Nuclear localisation of cytosolic phospholipase A_2-α in the EA.hy.926 human endothelial cell line is proliferation dependent and modulated by phosphorylation

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Summary

Cytosolic phospholipase A_2-α (cPLA$_2$-α) is a calcium-sensitive enzyme involved in receptor-mediated eicosanoid production. In resting cells, cPLA$_2$-α is present in the cytosol and nucleus and translocates to membranes via its calcium-dependent lipid-binding (CaLB) domain following stimulation. cPLA$_2$-α is also regulated by phosphorylation on several residues, which results in enhanced arachidonic acid release. Little is known about the factors controlling the nuclear localisation of cPLA$_2$-α. Here the nuclear localisation of cPLA$_2$-α in the EA.hy.926 human endothelial cell line was investigated. Nuclear localisation was dependent on proliferation, with subconfluent cells containing higher levels of nuclear cPLA$_2$-α than contact-inhibited confluent or serum-starved cells. The broad-range protein kinase inhibitor staurosporine caused a decrease in the nuclear level of cPLA$_2$-α, whereas the protein phosphatase inhibitor okadaic acid increased the level of nuclear cPLA$_2$-α. Using inhibitors for specific mitogen-activated protein (MAP) kinases, both p42/44^{MAPK} and p38^{MAPK} were shown to be important in modulating nuclear localisation. Finally, inhibition of nuclear import and export using *Agaricus bisporus* lectin and leptomycin B, respectively, demonstrated that cPLA$_2$-α contains functional nuclear localisation and export signals. Thus we have identified a novel mode of regulation of cPLA$_2$-α. This, together with the increasing body of evidence supporting the role of nuclear lipid second messengers in gene expression and proliferation, may have important implications for controlling the growth of endothelial cells in angiogenesis and tumour progression.

Key words: Nucleus, Endothelial, EA.hy.926, Cytosolic phospholipase A_2-α

Introduction

Endothelial cells form the inner lining of blood vessels and play important roles in controlling blood clotting, blood pressure and blood flow (Vane et al., 1990). More recently, the growth of these cells to form new blood vessels has been identified as a key step in the formation of tumours (Carmeliet, 2000). Many endothelial cell functions are dependent on the release of various bioactive substances produced by these cells. One substance of particular interest is prostacyclin, a member of the eicosanoid family of lipid mediators, which acts as a potent vasodilator and inhibitor of platelet aggregation. Prostacyclin is synthesised by endothelial cells from its lipid precursor, arachidonic acid, which is released from membrane phospholipids by the action of the enzyme, cytosolic phospholipase A_2-α (cPLA$_2$-α).

cPLA$_2$-α belongs to a growing family of phospholipase A_2 enzymes that catalyse the hydrolysis of the *sn*-2 fatty-acyl bond of phospholipids to liberate free fatty acids (Dennis, 1997). cPLA$_2$-α preferentially liberates arachidonic acid and thus is considered to be the key enzyme in receptor-mediated eicosanoid production. This 85 kDa calcium-sensitive protein is subject to complex regulation at the transcriptional and post-translational level (Clark et al., 1991). Previous studies have shown that cPLA$_2$-α is present in the cytosol of resting cells and relocates to cellular membranes following stimulation with a variety of agonists (Glover et al., 1995; Peters-Golden et al., 1996; Schievella et al., 1995; Sierra-Honigmann et al., 1996). This translocation process is mediated by its calcium-dependent lipid binding (CaLB) or C2 domain, which promotes binding to phospholipids upon elevation of intracellular calcium concentrations (Gijon et al., 1999).

Several studies have shown that cPLA$_2$-α is also subject to regulation by phosphorylation. Phosphorylation of cPLA$_2$-α on Ser505 by p38^{MAPK} enhances its intrinsic activity in platelets (Kramer et al., 1996); however this modification is not essential for catalytic activity. In addition, a MAPK-activated kinase that may be related to MNK1 has been shown to be responsible for the concomitant phosphorylation on Ser727 (Hefner et al., 2000). Other studies in fibroblasts have implicated the p42/44^{MAP} kinases in the regulation of cPLA$_2$-α (Mitchell et al., 1999; Nemenoff et al., 1993). More recently, Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) was

shown to activate cPLA$_2$-α in myeloblastic leukaemia U937 cells (Muthalif et al., 2001b). To date, the studies carried out on endothelial cells have demonstrated that both the p38^{MAPK} and the p42/44^{MAPK} members of the MAPK family, as well as protein kinase C (PKC) and an unknown kinase, are involved in cPLA$_2$-α mediated arachidonic acid release (Gliki et al., 2001; Gudmundsdottir et al., 2001; Houliston et al., 2001; Sa et al., 1995; Wheeler-Jones et al., 1997).

The effects of phosphorylation on the subcellular location of cPLA$_2$-α have not been investigated. A previous study on human umbilical vein endothelial cells (HUVECs) demonstrated that the distribution of cPLA$_2$-α was dependent on cell density, with subconfluent cells showing increased nuclear localisation of cPLA$_2$-α compared with confluent cells (Sierra-Honigmann et al., 1996). In addition, close inspection of data obtained from the recent direct labelling of cPLA$_2$-α with green fluorescent protein (GFP) reveals some degree of nuclear localisation (Evans et al., 2001; Gijon et al., 1999; Hirabayashi et al., 1999; Hirabayashi and Shimizu, 2000). Following on from this, we investigate the subcellular location of cPLA$_2$-α in the EA.hy.926 endothelial cell line and examine the effects of cell density, protein phosphorylation and inhibition of nuclear import and export on nuclear localisation.

Materials and Methods

Materials

Tissue culture media, enzymes and antibiotics were purchased from Gibco BRL (Paisley, Scotland). The specific kinase inhibitors, PD98059 and SB203580, were from Calbiochem (Nottingham, UK) and staurosporine, okadaic acid, leptomycin B and Agaricus bisporus lectin were from Sigma. Goat polyclonal antibodies to cPLA$_2$-α were obtained from Santa Cruz Biotechnology Inc. (California, USA). Goat polyclonal antibody to lactate dehydrogenase was obtained from Sigma, and mouse monoclonal antibody to NuMA was from Calbiochem. Secondary FITC-conjugated secondary antibodies were from Sigma and anti-goat HRP antibodies were from Pierce (Cheshire, UK). All other standard reagents and chemicals were from Sigma (Poole, Dorset, UK) or BDH (Poole, Dorset, UK).

Cell culture

The EA.hy.926 endothelial cell line, a hybrid of human umbilical vein endothelial cells (HUVECs) and A549 human lung carcinoma epithelial cells, was a generous gift from C. J. Edgell (University of North Carolina). Cells were cultured at 37°C in a humid atmosphere containing 5% CO_2 in air. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine).

Immunofluorescence microscopy

The method for immunofluorescence microscopy was adapted from Barwise and Walker (Barwise and Walker, 1996) and Heggeness et al. (Heggeness et al., 1977). Cells were grown on glass coverslips in six-well dishes overnight. Media was removed, and the cells were washed three times with pre-warmed (37°C) PBS and fixed in pre-warmed 10% formalin in neutral buffered saline (approximately 4% formaldehyde, Sigma) for 5 minutes. All subsequent steps were performed at room temperature. After fixation, the cells were permeabilised with 0.1% Triton X-100 in PBS for 5 minutes and fixed once again for 5 minutes. The cells were then washed three times with PBS and incubated in sodium borohydride solution (1 mg/ml in PBS) for 5 minutes. Following three further PBS wash steps, the cells were blocked in 5% rabbit serum in PBS for 3 hours. The cells were then incubated with primary antibody (diluted 1:100 into PBS-5% serum) overnight followed by the appropriate FITC-conjugated secondary for 3 hours. For antigenic adsorption, the antibody was incubated with its corresponding blocking peptide (1:5 ratio of mg antibody to mg antigen) for 30 minutes at room temperature prior to being added to the cells. The cells were then washed eight times with PBS and mounted onto slides in Citifluor mounting medium (Agar Scientific, Hertfordshire, UK).

Confocal imaging

Confocal fluorescence microscopy was performed using a Leica TCS NT spectral confocal imaging system coupled to a Leica DM IRBE inverted microscope. Each confocal section was the average of four scans to obtain optimal resolution, and the system was used to generate individual sections that were 0.485 μm thick. All figures shown in this study represent 0.485 μm sections taken through the centre of the nucleus and thus do not include cytosolic staining above and below the nucleus. Measurements of fluorescence intensity were performed using the integral quantification module of the Leica TCS NT software as recommended by the manufacturer (Brokstad et al., 2001). Briefly, capture levels were first adjusted so as to avoid saturation and then kept constant throughout experiments. For each section examined, a line was drawn through the nucleus in a random orientation. The pixel intensity value along this line (in arbitrary units) was measured and a mean value was obtained. Values shown in all figures are a mean value of intensity (n=90)±s.e.m., taken from cell populations investigated over three independent experiments.

SDS-PAGE and western blotting

Proteins (20 μg per well) were separated on SDS-polyacrylamide gels using a discontinuous buffer system (Laemmli, 1970). For western blot analysis, proteins were transferred to nitrocellulose (Towbin et al., 1979). Subsequently, the nitrocellulose blots were blocked in 5% non-fat milk in PBS-0.1% Triton X-100 for 1 hour. Primary antibody incubations (1:1000) were carried out overnight at room temperature, followed by 1 hour incubations with the appropriate horseradish-peroxidase-conjugated secondary antibody. For antigenic adsorption, the antibody was incubated with its corresponding blocking peptide (1:5 ratio of mg antibody to mg antigen) for 30 minutes at room temperature prior to being incubated with the nitrocellulose blot. Immunoreactive bands were visualised using an ECL detection kit (Pierce) according to the manufacturers instructions. Developed films were photographed and captured using the FujiFilm Intelligent dark Box II with the Image Reader Las-1000 package. The intensity of the bands was quantified densitometrically using the AIDA (Advanced Image Data Analyzer) 2.11 software package in accordance with the manufacturer's instructions. Average band intensities from three independent experiments and s.e.m.s were calculated.

Preparation of EA.hy.926 cell nuclei

This method was carried out as described previously (Compton et al., 1976). Briefly, cells were grown in flasks to the appropriate level of confluency. Cells were then washed twice with ice-cold PBS, scraped into ice-cold PBS (containing 1 mM PMSF and 0.1 mg/ml leupeptin) and pelleted by centrifugation at 160 ***g*** for 10 minutes at 4°C. The cells were then resuspended in a hypotonic solution (Buffer C: 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$) and allowed to swell on ice for 10 minutes. Cells were then lysed mechanically with a Dounce homogeniser. The lysate was layered onto 3 ml of 1.7 M sucrose in Buffer C and centrifuged for 1 hour at 650 ***g*** at 4°C. The supernatant was removed, and the nuclear pellet was resuspended in 1 ml PBS (containing 1 mM PMSF and 0.1 mg/ml leupeptin). This suspension

of nuclei was then layered onto 3 ml of a 2.0 M sucrose solution and centrifuged for 1 hour at 3000 ***g*** at 4°C. The resultant nuclear pellet was again resuspended in PBS and layered onto a 2.12 M sucrose solution and centrifuged. The final nuclear pellet obtained after this procedure was analysed by immunofluorescence microscopy and western blotting. To obtain nuclei from stimulated cells, the procedure was carried out using a Buffer C that contained 1 mM $CaCl_2$ instead of $MgCl_2$. For immunofluorescence microscopy, a small volume of the final suspension of nuclei obtained was placed onto polylysine-coated microscope slides in a moist chamber and allowed to adhere for 15 minutes. The slides were then washed three times in PBS before being subjected to the immunofluorescence microscopy procedure described above.

Results

Location of cPLA$_2$-α in confluent and subconfluent EA.hy.926 cells

Optimal fixation and permeabilisation for fluorescence microscopy was achieved using formaldehyde followed by 0.1% Triton X-100. Immunofluorescence microscopy studies were carried out using an antibody that is specific for the α-isoform of cPLA$_2$. Antigenic adsorption of this antibody using its corresponding blocking peptide completely abolished detection of cPLA$_2$-α both by western blotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1D).

Using this specific antibody, a comparison of the location of cPLA$_2$-α in confluent and subconfluent EA.hy.926 endothelial cells revealed that a higher level of nuclear staining was present in subconfluent cells (Fig. 1B). Measurements of fluorescence intensity across individual cells illustrated a distinct elevation of fluorescence staining in the region corresponding to the nucleus (Fig. 1C), in particular in the case of the subconfluent cells. The detection of a non-related nuclear protein, NuMA, revealed that the level of nuclear staining did not vary with cell density (Fig. 1E), indicating that the changes seen for cPLA$_2$-α were specific and proliferation dependent. Secondary antibody controls gave no staining (data not shown).

Isolation of purified nuclei from subconfluent and confluent EA.hy.926 cells also confirmed the presence of a higher level of nuclear cPLA$_2$-α in subconfluent cells. Immunofluorescence microscopy of isolated nuclei demonstrated elevated levels of staining in nuclei obtained from subconfluent cells compared with those obtained from confluent cells (Fig. 2A). Analysis of nuclei isolated from A23187-stimulated cells revealed that this pool of nuclear cPLA$_2$-α relocates to the nuclear membrane following elevation of intracellular calcium concentrations (Fig. 2A). Furthermore, western blots of identical amounts of nuclear fractions confirmed that subconfluent cells contained a higher proportion of nuclear cPLA$_2$-α than confluent cells (Fig. 2B). Analysis of the distribution of the cytosolic marker, lactate dehydrogenase (LDH) revealed that the nuclear fractions did not contain this protein (Fig. 2B). Quantification of the amounts of nuclear cPLA$_2$-α (Fig. 2C) showed that confluent cells contained approximately half the amount of nuclear cPLA$_2$-α that subconfluent cells contain (46.2%±7.4 compared with 83.4%±3.3 of the total amount). It was also noted that in all experiments the total cellular amount of cPLA$_2$-α did not show any change, indicating that only the subcellular location and not the overall expression levels of cPLA$_2$-α are dependent on cell density.

Effect of serum starvation on nuclear localisation

The effect of serum starvation on nuclear localisation of

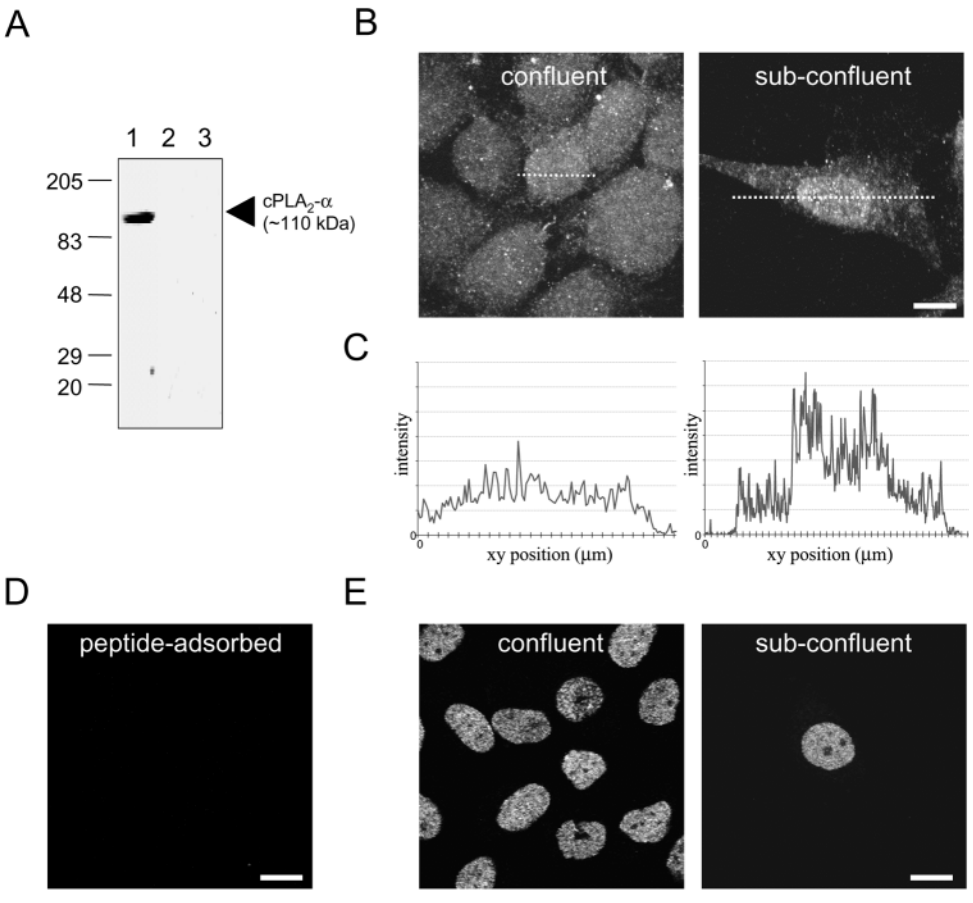
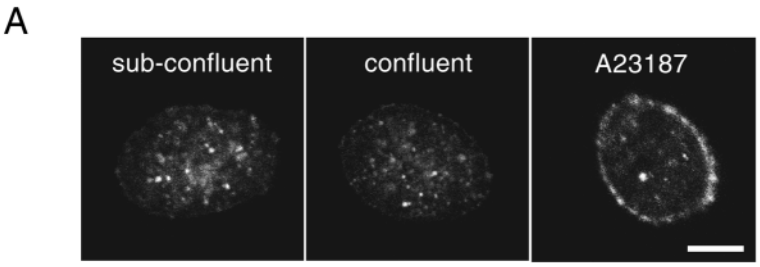


Fig. 1. Distribution of cPLA-α in confluent and subconfluent EA.hy.926 cells. (A) cPLA-α was detected by western blotting EA.hy.926 lysates (20 μg protein) using an affinity-purified goat polyclonal antibody (lane 1). Also shown are control lanes corresponding to antigen-adsorbed antibody (lane 2) and HRP anti-goat IgG controls (lane 3). (B) Cells (1×10 for subconfluent and 5×10 for confluent) were seeded onto glass coverslips, and cPLA-α was detected by immunofluorescence microscopy. Bar, 10 μm. (C) Densitometrical plot analysing the distribution of staining across individual cells (marked by dashed line in B). (D) Peptide-adsorbed antibody control. (E) Detection of NuMA in confluent and subconfluent cells.



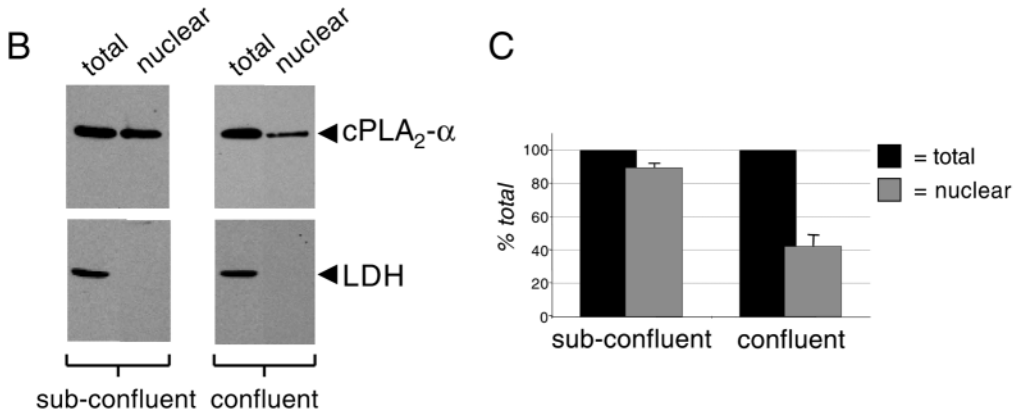


Fig. 2. cPLA$_2$-α in nuclear fractions. (A) Nuclei from subconfluent and confluent cells were isolated and analysed by immunofluorescence microscopy. Also shown are nuclei obtained from A23187-stimulated cells (5 μM, 10 minutes). (B) 20 μg of total homogenate and nuclear samples were western blotted, and LDH and cPLA-α were detected using goat polyclonal antibodies. (C) Quantification of the amounts of cPLA-α in nuclear fractions expressed as a percentage of the amount in the total sample±s.e.m. (*n*=3).

cPLA$_2$-α was studied by immunofluorescence microscopy. The results showed that the level of nuclear staining decreased following the removal of serum (Fig. 3A). Quantification of this reduction (Fig. 3B) showed that the intensity of nuclear staining in cells starved for 48 hours (34.4±1.5 arbitrary units) was approximately half that observed in control serum-fed cells (63.9±2.1 arbitrary units). Interestingly, although removal of serum caused a decrease in nuclear staining, it appeared to cause a change in morphology and an increase in staining around the periphery of the nucleus, particularly after the longer period of 48 hours of starvation.

In order to confirm that the presence of nuclear cPLA$_2$-α is dependent on factors present in serum, starved cells were re-fed with complete serum-containing medium for 24 hours. The results (Fig. 3C) demonstrated that cPLA$_2$-α in cells that had been starved for 24 or 48 hours returns to the nucleus upon the addition of serum. Interestingly, the morphology of the cells returned to normal; however, the cPLA$_2$-α-enriched cytosolic speckles that arose during the starvation period did not disappear completely.

The broad range kinase inhibitor, staurosporine, causes a decrease in the extent of nuclear localisation

Serum factors regulate cell function via phosphorylation of target proteins by protein kinases (Karin, 1992). In order to determine whether phosphorylation events were mediating nuclear localisation of cPLA$_2$-α, the effects of the broad-range kinase inhibitor, staurosporine, were studied using immunofluorescence microscopy. This cell-permeable inhibitor is known to inhibit CaM kinase, myosin light chain

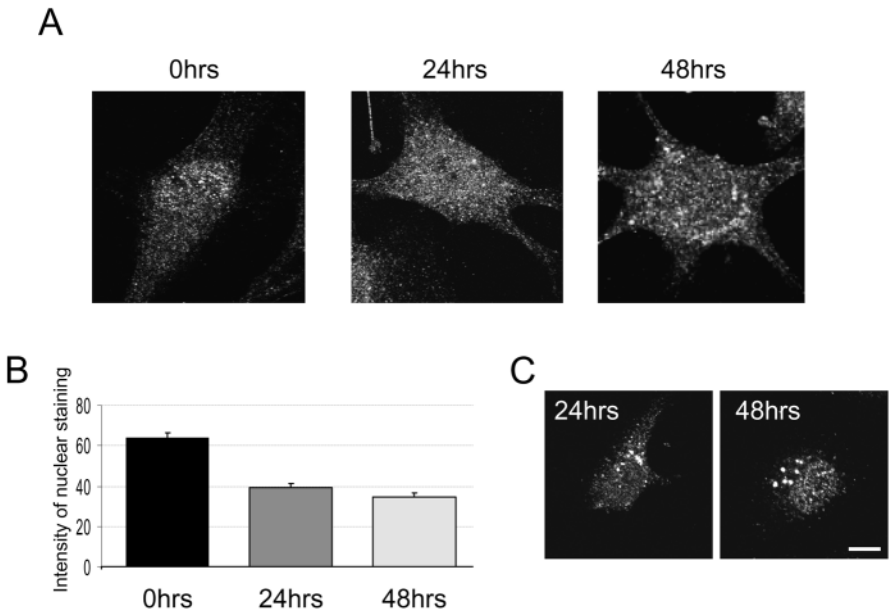


Fig. 3. Effects of serum starvation on cPLA-α localisation. (A) cPLA-α was detected by immunofluorescence microscopy in cells starved for 0, 24 and 48 hours. Bar, 10 μm. (B) Graph showing the changes in intensity of nuclear staining in sections taken through the nuclei of cells following serum starvation (in arbitrary units, *n*=90, data represents averages from three independent experiments±s.e.m.). (C) Cells starved for 24 and 48 hours were re-fed with complete medium, grown for a further 24 hours and viewed by immunofluorescence microscopy. Bar, 10 μm.

kinase, protein kinase A, protein kinase C and protein kinase G. Previous studies on HUVECs using this inhibitor have shown that it has no effect on histamine-induced arachidonic acid release (Gudmundsdottir et al., 2001). By contrast, studies on Chinese hamster ovary cells demonstrated that ATP-mediated phosphorylation and activity of $cPLA_2$-α could be inhibited by pre-treatment with staurosporine (Lin et al., 1992). To date, however, no studies characterising the effects of staurosporine on the subcellular location of $cPLA_2$-α have been performed.

The results (Fig. 4A) indicated that pre-treatment of cells with 1 μM staurosporine for 30 minutes led to a decrease in $cPLA_2$-α staining within the nuclei of both subconfluent and confluent cells. Quantification of the relative intensities demonstrated that in subconfluent cells, staurosporine treatment reduces the intensity of nuclear staining from 67.0±1.4 arbitrary units to 46.2±1.1 units (Fig. 4B). A similar 30% decrease in nuclear staining of confluent cells was evident (from 35.5±1.4 to 25.7±1.7 arbitrary units).

The protein phosphatase inhibitor, okadaic acid, increases nuclear localisation

The results above indicated that $cPLA_2$-α nuclear localisation could be reduced by inhibiting protein phosphorylation. Following this, the protein phosphatase inhibitor, okadaic acid, was applied to see if conditions that promote protein phosphorylation would lead to increased nuclear localisation of $cPLA_2$-α. In previous studies on coronary endothelial cells (Kan et al., 1996) and macrophages (Gijon and Leslie, 1999; Qiu et al., 1998), okadaic acid has been shown to cause a slight increase in arachidonic acid release. However, the effect of okadaic acid on the subcellular localisation of $cPLA_2$-α has not been studied.

Pre-treatment of confluent monolayers of EA.hy.926 cells with 1 μM okadaic acid for 30 minutes led to small but reproducible increases in the level of nuclear staining (Fig. 5A). Quantification of this increase demonstrated that the level of nuclear staining rises by approximately 35% (Fig. 5B). Studies on the effect of okadaic acid on subconfluent cells were also carried out. However, it was noticed that pre-treatment of subconfluent cells with okadaic acid lead to cell death and necrosis. Under these conditions, many of the cells no longer remained attached to the coverslip and of those that did, all were spherical in appearance (data not shown).

Effects of the specific MEK and $p38^{MAPK}$ inhibitors, PD98059 and SB203580, on the nuclear localisation of $cPLA_2$-α

Previous studies have shown that $cPLA_2$-α in platelets and in HeLa cells is phosphorylated on Ser505 and Ser727 (Borsch-Haubold et al., 1998). In platelets, $p38^{MAPK}$ has been shown to be responsible for phosphorylation on Ser505, and it is believed that a MAPK-activated kinase is involved in phosphorylation at Ser727 (Hefner et al., 2000). Studies on endothelial cells also demonstrate the importance of both p38 and p42/44 MAP kinases in the control of arachidonic acid release (Gudmundsdottir et al., 2001; Wheeler-Jones et al., 1997). Consequently, the effects of specific inhibitors of the $p42/44^{MAPK}$ activator, MEK-1, and $p38^{MAPK}$ were studied.

Treatment of subconfluent EA.hy.926 cells with the MEK-1 inhibitor, PD98059 (20 μM), for 30 minutes resulted in a dramatic decrease in the levels of nuclear $cPLA_2$-α (Fig. 6A). Similarly, the intensity of nuclear staining decreased following treatment with the $p38^{MAPK}$ inhibitor, SB203580 (Fig. 6B). Quantification of the relevant intensities of nuclear staining (Fig. 6C) demonstrated that treatment of cells with PD98059 and SB203580 decreased $cPLA_2$-α staining in the nucleus by approximately 39% and 36%, respectively.

The data above showed that treatment of cells with the kinase inhibitors, staurosporine, PD98059 and SB203580,

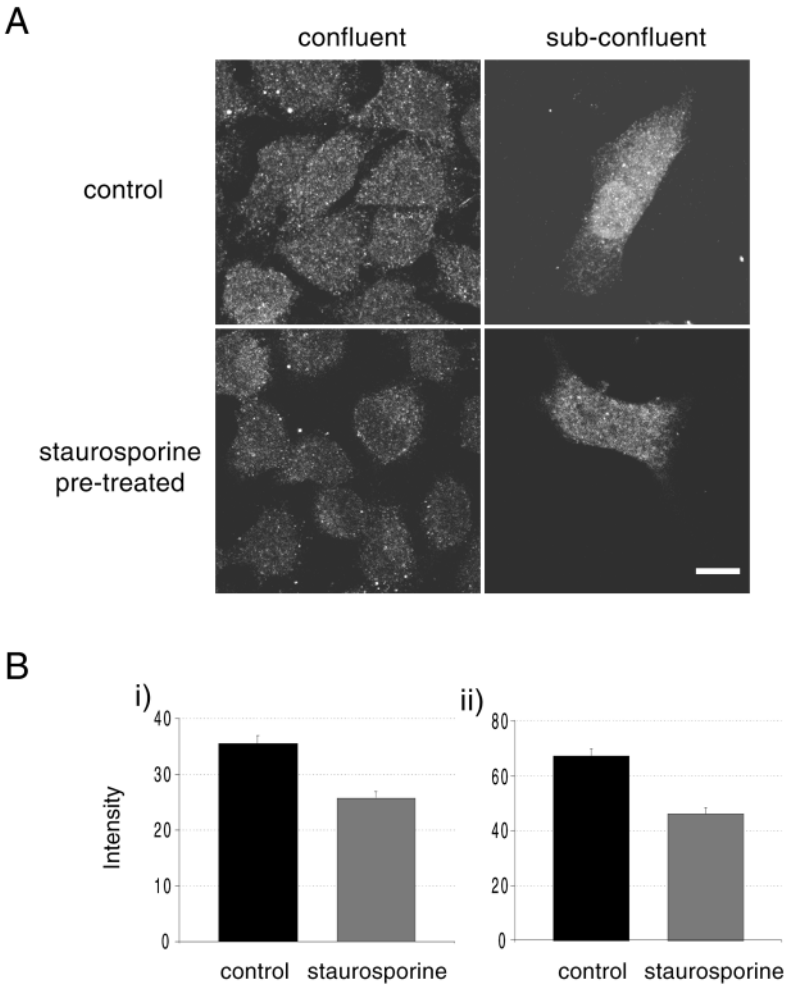


Fig. 4. Effects of staurosporine treatment on the nuclear localisation of cPLA-α. Cells (1×10 for sub-confluent, 5×10 for confluent samples) were seeded onto coverslips and grown overnight. Cells were then treated with 1 μM staurosporine in HEPES/Tyrode's buffer for 30 minutes at 37°C (control cells were incubated with buffer alone). cPLA-α was detected by immunofluorescence microscopy. Bar, 10 μm. (B) The intensity of cPLA-α staining in sections through the nuclei of confluent (i) and subconfluent (ii) cells was quantified. Plots represent average intensities (in arbitrary units)±s.e.m. (*n*=90, data from three independent experiments).

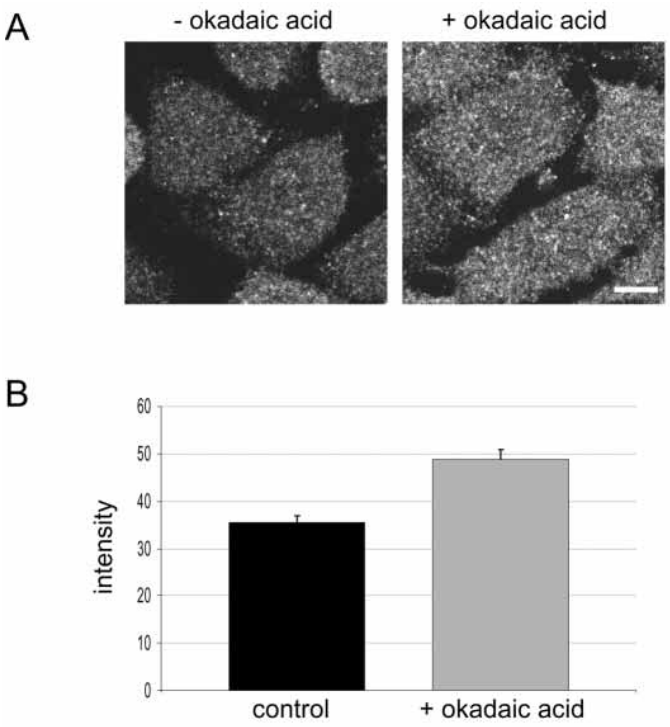


Fig. 5. Effects of okadaic acid pre-treatment on the levels of nuclear cPLA-α. **(**A) Cells (5×10) were seeded onto coverslips and grown to confluency. Cells were then washed with PBS and pre-treated with 1 μM okadaic acid in HEPES/Tyrode's buffer for 30 minutes at 37°C (controls cells were incubated with buffer alone). Cells were then fixed and permeabilised and cPLA-α was detected by immunofluorescence microscopy. Bar, 10 μm. (B) Plot showing the change in the level of nuclear intensity (±s.e.m., *n*=90, data from three independent experiments) in sections taken through control and okadaic acid treated cells.

results in a decrease in the intensity of nuclear cPLA$_2$-α staining. In order to demonstrate this biochemically, nuclei from control cells and cells treated with the inhibitors were isolated and analysed by western blotting. The result (Fig. 6C) confirmed that the level of cPLA$_2$-α in the nucleus decreases dramatically following treatment with PD98059, SB203580 or staurosporine. Furthermore, quantification confirmed that these inhibitors caused a decrease of greater than 50% in the amount of cPLA$_2$-α present within isolated nuclei (Fig. 6D).

These results demonstrate that the proliferation-dependent nuclear localisation of cPLA$_2$-α is mediated by phosphorylation events. In particular, both the p42/44 and the p38 MAP kinases appear to be critical in controlling the levels of cPLA$_2$-α present within the nucleus.

Agaricus bisporus lectin blocks re-entry of cPLA$_2$-α into the nucleus

In order to determine whether or not the nuclear import of cPLA$_2$-α is dependent on a functional nuclear localisation signal (NLS), the edible mushroom (Agaricus bisporus) lectin (ABL) was used as an inhibitor of NLS-dependent import. This Galβ1-3GalNAcα (TF antigen)-binding lectin has been shown to block NLS-dependent protein uptake into the nucleus without any apparent cytotoxic effects (Yu et al., 1999).

Cells were grown on coverslips overnight and serum starved for 24 and 48 hours, which resulted in a decrease in nuclear staining (Fig. 7A). Consistent with previous experiments, the levels of nuclear cPLA$_2$-α returned to normal following the addition of serum-containing media. However, pre-treatment of cells with ABL (20 μg/ml) for 6 hours prior to the re-addition of serum inhibited the re-entry of cPLA$_2$-α into the nucleus (Fig. 7A). Quantification of the relative levels of nuclear staining revealed that re-feeding following treatment with ABL did not cause a significant increase in nuclear cPLA$_2$-α (Fig. 7B). This effect suggests that the import of cPLA$_2$-α into the nucleus is indeed dependent on an NLS-mediated mechanism.

Leptomycin B blocks nuclear export of cPLA$_2$-α

Leptomycin B (LMB), an anti-fungal agent, inhibits nuclear export signal (NES)-dependent nuclear export by binding to CRM1, a NES receptor in the nuclear pore complex. Using this inhibitor the subcellular location of several proteins, including actin (Wada et al., 1998) and phospholipase C-δ1 (Yamaga et al., 1999), has been shown to be controlled through regulated nuclear export. Hence LMB was used to assess whether or not cPLA$_2$-α contains a functional NES. Cells were grown overnight on coverslips and prior to serum starvation, cells were treated with LMB (10 ng/ml) for 1 hour. The results (Fig. 8A) indicated that treatment with LMB inhibits the serum-starvation-induced export of cPLA$_2$-α from the nucleus. Quantification of the intensity of nuclear cPLA$_2$-α staining (Fig. 8B) showed that no significant decrease in nuclear staining was observed if cells were pre-treated with LMB prior to serum starvation.

Discussion

The nuclear localisation of cPLA$_2$-α in EA.hy.926 and other cells

To date, very few studies have investigated the presence of cPLA$_2$-α within the nuclei of cells. Here, the nuclear localisation of cPLA$_2$-α in the EA.hy.926 human endothelial cell line was studied and the factors that regulate this compartmentalisation were examined.

Using both confocal immunofluorescence microscopy and western blot analysis of isolated nuclei, we have shown that cPLA$_2$-α is present in the nuclei of EA.hy.926 endothelial cells. This finding correlates with previous data on human umbilical vein endothelial cells (HUVECs) presented by Sierra-Honigmann et al. (Sierra-Honigmann et al., 1996), which showed a higher degree of nuclear localisation in subconfluent cells compared with confluent cells. We have also investigated the localisation of cPLA$_2$-α in HUVECs and have seen high levels of nuclear cPLA$_2$-α in these cells (data not shown). Furthermore, immunofluorescence studies on HeLa and A549 epithelial cells, which do not exhibit contact-inhibition, also reveal a high degree of cPLA$_2$-α nuclear localisation in these cell types (data not shown).

Several recent studies have demonstrated that GFP-cPLA$_2$-α fusion proteins are also localised in the nuclei of cells in culture. Studies on MDCK, CHO and PtK2 cells show various levels of nuclear GFP-cPLA$_2$-α (Evans et al., 2001; Hirabayashi et al., 1999; Perisic et al., 1999), confirming that the nuclear staining observed by immunofluorescence

microscopy is not an antibody or fixation artefact. Interestingly, in some of these studies, cells were maintained in low levels of serum, perhaps explaining why the levels of nuclear GFP-cPLA2-α in these cells were comparatively low. In addition, we have also noted that such GFP-cPLA2-α fusion proteins reside in the nuclei of EA.hy.926 cells, HeLa cells and HEK 293 cells (data not shown).

Many previous immunofluorescence microscopy studies on the localisation of cPLA2-α showed little or no nuclear staining. It is possible that this may be due to the diverse range of cell types studied, since we have observed cell-specific variation in the extent of nuclear localisation of cPLA2-α (data not shown). Alternatively, these differences could be due to the variety of fixation methods employed. In many cases, in particular the less recent studies (Kan et al., 1996; Peters-Golden et al., 1996; Schievella et al., 1995), methanol and acetone were used to fix and permeabilise the cells. This method, however, has been shown to result in poor preservation of cell morphology and can often destroy protein antigens (Pastan and Willingham, 1985), suggesting a possible explanation for the absence of nuclear staining in these studies. Paraformaldehyde fixation followed by permeabilisation with a non-ionic detergent, such as Triton X-100, is also often used in immunofluorescence studies; however, previous work carried out in this laboratory indicates that prolonged fixation with paraformaldehyde can inhibit antibody penetration in to the nucleus (data not shown). Some studies on cPLA2-α (Glover et al., 1995; Liu et al., 2001) have used fixation periods of up to 30 minutes; hence it is possible that the absence of nuclear staining in these cases may be due to overfixing.

A previous study that did report the presence of nuclear cPLA2-α in HUVECs (Sierra-Honigmann et al., 1996) suggested that the nuclear species of cPLA2-α had a lower M_r of 70,000. No such species was observed in the study presented here. Interestingly, several more recent studies have shown that cPLA2-α is subject to caspase-mediated cleavage during apoptosis (Adam-Klages et al., 1998; Atsumi et al., 1998). The resulting 70-80 kDa fragment has been shown to be catalytically inactive and present solely within the nucleus (Atsumi et al., 2000). Following this, it is probable that the low molecular weight nuclear species observed previously corresponds to a proteolytic fragment.

We also show here that the pool of cPLA2-α present within the nucleus is capable of relocating to its phospholipid substrate at the nuclear membrane in a calcium-dependent manner. This would lead to the generation of nuclear arachidonic acid and arachidonic acid metabolites.

Nuclear localisation of cPLA2-α is dependent on proliferation

The studies carried out here demonstrate that the presence of cPLA2-α within the nuclei of EA.hy.926 endothelial cells is dependent on cell density. Thus, the nuclei of subconfluent

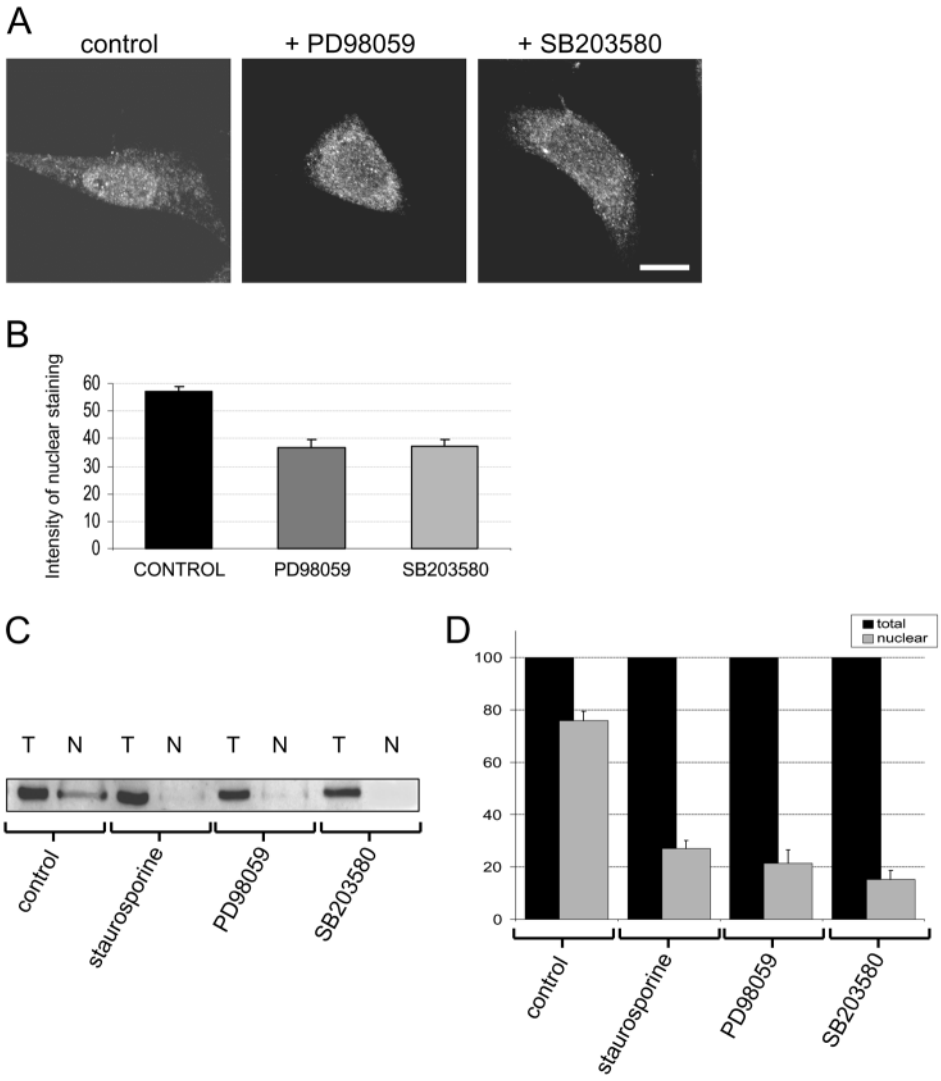


Fig. 6. Effects of the specific MEK and p38^{MAPK} inhibitors, PD98059 and SB203580, on the levels of nuclear cPLA-α. (A) Cells (1×10^5) were seeded onto coverslips and grown overnight. Cells were then pre-treated with PD98059 or SB203580 (20 μM in HEPES/Tyrode's buffer) for 30 minutes at 37°C (control cells were incubated with buffer alone). cPLA-α was detected by immunofluorescence microscopy. Bar, 10 μm. (B) The level of nuclear staining in sections taken through the control, PD98059- and SB203580-treated cells were quantified. The plot shows average intensity (in arbitrary units)±s.e.m. (n=90, data from three independent experiments). (C) Nuclei were isolated from control and PD98059-, SB203580- and staurosporine-treated cells. 20 μg of total homogenate (T) and nuclear (N) proteins were analysed by western blotting. (D) Quantification of the relative amounts of cPLA2-α present in the nuclear and total fractions (expressed as a percentage of the total±s.e.m., n=3)

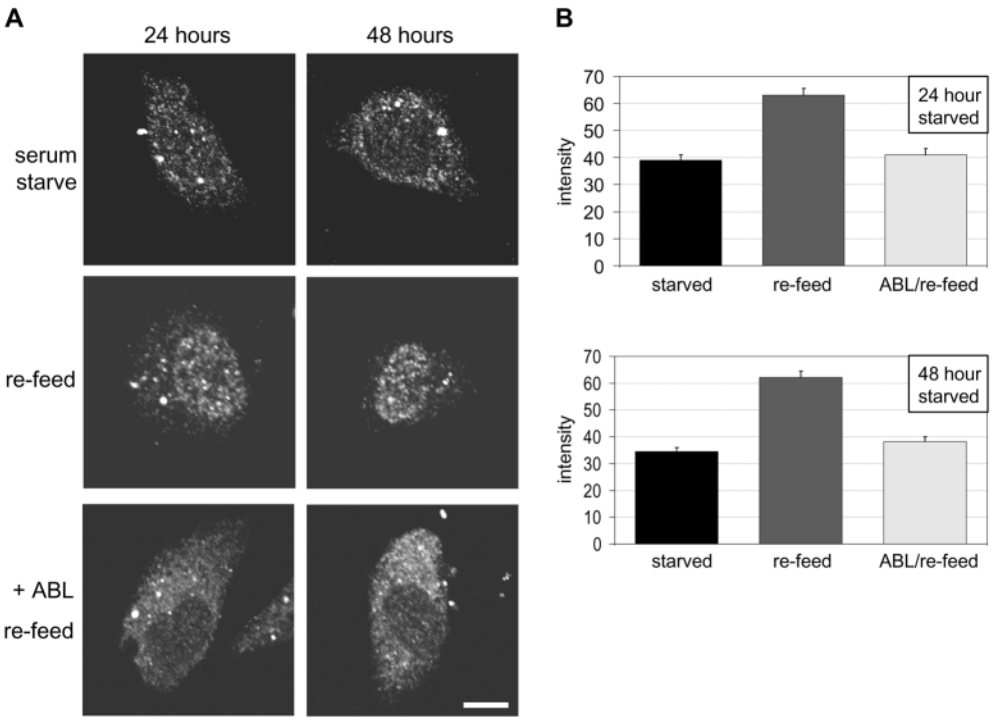


Fig. 7. Effects of the edible mushroom *Agaricus bisporus* lectin (ABL) on the serum-induced nuclear import of cPLA-α. Cells were grown on coverslips and starved for 24 or 48 hours. Cells were then re-fed with complete medium or pre-treated with ABL (20 μg/ml) for 6 hours prior to the addition of complete medium. (A) cPLA-α was detected by immunofluorescence microscopy. Bar, 10 μm. (B) Graph showing changes in intensity of nuclear staining (in arbitrary units, *n*=90, data represents averages from three independent experiments±s.e.m.) in sections taken through serum-starved, re-fed and ABL-treated re-fed cells.

cells were shown to contain higher levels of cPLA$_2$-α than those of contact-inhibited confluent cells. The reduced level of nuclear cPLA$_2$-α in confluent cells could be mimicked by removing serum from the growth medium of sub-confluent cells, indicating that a growth factor within the serum is necessary for cPLA$_2$-α nuclear localisation. In addition to this, immunofluorescence studies on HeLa cells demonstrated that these highly proliferating cells contained elevated levels of nuclear cPLA$_2$-α (data not shown), suggesting a possible link between nuclear cPLA$_2$-α and proliferation.

p38^{MAPK} and p42/44^{MAPK} mediate the nuclear localisation of cPLA$_2$-α

To date, various studies of many cell types have demonstrated that cPLA$_2$-α activity can be enhanced by exposure to diverse agonists that promote phosphorylation (Borsch-Haubold et al., 1999; Buschbeck et al., 1999; de Carvalho et al., 1996; Hernandez et al., 1997; Kramer et al., 1993; Nemenoff et al., 1993; Sa et al., 1995; Schalkwijk et al., 1995). The effects of phosphorylation on the subcellular location of cPLA$_2$-α, however, have not been examined. Here, studies using inhibitors that block activation of p42/44^{MAPK} and p38^{MAPK} demonstrate that the nuclear localisation of cPLA$_2$-α is dependent on these kinase activities. Vascular endothelial growth factor (VEGF), which is essential for endothelial cell growth and differentiation, is known to activate MAPK signalling cascades, including those involving the p42/44 kinases. In addition, VEGF-induced mitogenesis, cyclin D1 synthesis and cyclin-dependent kinase 4 activation were inhibited by PD98059 (Pedram et al., 1998); thus activation of MAP kinases by VEGF is likely to play a central role in the stimulation of endothelial cell proliferation. Although the precise signalling events that mediate the biological effects of VEGF remain unclear, it is possible that phosphorylation and nuclear localisation of cPLA$_2$-α play a role in VEGF-mediated proliferation.

One of the potential cPLA$_2$-α phosphorylation sites, Ser505, lies within the PXSP motif, which represents a consensus MAPK phosphorylation site. Phosphorylation on this residue enhances the intrinsic activity of cPLA$_2$-α, leading to increased

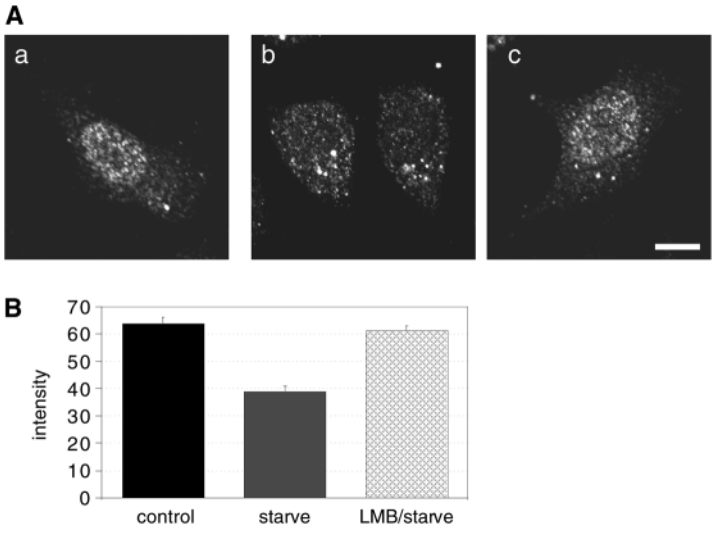


Fig. 8. Effects of leptomycin B (LMB) on the nuclear export of cPLA-α. Cells were grown overnight on coverslips then serum starved for 24 hours. The effects of LMB were examined by treating the cells for 1 hour with 10 ng/ml LMB prior to serum starving. (A) cPLA-α was detected in control cells (a), serum-starved cells (b) and LMB-treated serum-starved cells (c). Bar, 10 μm. (B) Graph showing changes in intensity of nuclear staining (in arbitrary units, *n*=90, data represents averages from three independent experiments±s.e.m.) in sections taken through the nuclei of cells.

levels of Ca^{2+}-induced arachidonic acid release in several different models (Abdullah et al., 1995; Lin et al., 1992; Nemenoff et al., 1993). Using specific inhibitors in platelets, $p38^{MAPK}$ has been shown to be responsible for phosphorylation of cPLA2-α on Ser505, and this causes a decrease in the level of arachidonic acid release (Kramer et al., 1996). Furthermore, transfected cells expressing S505A, S727 or double S505A S727A mutant cPLA2-α show significantly decreased levels of arachidonic acid release (Hefner et al., 2000). It is possible, however, that phosphorylation at these sites is also able to control the subcellular location of cPLA2-α. Alternatively, phosphorylation on the more recently identified Ser515 (Muthalif et al., 2001a), by either $p42/44^{MAPK}$ or $p38^{MAPK}$, may be involved in nuclear localisation. Thus, the activation of cellular kinases by growth factors together with activation of phosphatases induced upon contact-inhibition would provide complementary mechanisms of regulating the nuclear compartmentalisation of cPLA2-α.

cPLA2-α contains putative nuclear import and export signals

The nuclear localisation of proteins that are too large to simply cross the nuclear envelope by diffusion through nuclear pores is dependent on the presence of NLSs that target the protein specifically to the nucleus. Analysis of the amino-acid sequence of cPLA2-α reveals potential NLSs (amino acids 54-60: PDSRKRT and amino acids 269-283: PQKVKRYVESLWKKK) that may be involved in the targeting of this protein. Similarly, a putative NES can also be identified (amino acids 552-562: LTFNLPYPLIL). The inhibition of nuclear uptake and export using ABL and LMB, respectively, suggests that the nuclear compartmentalisation of cPLA2-α is indeed dependent on such targeting signals; however, the functionality of those signals suggested above remains to be investigated. One of the suggested NLSs lies within the C2 domain. Interestingly, it was observed that a GFP-C2 fusion protein shows a higher degree of nuclear localisation than a full-length fusion (Evans et al., 2001; Perisic et al., 1999), which is in agreement with the C2 domain containing a functional NLS. Furthermore, the proteolytic fragment of cPLA2-α generated during apoptosis (amino acids 1-522) contains only the two putative NLSs and not the NES. This caspase-mediated fragment was found to be exclusively intranuclear (Atsumi et al., 2000), further supporting the functionality of the potential NLSs. In addition, analysis of the 3D structure of cPLA2-α shows that both the potential NLSs are placed in exposed positions in the cleft between the catalytic and C2 domains (Fig. 9). Since these two domains are interconnected by a hinge region that has been shown to be highly flexible (Dessen et al., 1999), it is possible that phosphorylation results in rotation of the two domains, thereby varying the exposure of the NLSs. Phosphorylation-mediated regulation of such NLSs is a common phenomenon (reviewed in Jans and Hubner, 1996); hence it is feasible that phosphorylation of cPLA2-α regulates not only its activity but also its cellular localisation.

The role of nuclear cPLA2-α

The role of nuclear cPLA2-α in the EA.hy.926 endothelial cell line remains unclear. An increasing body of evidence shows

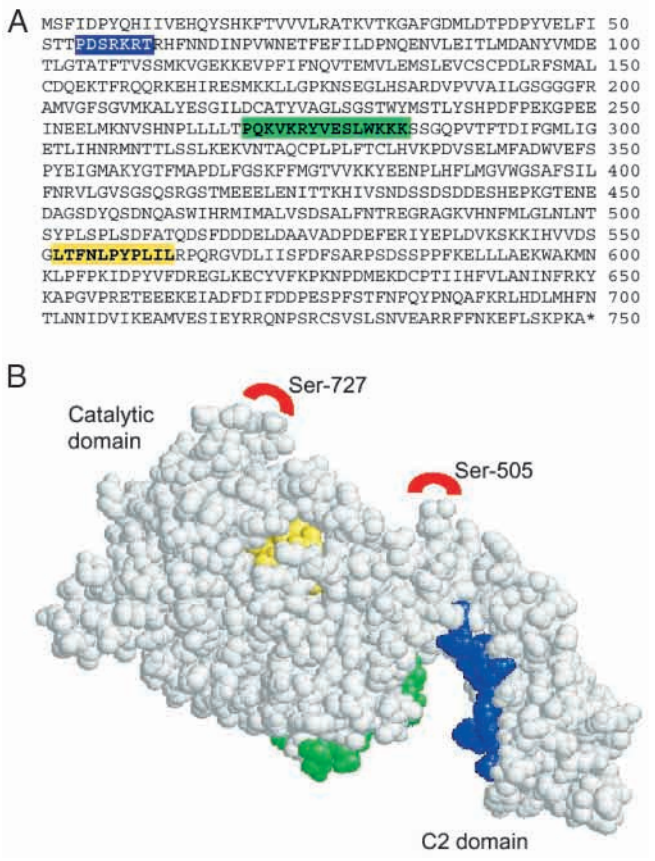


Fig. 9. Putative cPLA2-α nuclear localisation and export signals. (A) Amino-acid sequence of human cPLA2-α. The potential NLSs are highlighted in blue and green. The potential nuclear export signal is outlined in yellow. (B) 3D structure of cPLA2-α, showing the positions of the potential NLSs and NES in their respective colours. Also highlighted are the approximate locations of the phosphorylation sites, Ser505 and Ser727.

that many key lipids and the enzymes involved in their metabolism reside within the nucleus (reviewed in Maraldi et al., 1999). The nuclear lipid second messengers generated are thought to play crucial roles in processes such as gene expression, proliferation, differentiation and apoptosis. With regards to cPLA2-α metabolites, for example, arachidonic acid activates PPARα (peroxisome proliferator-activated receptor-α), a member of the PPAR family of nuclear receptor transcription factors (Delerive et al., 2000). These receptors bind to PPAR response elements (PPREs) and control transcription of genes involved in fatty acid metabolism and lipid homeostasis. Prostacyclin, on the other hand, has recently been shown to promote apoptosis by activating PPARδ (Hatae et al., 2001). In addition, activation of the serum response element (SRE), a primary nuclear target for many diverse signals, has also been shown to be dependent on cPLA2-α activity (Oh et al., 2000). These findings imply that cPLA2-α and its downstream products play significant roles in nuclear functions such as regulation of gene expression. In particular, the products of cPLA2-α action have been strongly implicated in proliferation. Increased lysophosphatidylcholine production, for example, has been shown to lead to proliferation of U937 cells (Muthalif et al., 2001b). Increased arachidonic acid levels

also have been associated with proliferation of A549 cells (Croxtall et al., 1998; Croxtall et al., 1996). The need for nuclear cPLA$_2$-α in proliferation is further supported by the high levels of nuclear cPLA$_2$-α observed in cancerous cells, such as HeLa and A549, which are continually proliferating.

Future perspectives

The elucidation of the mechanisms regulating cPLA$_2$-α nuclear localisation and the exact role of nuclear cPLA$_2$-α requires extensive further studies. Whether or not cPLA$_2$-α is associated with any accessory proteins or structures within the nucleus also remains to be investigated. It is possible that entry into the nucleus simply provides cPLA$_2$-α with an additional pool of phospholipid substrate. Previous data have shown that actively growing endothelial cells are able to liberate more arachidonic acid than growth-arrested cells (Whatley et al., 1994). Thus, while endothelial cells are proliferating, for example at sites of wounds, they can produce elevated levels of prostacyclin, resulting in decreased platelet activation. By contrast, the nuclear pool of cPLA$_2$-α may be involved more directly in the process of proliferation and control of gene expression. Hence, the transport of cPLA$_2$-α into the nuclei of proliferating cells may be a means of controlling gene expression in these cells. Interestingly, lipid second messengers such as arachidonic acid and lysophosphatidylcholine have been shown to stimulate proliferation, and inhibition of cPLA$_2$-α itself results in decreased proliferation in a number of cell types. The studies presented here suggest that the growth-regulated compartmentalisation of cPLA$_2$-α within the nucleus may be a mechanism for regulating the levels of such second messengers within the nucleus, implicating cPLA$_2$-α as a pivotal enzyme in controlling proliferation. With regards to angiogenesis in particular, it can be seen that cPLA$_2$-α may be playing a crucial role in controlling endothelial cell growth. A further understanding of the regulation of cPLA$_2$-α and the factors that control its nuclear localisation may be beneficial in controlling angiogenesis in the growth of tumours.

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