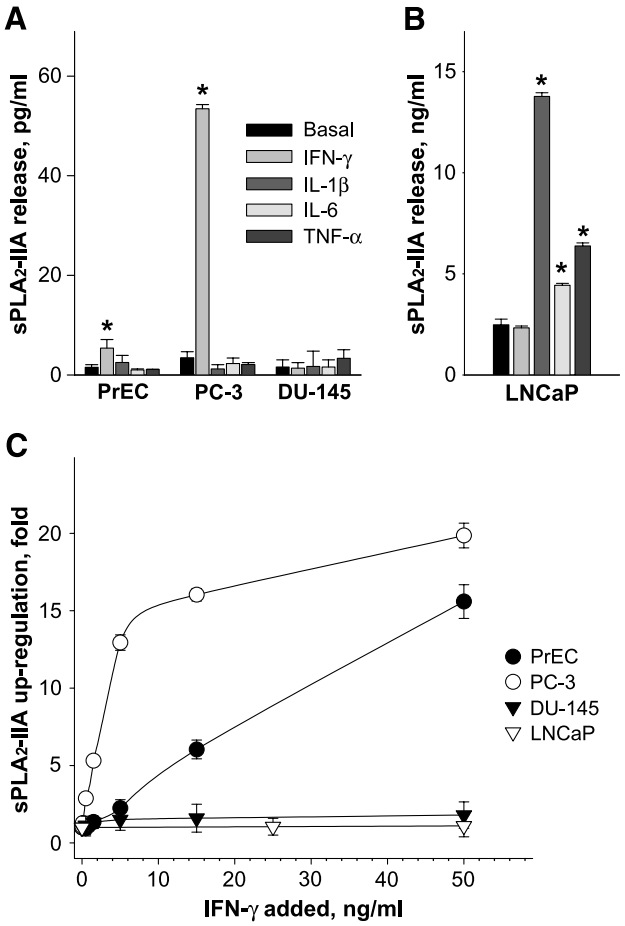

Figure 2. Effects of proinflammatory cytokines on sPLA$_2$-IIA expression in normal (PrEC) and malignant (DU-145, PC-3, and LNCaP) prostate cells. Cytokines were added in a final concentration of 25 ng/ml. The sPLA$_2$-IIA amounts released into the medium by PrEC, PC-3, and DU-145 cells (A) were measured after 48 hours of incubation. LNCaP cells (B) were incubated for 24 hours. *$P <$ .05 *versus* cells incubated without cytokines (basal expression). (C) Concentration dependence of IFN-γ on sPLA$_2$-IIA upregulation in prostate cells. The results are expressed as changes relative to the basal level of sPLA$_2$-IIA secretion without IFN-γ treatment. The basal levels of sPLA$_2$-IIA (pg/mg cell protein) in PrEC, PC-3, DU-145, and LNCaP cells were 10.05 ± 1.34; 13.11 ± 0.95; 2.50 ± 2.91, and 9898.15 ± 171.43, respectively. The prostate cells were incubated with indicated concentrations of IFN-γ for 48 hours. Results are the means ± SD of analysis in quadruplicate and are representative of three independent experiments.

Activities of Transcription Factors in IFN-γ–Treated Prostate Cells

Using a cell-based ELISA, we showed a marked difference in the time course of cellular STAT1 phosphorylation (P-STAT1) induced by IFN-γ (Figure 4). After 15 minutes of incubation, the highest reactivity in P-STAT1 values was found in PC-3 cells followed by PrEC and DU-145 cells. No increased P-STAT1 signals were detected during the whole incubation time-course in LNCaP cells (Figure 4). In addition to STAT1 phosphorylation, we studied the DNA binding activities of STAT1 using nuclear extracts of cells treated with IFN-γ (Figure 5). There was a ~20-fold activation of STAT1 in PC-3 cells after exposing to IFN-γ for 2 hours. In PrEC cells, this activation averaged ~10-fold, and in DU-145 cells, it averaged ~ 6-fold. In LNCaP cells, again no significant activation of STAT1 occurred.

Large differences were found in the basal binding activities of STAT1, NF-κB, C/EBP-β, and SP1 between the different prostate cell lines (Figure 5). In contrast to STAT1 activation, however, no significant changes in DNA binding activities of NF-κB, C/EBP-β, and SP1 were found, with the exception of SP1, the binding activity of which decreased in PC-3 cells after incubation with IFN-γ (Figure 5). In LNCaP cells, treatment with IL-1β did not result in STAT1 activation (data not shown).

Reexpression of sPLA$_2$-IIA in DU-145 Cells After Treatment with a DNA Methyltransferase Inhibitor

DU-145 cells do not express sPLA$_2$-IIA either constitutively or after exposure to cytokines. Therefore, we addressed the question of whether failure of sPLA$_2$-IIA expression in this cell line may be due to *PLA2G2A* gene mutations. For this reason, we analyzed the sequence of the proximal part of the promoter region [–319, +111] and exons 2 to 5 of the *PLA2G2A* gene (access codes M22431 and AY462114, transcription start site according to the study of Andreani et al. [31]). Our data did not show substantial differences between DU-145 DNA probes and the wild-type sequence (data not shown).

To define the contribution of epigenetic mechanisms, malignant DU-145 cells were incubated with the DNA methyltransferase inhibitor, 5-aza-dC, for 48 to 96 hours. No transcripts specific for sPLA$_2$-IIA were present in DU-145 cells treated with DMSO as a control, but transcripts became detectable after incubating with

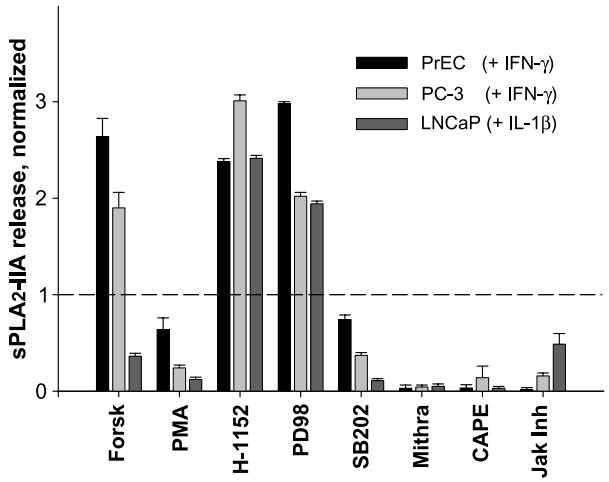

Figure 3. Effects of activators and inhibitors of cell signaling pathways on the sPLA$_2$-IIA expression in normal PrEC and malignant PC-3 cells stimulated with IFN-γ and in malignant LNCaP cells stimulated with IL-1β. Cells were incubated for 48 hours. The sPLA2-IIA levels (pg/mg cell protein) induced by corresponding cytokines were 19.57 ± 2.96; 268.49 ± 4.61; 23640.0 ± 130.0 in PrEC, PC-3, and LNCaP cells, respectively. Forskolin (Forsk; 10 μM), PMA (30 ng/ml), H-1152 (10 μM), PD98059 (PD98; 50 μM), SB-202190 (SB202; 50 μM), mithramycin A (Mithra; 250 nM), CAPE (25 μM), or JAK inhibitor-I (JAK Inh; 1 μM) was added simultaneously with cytokines. Results are expressed as changes relative to the control (treated just with cytokine) that was assigned a value of 1.0. The data shown are the means ± SD of analysis in triplicate and are representative of four independent experiments.

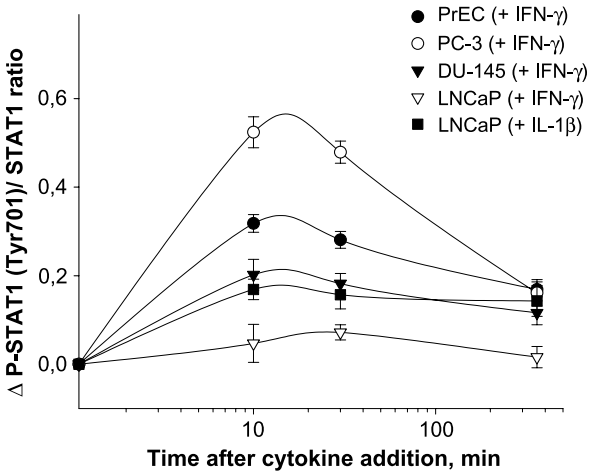

Figure 4. Signal transducer and activator of transcription-1 (STAT1) phosphorylation induced by IFN-γ in PrEC, PC-3, DU-145, and LNCaP cells. The STAT1 phosphorylation was determined by cell-based ELISA technique. Each point represents the mean of cellular STAT1 activation expressed as the ratio P-STAT1/STAT1 and measured after 0, 10, 30, and 360 minutes of incubation with 25 ng/ml IFN-γ or 25 ng/ml IL-1β. The results are the means ± SD of analysis in triplicate and are representative of three independent experiments.

5-aza-dC (Figure 6*A*). The recovery of $sPLA_2$-IIA expression after 48 to 96 hours of treatment with the DNA-demethylating agent was confirmed by measuring the $sPLA_2$-IIA protein released into the cell culture medium (Figure 6*B*).

Discussion

In the current study, we present data on the expression of three $sPLA_2$ isoforms (IIA, V, and X) in normal human prostate cells and in three cancer cell lines, PC-3, DU-145, and LNCaP, with differing tumorigenicity [32–34]. The results show that the $sPLA_2$ isozymes are differentially expressed and induced in different manners in prostate cells. In normal PrEC prostate cells no basal expression of $sPLA_2$-IIA and $sPLA_2$-V was detectable, whereas both isozymes were constitutively expressed in malignant LNCaP and PC-3 cell lines. Expression of $sPLA_2$-IIA, but not the expression of $sPLA_2$-V, was inducible by IFN-γ in PC-3 and PrEC cells. Here, the IFN-γ–mediated increase of $sPLA_2$-IIA expression correlated strongly with STAT1 activation.

In contrast to PC-3 and PrEC cells, in LNCaP cells, the expression of $sPLA_2$-IIA was completely insensitive to IFN-γ. This unresponsiveness agrees with the observation that LNCaP cells are unable to initiate IFN-γ signaling due to the lack of JAK1 expression caused by an epigenetic mechanism [35]. For this reason, it was impossible to find a significant STAT1 activation by IFN-γ in LNCaP cells. Nevertheless, IL-1β, TNF-α, and IL-6 increased $sPLA_2$-IIA expression in this cell line. In PC-3 and PrEC cells, synthesis of $sPLA_2$-IIA was completely unresponsive to IL-1β, TNF-α, and IL-6, implying that corresponding signaling events and transcription factors are not functionally active in these cell lines. The finding that the JAK inhibitor-I, which suppresses both JAK1 and JAK2, significantly decreased $sPLA_2$-IIA expression in LNCaP cells, although these cells do not possess JAK1 activity, suggests that in addition to JAK1, a JAK2-dependent signaling pathway is involved in the regulation of $sPLA_2$-IIA.

Besides STAT1, other transcription factors, such as NF-κB, SP1, and C/EBP-β, also exhibit specific binding sites in the $sPLA_2$-IIA promoter region [31,36]. Although the IFN-γ–mediated upregulation of $sPLA_2$-IIA in PrEC and PC-3 cells did not correlate with the activation of NF-κB, SP1, and C/EBP-β, for an optimal expression of $sPLA_2$-IIA, at least NF-κB and SP1 are absolutely necessary. This conclusion is based on a nearly complete inhibition of $sPLA_2$-IIA by mithramycin A, an inhibitor of SP1, and CAPE, an inhibitor of NF-κB, in PrEC, PC-3, and LNCaP cells. In the case of C/EBP-β, no specific inhibitors were available. Nevertheless, with the exception of DU-145 cells, the basal DNA binding activities of C/EBP-β correlated strongly with the $sPLA_2$-IIA expression in prostate cells. This underlines that C/EBP-β is also important for an optimal $sPLA_2$-IIA expression.

In DU-145 cells, genomic analysis showed that both the failure of basal $sPLA_2$-IIA expression (see also Sved et al. [6]) and the distinct lack of all tested cytokines to induce $sPLA_2$-IIA expression at mRNA and protein levels could not be explained by deletions in the *$sPLA_2$-IIA* gene. Our experiment on exposure of DU-145 cells to an inhibitor of methyltransferases (5-aza-dC) indicated, for the first time, that the involvement of epigenetic mechanisms in the regulation of in $sPLA_2$-IIA expression. In cancer cells, epigenetic silencing of different genes is caused by the aberrant methylation of cytosines belonging to CpG islands, stretches of DNA rich in CpG dinucleotides often associated with gene promoters [19,37–41]. This methylation may perturb the expression of genes critical to the regulation of cell proliferation. In this context, one possible explanation for the activation of the $sPLA_2$-IIA in 5-aza-dC–treated DU-145 cells was that this gene promoter contains CpG islands and that methylation of these may be responsible for *$sPLA_2$-IIA* gene silencing. However, analysis of the *PLA2G2A* gene promoter region (−1000 to +200 bp from the transcription start site according to the study of Andreani et al. [31]) performed using the EMBOSS-CpGPlot program (EMBL-European Bioinformatics Institute; http://www.ebi.ac.uk) reveals no CpG islands and a relatively low number of CpG sites. Nevertheless, it has been shown that the presence of a single methylated CpG dinucleotide within the binding sites of crucial transcription factors can be sufficient for gene inactivation as described, for example, in the case of the SP1 binding site in the *thymidine kinase* gene [42]. Therefore, this mechanism may also be responsible for *$sPLA_2$-IIA* gene silencing even when no CpG islands are present in the promoter region. In agreement with this suggestion, our results show that blockage of SP1 binding sites by mithramycin A treatment almost completely abolished the expression of $sPLA_2$-IIA in PrEC, PC-3, and LNCaP cells (Figure 3).

Additional investigations have to clarify whether an aberrant methylation in DU-145 cells is taking place directly at selected CpG sites within *PLA2G2A* promoter itself or indirectly through CpG methylation in the promoter of corresponding transcription factors that regulate $sPLA_2$-IIA transcription. The failed effect of DNA methyltransferase inhibition on $sPLA_2$-IIA expression in rat mesangial cells stimulated with cytokine IL-1β [43] suggests that perhaps such a mechanism is absent in cells that normally express $sPLA_2$-IIA.

A number of upstream pathways in both normal and malignant prostate cells are involved in the activation of STAT1, NF-κB, C/EBP-β, and SP1 transcription factors, with subsequent capacity to modulate $sPLA_2$-IIA expression. According to our data, p38 MAPK kinase activates the $sPLA_2$-IIA expression, whereas PKC, RhoA/Rho-kinase, and mitogen-activated/extracellular response protein kinase /

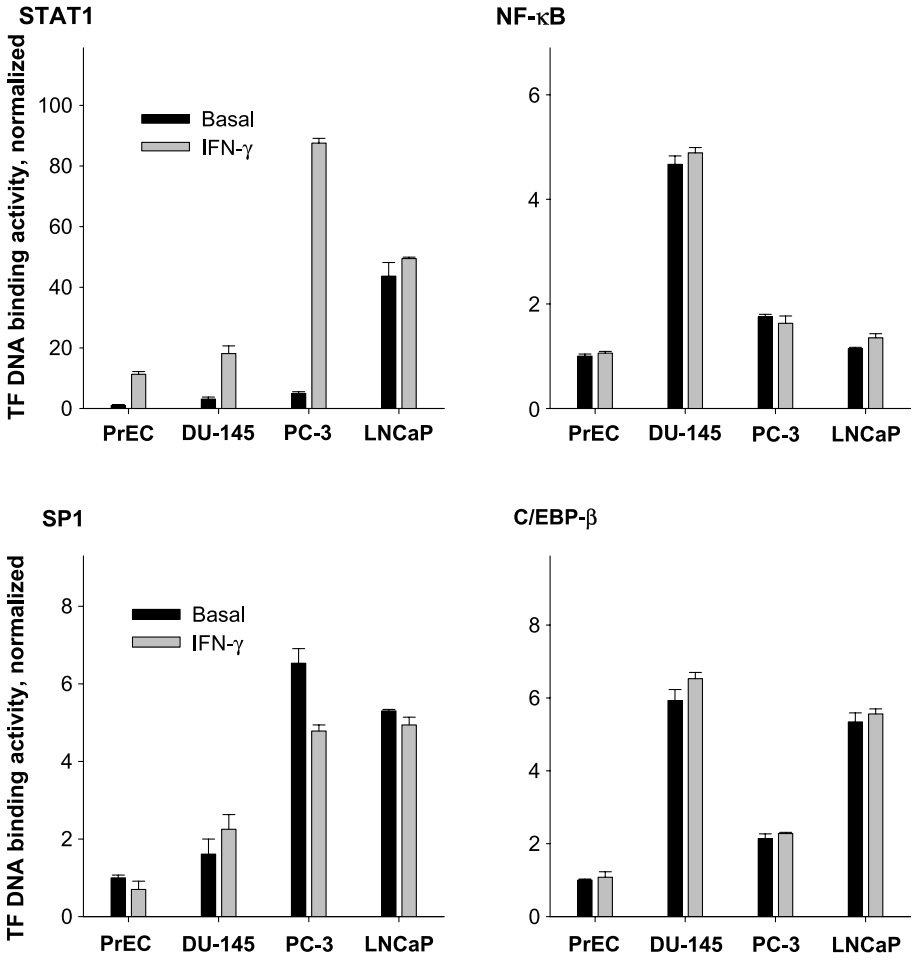

Figure 5. Effects of IFN-γ on cellular STAT1, NF-κB, SP1, and C/EBP-β activities in nuclear extract of normal (PrEC) and malignant prostate cells (PC-3, DU-145, and LNCaP). In nuclear cell extracts, the STAT1, NF-κB, SP1, and C/EBP-β DNA binding activities were measured after a 2-hour exposure of cells to 25 ng/ml IFN-γ. Obtained data for each transcription factor were normalized toward the signal level obtained in PrEC cells and are shown as means ± SD of the analyses in triplicate and are representative of three independent experiments.

extracellular signal–regulated kinase 1/2 pathways possess negative regulatory effects. It is known that PKC interferes with other signaling pathways leading to an inhibition of sPLA$_2$-IIA expression. This may relate to the sPLA$_2$-IIA downregulation found in mesangial cells [44]. Both p38 MAPK and survival kinase Akt pathways play critical roles as downstream effectors of PKC isozymes in prostate cancer cells [45]. Activation of PKC promotes the dephosphorylation and inhibition of the Akt pathway, which is constitutively activated in tumor cells.

In our study, we found a strong modulating effect of cAMP/protein kinase A pathway on sPLA$_2$-IIA expression in prostate cells. Forskolin, an activator of this signaling cascade, either facilitated the induction of sPLA$_2$-IIA expression by IFN-γ in PrEC and PC-3 cells or repressed the induction of sPLA$_2$-IIA expression by IL-1β in LNCaP cells. The reason of this forskolin-mediated opposite action remains unknown.

What relevance does different expression patterns of sPLA$_2$-IIA and sPLA$_2$-V in prostate cell lines have for tumorigenic potential? PC-3 and DU-145 cells have been identified as highly aggressive, whereas the LNCaP cell line has a low aggressive phenotype [32,33]. The metastatic potentials of these prostate cancer cell lines correlates with the expression of proangiogenic genes [34]. Based on these properties, the expression profiles of sPLA$_2$ isozymes suggest that the sPLA$_2$-IIA plays, if any, only a secondary role in tumorigenicity. DU-145 cells expressed, instead of sPLA$_2$-IIA, the sPLA$_2$-V isozyme, particularly after the exposure of cells to IFN-γ. Therefore, it is conceivable that sPLA$_2$-V compensates for the lack of expression of sPLA$_2$-IIA in DU-145 cells and contributes to oncogenesis in this cell line. In this context, it is noteworthy that sPLA$_2$-V isozyme acts similarly to sPLA$_2$-IIA at both arachidonic acid release [46] and arachidonic acid–mediated signaling transduction in sPLA$_2$-IIA-negative cell types [47,48]. Further studies are necessary to elucidate the contribution of various sPLA$_2$ isozymes to proliferation, migration, invasion, cell-to-cell interaction, and other activities in prostate cancer cells.

In summary, our study has elucidated several novel aspects about expression of sPLA$_2$ isozymes in normal (PrEC) and malignant (DU-145, PC-3, and LNCaP) human prostate cell lines: (1) sPLA$_2$-IIA, sPLA$_2$-V, and sPLA$_2$-X are expressed in both normal prostate cells and malignant prostate cell lines, but in a differential manner; (2) expression of sPLA$_2$-IIA and sPLA$_2$-V, but not of sPLA$_2$-X, is controlled by proinflammatory cytokines in prostate cells, but different cell signaling pathways are involved in the regulation of sPLA$_2$-IIA; (3) no significant expression of sPLA$_2$-IIA was found in the DU-145 cell line, and epigenetic mechanisms seem to be responsible for the silencing of sPLA$_2$-IIA in this cell line. Additional investigations may

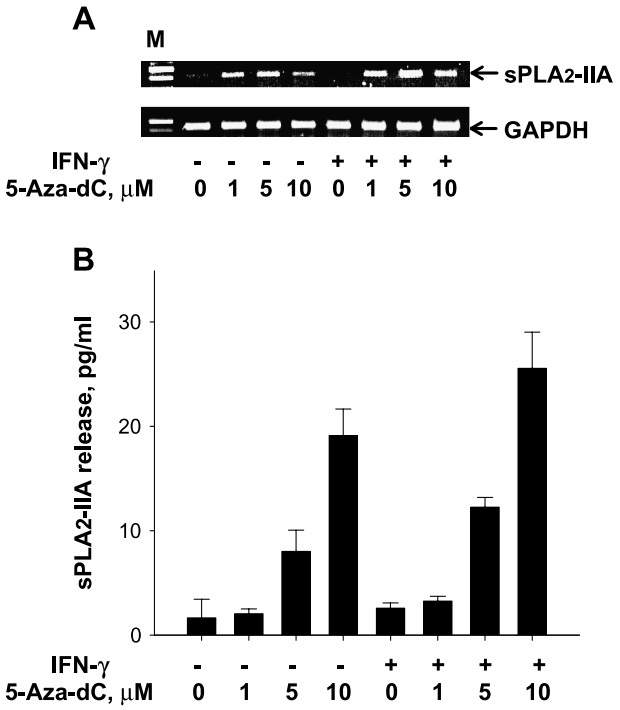

Figure 6. Reexpression of sPLA$_2$-IIA in DU-145 cells following the treatment with 5-aza-dC as inhibitor of DNA methyltransferase. (A) Agarose gel electrophoresis showing amplified sPLA$_2$-IIA mRNA of DU-145 cells treated with increased concentrations of 5-aza-dC. Cells were incubated for 72 hours without or with IFN-γ and indicated amounts of 5-aza-dC. M, 100-bp molecular weight ladder. (B) sPLA$_2$-IIA protein levels released by DU-145 cells into the medium at increased concentrations of 5-aza-dC. Cells were incubated for 72 hours without or with IFN-γ and indicated amounts of 5-aza-dC. Results are the means ± SD of analysis in triplicate and are representative of four independent experiments.

clarify whether an aberrant methylation in DU-145 cells is taking place directly at the promoter of the *sPLA$_2$-IIA* gene itself or indirectly through CpG methylation in the promoter of corresponding transcription factors that regulate the synthesis of sPLA$_2$-IIA in these cells.

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