Group X Phospholipase A2 Stimulates the Proliferation of Colon Cancer Cells
by Producing Various Lipid Mediators


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Abbreviations: AA, arachidonic acid; ATX, autotaxin; FAF BSA, fatty acid free bovine serum albumin; FCS, fetal calf serum; COX, cyclooxygenase; cPLA₂α, group IVA cytosolic PLA₂α; HETE, hydroxyeicosatetraenoic acid; LOX, lipooxygenase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; OS₁, Oxyuranus scutellatus scutellatus sPLA₂-1; OS₂, Oxyuranus scutellatus scutellatus sPLA₂-2; PA, phosphatidic acid; PGE₂, prostaglandin E₂; real-time Reverse Transcription-quantitative PCR (RT-qPCR); sPLA₂, secreted phospholipase A₂; a comprehensive abbreviation system for the various mouse and human sPLA₂s is used. Each sPLA₂ is abbreviated with the lowercase letter indicating the sPLA₂ species (m and h for mouse and human, respectively) followed by uppercase letters identifying the sPLA₂ group (GIBC, GIIC, GIID, GIIE, GIIF, GIIH, GV, GX, GXIIB).
Abstract

Among mammalian secreted phospholipases A₂ (sPLA₂s), the group X enzyme has the most potent hydrolyzing capacity toward phosphatidylcholine, the major phospholipid of cell membrane and lipoproteins. This enzyme has recently been implicated in chronic inflammatory diseases such as atherosclerosis and asthma, and may also play a role in colon tumorigenesis. We show here that group X sPLA₂ (mGX) is one of the most highly expressed PLA₂ in the mouse colon and that recombinant mouse and human enzymes stimulate proliferation and MAP kinase activation of various colon cell lines including Colon-26 cancer cells. Among various recombinant sPLA₂s, mGX is the most potent enzyme to stimulate cell proliferation. Based on the use of sPLA₂ inhibitors, catalytic site mutants, and siRNA silencing of cytosolic cPLA₂α and M-type sPLA₂ receptor, we demonstrate that mGX promotes cell proliferation independently of the receptor and via its intrinsic catalytic activity and production of free arachidonic acid (AA) and lysophospholipids which are mitogenic by themselves. mGX can also elicit the production of large amounts of PGE₂ and other eicosanoids from Colon-26 cells, but these lipid mediators do not play a role in mGX-induced cell proliferation since inhibitors of cyclooxygenases and lipoxygenases do not prevent sPLA₂ mitogenic effects. Together, our results indicate that group X sPLA₂ may play an important role in colon tumorigenesis by promoting cancer cell proliferation and releasing various lipid mediators involved in other key events in cancer progression.
Introduction

Phospholipases A\textsubscript{2} (PLA\textsubscript{2}s) catalyze the hydrolysis of the \textit{sn}-2 ester bond of glycerophospholipids to generate free fatty acids and lysophospholipids (Schaloske and Dennis, 2006; Lambeau and Gelb, 2008). Over the past few years, it has been realized that PLA\textsubscript{2}s constitute a superfamily of enzymes comprising several intracellular enzymes and secreted PLA\textsubscript{2}s (sPLA\textsubscript{2}s).

The group IVA cytosolic PLA\textsubscript{2} (cPLA\textsubscript{2}\textsubscript{α}) is the best known intracellular PLA\textsubscript{2}, and it clearly plays an important, yet not exclusive role in the release of arachidonic acid (AA) and subsequent production of eicosanoids in various biological settings (Kita et al., 2006). On the other hand, the biological functions of the different sPLA\textsubscript{2}s are slowly being unraveled. sPLA\textsubscript{2}s have been implicated in lipid digestion and obesity, activation of immune cells, asthma, atherosclerosis, acute respiratory distress syndrome, and host defense against bacteria, viruses and parasites (Touqui and Wu, 2003; Triggiani et al., 2006; Lambeau and Gelb, 2008; Nevalainen et al., 2008). Besides their catalytic activity, sPLA\textsubscript{2}s are also able to bind to specific soluble and membrane binding proteins including the M-type receptor (Rouault et al., 2007).

Several PLA\textsubscript{2}s have also been implicated in various cancers. Disruption of the cPLA\textsubscript{2}\textsubscript{α} gene decreases initiation and growth of intestinal tumors in Apc mutated mice (Takaku et al., 2000), but increases the number of tumors in the carcinogen azoxymethane-induced mouse model of colon cancer (Ilsley et al., 2005). The gene coding for mouse group IIA (mGIIA) sPLA\textsubscript{2} was identified as a gene modifier that reduces the number of intestinal polyps in Apc\textsuperscript{−/−} mice, but the mechanism of action is still unclear (Fijneman and Cormier, 2008). Distinct roles have also been proposed for human group IIA sPLA\textsubscript{2} in various cancers (Sved et al., 2004; Cummings, 2007). Recent data have shown differential expression of sPLA\textsubscript{2}s IID, III, and V, but not X in human colon cancer (Murakami et al., 2005; Mounier et al., 2008). In Apc\textsuperscript{Δ716} and azoxymethane mouse models of colon cancer, the expression of group X sPLA\textsubscript{2} may be increased in small intestinal tumors, but not in colon tumors (Takaku et al., 2000; Ilsley et al., 2005). \textit{In vitro}, group IB, IIA and III sPLA\textsubscript{2}s have been reported to stimulate cell proliferation and activation of MAP kinases in various cancer cells (Kinoshita et al., 1997; Sved et al., 2004; Murakami et al., 2005). Finally, subcutaneous injection into nude mice of
colon tumor cells overexpressing mGIIA sPLA₂ resulted in a 2.5-fold increase in tumor size (Belinsky et al., 2007).

Over the past few years, the group X sPLA₂ has appeared as the most potent sPLA₂ capable of hydrolyzing phosphatidylcholine and acting extracellularly on cellular membranes and non cellular phospholipid substrates such as lipoproteins (Lambeau and Gelb, 2008). Consequently, group X sPLA₂ has been proposed to play a role in atherosclerosis (Lambeau and Gelb, 2008), asthma (Henderson et al., 2007) and colon cancer (Morioka et al., 2000).

The above findings plus the fact that group X sPLA₂ is expressed at very high levels in human (Cupillard et al., 1997; Morioka et al., 2000; Mounier et al., 2008) and mouse colon ((Eerola et al., 2006) and this study) prompted us to analyze the proliferative effect of group X sPLA₂ on different colon cancer cells including the adenocarcinoma-derived mouse Colon-26 cancer cells. Among different mouse sPLA₂s, group X sPLA₂ was the most potent enzyme to stimulate cell proliferation. Using a combination of tools and methods, we found that its proliferative effect does not depend on binding to the M-type receptor or activation of cPLA₂α, but rather on its intrinsic catalytic activity and ability to release free fatty acids and lysophospholipids, which most likely act in concert to stimulate cell proliferation.


Materials And Methods

Materials. The mouse adenocarcinoma cell line Colon-26 was obtained from Cell Lines Service (Heidelberg, Germany). AJ02-nm0 cells (Belinsky et al., 2007), YAMC cells (Young Adult Mouse Colon (Whitehead et al., 1993)) and Apc⁺/Min cells (Forest et al., 2003) were generous gifts from Dr. D.W. Rosenberg (Farmington, USA), Dr. RH Whitehead (Melbourne, Australia), and Dr. F. Pierre and J. Menanteau (Toulouse and Nantes, France), respectively. RPMI 1640 and Dulbecco’s Modified Eagle Media (DMEM) were from Invitrogen (Cergy Pontoise, France). Fetal bovine serum (FCS) was from Dominique Dutscher (Brumath, France). Oleic acid (#O1008), linoleic acid (#L1376), linolenic acid (#L2376), oleoyl-L-α-lysophosphatidic acid sodium salt (#L7260), 1-oleyl-sn-glycero-3-phosphocholine (#L1881), PGE₂ (#P0409), AA (#A9798), Aspirin (#A5376), Ibuprofen (#I4883), Indomethacin (#I7378) and FAF BSA (#A7511) were from Sigma Aldrich (L’isle d’Abeau Chesnes, France). [Methyl ³H]-thymidine, [³H]-oleic acid and [³H]-AA were from Perkin Elmer Life Sciences (Courtaboeuf, France). Recombinant murine interferon γ (#31505) was from PeproTech EC (London, UK). ITS (Insulin, Transferrin, Selenious acid; #354352) was from BD Biosciences (Le Pont-De-Clai, France). Phospho-specific p42/p44 MAPK antibodies (clone E-4, #sc-7383), p42/p44 antibodies (#sc-153) and cPLA₂α antibodies (#sc-454) were from Santa Cruz Biotechnology (Tebu-Bio SA, Le Perray en Yvelines, France). MK886 (#10133), baicalein (#70610) and ATX antibody (#10005375) were from Cayman Chemicals (Interchim, Montluçon, France). Alkaline phosphatase conjugated secondary antibodies were from Jackson ImmunoResearch (Immunotech, Marseille, France) and ECF substrate was from GE Healthcare Life Sciences (Orsay, France). PGE₂ ELISA kit (#900-001) was from Assay Designs (Euromedex, Strasbourg, France). Prevalidated siRNAs were from QIAGEN (Courtaboeuf, France). Hoechst (#H3570) was from Molecular Probes (Invitrogen, Cergy Pontoise, France). LY329722 and Me-Indoxam sPLA₂ inhibitors were prepared as described (Singer et al., 2002; Smart et al., 2006). Lysing Matrix D beads (#6913-100) were from Qbiogene (Illkirch, France). Nucleospin RNA extraction kit was from Macherey Nagel (Hoerdt, France). All recombinant mouse and human sPLA₂s used in this study and catalytically-inactive hGX and mGX H48Q sPLA₂ mutants were prepared as described previously (Singer et al., 2002; Rouault et al., 2006; Rouault et al., 2007).
sPLA₂ expression in mouse intestinal tract analyzed by RT-qPCR. Tissue samples from duodenum, jejunum, ileum, and proximal and distal colon from two C57BL/6J female or three BALB/c female mice were snap-frozen in liquid nitrogen. Total RNA was isolated using the Nucleospin RNA extraction kit with DNase I treatment and Lysing Matrix D beads to homogenize the samples. Reverse-transcription and real-time quantitative PCR (RT-qPCR) were performed as described (Eerola et al., 2006). The abundance of each sPLA₂ mRNA target was calculated relative to the expression of GAPDH mRNA which was used as a reference gene. The tissue distribution of sPLA₂s and cPLA₂α was further normalized to the lowest Ct value accurately measured. A relative abundance of 1 (arbitrary unit = 1) was assigned to the expression level of the pancreatic group IB sPLA₂ mRNA in the colon which was detected with Ct values of 33 or higher. Standard deviation calculation was made according to the User Bulletin #2 from ABI.

Expression of mGX sPLA₂ by in situ hybridization. Samples for in situ hybridization were obtained from small and large intestines of C57BL/6J mice, fixed in 4% phosphate-buffered formalin and embedded in paraffin. A 0.45 kb mGX sPLA₂ cDNA insert was cloned into the pRc/CMV vector (Invitrogen) and used as a template to prepare digoxigenin (DIG)-labeled antisense (test) and sense (control) RNA probes by in vitro transcription using a commercial kit (DIG RNA labeling kit, Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Non radioactive in situ hybridization has been described in detail previously (Haapamaki et al., 1999). Briefly, 5-μm-thick paraffin sections were placed on silanated slides, dewaxed in xylene and rehydrated in a graded alcohol series. Pretreatment was performed sequentially with 200 mM HCl for 20 min, 20 μg/ml proteinase K at 37°C for 15 min, 0.2 % glycine in phosphate-buffered saline (pH 7.4) two times 3 min, freshly prepared 0.4% acetic anhydride in 100 mM triethanolamine two times 5 min, and finally with ficin (Zymed Digest-All Kit, San Francisco, CA) at 37°C for 20 min. The sections were then blocked in hybridization solution without probe. The solution was replaced with fresh hybridization solution containing the antisense or sense probe at a final concentration of 60 ng/ml. After overnight hybridization, the sections were washed in 2 X saline-sodium citrate (SSC) for 5 min, 0.2 X SSC, 60% formamide at 37°C three times 5 min and 2 X SSC two times 5 min. The sections were blocked with
3% bovine serum albumin in tris-buffered saline and the hybridized probe detected with alkaline phosphatase-labeled anti-DIG Fab-fragments (1/2,000; Roche) using a substrate solution containing 0.18 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 0.34 mg/ml 4-nitroblue tetrazolium phosphate (NBT/BCIP, Roche) and 0.24 mg/ml levamisole (Vector, San Francisco, CA) in 100 mM Tris pH 9.5, 100 mM NaCl and 50 mM MgCl2. The color reaction was allowed to develop at 4°C overnight and at 25°C for 6 h. The tissue sections were briefly counterstained with hematoxylin, washed with water and mounted in aqueous mounting medium.

**Cell culture.** Colon-26 cells were grown at 37°C in DMEM/10% FCS, antibiotics, and 2 mM glutamine. AJ02-nm0 cells were grown in RPMI/10% FCS with antibiotics, glutamine and supplementation with insulin (6.25 ng/ml), transferrin (6.25 µg/ml) and selenious acid (6.25 ng/ml) (ITS). YAMC and Apc+/-Min cells express the temperature-sensitive mutation of the simian virus 40 large tumor antigen gene (tsA58) and grow well at 33°C but become quiescent at 37°C. YAMC cells were grown at 33°C in RPMI supplemented with 5% FCS, antibiotics, ITS, and 5 U/ml recombinant IFNγ. Apc+/-Min cells were grown at 33°C in DMEM supplemented with 10% FCS, antibiotics, 10 U/ml recombinant IFNγ and 10 ng/ml recombinant EGF. We found that these cells do not release sPLA2 enzymatic activity in cell medium during their normal growth, nor express detectable amounts of mGX sPLA2 protein by time-resolved fluoroimmunoassays (Eerola et al., 2006). To determine whether these cell lines express or not a functional mGIIA sPLA2 gene (MacPhee et al., 1995), we set up conditions for the specific detection of wild-type and mutated mGIIA alleles by PCR on genomic DNA (see supplemental data). YAMC cells were found to be heterozygous for the pla2g2a gene, Colon-26 cells were found to be wild-type while AJ02-nm0 and Apc+/-Min cells were found to contain two mutated pla2g2a alleles (supplemental Fig. 1S).

**[³H]-thymidine incorporation and cell growth assays.** Colon-26 (5,000 cells/well), YAMC (10,000 cells/well), AJ02-nm0 (5,000 cells/well) and Apc+/-Min (4,000 cells/well) were plated into 48-well culture plates (Falcon), that were precoated (Colon-26, YAMC) or not (AJ02-nm0 and Apc+/-Min) with rat tail collagen to ensure tight adhesion of cells. Fifty % (Colon-26) or 80 % (YAMC, AJ02-nm0, Apc+/-Min) confluent cells were made quiescent by incubating them for 24 h in serum-free medium with 0.02% FAF BSA at 37°C. Importantly, we observed that the percentage of cell confluence can
dramatically affect the ability of the sPLA$_2$ to trigger cell proliferation. Quiescent cells were treated with medium containing 0.02% FAF BSA and the effectors, and further incubated for 24 h (Colon-26) or 48 h (YAMC, AJ02-nm0, Apc$^{+/Min}$) at 37°C (Colon-26, AJ02-nm0) or 33°C (YAMC, Apc$^{+/Min}$). During the last four hours, [methyl $^3$H]-thymidine was added to wells to a final concentration of 1 $\mu$Ci/ml, 1 $\mu$M cold thymidine. In some experiments, cell supernatant of Colon-26 cells was collected for PGE$_2$ assays. The cells were then washed twice with cold phosphate-buffered saline, incubated for 30 min in 5% ice-cold trichloroacetic acid, solubilized in 0.2 N NaOH, and analyzed for $[^3]$H]-thymidine incorporation into DNA. For cell growth assays, cells were seeded as above, starved at low density, and then treated with sPLA$_2$. Cells were dissociated and counted after trypan blue staining. Assays were done in triplicate.

**Immunoblot analysis.** Colon-26 cells grown and made quiescent as above were treated with effectors for various times, washed with PBS, lyzed in hot Laemmli buffer and boiled for 5 minutes at 95°C. Sonicated lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to immobilon-FL polyvinylidene fluoride membrane (Millipore, Saint-Quentin-en-Yvelines, France). Membranes were blocked for 2 h at room temperature in NETG buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 0.05% Triton X100, 0.25% gelatin), and probed for 1 h with phospho-specific p42/44 MAPK antibodies or cPLA$_2$,$\alpha$ antibodies (1/2,000). Blots were washed 5 times with PBS/0.1% Tween-20, incubated with alkaline phosphatase-conjugated secondary antibodies diluted in PBS/Tween (1/10,000), visualized by incubation for 5 min with the enhanced chemifluorescence substrate (ECF), and scanned with a Pro-Xpress imager (excitation 480 nm, emission 590 nm). For western-blot of the mouse M-type receptor, cells were lyzed in cold 20 mM Tris pH 7.4, 2 mM EDTA, 1 mM PMSF and sonicated. Unboiled proteins (60 $\mu$g) prepared in non-reducing Laemmli buffer were separated on a 7% SDS-PAGE gel and transferred to an immobilon-FL membrane. The membrane was blocked with 5% blocking agent (GE Healthcare Life Sciences) dissolved in TBS-Tween (Tris 25 mM pH 7.8, 150 mM NaCl, 0.15% Tween 20) for 1 h and then incubated with the anti-mouse M-type receptor antibody (1/5000 in TBS-Tween, (Rouault et al., 2007)) overnight at 4°C. The membrane was washed 6 times for 5 min and incubated with secondary
alkaline phosphatase goat anti-rabbit antibody (1/10,000 in TBS-Tween). The immunocomplex was visualized with ECF substrate as above.

**siRNA transfection.** siRNAs were designed using the QIAGEN program HP GenomeWide siRNA (HiPerformance Design Algorithm). Best siRNAs respectively targeting the mouse M-type receptor and mouse cPLA₂α were selected from two QIAGEN-designed siRNAs after validation by binding assays and immunocytochemistry for the M-type receptor and western blot analysis for cPLA₂α. For siRNA transfection, duplex siRNAs were first heated for 1 min at 90°C and cooled at 37°C for 30 min to allow for annealing. Cells were seeded on day 0 and transfected on day 1. Duplex siRNAs were pre-incubated with High Perfect Reagent (Qiagen) and added dropwise to each well to a final concentration of 5 nM. On day 2, cells were made quiescent for 24 h before stimulation with sPLA₂ and subsequent western blotting, binding assays, proliferation experiments or immunocytochemistry. Transfection efficiency was evaluated by transfection with an Alexa546-coupled siRNA and fluorescence visualization with a microscope. The effect of the best selected siRNAs for M-type receptor (Forward : r(GGU ACA CUC GAU ACA UUA A)dTdT Reverse : r(UUAAUGUAUCGAGUGUACC)dTdA) and cPLA₂α (Forward : r(GGA GAU UAA UGA AGA GCU A) dTdT Reverse : r(UAG CUC UUC AUU AAU CUC C)dTdC) were compared with the effect of the non relevant GFP-directed siRNA (Forward : GCA AGC UGA CCC UGA AGU UCA U, Reverse : GAA CUU CAG GGU CAG CUU GCC G).

**Binding assays and immunocytochemistry.** The expression of the mouse M-type receptor in various cell lines was determined by binding experiments using iodinated OS₁ as described (Rouault et al., 2007). Cells were seeded in 6-well plates, and 90% confluent cells were incubated for 1 h in binding buffer (140 mM NaCl, 1 mM CaCl₂, 20 mM Tris pH 7.4 and 0.1% BSA) at room temperature, with 200,000 cpm iodinated OS₁ in the absence (total binding) or presence (non specific binding) of 100 nM unlabeled OS₁. Cells were washed, lyzed with 0.2 M NaOH and analyzed for bound iodinated OS₁. The expression of the mouse M-type receptor after siRNA transfection was evaluated by immunocytochemistry as follows. Colon-26 cells were seeded in 24-well culture plates (4,000 cells/well) on coverslips coated with rat tail collagen. Twenty-four hours after transfection with
siRNA, the medium was changed, and cells were incubated in FCS-free medium for 24 h to mimic conditions of proliferation assays. Cells were then fixed with 3.7% paraformaldehyde for 25 min at RT, and washed twice with PBS. Free aldehyde groups were quenched with 50 mM NH₄Cl in PBS, cells were washed once with PBS and permeabilized with 0.5% saponin (twice 5 min). Non-specific sites were blocked with 10% horse serum/0.05% saponin. Anti-mouse M-type receptor rabbit serum (Rouault et al., 2007) was diluted 1/1,000 in 5% horse serum/0.05% saponin and incubated for 30 min at room temperature. After 3 washes with PBS/0.05% saponin, FITC-linked secondary antibodies were diluted 1/600 in 5% horse serum/0.05% saponin and incubated for 1 h at room temperature under dim light. Nuclei were stained with Hoechst 33342 (5 µg/ml). Cells were washed once in PBS/0.05% saponin, twice in PBS and once in H₂O, then mounted with Dako mounting medium (Dako, Trappes, France) and visualized under a microscope.

AA release. AA release was performed under the same conditions of culture as those used for proliferation assays. During the starvation time, cells were labeled with [³H]-AA (0.2 µCi/well) in FCS-free DMEM/0.02% FAF BSA for 24 h at 37°C. Cells were washed twice with FCS-free medium/0.1% FAF BSA, and incubated for the indicated periods of time in 200 µl of serum-free medium with 200 nM sPLA₂. Cell-free supernatants and cell monolayers lyzed in 400 µl of 0.2 N NaOH, were submitted to scintillation counting. [³H]-AA release is expressed as the percentage of cpm in cell supernatant versus total cpm (supernatant and cell lysate).

Eicosanoid assays. Colon-26 cells (10⁶ cells) were grown in 6-well culture plates for 2 days, starved for 24 h and stimulated for 24 h with 200 nM mGX sPLA₂ in 1 ml of serum-free medium containing 0.02% FAF BSA. Total eicosanoid production in combined supernatant and cell pellet was assayed by HPLC/MS following the procedure described previously (Kita et al., 2005; Henderson et al., 2007). For specific PGE₂ assay, cell supernatants from proliferation assays were centrifuged, appropriately diluted (1/10 to 1/500) and PGE₂ levels were determined using the PGE₂ enzyme immunoassay kit (Assay Designs) according to the manufacturer’s instructions.

Lysophospholipid analysis. Colon-26 cells (2.10⁶ cells) were seeded in 10-cm Petri dishes and grown for 2 days, then starved for 24 h in FCS-free medium/0.02% FAF BSA and stimulated with effectors in 4 ml of FCS-free medium/0.02% FAF BSA for 6 and 24 h. After incubation, the cell
supernatants were collected and lyophilized. The cell monolayer (about $10^7$ cells) was scraped in PBS, centrifuged and stored as a dried pellet at -70°C. The following procedure was adapted from previously published methods of LPA analysis (Baker et al., 2001). Lyophilized supernatants were resuspended in 0.5 ml Milli-Q water and cell pellets were thawed on ice in 0.5 ml Milli-Q water. To each sample, 1.5 ml of 30 mM Citric Acid, 40 mM Na$_2$HPO$_4$ (pH~4) was added and vortexed for 30 seconds. Samples were then spiked with 500 pmoles 17:0 LPA and 1 nmole d$_{31}$-16:0 LPC and extracted using 4 ml of 1-Butanol followed by re-extraction with 2 ml of 1-Butanol. Organic phases were dried in vacuo overnight. The resulting residues were reconstituted in 1.5 ml CHCl$_3$:MeOH (2:1), dried again, and finally reconstituted in 100 µl of CHCl$_3$:MeOH (2:1). LC/MS-MS analysis was performed using a Waters Micromass Quattro Micro Tandem Quadrupole Mass Spectrometer coupled with a Waters 2795 Chromatography System. Twenty µl of concentrated lipid extract was injected onto a Luna Silica column (Phenomenex # 00G-4274-E0, 5 µm particle size, 4.6 X 250 mm, Torrance, USA) equilibrated with 65% Solvent A (30:40 n-Hexane:Isopropanol) and 35% Solvent B (30:60:15 n-Hexane:Isopropanol:H$_2$O) at 30°C with a flow rate of 1 ml/min. After Injection, 35% solvent B was maintained for 6 minutes. A gradient of 35-100% Solvent B was run over 24 minutes. The gradient was held at 100% Solvent B for an additional 20 minutes. Mass transitions, cone voltages, and collision energies are available on request. Transitions were monitored in the negative mode for the first 30 minutes with 40 msec dwell times and in the positive mode for the final 20 minutes with 100 msec dwell times. In both modes, 10 msec Inter-Channel and 100 msec Inter-Scan delays were employed.

**Statistics.** Data are expressed as mean ± S.D.. Statistical analyses were performed with PRISM (GraphPad, San Diego, CA) using Student’s t test and one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons. P values lower than 0.05 were considered as statistically significant.
Results

Group X sPLA₂ is expressed at high levels in mouse colon. We have recently found that mGX sPLA₂ is expressed at high levels in the small intestine and colon at both mRNA and protein levels (Eerola et al., 2006). To further analyze the expression of mGX sPLA₂ and other PLA₂ genes in the different sections of the intestine of C57BL/6J mice, we have measured by RT-qPCR the relative abundance of mRNAs coding for the full set of mouse sPLA₂s (except IIC), for cPLA₂α (Fig. 1A) and for the M-type receptor (not shown). Most sPLA₂s were expressed in the different gut sections, but marked differences were found. Among catalytically-active sPLA₂s, mGX sPLA₂ is by far the most highly expressed enzyme in the intestine of C57BL/6J mice, with highest expression in the distal colon. For instance, its level of expression in the colon is about 10-fold higher than that of mGV sPLA₂. Interestingly, mGX sPLA₂ mRNA levels vary along the intestine with up to 120-fold increase in expression from duodenum to distal colon. Second, the levels of mRNA for the catalytically-inactive group XIIB sPLA₂-like protein are similar to those of mGX sPLA₂, but the expression pattern is the inverse. Third, group IID, IIF, III and XIIB sPLA₂s are expressed in the intestine at significant but low levels, and group IB and IIE sPLA₂s are barely detectable. Expression of group XIIA sPLA₂ is rather constitutive all along the intestine. Conversely, that of group IIF, III, and V sPLA₂s increases while that of group IID sPLA₂ decreases when moving from the proximal to the distal part of the gut. Fourth, the expression of cPLA₂α is 5-fold lower than that of mGX sPLA₂, but follows a similar gradient of expression along the intestine. Finally, the expression of the M-type receptor was low along the gut sections (not shown), but interestingly follows the same gradient of expression of mGX sPLA₂.

Importantly, C57BL/6J mice have a disrupted mGIIA sPLA₂ gene (MacPhee et al., 1995), which likely explain the low and aberrant amount of mRNA observed in the small intestine (Fig. 1A). When RT-qPCR assays for the different sPLA₂s were done as above on intestine sections from BALB/c mice expressing a functional gene, the expression of mGIIA sPLA₂ in jejunum and ileum was found to be much higher than that of all other sPLA₂s including mGX sPLA₂ (Fig. 1B). However, the
mRNA levels of mGIIA was fairly low in proximal and distal colon, where mGX sPLA2 remained the most abundantly expressed sPLA2 (Fig. 1B).

To determine more precisely the cellular site of mGX sPLA2 expression in small and large intestine, we performed in situ hybridization. Figs. 1C and 1D show strong labeling of columnar epithelial cells of mucosal villi, Paneth cells in the crypts of Lieberkühn and in ganglion cells of the myenteric plexus between smooth muscle cell fibers. Fig. 1E illustrates the absence of labeling from a section of small intestine hybridized with sense probe. Fig. 1F shows labeling of epithelial cells in the mucosal glands of the large intestine adjacent to empty looking goblet cells.

We also analyzed the expression level of group X sPLA2 in the ileum and colon of mouse models of colon cancer (ApcΔ14 and azoxymethane) and found no dramatic change in the expression of group X sPLA2 (see supplemental Fig. 2S).

**Group X sPLA2 stimulates the proliferation of mouse colon cells.** Since group X sPLA2 is expressed in both normal colon and colon adenocarcinoma (Cupillard et al., 1997; Morioka et al., 2000; Takaku et al., 2000; Osterstrom et al., 2002; Ilsley et al., 2005; Mounier et al., 2008), we analyzed the effect of exogenous recombinant group X sPLA2 on the proliferation of four different mouse colon cell lines (YAMC, Apc+/Min, AJ02-nm0 and Colon-26 cells). Both mGX and hGX sPLA2s were able to stimulate [3H]-thymidine incorporation in the four tested cell lines (Fig. 2). At 200 nM, group X sPLA2 increased [3H]-thymidine incorporation by up to 3-fold, depending on the cell line and conditions of stimulation. This proliferative effect is important as it corresponds to about 50% of the maximal effect observed with fetal calf serum. We decided to use the Colon-26 cell line for the experiments described below because these cells are adenocarcinoma-derived and were easier to grow and transfect than the other cell lines. We also found that these cells do not express mGX sPLA2 at the mRNA level by RT-qPCR analysis and at the protein level by both a highly specific and sensitive time-resolved fluoroimmunoassay (Eerola et al., 2006) and E. coli sPLA2 enzymatic assays (data not shown). When compared to various mouse sPLA2s, we found that group X sPLA2 has the strongest mitogenic capacity on Colon-26 cells (Fig. 3A), and dose-dependently induced cell proliferation (Fig. 3B). mGV and mGIIA sPLA2 also significantly induced cell proliferation while the other sPLA2s were relatively poor inducers. To further demonstrate that group X sPLA2 can trigger cell proliferation, we
measured the growth of Colon-26 cells treated with mGX and hGX sPLA2s by direct cell counting. As indicated in Fig. 3C, cells proliferated more rapidly over time in the presence of group X sPLA2. In accordance with their proliferative effects, both mGX and hGX sPLA2s were capable of inducing a time-dependent phosphorylation of p42/44 MAPK with a sustained induction at 4 h (Fig. 3D).

**Group X sPLA2 stimulates the proliferation of Colon-26 cells via its catalytic activity and independently of binding to the M-type receptor.** We next analyzed the molecular mechanisms involved in the proliferative effect of group X sPLA2. To evaluate the role of sPLA2 enzymatic activity, we first used the specific sPLA2 inhibitors Me-indoxam (Singer et al., 2002) and LY329722 (compound B in (Smart et al., 2006)), which inhibit the catalytic activity of mGX sPLA2 with IC50 values of 500 nM and 75 nM, respectively. Both inhibitors clearly suppressed the proliferation and MAP kinase phosphorylation induced by mGX sPLA2 (Figs. 4A and B). We then tested the mitogenic activity of catalytically-inactive mutants of mGX and hGX sPLA2s as well as of OS2, a snake venom sPLA2 with high catalytic activity on phosphatidylcholine (Rouault et al., 2006). The H48Q mutants of mGX and hGX sPLA2s have less than 0.1% of WT catalytic activity (not shown) and the D49K mutant of OS2 is fully inactive (Rouault et al., 2006). Results shown in Fig. 4C further demonstrates that the catalytic activity of group X sPLA2 is required for its mitogenic effect. Finally, we evaluated the role of cPLA2α and found that the mitogenic effect of group X sPLA2 on Colon-26 cells does not depend on cPLA2α activation as siRNA silencing of cPLA2α efficiently suppressed protein expression, but has little effect on the fold-increase factor of [3H]-thymidine incorporation induced by hGX sPLA2 (Fig. 4D). However, our results also suggest that cPLA2α is involved in the proliferation of Colon-26 in the absence of exogenously added sPLA2 since siRNA silencing slightly decreased the basal level of incorporation of [3H]-thymidine (Fig. 4D).

Since the M-type sPLA2 receptor was previously proposed to play a role in cell proliferation (Kinoshita et al., 1997) and since both mGX and hGX sPLA2s bind to the mouse M-type receptor (Rouault et al., 2007), we sought to determine whether this receptor is expressed in the above different colon cells and plays a role in the proliferative effect of group X sPLA2. We first screened the four colonic cell lines for the expression of the receptor using iodinated OS1, the snake venom sPLA2 that
binds to the M-type receptor with very high affinity and specificity (Rouault et al., 2007). Apc+Min cells were found to express high levels of the receptor, while Colon-26 and YAMC cells express low but clearly detectable levels (Fig. 5A). Conversely, AJ02-nm0 cells do not express the receptor. Scatchard plot analysis indicated that Colon-26 cells contain a single population of binding sites for iodinated OS1 with a K_d value of 90 pM and a maximal binding capacity (B_max) of 0.016 pmol/mg of total protein (not shown). These binding data were confirmed by western-blot analysis (Fig. 5B) and RT-qPCR analysis (not shown). The fact that group X sPLA2 stimulates the proliferation of AJ02-nm0 cells (Fig. 2B) which do not express the M-type receptor (Fig. 5A) represents a first indication that the M-type receptor is not required for the proliferative effect of group X sPLA2. This view was further supported by siRNA experiments targeting the M-type receptor expressed in Colon-26 cells. As shown in Fig. 5C, we observed by both binding assays and immunocytochemistry that the expression of the M-type receptor was dramatically reduced at 48 h and 72 h after siRNA transfection, at the time window where the Colon-26 cells were stimulated with exogenous hGX and mGX sPLA2s for cell proliferation (Fig. 5D). The specificity of siRNA silencing of the receptor was validated using a non-relevant GFP-siRNA that did not suppress M-type receptor expression (Fig. 5C). The capacity of group X sPLA2 to increase Colon-26 proliferation was unaffected by knocking-down the M-type receptor, indicating that receptor binding is not required for the proliferative effect of group X sPLA2 (Fig. 5D).

**Group X sPLA2 produces various lipid mediators from Colon-26 cells.** Based on the above findings, we sought to determine whether group X sPLA2 can release various lipid mediators including free AA, eicosanoids and lysophospholipids from Colon-26 cells. As previously found in other cells (Singer et al., 2002), exogenously added mGX, but not mGIB, mGIIA, mGV and mGXIIA sPLA2s, can release significant amounts of free AA from Colon-26 cells radiolabeled with [3H]-AA (Fig. 6A). Similar results were obtained when cells were radiolabeled with [3H]-oleate (not shown). We also monitored the ability of group X sPLA2 to produce various eicosanoids derived from free AA by combined liquid chromatography/mass spectrometry after stimulation of Colon-26 cells by the sPLA2 for 6 h (not shown) and 24 h (Fig. 6B). Group X sPLA2 increases the production of several eicosanoids, with PGE2 being by far the most prominent eicosanoid product, with up to 74 ng/10^6 cells.
PGD₂, PGF₂α, 5-, 12- and 15-HETEs, and to a lesser extent LTC₄ and LTE₄, are also produced by group X sPLA₂ at 6 h (not shown) and 24 h.

In parallel experiments, we monitored the ability of group X sPLA₂ to release various lysophospholipids, namely LPA, LPC, LPE, LPG, LPI and LPS. We found that group X sPLA₂ can release a variety of lysophospholipids with different fatty acids at the sn-1 position from Colon-26 cells at 6 h (Fig. 7) and 24h (not shown). The lysophospholipids produced in highest quantities were LPC and LPE, a result which is in accordance with the relative abundance of these lipids in cell membranes and the ability of group X sPLA₂ to efficiently hydrolyze zwitterionic phospholipids (Singer et al., 2002; Mitsuishi et al., 2007). For instance, treatment with group X sPLA₂ for 6 hours led to 25- and 36-fold increases in the release of total LPC and LPE compared to untreated cells (Fig. 7A).

Importantly, we also found that group X sPLA₂ increases by 3.7-fold the overall production of LPA. The detailed data for production of the different acyl chain species for LPC and LPA are shown in Fig. 7B. They indicate for instance 41- and 26-fold increases for the two main products, oleoyl-LPC and palmitoyl-LPC.

**Effects of various lipid mediators released by group X sPLA₂ on the proliferation of Colon-26 cells.** The above results led us to analyze the proliferative effect of each type of lipid mediators that are generated by the action of group X sPLA₂. Since LPC can be further converted into lysophosphatidic acid (LPA) by autotaxin (Ferry et al., 2008), we also tested the effect of LPA on cell proliferation. As shown in Fig. 8, free AA, LPC and LPA can all stimulate the incorporation of [³H]-thymidine in Colon-26 in a dose-dependent manner. Various other fatty acids were also able to stimulate cell proliferation, although with a lower efficacy than free AA (not shown). For instance, Oleic (50 µM), linoleic (50 µM) and α-linolenic (20 µM) acids could stimulate the incorporation of [³H]-thymidine by factors of 1.4, 1.6 and 2.1, respectively. Furthermore, both AA and LPA were able to phosphorylate p42/44 MAPK (Fig. 8). To test the hypothesis that PGE₂ production could explain at least in part the mitogenic effects of group X sPLA₂, we treated Colon-26 cells with four non selective or COX-2 selective inhibitors (Aspirin, Indomethacin, Ibuprofen and Rofecoxib). We found that all four inhibitors were able to dramatically reduce PGE₂ production (Fig. 9A), but were unable to suppress the cell proliferation induced by mGX sPLA₂ (Fig. 9B). Interestingly, the COX inhibitors
were also ineffective at suppressing the proliferative effect of free AA (Fig. 9C). Furthermore, we found that exogenously added PGE$_2$ (1-1000 nM) did not induce cell proliferation (not shown). Finally, incubation of Colon-26 cells with MK886 or baicalein, that respectively inhibit 5- and 12-LOXs, did not alter the effect of mGX sPLA$_2$ on cell proliferation (Fig. 9D). Together, these results indicate that although group X sPLA$_2$ can release large amounts of PGE$_2$ (and small amounts of other eicosanoids) from Colon-26 cells (Fig. 6), this PGE$_2$ is at best a minor effector of the sPLA$_2$ proliferative effect. Rather, the sPLA$_2$ proliferative effect appears to be due to the combined production and direct action of free AA and lysophospholipids including LPC and LPA.
Discussion

This work shows for the first time that group X sPLA₂ can stimulate the proliferation of colon cancer cells and activate the phosphorylation of p42/44 MAPK via its potent catalytic activity and ability to produce various lipid mediators. We found that the proliferative effect of group X sPLA₂ was much higher than that of other mouse sPLA₂s. This effect was as strong as or higher than that of sPLA₂-IB, -IIA or -III acting on different cell types (Kinoshita et al., 1997; Sved et al., 2004; Murakami et al., 2005; Belinsky et al., 2007). mGV and mGIIA sPLA₂s, but not other enzymes were also able to promote cell proliferation. Except for mGIIA, the ability of the different sPLA₂s to stimulate proliferation appears to be linked to their direct capacity to hydrolyze phosphatidylcholine and release AA and LPC from cells (Singer et al., 2002; Masuda et al., 2005). As previously found in LNCaP prostatic cancer cells (Sved et al., 2004), it is possible that the proliferative effects mediated by mGIIA sPLA₂ is due to activation of cPLA₂α, but we did not further investigated this point.

The proliferative effect of various sPLA₂s has been associated with binding to the M-type receptor (Kinoshita et al., 1997) or enzymatic activity of sPLA₂ and subsequent activation of cPLA₂α (Sved et al., 2004; Murakami et al., 2005). We found here that the proliferative effect of group X sPLA₂ fully depends on its intrinsic catalytic activity and not on binding to the M-type receptor or cPLA₂α activation. First, two specific sPLA₂ inhibitors suppressed the proliferative effect of group X sPLA₂. Second, the H48Q mutants of mGX and hGX sPLA₂s and the D49K mutant of OS2 (Rouault et al., 2006) which have less than 0.1% of wild-type enzymatic activity but still bind with high affinity to the M-type receptor (not shown and (Rouault et al., 2006)), had no or very modest effects on cell proliferation. Third, mGX and hGX sPLA₂s can trigger proliferation on cells expressing or not the M-type receptor. Fourth, siRNA silencing of the M-type receptor in Colon-26 cells did not affect group X sPLA₂-induced cell proliferation. Finally, because of the possible role of cPLA₂α (Sved et al., 2004), we analyzed its role by siRNA silencing and found that group X sPLA₂ stimulates cell proliferation independently of cPLA₂α.

In line with the role of group X sPLA₂ enzymatic activity in proliferation of Colon-26 cells,
we found that group X sPLA₂ can release relatively large quantities of free AA, LPC and LPA, which are all able to stimulate cell proliferation and activation of MAP kinase phosphorylation. AA is the precursor of numerous eicosanoids including PGE₂ and LTD₄ which contribute to colon cancer cell proliferation (Wang and Dubois, 2006). Group X sPLA₂ increases the production of various prostaglandins, leukotrienes and HETEs, but these mediators do not explain the sPLA₂ mitogenic effect (Fig. 9). It has been shown that Colon-26 cells express the four PGE₂ receptors (EP1 to EP4), and that PGE₂ is mitogenic on these cells (Pozzi et al., 2004). The fact that exogenous PGE₂ or sPLA₂-produced PGE₂ did not trigger the proliferation of our Colon-26 cells may be linked to the high basal concentrations of PGE₂ (about 30 nM) that already saturate EP receptors. Treatment of Colon-26 cells with 5- and 12-LOX inhibitors also does not prevent the mitogenic effect of sPLA₂ or free AA, indicating that the proliferative effect of the sPLA₂ is in part mediated by free AA without conversion into prostaglandins or leukotrienes.

Based on lysophospholipid analyses of cells treated with hGX sPLA₂ (Fig. 7), we could estimate that the sPLA₂ can release LPA and LPC at nM and low μM concentrations, respectively. Although these concentrations are lower than those of exogenously added LPA and LPC (Fig.8), lysophospholipids produced endogenously may be more effective as they are released locally at the cell surface. The proliferative effect of LPA is likely explained by its binding to receptors LPA₁, LPA₂ and LPA₄ which are expressed in Colon-26 cells (supplemental Fig. 3S). Whether the proliferative effects of exogenous LPC is due to conversion into LPA by autotaxin (ATX) and subsequent binding to LPA receptors or to a direct action via other G-protein coupled receptors and/or transactivation of tyrosine kinase receptors (Ikeno et al., 2005; Fujita et al., 2006) remains an open question. We analyzed Colon-26 cells for expression of ATX at the mRNA and protein levels (supplemental Fig. 3S). Although detectable amounts of ATX mRNA were observed by RT-qPCR analysis, no ATX protein could be detected by western-blot analysis using two distinct antibodies and a radioactive lysophospholipase D enzymatic assay (Ferry et al., 2008). We also analyzed the effect of the recently described ATX inhibitor S32826 (Ferry et al., 2008) on the production of LPA triggered by hGX sPLA₂ in conditions identical to those used in Fig.7. The ATX inhibitor S32826 had no effect on LPA production at 1 and 10 μM (supplemental Fig. 3S), further indicating that Colon-26 cells do
not express ATX. It thus appears unlikely that the LPA produced by group X sPLA2 comes from released LPC being converted into LPA by ATX. Although we cannot rule out the presence of another lysophospholipase D-like activity in Colon-26 cells, our finding raises the possibility that group X sPLA2 directly hydrolyzes cellular PA to generate LPA by acting either at the plasma membrane or after shuttling into intracellular compartments enriched into PA. Of note, the total amount of LPA released by group X sPLA2 was less than 3% of the LPC produced (Fig. 7A), suggesting that the sPLA2 may have access to little amounts of PA substrate. We have shown that group X sPLA2 can efficiently hydrolyze PA in mixed phospholipid vesicles (Singer et al., 2002), but there has been so far no report on the capacity of this enzyme to release LPA from cells. Only a few studies have suggested that group IIA sPLA2 may be involved in LPA release (Fourcade et al., 1995; Snitko et al., 1997).

Together, the above data indicate that the proliferative effects of group X sPLA2 are likely to be dependent on the combined production of both free fatty acids including AA and lysophospholipids including LPC and LPA.

We also found by RT-qPCR that group X sPLA2 is expressed at very high levels in the small intestine and more particularly in the colon of C57BL/6J and BALB/c mice. The cellular sites of mGX sPLA2 expression include columnar epithelial cells, Paneth cells and ganglion cells. It should be noted that the very low level of expression measured for mGIIA sPLA2 mRNA in C57BL/6J mice is due to the natural disruption of the pla2g2a gene. In the small intestine of BALB/c mice harboring a functional gene, the expression level of mGIIA was much higher than those of group X sPLA2 and other sPLA2s (Fig. 1B). However, the expression of mGIIA dramatically decreased in the colon while that of mGX sPLA2 increased, making mGX sPLA2 among the most highly expressed sPLA2 gene in the proximal and more particularly in the distal colon of both C57BL/6J and BALB/c mice (Fig. 1A and B). In good accordance, group IIA and X sPLA2s are also the most highly expressed sPLA2s in human colon (Mounier et al., 2008). These observations raise the question as to whether the two enzymes play redundant or divergent functions within the small intestine and colon. Based on their unique molecular and functional features, it is likely that the two sPLA2s have distinct roles. First, group IIA sPLA2 is a very basic protein while group X is the most acidic sPLA2 (Lambeau and Gelb, 2008). Second, group IIA sPLA2 binds tightly to anionic phospholipid interfaces but not zwitterionic
ones, while group X sPLA₂ shows similar binding (Singer et al., 2002). Third, their expression in normal intestine is different in both mouse and human species (see above). Fourth, there is a strong upregulation of group IIA sPLA₂ at either mRNA or protein levels in inflammatory bowel diseases and likely in mouse and human colon tumors (Ilsley et al., 2005; Cummings, 2007). On the other hand, there is no conclusive evidence for upregulation of group X sPLA₂ in the small intestine and colon of ApcΔ⁷¹⁶ (Takaku et al., 2000) and ApcΔ¹⁴ mice (supplemental Fig. 2S), two models of human familial adenomatous polyposis. No upregulation was observed in the colon tumors of mice treated with the carcinogen azoxymethane (Ilsley et al., 2005) and in human colorectal adenocarcinomas (Osterstrom et al., 2002; Mounier et al., 2008). This is probably reminiscent of the fact that the catalytic activity of group X sPLA₂, but not group IIA, may be regulated at the post-translational level by maturation of its N-terminal propeptide by a still poorly defined proteolytic mechanism (Cupillard et al., 1997; Masuda et al., 2005). Finally, group IIA sPLA₂ likely plays an important antibacterial role in the intestine and is accordingly highly expressed in Paneth cells of the small intestine and epithelial cells of colonic mucosa (Nevalainen et al., 2008). Its antitumoral role in colon cancer has been proposed to be linked to this antibacterial activity, but the mechanism is still enigmatic (Fijneman and Cormier, 2008). On the other hand, group X sPLA₂ has been proposed to play a central role in AA release and PGE₂ production in the colon, but not in the small intestine where cPLA₂α would play the major role (Morioka et al., 2000; Takaku et al., 2000). This hypothesis fits well with our RT-qPCR data showing a 5-fold higher expression of group X sPLA₂ over cPLA₂α in the colon, but not in the small intestine (Fig. 1). Whether autotaxin and/or LPA receptors which are over-expressed in cancer (Parrill and Baker, 2008) contribute to the effects of group X sPLA₂ is unknown. The fact that group X sPLA₂ can produce various lipid mediators suggests that this enzyme may also regulate other key events in tumorigenesis (Wang and Dubois, 2006). Finally, the presence of mGX sPLA₂ mRNA in ganglion cells may suggest additional functions in the enteric nervous system including neuritogenesis (Masuda et al., 2005), peristaltic reflex or nociception.

In conclusion, we have shown that group X sPLA₂ can stimulate the in vitro proliferation of colon cancer cells via its enzymatic activity and production of free AA and lysophospholipids. The
high expression of group X sPLA₂ in normal colon and tumors in both mouse and human species and its ability to produce various lipid mediators suggests that this enzyme plays a similar role in vivo.
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Fijneman RJ and Cormier RT (2008) The roles of sPLA2-IIA (Pla2g2a) in cancer of the small and large intestine. *Front Biosci* **13**:4144-4174.


Footnotes

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Legend for Figures

Fig. 1. Expression of the different mouse sPLA₂s in the intestine of C57BL/6J and BALB/c mice by RT-qPCR and in situ hybridization of mGX sPLA₂. A and B. RT-qPCR in C57BL/6J (A) and BALB/c (B) mouse intestine sections using specific sets of PLA₂ primers. To facilitate the comparison of expression between the different sPLA₂s, the data were first normalized to GAPDH mRNA, which was used as a reference gene, and then expressed relative to the lowest expression level that can be accurately measured in our RT-qPCR assay conditions, ie the expression of pancreatic group IB sPLA₂ in the colon (relative abundance of 1 (arbitrary unit = 1). Note that two different ordinate axes have been used in panel B. mGIIE sPLA₂ could not be detected in all intestine sections from BALB/c mice.

C to F. In situ hybridization of mGX sPLA₂ in small intestine (ileum) showing labeling of columnar epithelial cells in mucosal villi (V), Paneth cells (P) and ganglion cells (G) of the myenteric plexus. Hybridized with antisense probe. D. Absence of reaction product from ileal tissue when hybridized with sense probe. E, in situ hybridization of mGX sPLA₂ in large intestine (cecum) showing labeling in epithelial cells. Hybridized with antisense probe.

Fig. 2. Effect of group X sPLA₂ on [³H]-thymidine incorporation in mouse colon cell lines. After starvation for 24 h at 37°C in serum-free and additive-free medium, all cell lines were incubated in the presence of mGX and hGX sPLA₂s (200 nM) or FCS in the following conditions : A. Colon-26 were grown at 37°C for 24 h in DMEM/0.02% FAF BSA; B. AJ02-nm0 cells were grown at 37°C for 48 h in RPMI/0.02% FAF BSA; C. Apc⁺/Min cells were grown at 33°C in DMEM/0.02% FAF BSA/10 ng/ml recombinant EGF; D. YAMC cells were incubated at 33°C for 48 h in serum-free RPMI/0.02% FAF BSA. [³H]-Thymidine was added during the last 4 h of sPLA₂ stimulation, and cells were processed as described in methods. The increase in [³H]-thymidine incorporation induced by sPLA₂s was compared to untreated cells (-) and FCS-treated cells. In all panels, values are representative of at least two experiments performed in triplicates. Significant differences between untreated and sPLA₂-treated cells were found (**, P <0.001; Student’s t test).

Fig. 3. Effect of various mouse sPLA₂s and hGX sPLA₂ on DNA synthesis, cell growth and
**p42/44 MAP kinases phosphorylation in Colon-26 cells.** **A and B.** Quiescent cells were incubated for 24 h with the different mouse sPLA2s (A) or various concentrations of hGX sPLA2 (B). [3H]-Thymidine incorporation is expressed as fold-increase over untreated cells (incorporation of [3H]-thymidine under untreated conditions was 44,962 dpm). Values represent average values of at least three experiments performed in triplicate (A) or are representative of at least three experiments (B). Group IIA, V and X sPLA2s significantly stimulated [3H]-thymidine incorporation when compared to untreated cells (**, P <0.001; One-Way ANOVA, Bonferroni adjustment). No significant difference was found between cells treated with group V and X sPLA2s (P = 0.2176, Student’s t test). **C.** Quiescent Colon-26 cells were cultured for up to 3 days in the presence or absence of hGX or mGX sPLA2s (200 nM) and counted every day. The difference in cell number between cells cultured in the absence and presence of sPLA2 at different timepoints is statistically significant (**, P<0.05, Student’s t test). **D.** Cells were incubated for various times at 37°C with mGX and hGX sPLA2s (200 nM). The cell lysates were subjected to immunoblotting with anti-phosphospecific antibodies which specifically recognize tyrosine phosphorylated p42/44 MAPK. Equal amount of proteins were loaded, which was verified by immunoblotting of total p42/44 MAPK proteins.

**Fig. 4. The proliferative effect of group X sPLA2 on Colon-26 cells is dependent on sPLA2 catalytic activity.** **A and B.** Effect of sPLA2 inhibitors on the proliferative effect of mGX sPLA2 and MAP kinase activation. Me-indoxam (10 µM) and LY329722 (5 µM) were pre-incubated with mGX sPLA2 (200 nM) in DMEM/0.02% FAF BSA for 20 min before incubation with Colon-26 cells. Under untreated conditions, incorporation of [3H]-thymidine was 54,669 dpm; mGX sPLA2 alone but not mGX sPLA2 pre-incubated with Me-indoxam or LY329722 significantly stimulated [3H]-thymidine incorporation versus untreated cells (**, P <0.001; One-Way ANOVA, Bonferroni adjustment). Although significative (P <0.01), the effect of Me-indoxam was likely due to a toxic effect which was not observed with LY329722 (P>0.05). **C.** Effect of catalytically-inactive mGX H48Q, hGX H48Q and OS2 D49K sPLA2s (200 nM) on the proliferation of Colon-26 cells (**, P <0.001; One-Way ANOVA, Bonferroni adjustment). Under untreated conditions, the incorporation of [3H]-thymidine was 74,679 dpm; **D.** Effect of cPLA2α siRNA silencing on the proliferative effect of hGX sPLA2. Two
different commercially available siRNAs (Qiagen) targeting mouse cPLA\(_2\alpha\) were tested for silencing by western-blotting (inset), and the effect of the best cPLA\(_2\alpha\) siRNA (n°1) versus an irrelevant GFP-directed siRNA was tested for incorporation of \([^3H]\)-thymidine triggered by hGX sPLA\(_2\). Colon-26 cells were transfected with siRNA on day 1, starved the next day for 24 h and then assayed for cPLA\(_2\alpha\) expression or incorporation of \([^3H]\)-thymidine triggered by hGX sPLA\(_2\) (200 nM). Under untreated conditions, the incorporation of \([^3H]\)-thymidine was 45,825 dpm. The differences in incorporation of \([^3H]\)-thymidine in the absence and presence of sPLA\(_2\) or between untreated cells transfected with the two siRNA are statistically significant (**, \(P<0.01\), Student’s t test). In all panels, data are representative of two experiments.

**Fig. 5. The proliferative effect of group X sPLA\(_2\) on Colon-26 cells is not dependent on binding to the M-type receptor.** A and B. Expression of the M-type receptor in Colon-26, YAMC, AJ02-nm0 and Apc\(^{+\text{Min}}\) cells. Mouse M-type receptor expression was measured by binding experiments (A) using the specific ligand \([^{125}\text{I}]\)-OS\(_1\) and by western blotting (B) of cell lysates as described in methods. C. Validation of M-type receptor silencing by siRNA. Two different commercially available siRNAs (Qiagen) targeting the mouse M-type receptor were tested for silencing by binding assays and immunocytochemistry to measure the percentage of receptor silencing. The two siRNAs were effective, and representative results of at least two experiments obtained with siRNA1 versus an irrelevant GFP-directed siRNA are shown. Colon-26 cells were transfected with siRNAs on day 1, starved on day 2 for 24 h and then assayed on day 3 (48 h) and day 4 (72 h) after transfection for M-type receptor expression by binding experiments using \([^{125}\text{I}]\)-OS\(_1\) ligand. The decrease in binding at 48 and 72 h compared to GFP siRNA is statistically significant (**, \(P<0.01\), Student’s t test). Immunocytochemistry using mouse M-type receptor antiserum were performed 48 h after transfection. D. Effect of M-type receptor siRNA1 versus GFP-directed siRNA on incorporation of \([^3H]\)-thymidine triggered by hGX sPLA\(_2\). Colon-26 cells were transfected with siRNAs on day 1, starved on day 2 for 24 h and then stimulated on day 3 for 24 h in the absence (-) or presence of mGX and hGX sPLA\(_2\)s (200 nM). \([^3H]\)-thymidine was added during the last 4 h of sPLA\(_2\) stimulation, and cells were processed as described in methods. Under untreated conditions, the incorporation of \([^3H]\)-thymidine
was 42,395 dpm. Incorporation of $[^3\text{H}]$-thymidine in sPLA$_2$-treated cells over untreated is statistically significant (**, $P<0.05$, Student’s t test), but no significant difference was found between the two siRNAs. Data are representative of two experiments.

**Fig. 6. Release of AA by various mouse sPLA$_2$s and production of eicosanoids triggered by mGX sPLA$_2$ from Colon-26 cells.** A. Release of AA by various mouse sPLA$_2$s was measured on quiescent Colon-26 cells prelabeled with $[^3\text{H}]$-AA for 24 h as described in methods. Mouse recombinant sPLA$_2$s (200 nM) were incubated with cells for 6 h in DMEM with 0.02% FAF BSA. $[^3\text{H}]$-AA release is expressed as the percentage of radioactivity present in cell medium relative to the total radioactivity incorporated into cells. sPLA$_2$-X treated cells release significantly more $[^3\text{H}]$-AA (**, $P<0.001$; One-Way ANOVA, Bonferroni adjustment). A representative experiment out of at least two experiments is shown; B. Eicosanoid production triggered by mGX sPLA$_2$. Colon-26 cells ($10^6$ cells) were starved for 24 h and treated with mGX sPLA$_2$ (200 nM) for 24 h in DMEM/0.02% BSA. The cell medium and the cell monolayer were collected, extracted with organic solvent, combined and analyzed for eicosanoid production by liquid chromatography/mass spectrometry as described in methods. A representative experiment out of two experiments is shown.

**Fig. 7. Release of lysophospholipids by group X sPLA$_2$ from Colon-26 cells.** A. Total amount of LPX acyl chain species released by hGX sPLA$_2$. B. Release of the different LPC and LPA acyl chain species by hGX sPLA$_2$. Colon-26 cells were starved for 24 h and treated with hGX sPLA$_2$ (200 nM) for 6 h in DMEM/0.02% BSA. The cell medium and the cell monolayer were collected, extracted with organic solvent, combined and analyzed for lysophospholipid production by liquid chromatography/mass spectrometry as described in methods. A representative experiment out of two experiments is shown.

**Fig. 8. Effect of AA, LPA and LPC on Colon-26 proliferation.** Effect of free AA (A), LPA (B) and LPC (C) on $[^3\text{H}]$-thymidine incorporation and p42/44 MAPK phosphorylation. Quiescent Colon-26 cells were stimulated with various concentrations of the different lipid mediators, and $[^3\text{H}]$-thymidine incorporation was evaluated as described in methods. Under untreated conditions, the incorporation of $[^3\text{H}]$-thymidine was 42,493 dpm. The effect of AA and LPA on p42/44 phosphorylation was measured.
by incubating Colon-26 cells for the indicated periods of time at 37°C with 20 µM AA and 5 µM LPA as described in Fig. 3. A representative experiment out of at least two separate experiments is shown (*, P <0.05; **, P <0.001 over untreated cells; One-Way ANOVA, Bonferroni adjustment).

**Fig. 9. Effect of COX and LOX inhibitors on PGE₂ release and proliferation in Colon-26.**

**A.** Effect of COX inhibitors on PGE₂ release. Quiescent Colon-26 cells were preincubated with 20 µM Aspirin, Ibuprofen, Indomethacin or Rofecoxib for 20 min before adding mGX sPLA₂ (200 nM) for 24 h. Supernatants were collected and analyzed for PGE₂ by ELISA as described in methods.

**B and C.** Effect of COX inhibitors on mGX- and AA-induced cell proliferation. Quiescent Colon-26 cells were preincubated with COX inhibitors for 20 min before adding mGX sPLA₂ (B) or AA (C). The fold-increase in [³H]-thymidine incorporation over untreated cells is shown. Under untreated conditions, the incorporation of [³H]-thymidine was 41,719 dpm.

**D.** Effects of the 5-LOX inhibitor MK886 (10 µM) and the 12-LOX inhibitor baicalein (10 µM) on Colon-26 cell proliferation. Under untreated conditions, the incorporation of [³H]-thymidine was 39,550 dpm. A representative experiment out of at least two experiments is shown in the different panels and results statistically significant over their respective controls are indicated (**, P <0.001; One-Way ANOVA, Bonferroni adjustment).
Fig. 6

A

[^3]H-arachidionate release (%)

sPLA2 (200 nM)

- IB IIA V X XIIA

B

Eicosanoid production (ng/10^6 cells)

- PGE2
- PGD2
- PGF2\_
- PGI2
- TXB2
- LTB4
- LTC4
- LTD4
- LTE4
- 5-HETE
- 8-HETE
- 12-HETE
- 15-HETE

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Fig. 9

A

B

C

D

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