

## Secreted Phospholipase A<sub>2</sub>-IIA Modulates Key Regulators of Proliferation on Astrocytoma Cells

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**Running Title:** sPLA<sub>2</sub>-IIA signalling in proliferation

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**Abbreviations:** sPLA<sub>2</sub>-IIA, secreted phospholipase A<sub>2</sub>-type IIA; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; S6, S6 ribosomal protein; p70S6K, ribosomal S6 kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase.

## **Abstract.**

Human group IIA secreted phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-IIA) has been characterized in numerous inflammatory and neoplastic conditions. sPLA<sub>2</sub>-IIA can either promote or inhibit cell growth depending on the cellular type and the specific injury. We have previously demonstrated that exogenous sPLA<sub>2</sub>-IIA, by engagement to a membrane structure, induces proliferation and activation of MAPKs cascade in human astrocytoma cells. In this study we used human astrocytoma 1321N1 cells to investigate the key molecules mediating sPLA<sub>2</sub>-IIA-induced cell proliferation. We found that sPLA<sub>2</sub>-IIA promoted ROS accumulation, which was abrogated in the presence of allopurinol and DPI, but not by rotenone, discarding mitochondria as a ROS source. In addition, sPLA<sub>2</sub>-IIA triggered Ras and Raf-1 activation, with kinetics that paralleled ERK phosphorylation, and co-immunoprecipitation assays indicated an association between Ras, Raf-1 and ERK. Additionally, Akt, p70S6K, and S6 were also phosphorylated upon sPLA<sub>2</sub>-IIA treatment, effect that was abrogated by NAC or LY294002 treatment indicating that ROS and PI3K are upstream signaling regulators. Since the inhibitors NAC, PD98059, LY294002 or rapamycin blocked sPLA<sub>2</sub>-IIA-induced proliferation without activation of the apoptotic program, we suggest that inhibition of these intracellular signal transduction elements may represent a mechanism of growth arrest. Our results reveal new potential targets for therapeutic intervention in neuroinflammatory disorders and brain cancer in particular.

**Keywords:** Astrocytoma cells, human group IIA secreted phospholipase A<sub>2</sub>, reactive oxygen species, Akt, p70 S6 kinase, proliferation.

## **Introduction.**

Human group IIA secreted phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-IIA) belongs to a highly conserved family of enzymes with lipolytic activity (Kramer et al., 1989). It is secreted from mammalian cells, being its expression regulated by proinflammatory cytokines and growth factors. Primarily known for its role in acute and chronic inflammatory diseases such as atherosclerosis (Webb 2005; Menschikowski *et al.* 2006), it has also become evident its role in the pathogenesis of CNS injuries and certain neurological disorders (Sun *et al.* 2004; Adibhatla and Hatcher 2007). In addition, the recognition that it is overexpressed in numerous tumoral tissues has formed the basis of the sPLA<sub>2</sub>-IIA pro-oncogenic activity concept (Abe *et al.* 1997; Menschikowski *et al.* 2008). So far, this increased sPLA<sub>2</sub> presence in tumor tissues has even opened new therapeutical approaches (Jensen et al. 2004). Nevertheless, while many studies have associated the increased protein levels of the enzyme with the severity of the diseases, its precise function in tumorigenesis remains unclear, having been related either to malignant potential (Dong *et al.* 2002; Belinski *et al.* 2006; Menschikowski *et al.* 2008) or to reduced tendency towards metastasis and longer postoperative survival of patients (Leung *et al.* 2002).

Accumulating evidence indicates that secreted PLA<sub>2</sub>s and specially sPLA<sub>2</sub>-IIA, in addition to its esterase activity, may also exert its biological effects by interacting with different membrane targets (Lambeau and Lazdunski 1999; Krizaj and Gubensek 2000; Boilard *et al.* 2003; Saegusa *et al.* 2008) and behave as cytokine-like molecules. Thus, different studies mainly performed on inflammatory and hematopoietic lineage cells with either bee venom or mammalian secreted PLA<sub>2</sub>s have identified several signaling pathways regulated by the phospholipases such as protein kinase C, MEK/ERK1/2, p38 MAPK and PI3-K/Akt cascades, where their capacity to generate

biologically active lipids is not required. (Silliman *et al.* 2002; Trigglioni *et al.* 2002, 2003; Gambero *et al.* 2004).

However, although sPLA<sub>2</sub>-IIA acting extracellularly has emerged as an important regulator of survival and growth on astrocytoma cells (Hernandez *et al.* 1998), to our knowledge little research has been conducted to examine mechanistically their functional role in neoplastic disorders of the CNS.

In 1321N1 human astrocytoma cells, we have previously described the existence of a signaling pathway triggered by exogenous sPLA<sub>2</sub>, that induces phospholipase C $\gamma$ -1 activation and Ca<sup>2+</sup> mobilization and modulates the MAPK cascade resulting in a mitogenic response (Hernández *et al.* 1998, 1999). To obtain further insight into the aforementioned signaling effects, we considered the fact that reactive oxygen species (ROS), prototypical molecules associated with a variety of diseases such as cancer or ischemia, may contribute to increase growth and DNA synthesis, and that mitogen-activated protein kinases (ERK/MAPK), as well as the serine/threonine protein kinase Akt are key mediators of ROS-induced growth in response to different stimuli (Chen *et al.*, 1995; Wang *et al.* 2000). Therefore, we addressed the actions of exogenous sPLA<sub>2</sub>-IIA on ROS buildup, as well as on activation of key molecules of the proliferative networks: Ras/Raf-1/MEK/ERK and PI3K/Akt/mTOR.

Hence, this research greatly increases the knowledge of the intracellular networks by which secreted phospholipases, especially sPLA<sub>2</sub>-IIA, may functionally modulate the evolution of CNS tumors, improving the understanding, not only of the underlying molecular biology of astrocytic tumor cells, but also of the signaling mechanisms modulated by sPLA<sub>2</sub>-IIA.

## **Materials and Methods.**

**Materials.** See Supplementary Material online for details

**Cell culture and transfection.** 1321N1 human astrocytoma cells were grown in DMEM supplemented with 5% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin in a 37°C incubator with 5 % CO<sub>2</sub>. Cells were transfected by the calcium-phosphate method with pMT2-HA-Ras or empty vector expressed in *E. coli* DH5alpha cells and purified with a Qiagen maxiprep kit following the manufacturer's instructions.

### **ROS production and mitochondrial inner transmembrane potential detection.**

Measurements of superoxide-derived ROS accumulation in DCFH-DA-preloaded cells in response to 1 µg/ml of sPLA<sub>2</sub>-IIA were carried out as previously described (Martin *et al.*, 2007). In some experiments, cells were treated for 30 min with the indicated antioxidant before incubation with the phospholipase.

To evaluate mitochondrial transmembrane potential ( $\Delta\psi_m$ ), cells were treated with 1 µg/ml of sPLA<sub>2</sub>-IIA for 6 or 18 h. and then 1 µM rhodamine 123 was added to the culture. Cells were analysed immediately by flow cytometry.

**Cell proliferation.** Cell proliferation was evaluated by a [<sup>3</sup>H]-thymidine incorporation assay. Cells were starved for 24 h and then treated with 1 µg/ml of sPLA<sub>2</sub>-IIA in the presence or absence of the indicated inhibitors. After 24 h of incubation, cells were labeled with 0.5 µCi [<sup>3</sup>H]-thymidine/ml during 4 additional hours, and incorporation was assessed by scintillation counting. Values were normalized by setting sPLA<sub>2</sub>-IIA-stimulated cells to 100%. Numerical data are mean ± SD of 4 different experiments, each performed in triplicate.

**Trypan blue dye assay.** See Supplementary Material online for details.

**Analysis of apoptosis.** After a 24 h treatment with 1  $\mu\text{g/ml}$  of sPLA<sub>2</sub>-IIA in the presence or absence of the indicated inhibitors, cells were visualized and photographed with a 40 $\times$  objective in a Nikon Eclipse 80i microscope, or used for an Annexin V-PE Apoptosis Detection Assay. Briefly, cells were resuspended in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 4% BSA), and incubated for 30 min with Annexin V-PE, followed by flow cytometric analysis using an EPICS XL cytofluorometer, Beckman-Coulter.

**Analysis of endogenous Ras activation.** Ras activation was determined using bacterially produced GST-Raf-RBD (kindly provided by Dr. J.L. Bos). After stimulation, cells were lysed on ice in lysis buffer (15% glycerol, 50 mM Tris-pH 7.4, 1% NP-40, 200 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{M}$  leupeptin, 0.1 $\mu\text{M}$  aprotinin). Cellular debris was removed and supernatants were incubated for 30 min at 4 °C with GST-Raf-RBD (15  $\mu\text{g/sample}$ ) coupled to glutathione-Sepharose beads (10  $\mu\text{l/sample}$ ). The precipitated complexes were boiled in SDS sample buffer. Total lysates and precipitates were analysed by Western Blot using an antibody against Ras.

**Immunoblotting and Immunoprecipitation.** This is described in the Supplemental Material.

**Data Presentation** Each set of experiments was repeated at least three times. Unless otherwise indicated, the data presented are from representative experiments. Numerical data are expressed as means  $\pm$  SD and analyzed with the SPSS software for statistical significance using Student's *t* test. A *p* value  $<0.05$  was considered significant.

## Results

**sPLA<sub>2</sub>-IIA induces ROS generation in 1321N1 cells.** Enhanced ROS levels have been observed in cells in response to growth factors or cytokines, and numerous studies suggest their participation in cell proliferation. To determine their role in sPLA<sub>2</sub>-IIA-mediated 1321N1 astrocytoma cell growth, we first examined the ability of the phospholipase to elicit ROS production using the probe DCFH-DA. The results obtained demonstrated that cell exposure to sPLA<sub>2</sub>-IIA led to a remarkable increase in DCF fluorescence in a dose- and time-dependent manner compared to control cells. Figure 1A shows that accumulation of superoxide-derived ROS was observed around 5 min after stimulation with 1 µg/ml of sPLA<sub>2</sub>-IIA and rapidly increased to its maximum levels around 15-30 min. Although we detected ROS generation at 0.1 µg/ml (18 % cells were positive), maximal effect was observed between 1-5 µg/ml (47 % cells were positive) compared to untreated cells (5 % cells were positive) (Fig. 1B). Hence, in subsequent experiments 1 µg/ml of sPLA<sub>2</sub>-IIA was used as optimal concentration for signaling studies. We also performed experiments with human recombinant sPLA<sub>2</sub>-IIA obtained from C127 cells stably transfected with the coding sequence of sPLA<sub>2</sub>-IIA from human placenta with a very similar, if not identical results (data not shown).

Next, we examined the effect of some scavenger enzymes, such as catalase (CAT) or superoxide dismutase (SOD), and synthetic antioxidants, such as N-acetylcysteine (NAC), or the radical scavenger pyrrolidine dithiocarbamate (PDTC). For this purpose, 1321N1 cells were incubated for 30 min with either 50 or 100 U/ml of CAT, 50 or 500 U/ml of SOD, 5 or 10 mM of NAC, or 50 or 100 µM of PDTC prior to the addition of the phospholipase. Under these conditions, all the antioxidants notably attenuated sPLA<sub>2</sub>-IIA-induced ROS upregulation (Fig. 2 A,B).

Then, we investigated the source of phospholipase-induced ROS production in 1321N1 cells. We used various inhibitors of possible enzymatic sources: DPI (10-20  $\mu\text{M}$ ) an inhibitor of flavoenzymes, such as xanthine oxidase or NADPH oxidase (NOX) isozymes, allopurinol (50-100  $\mu\text{M}$ ) a potent xanthine oxidase inhibitor, and rotenone (5-10  $\mu\text{M}$ ) a mitochondrial complex I inhibitor. As shown in Figure 2C, the presence of either 100  $\mu\text{M}$  allopurinol or 20  $\mu\text{M}$  DPI abolished DCF fluorescence of sPLA<sub>2</sub>-IIA-treated cells, therefore, ROS production. In contrast, no concentration of rotenone was able to alter DCF fluorescence, making mitochondrial electron transport an improbable ROS source.

Finally, we evaluated whether this ROS overproduction led to changes in  $\Delta\psi\text{m}$  that would result in alteration of mitochondrial function using Rhodamine 123, a specific fluorescent probe, whose uptake and retention into the mitochondrial matrix depends on the  $\Delta\psi\text{m}$ . In this study we also used a ROS inducer, oleanolic acid (OA), as a positive control for  $\Delta\psi\text{m}$  depolarization (Martín *et al.*, 2007) As shown in Figure 2B, sPLA<sub>2</sub>-IIA did not affect Rhodamine 123 accumulation in 1321N1 cells after 6 or 18 h of stimulation compared to untreated control cells, while in OA-treated cells a reduced uptake and retention of the dye was detected. In fact, after 6 or 18 h of incubation with 25  $\mu\text{M}$  OA, a decrease of 82% and 96% in fluorescence was noted, whereas in sPLA<sub>2</sub>-IIA-treated cells it remained at similar levels to those found in untreated cells.

**Effect of sPLA<sub>2</sub>-IIA on the classical Akt and ERK signaling pathways.** Several studies have suggested that Ras plays an important role in a variety of cells through the sequential activation of the Raf-1 /MEK1/2/MAPK pathway, and in 1321N1 astrocytoma cells we have already demonstrated MAPK activation upon cell exposure to sPLA<sub>2</sub>-IIA (Hernández *et al.* 1998). To elucidate whether the activation of Ras/Raf were also signaling elements of the phospholipase action, cells were incubated in the absence or presence of an optimal concentration of sPLA<sub>2</sub>-IIA



(1  $\mu\text{g/ml}$ ) for different times. As it can be seen in Figure 3A, sPLA<sub>2</sub>-IIA treatment increased Ras-GTP formation in a time dependent manner, reaching the maximum level around 5 min. At similar time points, Raf-1 phosphorylation and translocation to the plasma membrane was also assessed. Figure 3B shows that both kinetics of Raf phosphorylation and translocation correlated with that of Ras activation (Fig.3 A,B) and also paralleled the ERK activation/phosphorylation profile (data not shown) (Hernandez *et al.* 1998), supporting a relationship between Ras/Raf/ERK. To get an insight into this process, cells were subjected to an immunoprecipitation assay with an anti-Raf-1 antibody. Figure 3C shows the results of a representative experiment in which the association of Raf with Ras and ERK was examined in immunoprecipitates from cells exposed to sPLA<sub>2</sub>-IIA. We found that 1  $\mu\text{g/ml}$  of sPLA<sub>2</sub>-IIA pulled together Ras, ERK and Raf-1. In addition, 1321N1 cells were transfected with HA-Ras or empty vector, treated with sPLA<sub>2</sub>-IIA, and then total cell extracts were subjected to an immunoprecipitation assay with an anti-HA antibody. As shown in Figure 3D, sPLA<sub>2</sub>-IIA induced Ras association with p-Raf-1 and p-ERK.

Next, considering that Akt is another key signal-transduction enzyme that has been implicated in the promotion of cell survival as well as growth (Cantley 2002), we investigated whether sPLA<sub>2</sub>-IIA affected Akt activity on astrocytoma cells. To address this question, we examined the Akt phosphorylation status at Thr<sup>308</sup> and Ser<sup>473</sup> upon exposure of 1321N1 cells to sPLA<sub>2</sub>-IIA, since phosphorylation at both residues is required for its full activation. As shown in Figure 4A, we observed a significant and sustained increase in Akt phosphorylation on both residues in the presence of sPLA<sub>2</sub>-IIA. The phosphatidylinositol 3 kinase (PI3K) inhibitor, LY294002, abrogated the phosphorylation of Akt at both sites -in order to simplify the figure, we only show the western blot for Ser 473, phosphorylation, required to get maximum activation of Akt-, suggesting that

PI3K is an upstream mediator of Akt activation in 1321N1 astrocytoma cells. However, this inhibitor did not affect sPLA<sub>2</sub>-IIA-induced ERK phosphorylation.

To further characterize the relationship between ERK and PI3K signaling pathways, we also evaluated the effect of the MEK inhibitor, PD98059, on Akt phosphorylation. We observed that pre-treatment of 1321N1 cells with PD98059 had no significant effect on phospholipase-induced Akt phosphorylation (both at Thr<sup>308</sup>, fig 4A, and Ser<sup>473</sup>, not shown), indicating that both kinases can be activated independently in separate signaling pathways.

Next, we studied downstream Akt targets in the context of cell proliferation. In particular, we focused on the rapamycin-sensitive molecules p70 ribosomal protein S6 kinase (p70S6K) and S6 ribosomal protein (S6). We found that upon sPLA<sub>2</sub>-IIA treatment, both proteins were persistently phosphorylated in 1321N1 cells, with a maximal effect reached at 30 min that was sustained for at least 240 min (Fig 4B). In untreated samples, a low level of phosphorylated S6 protein was also detected and its functional significance needs to be determined. Then, we evaluated the effects of the PI3K inhibitor, LY294002, and the mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAP), on p70S6K and S6 phosphorylation. We found that the presence of these compounds considerably decreased the phosphorylation of both proteins (Fig. 4B).

Interestingly, the presence of the MEK inhibitor, although ineffective in blocking Akt phosphorylation, it significantly reduced p70S6K and S6 phosphorylation induced upon sPLA<sub>2</sub>-IIA treatment (Fig 4C).

In addition, to establish the selectivity and specificity of the sPLA<sub>2</sub>-IIA on the aforementioned effects, we first investigated the signaling capabilities of the human recombinant sPLA<sub>2</sub>-IIA, as well as of the other sPLA<sub>2</sub>. As shown in Figure S1A, astrocyte stimulation with 1 µg/ml of

human recombinant-sPLA<sub>2</sub>-IIA, sPLA<sub>2</sub>-IB or sPLA<sub>2</sub>-IIIA, also resulted in ERK and Akt phosphorylation while not major effects were observed with sPLA<sub>2</sub>-V. Next, to determine whether the catalytic activity affects the ability of sPLA<sub>2</sub>-IIA to induce these cytokine-like responses, the actions of the phospholipase were evaluated in the presence of EGTA (calcium is mandatory for its catalytic activity) or oleanolic acid (pharmacological inhibitor IC<sub>50</sub>: 3-7μM) (Dharmappa *et al.* 2009), and using sPLA<sub>2</sub>-IIA irreversibly inactivated with BPB (Hernandez *et al.* 1998). Figure S1B shows that in none of those conditions, where the catalytic activity of the phospholipase is compromised, its ability to promote ERK and Akt phosphorylation is affected. Taken together, those results indicate that the cellular responses induced by the sPLA<sub>2</sub>-IIA are independent of its enzymatic activity, and can be reproduced by other isoforms.

**Effect of ROS on sPLA<sub>2</sub>-IIA downstream signaling in 1321N1 cells.** ROS production, similarly to second messengers, has been suggested to indirectly affect signal transduction processes. To determine whether ROS could serve as mediators of sPLA<sub>2</sub>-IIA downstream signaling, we assessed the effect of disrupting ROS generation on Ras activation and on ERK, cPLA<sub>2</sub> and Akt phosphorylation. Phosphorylated cPLA<sub>2</sub> was detected by the retardation of its electrophoretic mobility (gel-shift), while phosphorylated ERK, Akt and p70S6K were analysed using phospho-specific antibodies. As shown in Figure 5A and B, NAC, a general ROS scavenger, was neither effective in blocking sPLA<sub>2</sub>-IIA-induced Ras-GTP, nor in abrogating the phosphorylation of its downstream signaling molecules, ERK or cPLA<sub>2</sub>. Another ROS scavenger, PDTC, did not show either an effect. However, sPLA<sub>2</sub>-IIA-induced Akt/p70S6K phosphorylation was significantly attenuated at all three NAC doses tested (Fig. 5C).

**sPLA<sub>2</sub>-IIA-induced 1321N1 cell proliferation requires ROS induction and activation of ERK and PI3K/mTOR pathways.** Consistent with previously reported data, exposure of

1321N1 cells to sPLA<sub>2</sub>-IIA caused a significant increase in DNA synthesis (Hernández *et al.* 1998). Thus, within 24 h of treatment, 1321N1 cells increased [<sup>3</sup>H]-thymidine incorporation up to  $34,470 \pm 2,299$ , from  $5,430 \pm 1,680$  in control cells. To investigate the signaling pathways that might be involved in this proliferative response, [<sup>3</sup>H]-thymidine incorporation was measured in the presence of inhibitors acting at different levels of the cascades regulated by sPLA<sub>2</sub>-IIA. We found that NAC, PD98059, LY294002 or rapamycin, were all effective preventing the stimulatory effect of the phospholipase on cell growth ( $n = 3$ ;  $p < 0.05$ ) (Fig. 6A) In parallel, cell viability was determined by the Trypan blue dye exclusion assay. As shown in Figure 6B, we found no major differences in the proportion of Trypan blue-positive cells at any experimental condition, suggesting that the population of dead cells is not increased under these culture settings in which the proliferation is blocked. In addition, microscopic observations of the cells, using a phase-contrast microscope revealed that these treatments did not affect either cellular appearance or attachment (Fig. 6C).

Finally, to explore whether apoptosis was involved in this inhibitory effect, we analysed phosphatidylserine externalization (an early event in apoptosis) by an annexin-V staining assay. As shown in Figure 6 D, flow cytometry analysis showed no annexin V-positive cells upon sPLA<sub>2</sub>-IIA treatment, neither in the presence, nor in the absence of the different inhibitors, meaning no phosphatidylserine exposure on the outer membrane of the cells.

## Discussion

Tumor progression frequently depends on interactions between tumoral cells and molecules present in its microenvironment, the “seed and soil” hypothesis developed by Paget (Paget et al. 1889). Microenvironmental factors influence the biological behaviour of tumor cells and, in many cases the same molecule operating under different circumstances may exert opposing effects, acting as a double-edged sword. In searching for such regulatory agents, sPLA<sub>2</sub>-IIA surges as a multifunctional protein playing promalignancy and antimalignancy roles in tumor progression depending on the cell type and context (Dong *et al.* 2002; Belinski *et al.* 2006; Menschikowski *et al.* 2008; Leung *et al.* 2002). In this study, we show that sPLA<sub>2</sub>-IIA mediates astroglial tumor cell growth and involves the activation of the major intracellular signaling cascades: Ras/MEK/ERK and PI3K/AKT/mTOR pathways. To our knowledge, this is the first report to demonstrate that exogenous sPLA<sub>2</sub>-IIA promotes ROS accumulation in brain tumor cells and that the activated PI3K/AKT/mTOR signaling pathway is ROS sensitive. Our data also suggest that both cascades converge to regulate cellular growth, since the inhibition of any of those pathways prevents sPLA<sub>2</sub>-IIA proliferative effect without affecting cell survival.

Involvement of these molecules in cell survival, growth, as well as in malignant transformation, in response to extracellular signals has been previously established in different cellular types. However, the relationship between these intracellular mediators and the mechanisms of action of human sPLA<sub>2</sub>-IIA on brain tumor cells remained to be determined, since, besides activation of MAPKs, arachidonate metabolism or Ca<sup>2+</sup> mobilization (Hernández *et al.* 1998), little is known about the pathways activated by this particular phospholipase isoform.

Group IIA secreted phospholipase A<sub>2</sub> is an acute phase protein that has been characterized in different pathological processes, being its presence usually linked to inflammatory disorders (Abe *et al.* 1997; Webb 2005).

It has been shown that sPLA<sub>2</sub>-IIA induces an apoptotic response restricted to neurons (Yagami *et al.* 2002), being recently found that in cerebellar neural cells the mitochondrial sPLA<sub>2</sub> mediates apoptotic activities through ROS generation (Mathisen *et al.* 2007). However, in non-neuronal cells, including astrocytes, it triggers survival, differentiation or even proliferative signals (Fonteh *et al.* 2001; Yagami *et al.* 2002; Saegusa *et al.* 2008), remaining the precise molecular mechanism involved an enigma. Thus, although the contribution of sPLA<sub>2</sub>-IIA, as well as the other sPLA<sub>2</sub> isozymes, to these degenerative disorders is increasingly relevant, clear information about their functions is not available yet.

In this study we have made use of the well characterized 1321N1 astrocytoma cells, where sPLA<sub>2</sub>-IIA acts as a mitogen to go further inside its mechanisms of action. The first finding of this paper is that 1321N1 cells are prone to generate ROS upon phospholipase stimulation. NOX isozymes, and possibly, xanthine oxidase contribute to this response, probably acting synergistically, as previously described (Isabelle *et al.* 2007), since both DPI and allopurinol were effective drugs blocking ROS levels. In contrast, the presence of rotenone, an inhibitor of complex I of the mitochondrial respiratory chain, did not affect intracellular ROS; therefore we can assume that mitochondria are not a probable source of ROS in this context. In addition, we neither observed that rotenone promotes cellular ROS accumulation, results in conflict with previously reported studies that show that rotenone triggers mitochondrial ROS induction (Li N *et al.* 2003). This discrepancy may be due to a different antioxidant capacity of the cellular systems used. In fact MnSOD, a primary mitochondrial antioxidant, is strongly expressed in glial

tumors and, in agreement with the above mentioned study, when MnSOD is overexpressed, rotenone-induced increase of cellular ROS is inhibited.

Besides,  $\Delta\psi_m$  was not either disrupted upon sPLA<sub>2</sub>-IIA treatment, discarding also mitochondria as a ROS target. Moreover, we have observed that sPLA<sub>2</sub>-IIA-induced ROS generation is involved in growth rather than in apoptosis, since the presence of NAC abrogated the proliferative response. The fact that both catalase and SOD were effective blocking ROS accumulation, suggests a reaction route starting with the sPLA<sub>2</sub>-IIA-mediated reduction of molecular oxygen to superoxide, followed by spontaneous dismutation to H<sub>2</sub>O<sub>2</sub>.

Mechanistically, ROS have been linked to tyrosine kinases, ERK and Akt signaling to regulate diverse biological functions, including survival and growth (Chen et al., 1995; Wang *et al.* 2000).

It is well established that ERK is a component of the Ras signal transduction pathway and a considerable number of data consider this cascade as one of the primordial signaling network that governs cell survival, growth or differentiation. Previous studies have shown that Ras is crucial for thrombin-induced mitogenesis in 1321N1 cells (LaMorte *et al.* 1993). Therefore, our earlier results showing ERK activation in 1321N1 cells upon exposure to sPLA<sub>2</sub>-IIA led us to characterize the activation of classical upstream effectors: Ras and Raf-1. However, although this cascade could be activated by ROS, here we show that treatment of 1321N1 cells with ROS scavengers does not affect the sPLA<sub>2</sub>-IIA-activated Ras/Raf/ERK network, suggesting a ROS-independent regulation. On the other hand, the Ras/ERK cascade itself might be involved in the generation of high ROS levels (Serù *et al.*, 2004); however, in our system, the presence of MEK inhibitors does not affect ROS production (data not shown), indicating that the ERK cascade is not either an upstream signaling system implicated in ROS accumulation.

The PI3K/Akt system, another effector of intracellular ROS, classically participates in pathways targeting cellular proliferation in both normal and tumoral cells. In particular, a critical role for Akt has been ascribed to the survival of astrocytes and glioblastomas in different model systems (Sonoda *et al.* 2001). In fact, current evidence supports the concept that Akt, through a complex network with multiple components, such as mTOR, p70S6K or S6, plays an essential role in cell growth and protein translation (Fingar *et al.* 2004). However, the sequential activation of this transduction pathway is complex and not completely understood, being probably context-specific. In this way, it has been shown in cortical neurons, that phosphorylation of p70S6K and its substrate, S6, can take place in a rapamycin-dependent manner (Ishizuka *et al.*, 2008), or in ovarian cancer cells PI3K transmits the mitogenic signal through Akt and mTOR to p70S6K (Gao *et al.*, 2004). However, in other systems, the p70S6K/S6 cascade can be activated independently of mTOR, being Akt, ERK or even phosphatidic acid, direct upstream effector molecules (Riemenschneider *et al.* 2006; Lehman *et al.* 2007).

Here we report that in human 1321N1 cells, sPLA<sub>2</sub>-IIA elicits a sustained phosphorylation of Akt, p70S6K and S6, which is prevented by the presence of the antioxidant NAC and by the inhibitor LY294002, suggesting a ROS-dependence, and a role for PI3-kinase in this pathway. In addition, we find that inhibition of mTOR signaling with rapamycin abrogates p70S6K/S6 phosphorylation. This demonstrates the induction of an active and functional PI3K/Akt/mTOR/p70S6K/S6 redox-sensitive cascade in 1321N1 cells upon exposure to sPLA<sub>2</sub>-IIA. These data agree with previously reported studies with either human sPLA<sub>2</sub>-IIA (on murine macrophages), or other sPLA<sub>2</sub> isozymes, such as mammalian group IB (on mouse fibroblasts) or snake venom group IA (on human lung macrophages), which link sPLA<sub>2</sub>-biological activity to PI3K and Akt activation (Park *et al.*. 2003; Choi *et al.* 2004; Granata *et al.* 2006). In contrast, it has been shown that bee venom sPLA<sub>2</sub>-III treatment induces on renal cancer cells a remarkable



inhibition of the Akt activity that prevents the transduction of survival signals (Putz *et al.* 2007). This points to a certain degree of selectivity displayed by distinct sPLA<sub>2</sub>s, and highlights the need to know the cascade of intracellular events regulated by each particular phospholipase.

In astrocytoma cells, the intracellular signals induced by sPLA<sub>2</sub>-IIA can be reproduced by other isozymes, although not by group V, remaining unknown their functional and mechanistic significance. These results underscore the importance of future studies aimed at characterizing the biological effects of the different sPLA<sub>2</sub>s on glioma cells, and the role therein of binding versus enzymatic activity, especially for group V because of its demonstrated activity on the phosphatidylcholine-rich outer plasma membrane of mammalian cells. In contrast, sPLA<sub>2</sub>-IIA is barely efficient hydrolyzing membrane phospholipids from the outer layer of mammalian cells due to its preference for anionic phospholipids, probably grounding the distinct roles of these two enzymes. In this way, we have observed that the effects induced by sPLA<sub>2</sub>-IIA cannot be attributed to its enzymatic activity, since the cytokine-like characteristics of sPLA<sub>2</sub>-IIA are maintained in a catalytically inactive environment. Therefore, although the proposed roles of sPLA<sub>2</sub> enzymes have largely been inferred from their enzymatic properties, their actions through interaction with membrane targets should be considered and defined.

Regarding sPLA<sub>2</sub>-IIA actions, assays using specific kinase inhibitors show that sPLA<sub>2</sub>-IIA needs the simultaneous functional activity of the ERK and Akt/mTOR systems to induce a proliferative phenotype in 1321N1 cells. The PI3-kinase inhibitor, LY294002, and the MEK inhibitor, PD98059, suppress sPLA<sub>2</sub>-IIA-induced cell growth. However, LY294002 does not affect ERK and PD98059 does not abolish Akt phosphorylation. Therefore, components of these pathways are probably converging on factors that regulate proliferation, at a different level than ERK and Akt.

In this sense, there is molecular evidence that growth factor-activated MAPK and PI3K cascades activate RSK and p70S6K proteins, respectively, and converge at the level of eIF4B phosphorylation to stimulate translation (Shahbazian *et al.* 2006). In addition, recent studies have shown that pharmacological inhibition of ERK does not affect phosphorylation of Akt, while it elicits a reduction of p70S6K activity and prevents S6 and 4E-BP1 phosphorylation to a similar extent to that induced with rapamycin, decreasing ribosomal biogenesis and protein translation efficiency. Interestingly, in our studies PD98059 pretreatment also blocks the sPLA<sub>2</sub>-IIA induced phosphorylation of p70S6K/S6. This clearly suggests that MEK1/2 is a critical intermediary of phospholipase-mediated p70S6K activation, therefore connecting Ras and PI3K pathways. The molecular level of interaction between these two signaling pathways should be studied in depth, considering the different recent hypothesis: (i) ERK is a direct p70S6K kinase (Iijima *et al.* 2002) (ii) other kinases regulated by MEK are necessary for p70S6K activation or, (iii) the tuberin/hamartin complex acts as a signal integrator, where the Ras and PI3K pathways converge to modulate growth (Shahbazian *et al.* 2006).

It is noteworthy that, although sPLA<sub>2</sub>-IIA proliferation is abrogated by inhibiting any component of the ERK or Akt signaling pathways, survival is not affected. It is accepted that inhibition of cellular growth in the absence of apoptosis would commit the cell into a differentiation program (Mijatovic *et al.* 2005); defining whether this machinery is triggered by sPLA<sub>2</sub>-IIA demands further investigation.

Taken together, our findings put forward that, in 1321N1 cells, sPLA<sub>2</sub>-IIA acts as an agonist to which cells respond by activating ROS-independent Ras/ERK and ROS-dependent Akt/mTOR pathways, which converge, ultimately leading to cell proliferation; and where MEK and/or ERK play an essential role connecting these two major signaling networks.

## **Acknowledgments.**

This work was supported by the Ramón y Cajal Program (to M. H.), F. P. I. Program from the Government of Castilla y Leon. (to R. M.) both co-funded by F. S. E, Grant SAF2005-01242 from the Ministry of Science and Technology, and Grant CSI11A08 from the Government of Castilla y León.

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## Figure Legends

FIG. 1.- sPLA<sub>2</sub>-IIA induces intracellular ROS generation in 1321N1 cells. 1321N1 cells were incubated with 1 µg/ml sPLA<sub>2</sub>-IIA for different times (A) or for 30 min with different doses (B). ROS levels were measured by flow cytometry analysis after staining with the fluorescent probe DCFH-DA. Solid gray curves, cells without treatment (control); empty black curves, sPLA<sub>2</sub>-IIA-treated cells. Representative histograms are shown.

FIG. 2.- A, Effect of antioxidants on sPLA<sub>2</sub>-IIA-induced ROS production. 1321N1 cells were incubated without or with 1 µg/ml of sPLA<sub>2</sub>-IIA for 30 min in the presence or absence of the indicated antioxidants (A, B, C). ROS levels were measured by flow cytometry analysis after staining with the probe DCFH-DA. Solid gray curves, cells in the absence of treatment (control); empty black curves, sPLA<sub>2</sub>-IIA-treated cells; and empty gray curves, sPLA<sub>2</sub>-IIA-treated cells in the presence of antioxidants. D, Effect of sPLA<sub>2</sub>-IIA on mitochondrial membrane potential. The mitochondrial membrane potential was analysed using the dye, rhodamine 123. 1321N1 cells were stimulated without or with 1 µg/ml of sPLA<sub>2</sub>-IIA or 25 µM of OA for 6 or 18 h, and mitochondrial dysfunction was determined by flow cytometry analysis.

FIG. 3.- sPLA<sub>2</sub>-IIA induces activation of the Ras/Raf/ERK cascade in 1321N1 cells. 1321N1 cells were incubated with 1 µg/ml sPLA<sub>2</sub>-IIA for different times. Protein lysates were utilized for a Ras-pulldown assay (A), for Western blotting with a phospho-specific Raf-1 antibody (B, upper panel) or for immunodetection of Raf-1 in the membrane fraction (B, lower panel). C, 1321N1 cells were stimulated for the indicated times with 1 µg/ml sPLA<sub>2</sub>-IIA, subjected to immunoprecipitation with anti-Raf-1, and blotted against the specified antibodies. Membranes

were reprobated with anti-Raf-1 to demonstrate equal loading of protein. D, 1321N1 cells transfected with HA-Ras were stimulated for 2 or 5 min with 1  $\mu\text{g/ml}$  sPLA<sub>2</sub>-IIA, subjected to immunoprecipitation with an anti-HA antibody, and blotted against anti-p-Raf-1 and anti-p-ERK1/2 antibodies. The higher molecular weight IgG band was used as reference for equal loading of samples. Actin present in the total cell extract also is shown. In addition, equal gel loading and membrane transference was confirmed by Ponceau S and Coomassie staining, respectively. These are representative experiments of 4 similar ones.

FIG. 4.- sPLA<sub>2</sub>-IIA activates Akt and the downstream targets p70S6K and S6 in 1321N1 cells. 1321N1 cells were incubated without or with 1  $\mu\text{g/ml}$  sPLA<sub>2</sub>-IIA for 15 min in the presence or absence of the indicated inhibitors. Lysates were analysed by Western blotting with the specified antibodies.

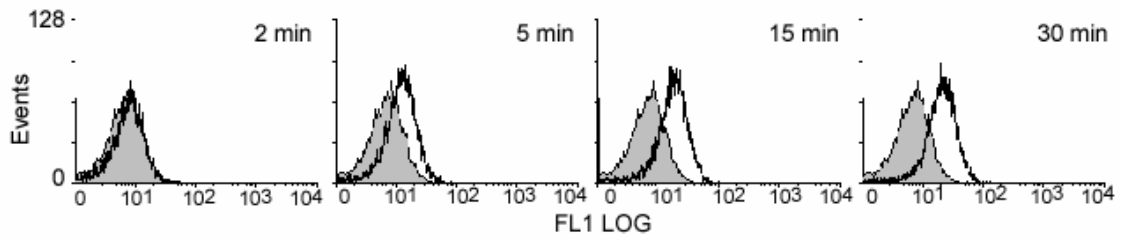
FIG. 5.- Effect of NAC on the intracellular signaling mediators activated in sPLA<sub>2</sub>-IIA-treated cells. 1321N1 cells were incubated without or with 1  $\mu\text{g/ml}$  sPLA<sub>2</sub>-IIA for different times (A) or for 15 min (B,C), in the presence or absence of antioxidants. Protein lysates were: (A) utilized for Ras-pulldown assay followed by immunoblotting detection with anti-Ras, or (B, C) analysed by Western blotting with phospho-specified ERK, Akt or p70S6K antibodies. Prolonged SDS-PAGE electrophoresis was used to detect the band-shift characteristic of cPLA<sub>2</sub> phosphorylation (B, ii).

FIG. 6.- Effect of different inhibitors on cellular growth and survival in sPLA<sub>2</sub>-IIA-treated cells. 1321N1 cells were incubated with 1  $\mu\text{g/ml}$  sPLA<sub>2</sub>-IIA in the presence or absence of different doses of antioxidants/inhibitors for 24 h. Cell proliferation was determined with a [<sup>3</sup>H]-thymidine

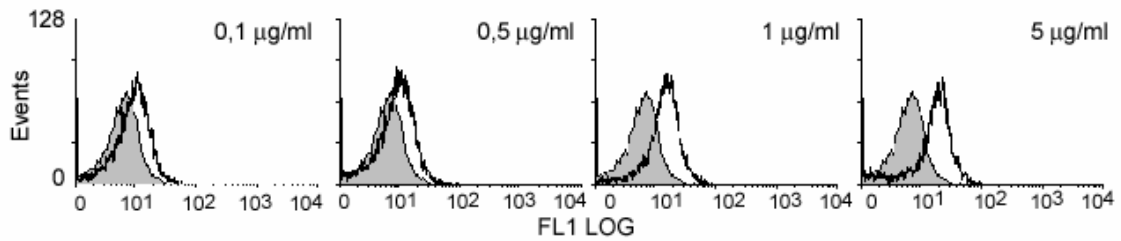
assay (A), and cell viability by Trypan blue exclusion (B). Values are means  $\pm$  SD from 4 experiments. C, Representative phase micrographs of cells treated with control solvents, or the indicated inhibitors in the presence of sPLA<sub>2</sub>-IIA for 24 h. D, Apoptosis was determined by using the annexin-V binding assay and flow cytometry analysis. Solid grey curves, cells without any treatment (control); empty black curves, sPLA<sub>2</sub>-IIA-treated cells without antioxidant/inhibitor; and empty grey curves, sPLA<sub>2</sub>-IIA-treated cells in the presence of the antioxidant/inhibitor.

Figure 1

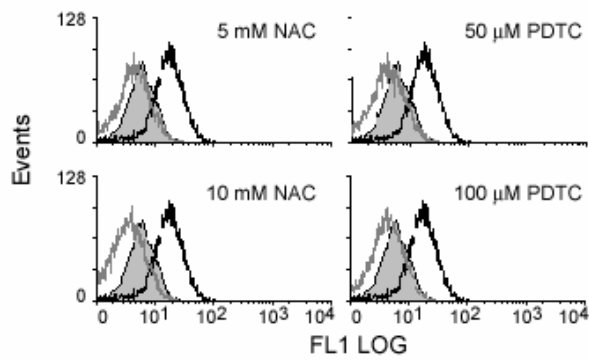
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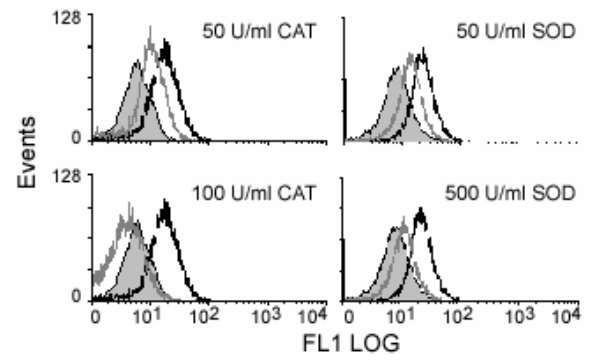
B



C

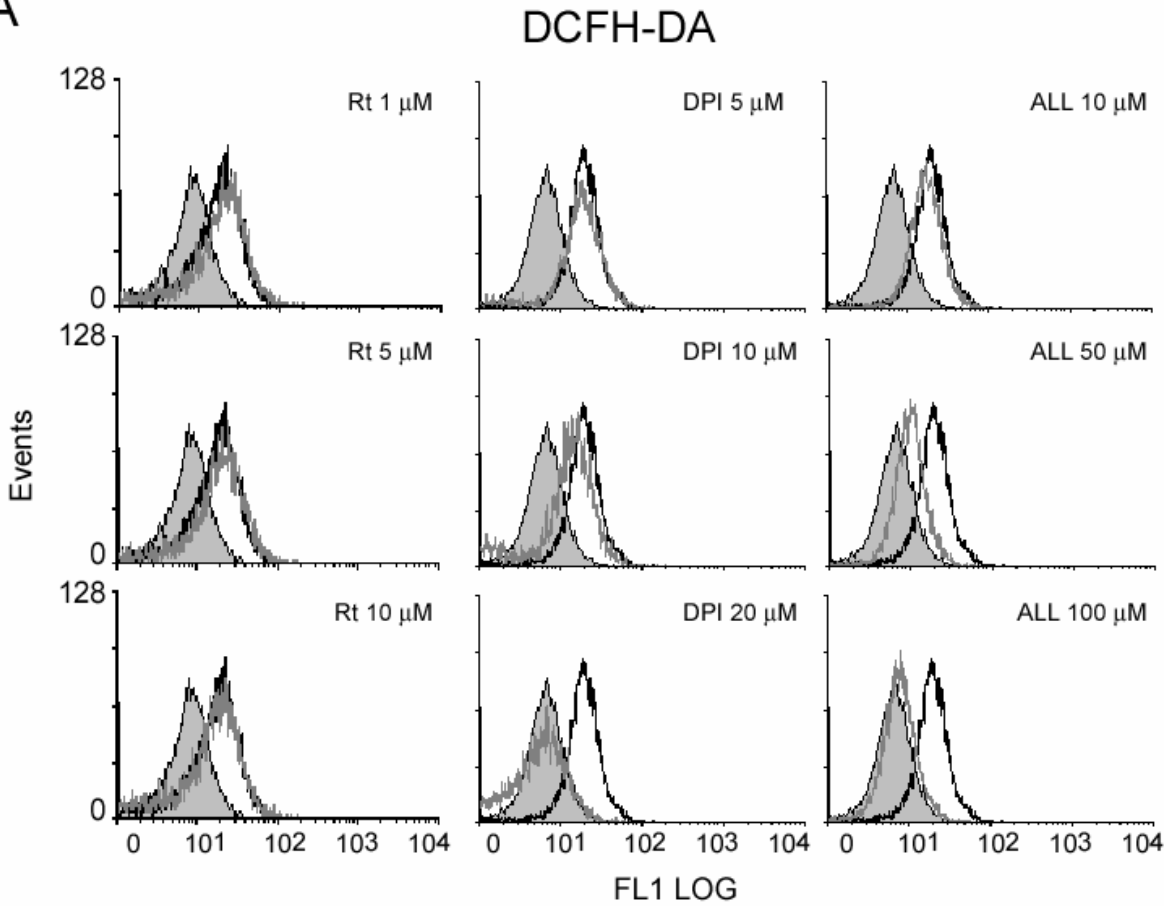


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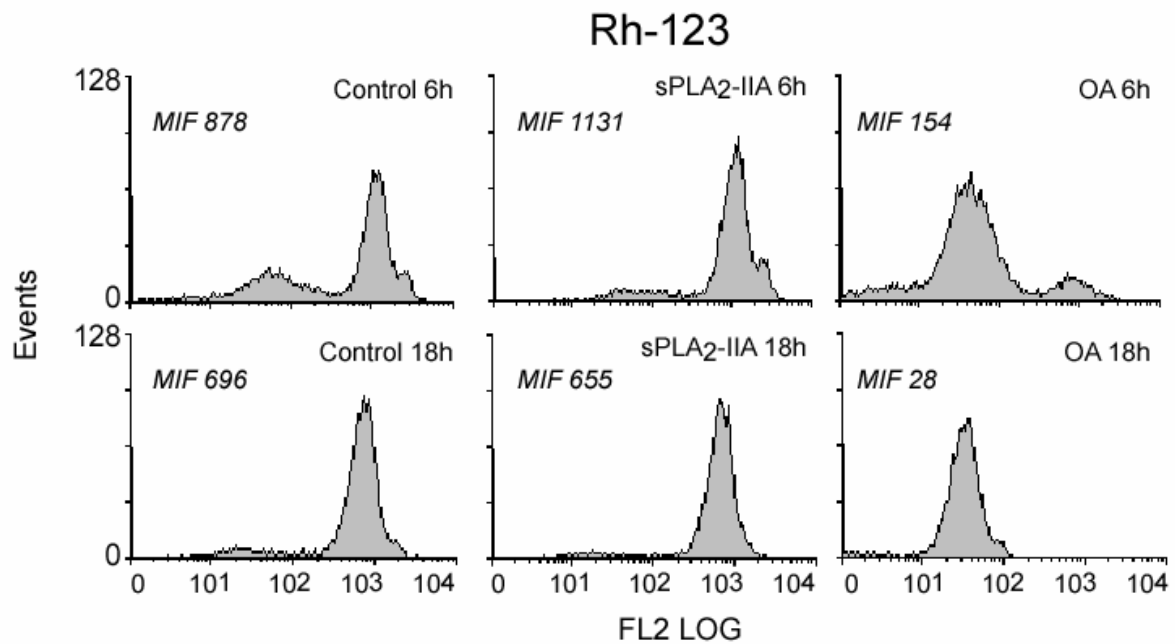


# Figure 2

## A



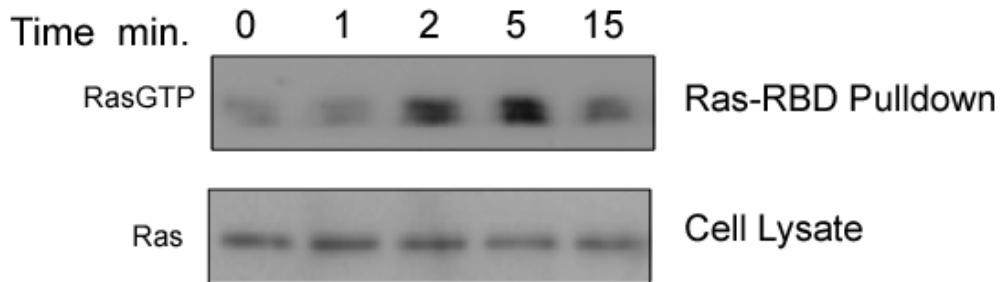
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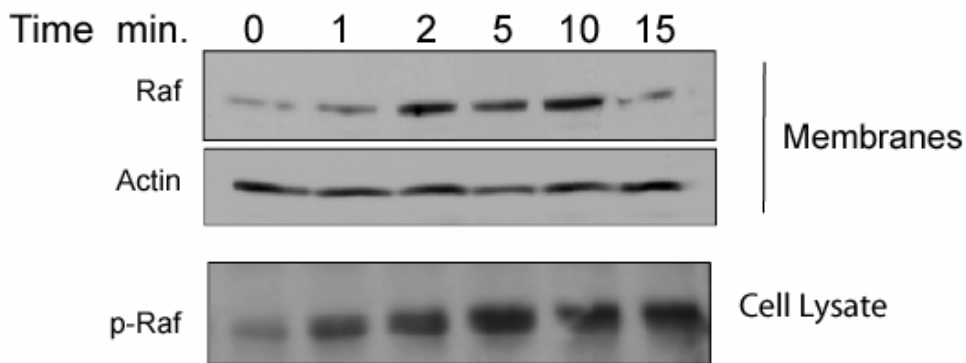


# Figure 3

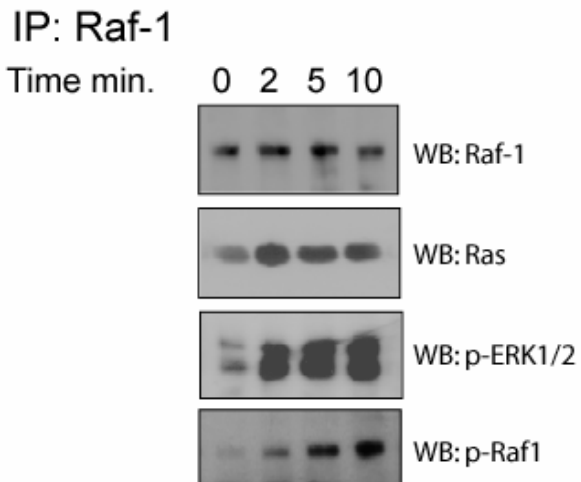
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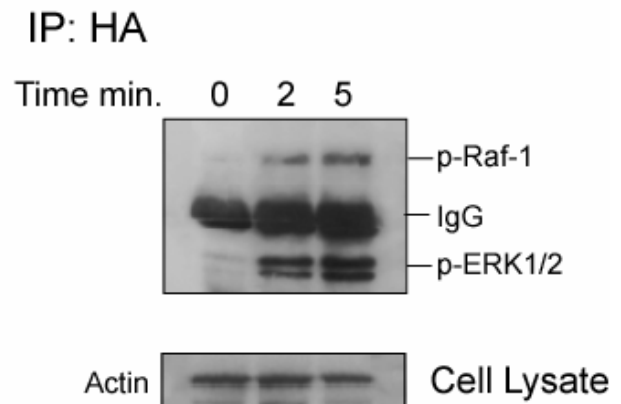
B



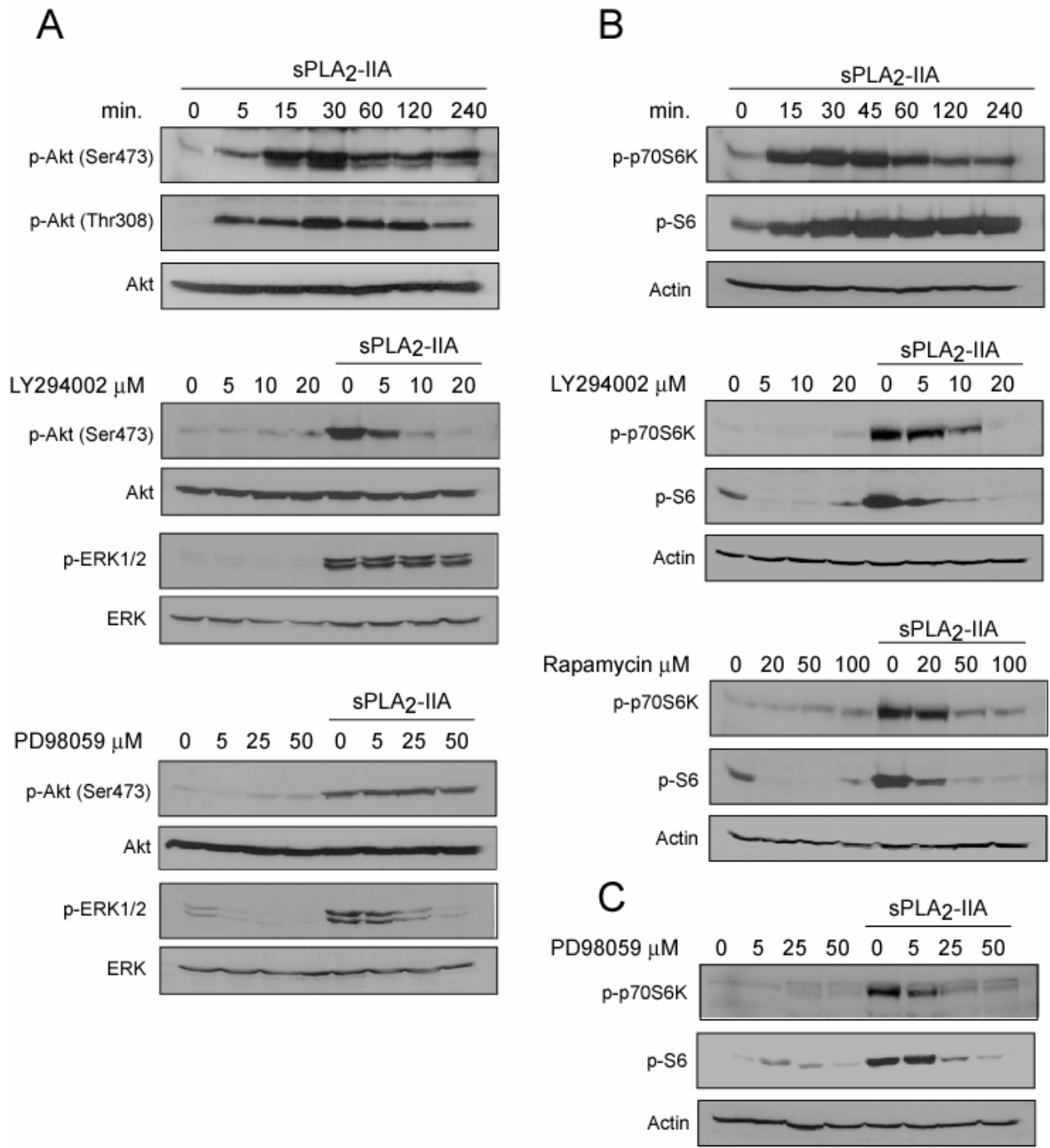
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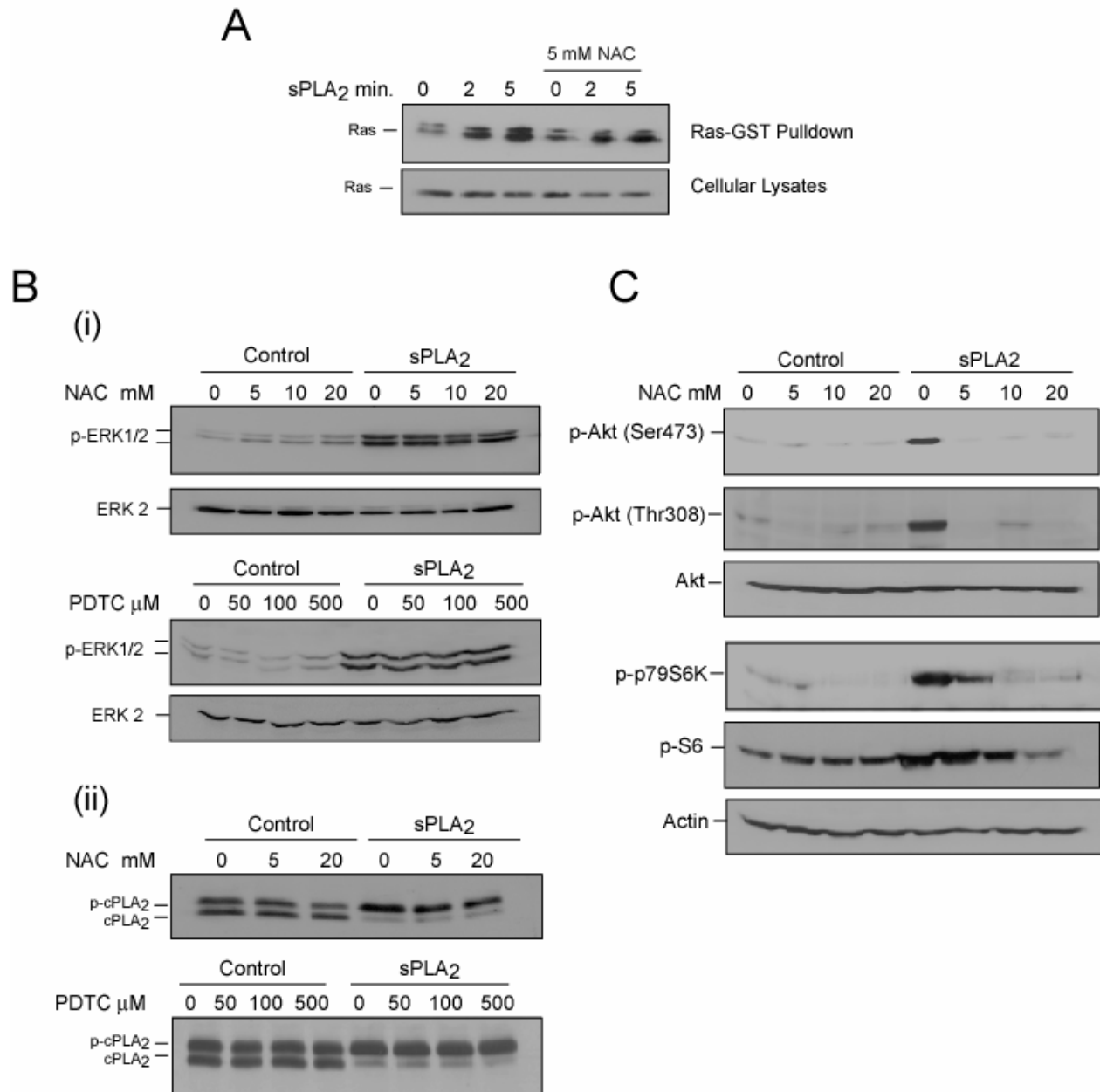
D



# Figure 4



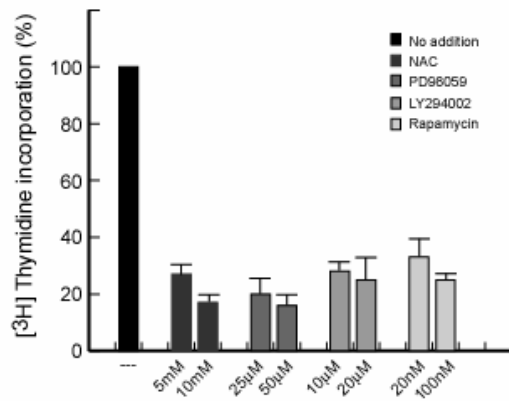
# Figure 5



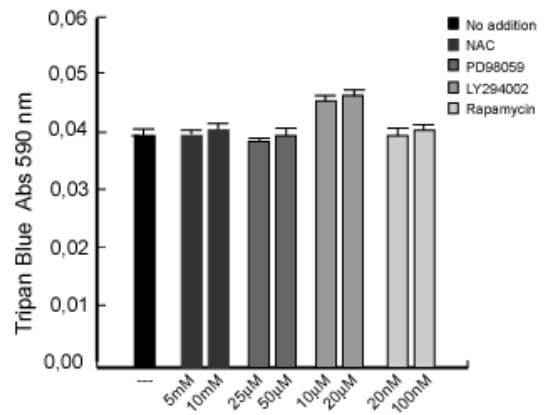


# Figure 6

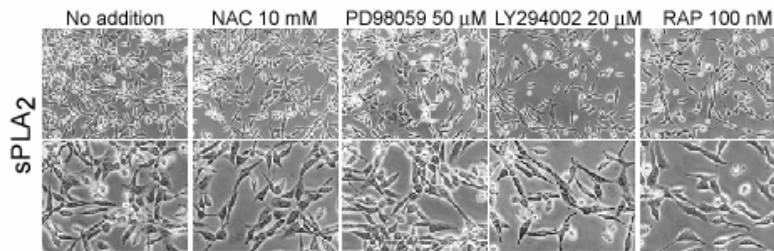
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