

# Secreted PLA<sub>2</sub> induces proliferation in astrocytoma through the EGF receptor: another inflammation-cancer link

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We have investigated mechanisms that contribute to reinforce the relationship between inflammation and cancer. Secreted phospholipase A<sub>2</sub> group IIA (sPLA<sub>2</sub>-IIA) is a molecule relevant in inflammatory events and has been proposed as a marker for some of these. Previously, we reported the mitogenic properties of this sPLA<sub>2</sub> in the human astrocytoma cell line 1321N1. Here, we go deeper into the mechanisms that link this inflammatory protein with proliferation in one of the most aggressive types of tumors. We found that phosphorylation of the extracellular regulated kinase (ERK) was preceded by the activation of the small GTPase Ras, and both failed to be activated by inhibiting protein kinase C (PKC). Fractionation and immunofluorescence studies revealed translocation of PKC alpha, delta, and epsilon to the membrane fraction upon stimulation with sPLA<sub>2</sub>-IIA. Immunoprecipitation analysis showed that sPLA<sub>2</sub>-IIA induces phosphorylation of the epidermal growth factor receptor (EGFR) through a PKC-dependent pathway. We found that phosphorylation of this receptor contributed to Ras and ERK activation and that inhibition of ERK, PKC, and EGFR blocked the mitogenic response induced by sPLA<sub>2</sub>-IIA. This study showed that sPLA<sub>2</sub>-IIA is able to bring into play EGFR to trigger its signaling and that PKC leads the distribution of resources. Interestingly, we found that this is not a cell-specific response, because sPLA<sub>2</sub>-IIA was also able to transactivate EGFR in MCF7 human breast cancer cells. Therefore, this mechanism could contribute to worsen the prognosis of a tumor in an inflammatory microenvironment. We also present

more links of the tumor chain possibly susceptible to targeting.

**Keywords:** cancer, epidermal growth factor receptor, inflammation, secreted phospholipase A<sub>2</sub>-IIA, signal transduction.

Although inflammation is part of the defense response, paradoxically it can lead to important health disorders. Since it was discovered in the 19th century, chronic inflammation has been proven to be a risk factor for cancer (for review see refs 1,2), both at the onset and as a prognostic marker. The tumor microenvironment is importantly orchestrated by inflammatory cells and mediators, which participate in key processes, such as proliferation, survival, and migration. Inflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor- $\alpha$ , have been found to be increased in patients with metastatic cancer. This finding makes anti-inflammatory drugs, as well as mechanisms triggered by proinflammatory mediators, of great clinical interest in the potential prevention and treatment of some tumors.

Secreted phospholipase A<sub>2</sub> type IIA (sPLA<sub>2</sub>-IIA) is an acute-phase protein expressed in response to a variety of proinflammatory cytokines by a number of different tissues and cell types. Increased sPLA<sub>2</sub>-IIA plasma levels have been reported in patients with various acute and chronic inflammatory conditions<sup>3,4</sup> and are a marker for some of these.<sup>5,6</sup>

sPLA<sub>2</sub>-IIA expression and activity are increased in numerous cancers, including breast, pancreatic, skin, liver, and prostate cancers (for review see ref. 7). Several investigations have related this expression to prognosis, for example, in human breast cancer.<sup>8,9</sup> sPLA<sub>2</sub>-IIA is also a marker of metastasis in patients with ovarian or gastrointestinal cancer,<sup>10</sup> and in prostate adenocarcinoma the expression level increases along with the tumor grade.<sup>11</sup> In mice, the sPLA<sub>2</sub>-IIA gene,

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Pla2g2a, has been identified as a susceptibility gene for cancers of the small and large intestines, although its role in this tumor type is still controversial.<sup>12</sup> Also, subcutaneous injection of cells overexpressing PLA2g2a resulted in increased tumor size, and these cells were found to be more infiltrative than controls.<sup>13</sup> The importance of these findings has drawn a claim for sPLA<sub>2</sub>-IIA and other PLA<sub>2</sub> isoforms to be studied and considered as possible targets for anticancer drugs.<sup>7</sup> Thus, there is interest in the mechanisms by which sPLA<sub>2</sub>-IIA influences the tumor, which could contribute to the design of more effective chemotherapeutic approaches.

For decades, sPLA<sub>2</sub>-IIA has been considered relevant in different pathologies owing to its capacity to release lipid mediators. In recent years, sPLA<sub>2</sub>-IIA has also been revealed as a signaling molecule itself, independent of its catalytic activity,<sup>14,15</sup> and able to trigger different pathways resulting in opposing cellular responses, such as proliferation<sup>15,16</sup> and apoptosis.<sup>17</sup> In previous studies, we demonstrated that in the human astrocytoma cell line 1321N1, sPLA<sub>2</sub>-IIA triggered signaling cascades that involve members of the mitogen activated protein kinase (MAPK) family, resulting in proliferation of the astrocytoma cells.<sup>15</sup>

The astrocytoma capacity of growth and invasion is closely correlated with the expression of cell surface receptors that sense the signals present in the tumor microenvironment. One of the receptors found overexpressed in this tumor and many others is the epidermal growth factor receptor (EGFR).<sup>18</sup> Also, overexpression of other signaling elements that can lead to proliferation, such as protein kinase C (PKC), has been reported and related to malignant progression.<sup>19</sup> Both EGFR and PKC could have a role in the sPLA<sub>2</sub>-IIA-induced activation of extracellular regulated kinase (ERK) and mitogenesis. Therefore, we proposed to more deeply characterize the signaling machinery triggered by this inflammatory mediator, which ultimately leads to cell proliferation. This study may shed new light on the general machinery that modulates cancer and/or cancer risk, providing a better understanding of the linkages between inflammation and cancer.

## Materials and Methods

### Reagents

A C127 mouse fibroblast cell line stably transfected with the coding sequence of sPLA<sub>2</sub>-IIA from human placenta was used as a source of human recombinant enzyme (cell line kindly provided by Dr. Bereziat, France). sPLA<sub>2</sub>-IIA was purified as described previously,<sup>20</sup> and the dose for all experiments was 1 µg/ml, as determined in previous studies.<sup>21</sup> Farnesyl transferase inhibitor (FTI) 227, atorvastatin, PD98059, GF109203X, and AG1478 were from Calbiochem. Radiolabeled thymidine and antirabbit secondary HRP antibody were from GE Healthcare. Antimouse secondary HRP antibody was from Bio-Rad. Rabbit anti-ERK2 was from Zymed Laboratories. Rabbit phospho-ERK1/2 (Thr202/

Tyr204), cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>), and Ras were from Cell Signaling Technology. Antihuman actin antibody, antibodies against the different PKC isoforms (alpha, delta, epsilon, and zeta), and phospho-EGFR (Tyr-1173) antibody were all from Santa Cruz Biotechnology. Antiphosphotyrosine antibody, clone 4G10, was from Upstate (Millipore). EGFR antibody was a kind gift from Dr. Pandiella (Salamanca, Spain). Both antirabbit and antimouse FITC secondary antibodies, phorbol myristate acetate (PMA), and other chemicals were from Sigma.

### Cell Culture and Transfection

1321N1, MCF7, and Calu-3 cells were cultured at 37°C under a 5% CO<sub>2</sub> atmosphere in DMEM (1321N1 and MCF7) or RPMI (Calu-3) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 2 mM glutamine, and FCS (5% for 1321N1 and MCF7 cells or 10% for Calu-3 cells). The 1321N1 cells were transfected using Lipofectamine (Invitrogen):DNA (ratio 1.2:0.4) 24 hours before the assay, and cells were serum starved overnight.

### Immunoblotting

Once cells reached semiconfluence, they were serum starved overnight. After stimulation, cells were harvested directly in Laemmli-dithiothreitol (DTT) loading buffer and boiled for 5 minutes. SDS-PAGE of all protein samples was then performed using polyvinylidene difluoride membranes, followed by detection using the GE ECL system.

### Ras Activation Assay

Cells were seeded onto 100-mm plates, and when they reached semiconfluence, they were left in serum-free medium overnight. After stimulation with the agonists, they were harvested in 750 µL of lysis buffer (50 mM Tris pH 7.4, 200 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP40, 15% glycerol, 10 µg/mL leupeptin, 5 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM orthovanadate). After clearing the lysates by centrifugation, we incubated the supernatants with GST-RBD-Raf-1 (kindly provided by Dr. J. L. Bos, UMC, Utrecht, The Netherlands) for 20 minutes at 4°C. After coupling, the GTP-bound form of Ras was recovered by centrifugation using GST beads and washed with a lysis buffer. The samples were then boiled in Laemmli-DTT buffer and analyzed by SDS-PAGE.

### Cell Fractionation Assay

Membrane-enriched fractions were obtained after permeabilization of the cells with saponin.<sup>22</sup> Briefly, cells were seeded onto 100-mm plates and harvested in 400 µL of Buffer A (150 mM KCl, 2 mM MgCl<sub>2</sub>, 20 mM HEPES, 10% glycerol, pH 7.2, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL

aprotinin) containing 0.02% (w/v) saponin. After centrifugation, the supernatants (cytosolic fraction) were separated and boiled for 5 minutes in Laemmli-DTT buffer. Pellets (membrane fraction) were washed and resuspended in Buffer A containing Triton X-100 and then boiled in Laemmli-DTT buffer.

### Immunofluorescence

Cells were grown on glass coverslips, serum starved overnight, and stimulated as indicated in each case. After the cells were washed with PBS, they were fixed and permeabilized with 4% PFA–0.1% Triton X-100 PBS and blocked with PBS 1% BSA. The cells were then incubated at 4°C overnight with the primary antibody. Following washing, coverslips were incubated with the secondary antibody for 1 hour; after thorough washing, they were mounted with Vectashield mounting medium for fluorescence (Vector Laboratories). Images were captured with a Nikon Eclipse TS100 microscope using a 20× or 40× objective lens.

### EGFR Immunoprecipitation

Cell lysates were immunoprecipitated with 4 μL/mL anti-EGFR antibody at 4°C for 4 hours, as described previously.<sup>23</sup> The immune complex was recovered using GammaBind G-Sepharose. After washing, the beads were resuspended in Laemmli-DTT loading buffer and subjected to SDS-PAGE. The extent of tyrosine phosphorylation of EGFR was determined by immunoblotting with an antiphosphotyrosine antibody.

### Measurement of DNA Synthesis Reinitiation

Quiescent cells were treated in serum-free DMEM for 24 hours with sPLA<sub>2</sub>-IIA after preincubation with either the inhibitor or vehicle. Four hours before the cells were harvested, 0.5 μCi/mL [<sup>3</sup>H]thymidine was added to the medium. The incubation was then terminated by washing with ice-cold 0.1 M MgCl<sub>2</sub>, and the radioactivity incorporated into the trichloroacetic acid precipitable fraction was measured.

## Results

### sPLA<sub>2</sub>-IIA Activation of Ras is Essential for Full Activation of ERK

One of the best-known and well-described pathways that leads to ERK activation results from the activation of the small GTPase Ras. A pull-down assay, using a fusion protein with the Ras-binding domain of Raf-1 (GST-RBD), showed a transient activation of the small GTPase Ras after sPLA<sub>2</sub>-IIA incubation of 1321N1 cells (Fig. 1A). This activation was already visible after 2 minutes of stimulation and reached its peak between 5 and 10 minutes, which preceded the phosphorylation previously found in ERK by sPLA<sub>2</sub>-IIA in 1321N1 cells.

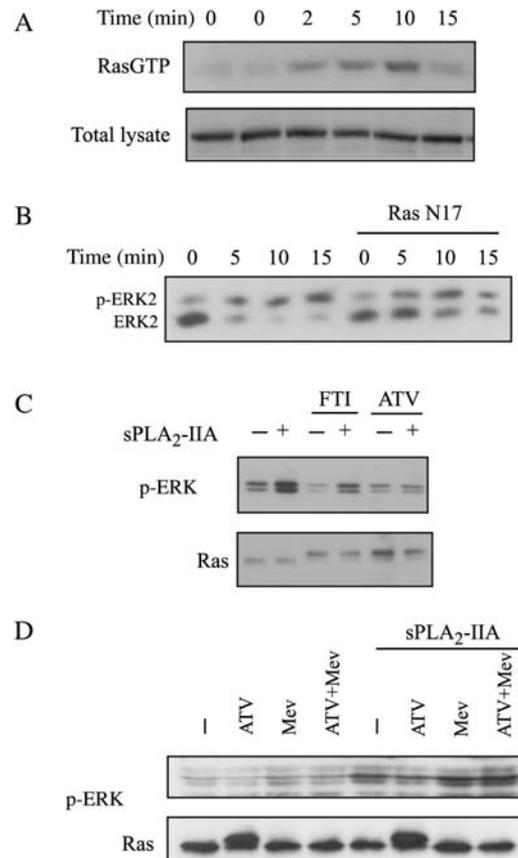


Fig. 1. Ras participation in sPLA<sub>2</sub>-IIA-induced phosphorylation of ERK. (A) Serum-starved 1321N1 cells were stimulated with sPLA<sub>2</sub>-IIA for the indicated times. RasGTP was determined using the RBD pull-down assay (upper), and total lysates were also incubated with anti-Ras antibody (lower). (B) Cells were transfected with RasN17 and then serum starved and stimulated with sPLA<sub>2</sub>-IIA for the indicated times. ERK2 phosphorylation was checked by retardation on a Western blot. (C and D) Cells were preincubated overnight with 10-μM FTI, 10-μM atorvastatin, 100-μM mevalonate, or vehicle and then stimulated with sPLA<sub>2</sub>-IIA for 5 minutes. Western blots of the lysates were incubated with phospho-ERK or Ras antibodies (upper band, unprocessed Ras; lower band, processed Ras). The experiments shown are representative of the results obtained in at least 3 trials for each condition.

We proved the participation of Ras in the phosphorylation of ERK by transfecting the cells with a plasmid that encodes for Ras N17, a dominant negative mutant for Ras. A gel shift of ERK showed that the activation obtained in Ras N17-transfected cells was clearly lower than in mock-transfected cells (Fig. 1B). Either inhibition of the farnesyl transferase or the availability of its substrate would interfere.

Aware of the limits of dominant negative mutants and transient transfection itself, since the wild-type form is still functional, we further confirmed the Ras-ERK relationship using a different approach related to Ras processing. When newly synthesized, Ras GTPases are soluble cytosolic proteins that undergo prenylation to

obtain the fully active enzyme. Either inhibition of the farnesyl transferase or inhibition of its substrate availability would interfere in the isoprenylation of Ras and, accordingly, in the processing to yield a functional protein.<sup>24</sup> We used 2 different compounds for this purpose: FTI and atorvastatin, a permeable statin that inhibits the 3-hydroxy-3-methylglutaryl coenzyme A reductase and therefore inhibits synthesis of mevalonate and the production of farnesyl pyrophosphate. Preincubation with either of these compounds resulted in an augmentation of unprocessed Ras in the cytosol, unable to locate in the membrane (not shown). Unprocessed Ras runs slower on SDS-PAGE, and both the FTI- and atorvastatin-preincubated cells showed more abundant unprocessed Ras after overnight treatment (Fig. 1C). Phosphorylation of ERK by sPLA<sub>2</sub>-IIA was clearly diminished in the presence of either atorvastatin or FTI (Fig. 1C, upper panel), concordant with the Ras N17 assay mentioned above. When mevalonate was added to the culture medium at the same time as atorvastatin, the effect of atorvastatin was bypassed (Fig. 1D), while we found that in total protein the processed/unprocessed Ras levels had returned to basal (Fig. 1C).

#### PKC is Upstream of Ras and ERK

Another pathway that could be activating ERK, and Ras itself, comprises the PKC family. We preincubated 1321N1 cells with GF 109203X, a PKC inhibitor, for 30 minutes prior to stimulation with sPLA<sub>2</sub>-IIA. This inhibitor showed a clear dose-dependent inhibition of sPLA<sub>2</sub>-IIA-induced ERK phosphorylation (Fig. 2A). We also found this inhibition downregulating DAG-PKC $\alpha$  after overnight incubation with 1- $\mu$ M PMA (Fig. 2B), again confirming PKC participation in ERK activation by sPLA<sub>2</sub>-IIA.

sPLA<sub>2</sub>-IIA activation of the cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) is ERK dependent in this cell line<sup>15</sup>; therefore, we checked whether PKC was, in turn, upstream of cPLA<sub>2</sub>. We incubated 1321N1 cells with different doses of GF109203X for 30 minutes prior to a 10-minute stimulation with sPLA<sub>2</sub>-IIA. The phosphorylated form of cPLA<sub>2</sub> runs slower during electrophoresis, as shown in Fig. 2C, and the PKC inhibitor also blocked the activation of cPLA<sub>2</sub> by sPLA<sub>2</sub>-IIA in a dose-dependent manner.

Next, we wondered whether Ras, which is necessary for full activation of ERK, also needed PKC to be activated by sPLA<sub>2</sub>-IIA. For that purpose, we incubated the cells with 1- $\mu$ M PMA overnight and found that PMA downregulation of DAG-dependent PKCs impeded Ras activation by sPLA<sub>2</sub>-IIA (Fig. 2D). Thus, we can conclude that PKC activation precedes Ras, thereby suggesting that PKC activation is upstream of Ras and ERK in this system.

#### sPLA<sub>2</sub>-IIA Stimulation Induces PKC Translocation to the Membrane

After activation, PKC translocates to the membrane to find its substrate. We examined PKC alpha, delta, epsilon, and zeta, and all expressed in 1321N1. After stimulating the cells with sPLA<sub>2</sub>-IIA, we performed

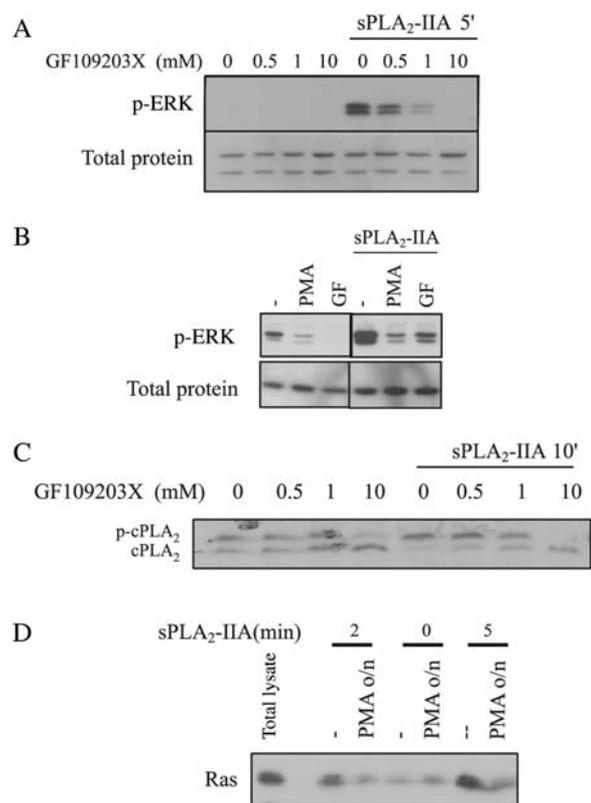


Fig. 2. PKC participation in sPLA<sub>2</sub>-IIA-induced activation of ERK, cPLA<sub>2</sub>, and Ras. (A and C) 1321N1 cells were incubated with the indicated doses of GF109203X 30 minutes prior to stimulation with sPLA<sub>2</sub>-IIA. Western blots of total lysates were incubated with either phospho-ERK (upper A) or actin (lower A) antibodies or cPLA<sub>2</sub> antibody (C). (B) 1321N1 cells were incubated with 1- $\mu$ M PMA overnight or with 1- $\mu$ M GF109203X for 30 minutes, prior to the addition of sPLA<sub>2</sub>-IIA for 5 minutes. Western blots of the lysates were incubated either with phospho-ERK (upper) or with actin antibody (lower). Samples shown belong to the same experiment and were run simultaneously but not consecutively; therefore, a lane represents the gap. (D) 1321N1 cells were incubated with 1- $\mu$ M PMA overnight and then with sPLA<sub>2</sub>-IIA for the indicated times, and RasGTP was determined using the RBD pull-down assay. The first lane shows a sample of total lysate; the other lanes are Ras bound to the RBD, which represents RasGTP. In all cases, cells were serum starved overnight and the experiments shown are representative of the results obtained in at least 3 trials for each condition.

fractionation studies. We found a quick and clear translocation of PKC alpha and epsilon to the membrane fraction in the presence of sPLA<sub>2</sub>-IIA (Fig. 3A). A 5-minute incubation with 1- $\mu$ M PMA was used as a classical positive control. We performed a detailed time course for PKC delta (Fig. 3B) and found that translocation was already visible 40 seconds after sPLA<sub>2</sub>-IIA was added to the culture medium, and after 10 minutes it had reverted to basal levels. PKC translocation induced by sPLA<sub>2</sub>-IIA was not restricted to the outer membrane; by immunofluorescence analysis, we found that, upon stimulation, aggregates of PKC epsilon were formed in

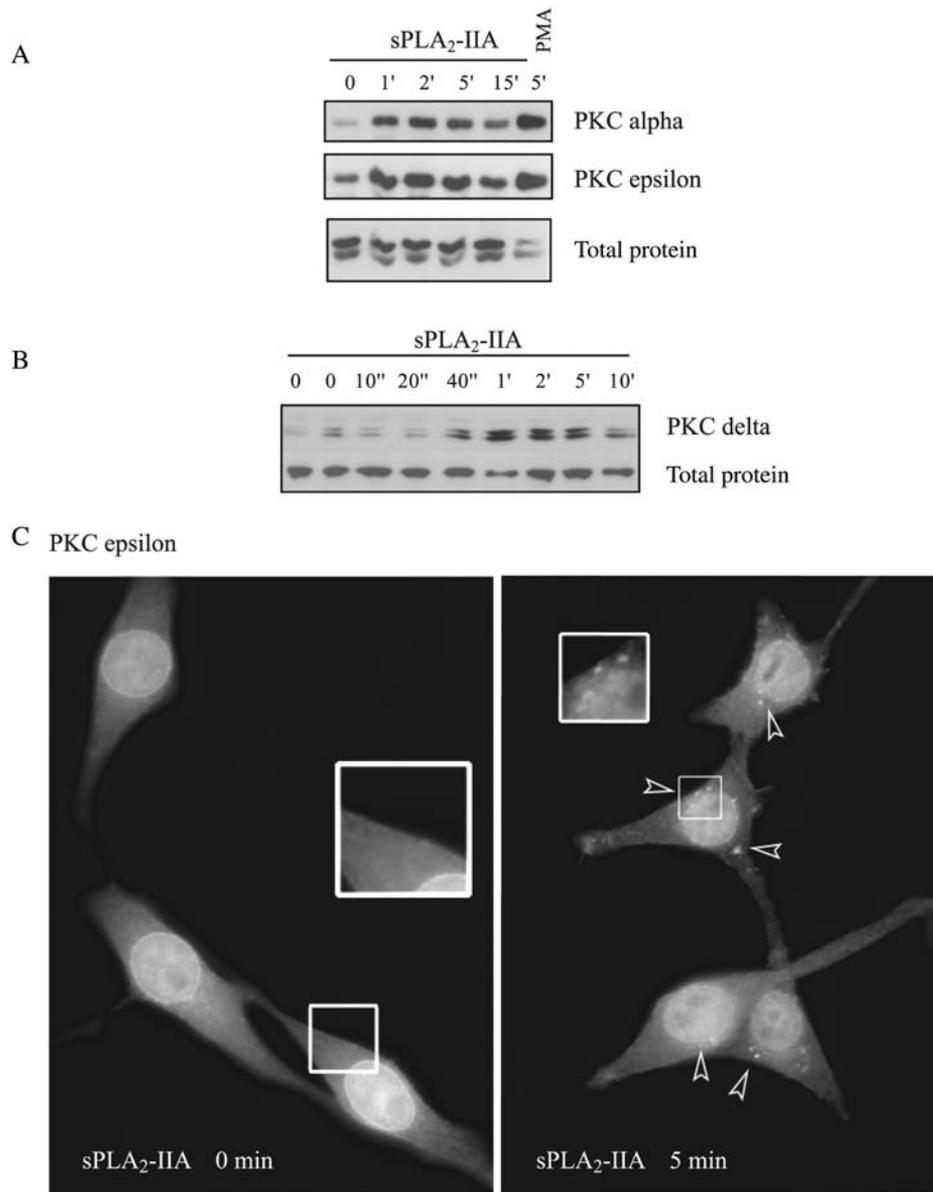


Fig. 3. PKC translocation to the membrane fraction. Serum-starved 1321N1 cells were stimulated with sPLA<sub>2</sub>-IIA or with 1- $\mu$ M PMA (positive control) for the indicated times, and the membrane-enriched fraction was separated by centrifugation as described in the Methods section. SDS-PAGE was performed, and membranes were incubated with polyclonal antibodies against different PKC isoforms: PKC alpha and epsilon (A) and PKC delta (B). (C) Cells were fixed after stimulation with sPLA<sub>2</sub>-IIA or vehicle for the indicated times and incubated with a polyclonal antibody against PKC epsilon. Then an FITC secondary antibody was used, and the samples were analyzed and photographed using a Nikon Eclipse TS100 microscope at  $\times 40$ . The experiments shown are representative of the results obtained in at least 3 trials for each condition.

other membrane structures in the cell (in Fig. 3C, arrows indicate some of these aggregates).

Therefore PKC alpha, delta, and epsilon underwent a quick and transient translocation to the membrane, whereas PKC zeta remained unaffected (not shown), when 1321N1 cells were exposed to sPLA<sub>2</sub>-IIA.

#### *sPLA<sub>2</sub>-IIA Transactivates EGFR, with the involvement of PKC*

Because PKCs can also activate Ras and ERK through transactivation of other receptors and because we had

found tyrosine phosphorylation by sPLA<sub>2</sub>-IIA,<sup>25</sup> we investigated the possibility of transactivation of the EGFR upon sPLA<sub>2</sub>-IIA stimulation in 1321N1 cells.

Tyrphostin AG1478 (AG) is an inhibitor of EGFR, so we preincubated 1321N1 astrocytoma cells with AG1478 prior to stimulation with sPLA<sub>2</sub>-IIA. The dose response of the inhibitor in a Ras pull-down assay suggested a role for EGFR in sPLA<sub>2</sub>-IIA activation of Ras, a partial inhibition of its activation (Fig. 4A). As expected, ERK behaved the same way (Fig. 4B), and only partial activation was achieved by sPLA<sub>2</sub>-IIA in the presence of AG1478.

EGFR undergoes tyrosine phosphorylation when activated, so we analyzed it by Western blotting using an antibody that recognizes phosphorylated EGFR. Results of this assay indicated that sPLA<sub>2</sub>-IIA induced a quick EGFR phosphorylation, which was sustained for about 6 hours and then diminished (Fig. 4C), returning to basal levels 12 hours after stimulation (not shown).

These results showed that the sPLA<sub>2</sub>-IIA signaling cascade activates EGFR and that this transactivation contributes to triggering Ras and ERK signaling.

We checked whether PKC was responsible for the phosphorylation of EGFR by sPLA<sub>2</sub>-IIA in 1321N1 cells. We downregulated DAG-PKC with a 1- $\mu$ M PMA treatment overnight and found that EGFR phosphorylation on tyrosine by sPLA<sub>2</sub>-IIA was considerably diminished in PMA-preincubated cells compared with cells preincubated with only vehicle (Fig. 4D). Therefore, we show a role for PKC in this transactivation.

#### sPLA<sub>2</sub>-IIA-Induced Proliferation Depends on EGFR, PKC, and ERK Activation

Proliferation is the cellular outcome after incubation of 1321N1 cells with sPLA<sub>2</sub>-IIA.<sup>15</sup> All the members of the signaling cascades studied so far are closely related

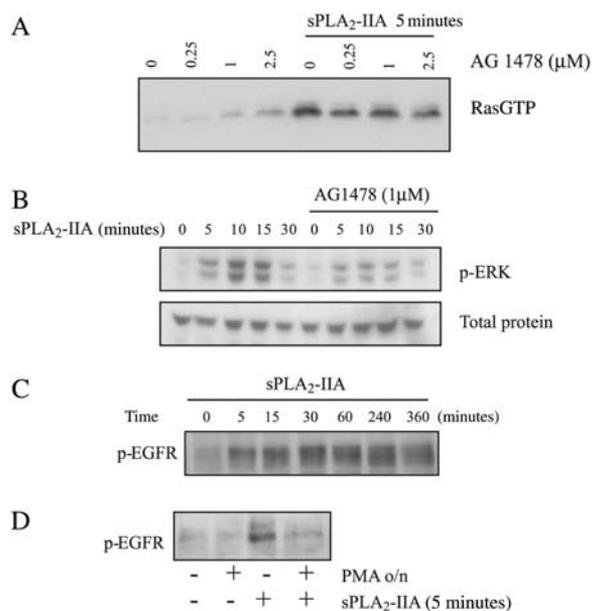


Fig. 4. EGFR activation and participation in sPLA<sub>2</sub>-IIA-induced Ras and ERK activation: role of PKC. Serum-starved 1321N1 cells were preincubated with the inhibitors, when indicated, and stimulated with sPLA<sub>2</sub>-IIA or vehicle for the indicated times. RasGTP was analyzed after a 30-minute preincubation with AG1478 (A), ERK phosphorylation was analyzed by Western blot with phospho-ERK antibody in a time course after preincubation with 1- $\mu$ M AG1478 for 30 minutes (B), EGFR phosphorylation was also checked by Western blot with antiphospho-EGFR, (C) a time course of sPLA<sub>2</sub>-IIA, and (D) a 5-minute stimulation with sPLA<sub>2</sub>-IIA in the absence or presence of 1- $\mu$ M PMA overnight. The experiments shown are representative of the results obtained in at least 3 trials for each condition.

to mitogenic pathways in several systems. To check whether they were also involved in this system, assays with [<sup>3</sup>H]thymidine were performed in the absence or presence of their respective inhibitors: AG1478 for EGFR, GF109203X for PKC, and PD098059 for MAPK kinase (MEK) that consequently impedes activation of ERK.

We preincubated the cells with the indicated doses of the inhibitors for 30 minutes prior to the addition of sPLA<sub>2</sub>-IIA and found that the presence of each inhibitor resulted in a clear and dramatic reduction of the mitogenesis induced by sPLA<sub>2</sub>-IIA, as judged from the amount of [<sup>3</sup>H]thymidine incorporated (Fig. 5A).

Therefore, the proliferation induced by sPLA<sub>2</sub>-IIA in this human astrocytoma cell line is triggered by a cascade depending on the activation of EGFR, ERK, and PKC.

#### sPLA<sub>2</sub>-IIA Also Transactivates EGFR in MCF7 Human Breast Cancer Cells

We wondered whether transactivation of this receptor by sPLA<sub>2</sub>-IIA could be considered as a possible

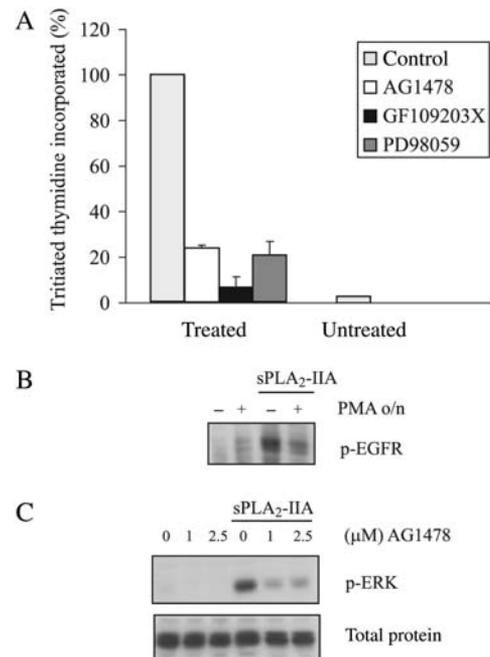


Fig. 5. sPLA<sub>2</sub>-IIA-induced proliferation in 1321N1 cells and sPLA<sub>2</sub>-IIA signaling induced in MCF7 cells. (A) Proliferation was measured as described in the Methods section. Serum-starved 1321N1 cells were stimulated with sPLA<sub>2</sub>-IIA for 24 hours after preincubation for 30 minutes with the indicated inhibitors (1- $\mu$ M AG1478, 1- $\mu$ M GF109203X, or 25- $\mu$ M PD98059) or vehicle. [<sup>3</sup>H]Thymidine incorporated in sPLA<sub>2</sub>-IIA-stimulated cells was taken as 100%, and the rest of the values were related to this. Bars represent the means  $\pm$  S.E. of 3 independent experiments performed in triplicate. (B and C) Serum-starved MCF7 cells were stimulated with sPLA<sub>2</sub>-IIA for 5 minutes after an overnight incubation with 1- $\mu$ M PMA (B) or a 30-minute incubation with AG1478 (C) at the indicated doses. Total lysates were subjected to SDS-PAGE and incubated with phospho-EGFR (B) or phospho-ERK (C).

mechanism to induce mitogenesis by sPLA<sub>2</sub>-IIA in other systems.

We performed our experiments in MCF7 cells, a human breast cancer cell line that expresses EGFR. Incubation with sPLA<sub>2</sub>-IIA induced the phosphorylation of EGFR in MCF7 cells (Fig. 5B), as was found for the astrocytoma cells. Similarly, downregulation of DAG-PKCs by overnight incubation of the cells with 1- $\mu$ M PMA diminished the level of transactivation.

Not only did this result parallel the findings of our astrocytoma study, but we also found that sPLA<sub>2</sub>-IIA induced phosphorylation of ERK. This phosphorylation was not detected after preincubation of the cells with different doses of the EGFR inhibitor AG1478 (Fig. 5C), suggesting a role for EGFR in the signaling triggered by sPLA<sub>2</sub>-IIA in the human breast cancer cell line MCF7 as well.

Next, we wondered whether the cellular outcome after incubation with sPLA<sub>2</sub>-IIA would be similar to that for the astrocytoma cell line. We performed DNA synthesis reinitiation assays and found that [<sup>3</sup>H]thymidine incorporation was also greater in the presence of sPLA<sub>2</sub>-IIA than in the presence of a vehicle (100% vs 18%), and preliminary data indicated that sPLA<sub>2</sub>-IIA-induced proliferation was also both PKC and ERK sensitive (from 100% to 46% and 70%, respectively).

We also performed preliminary studies in the Calu-3 human lung adenocarcinoma cell line, which lacks expression of the EGFR,<sup>26</sup> and these studies found that sPLA<sub>2</sub>-IIA was unable to induce a mitogenic response, resulting in comparable values of [<sup>3</sup>H]thymidine incorporated in the presence or absence of the enzyme. Likewise, no phosphorylation of ERK was found upon exposure of Calu-3 cells to sPLA<sub>2</sub>-IIA, indicating that it is unable to trigger this key signaling pathway. Further investigation should follow in order to certify a possible link between the level of EGFR expression and the proliferative properties of sPLA<sub>2</sub>-IIA.

## Discussion

Although there is plenty of evidence to ascertain that inflammation can promote and influence cancer, the cause of this relationship is not fully understood. In this report, we showed how sPLA<sub>2</sub>-IIA, an acute-phase protein present in inflammatory events and a wide range of pathologies, including cancer, engages the EGFR, which is frequently overexpressed in tumors, to induce proliferation. We also showed how PKC, a key molecule of many signaling cascades, plays a determining role. Because both EGFR and PKC are important signaling elements in many tumor types, we consider this a relevant finding in the event of inflammation, which is frequently present in the tumor microenvironment.

We performed our studies in the 1321N1 human astrocytoma cell line, in which incubation with sPLA<sub>2</sub>-IIA induces proliferation.<sup>15</sup> We wondered how sPLA<sub>2</sub>-IIA is able to elicit this response and the related mechanisms.

ERK is involved in the regulation of cell proliferation, survival, and differentiation; its aberrant regulation can contribute to cancer, and therefore, all the members of the Raf/MEK/ERK pathway have become classical targets in the field.<sup>27</sup> sPLA<sub>2</sub>-IIA induces phosphorylation of ERK in 1321N1 astrocytoma cells<sup>15</sup> and inhibition of this pathway stops proliferation.<sup>28</sup> ERK is, therefore, a key element in sPLA<sub>2</sub>-IIA-induced mitogenesis; consequently, we concentrated on unravelling the upstream components that lead to ERK activation in this system.

Ras proteins have been found to be overexpressed or mutated in a wide variety of tumors, and they play a pivotal role in the transduction of several growth or differentiation stimuli (for review see refs 29,30). The switch of the small GTPase Ras from GDP to GTP can trigger the activation of the Raf/MEK/ERK cascade.<sup>31</sup> We found activation of Ras when sPLA<sub>2</sub>-IIA was present, and it is necessary for full activation of ERK, as we showed by transfecting a dominant negative mutant of Ras or inhibiting Ras farnesylation with statins. In this situation, the presence of mevalonate restores Ras conformation and allows ERK full activation by sPLA<sub>2</sub>-IIA; therefore, we discard statins effects, other than the inhibition of farnesylation. Processing of other proteins can be also inhibited by the blockage of farnesylation; related to proliferation, we find the small GTPases Rheb (Ras homolog enriched in brain) and RhoB<sup>32</sup> and the centromeric proteins CENP-E and CENP-F.<sup>33</sup> Rheb has been described as regulating B-Raf, inhibiting its activation,<sup>34</sup> whereas RhoB has been related to ERK but as an inhibitor.<sup>35</sup> The centromere-associated proteins CENP-E and CENP-F are regulated during mitosis, and the inhibition of farnesylation results in accumulation of cells prior to metaphase.<sup>33</sup> To our knowledge, there are no reports linking these farnesylated proteins with ERK phosphorylation, thereby reinforcing the role of Ras as an activator of ERK in this system.

The activation of Ras as well as ERK itself can also be achieved through the participation of members of the extended PKC family, which have been linked to cancer and represent a target for therapy.<sup>36</sup> They could act on the cascade leading to ERK activation at different levels, activating Ras,<sup>37</sup> Raf,<sup>38</sup> or MEK<sup>39</sup> or transactivating other receptors that trigger the cascade Ras/Raf/MEK/ERK.<sup>40</sup>

Several PKC isoforms were expressed and translocated to the membrane upon sPLA<sub>2</sub>-IIA stimulation of the astrocytoma cells: PKC alpha, PKC delta, and PKC epsilon. In all cases, we found a very quick and transient translocation. For PKC epsilon, we even found that sPLA<sub>2</sub>-IIA induces translocation to cell compartments other than the plasma membrane. PKC isoforms have been described as presenting a particular pattern of activation depending on the stimuli, undergoing translocation to different cellular compartments<sup>41</sup> for a yet unknown purpose. The binding elements and precise localization of PKC epsilon demand further investigation. It has been stated that the expression and activity of various PKC isoforms are increased in malignant astrocytomas, suggesting that PKC activity contributes

to tumor progression.<sup>19</sup> In our system, inhibition of PKC with GF109203X blocked proliferation; therefore, PKC plays a determining role in the mitogenesis induced by sPLA<sub>2</sub>-IIA. Also, the inhibition of ERK blocked proliferation in 1321N1, and we found a link between both kinases: PKC is not only upstream of ERK and, as expected, of the cytosolic PLA<sub>2</sub>, cPLA<sub>2</sub> (downstream effector of ERK<sup>15</sup>), but also of the activation of the small GTPase Ras. These facts propose a cascade in which PKC has a leading role and induces the activation of the Ras/Raf/MEK/ERK canonical pathway; we therefore considered the above-mentioned possibility of transactivation of other receptors.

Murakami et al.<sup>42</sup> proposed the involvement of protein-tyrosine phosphorylation reactions in sPLA<sub>2</sub>-mediated cell activation in view of inhibitors' ability to block this reaction. In our system, sPLA<sub>2</sub>-IIA also triggers a pathway dependent on tyrosine phosphorylation,<sup>25</sup> supporting the possibility of transactivation of a growth factor receptor.

EGFR is one of the most important growth-stimulating receptors in a great variety of malignant tumors.<sup>18</sup> Growth factor receptors activate ERK by a canonical mechanism: RTK-Shc-Grb2-Sos-Ras-Raf1-Mek-ERK.<sup>43</sup> In this report, we showed that sPLA<sub>2</sub>-IIA induces a quick and sustained phosphorylation of EGFR. This activation is less strong than that induced by its natural ligand, epidermal growth factor, but similar in intensity, kinetics, and profile to those found for EGFR transactivation in other systems.<sup>44–46</sup> We showed that this transactivation of the EGFR is necessary for sPLA<sub>2</sub>-IIA-induced proliferation in 1321N1 astrocytoma cells. The relevance of this mechanism becomes more apparent when we consider that the most frequent genetic alteration associated with glioblastoma is amplification of the EGFR gene, which results in overexpression of the receptor,<sup>47,48</sup> believed to contribute to the aggressive phenotype seen in these tumors.<sup>49</sup> Because Ras is not a frequent mutation in glioblastoma, the use of FTI inhibitors could be considered in the event of activation, as in this situation, through the transactivation of the EGFR by sPLA<sub>2</sub>-IIA or any other factor in the tumor microenvironment.

There are reports that state the relationship between EGFR and PKC in both directions, EGFR-activating PKC,<sup>50</sup> and PKC-activating EGFR,<sup>40</sup> as we find in this system. PKC can induce activation of EGFR by several mechanisms: ligand independent, through the activation of Src by a GPCR, or ligand dependent, by activating metalloproteinases, responsible for the release of extracellular membrane-bound epidermal growth factor-like ligands.<sup>51,52</sup>

How sPLA<sub>2</sub>-IIA is able to trigger all these pathways at the membrane level remains unknown. The role of this enzyme has been classically attributed to its catalytic activity, because it releases bioactive lipids, such as arachidonic acid, platelet-activation factor, and lysophosphatidic acid.<sup>53</sup> In previous studies, we had shown that LPA is able to induce coupling of PLC to the receptor, but it does not modify the phosphorylation status of EGFR on its own in 1321N1 cells,<sup>23</sup> either discarding LPA or stimulating the search of a signaling partner

for LPA to accomplish the task. Nevertheless, when that study took place, we could not find sPLA<sub>2</sub>-IIA metabolites being released. Perhaps this finding should be revisited using the newer technologies available, even though sPLA<sub>2</sub>-IIA catalytic activity towards the cell membrane is apparently restricted to bacteria and modified eukaryotic membranes.<sup>53</sup>

On the other hand, the time course of EGFR phosphorylation shows an immediate response, discarding prostaglandins as the inducers of EGFR transactivation, as indicated in other reports.<sup>54</sup> It is precisely the immediateness of the response that leaves the direct interaction of sPLA<sub>2</sub>-IIA with a membrane structure as the most plausible mechanism to trigger the signaling cascade of concern. Heparan sulfate proteoglycans (glypican, decorin, biglycan, M-type, N-type, or mannose receptors) are claimed to be binding motifs for sPLA<sub>2</sub>-IIA.<sup>55</sup> Interestingly, a recent report has shown sPLA<sub>2</sub>-IIA induction of a mitogenic response linked to integrin engagement.<sup>16</sup> The interaction of sPLA<sub>2</sub>-IIA with a specific receptor is also feasible, but not with those described so far, because the M-type sPLA<sub>2</sub> receptor is not expressed in these cells and sPLA<sub>2</sub>-IIA has low affinity for the N-type receptor.<sup>55</sup> The search for the binding partner of sPLA<sub>2</sub>-IIA, able to trigger the signaling cascades in these cells, is the subject of future investigations.

In this study, we identified key elements (PKC and EGFR) of the signaling cascade of sPLA<sub>2</sub>-IIA that lead to proliferation in astrocytoma cells, since inhibition of any of them blocks this cellular response. As noted in the introduction, sPLA<sub>2</sub>-IIA is present in a wide range of tumors of different origin. In breast cancer, it is now upgraded to the status of a prognostic marker,<sup>8,9</sup> and also in MCF7 we find that sPLA<sub>2</sub>-IIA transactivates EGFR in a PKC-dependent manner. Again, sPLA<sub>2</sub>-IIA induces proliferation in these cells, which is abrogated by inhibition of PKC and ERK, conferring on EGFR a responsibility in the activation of ERK.

Regardless of evident cell-line differences, sPLA<sub>2</sub>-IIA triggers a similar cascade that results in a mitogenic response through the transactivation of EGFR. Further work could determine whether sPLA<sub>2</sub>-IIA mitogenic potential directly depends on the EGFR expression (preliminary work has shown that sPLA<sub>2</sub>-IIA is unable to induce proliferation in a human cell line that lacks EGFR) and also whether we are here presented with a general mechanism to be taken into consideration in tumors where sPLA<sub>2</sub>-IIA could be expressed due to an inflammatory environment. Inflammation and EGFR overexpression could occur in tandem to promote proliferation and worsen the prognosis, not only at the onset or development of the tumor. The resistance of some tumors to radiotherapy has been related to a higher expression of EGFR.<sup>56</sup> The microenvironment of the tumor could be playing a role in this event because inflammation markers are found in patients receiving radiotherapy.<sup>57</sup>

The value of EGFR as a prognostic marker in gliomas is still under debate. Some studies point to differences regarding other factors,<sup>58</sup> which might suggest considering additional factors not strictly confined to the plasma

membrane. Treatments for cancer could possibly be made more effective by taking into account the characteristics of not only the tumor itself but also its microenvironment at each stage.

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