Cytosolic Phospholipase A$_2$-α Mediates Endothelial Cell Proliferation and Is Inactivated by Association with the Golgi Apparatus

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Arachidonic acid and its metabolites are implicated in regulating endothelial cell proliferation. Cytosolic phospholipase A$_2$-α (cPLA$_2\alpha$) is responsible for receptor-mediated arachidonic acid evolution. We tested the hypothesis that cPLA$_2\alpha$ activity is linked to endothelial cell proliferation. The specific cPLA$_2\alpha$ inhibitor, pyrrolidine-1, inhibited umbilical vein endothelial cell (HUVEC) proliferation in a dose-dependent manner. Exogenous arachidonic acid addition reversed this inhibitory effect. Inhibition of sPLA$_2$ did not affect HUVEC proliferation. The levels of cPLA$_2\alpha$ did not differ between subconfluent and confluent cultures of cells. However, using fluorescence microscopy we observed a novel, confluence-dependent redistribution of cPLA$_2\alpha$ to the distal Golgi apparatus in HUVECs. Association of cPLA$_2\alpha$ with the Golgi was linked to the proliferative status of HUVECs. When associated with the Golgi apparatus, cPLA$_2\alpha$ activity was seen to be 87% inhibited. Relocation of cPLA$_2\alpha$ to the cytoplasm and nucleus, and cPLA$_2\alpha$ enzyme activity were required for cell cycle entry upon mechanical wounding of confluent monolayers. Thus, cPLA$_2\alpha$ activity and function in controlling endothelial cell proliferation is regulated by reversible association with the Golgi apparatus.

INTRODUCTION

Endothelial cells line the luminal surface of all blood vessels and are essential for vascular processes including blood clotting, blood pressure, and blood flow (Vane *et al.,* 1990). New blood vessel formation mediated by endothelial cells (angiogenesis) is also a crucial step in wound healing and the establishment of tumors (Folkman and Shing, 1992; Carmeliet, 2000). Antiangiogenic therapy is seen as one of the most promising strategies to restrict tumor growth and prevent metastases (Brem *et al.,* 1993). During the process of angiogenesis endothelial cells migrate, proliferate, and differentiate to form new capillaries (Carmeliet, 2000). Numerous reports have implicated arachidonic acid (AA) in the control of proliferation in a variety of cell types. Actively proliferating endothelial cells in culture liberate elevated levels of AA and its metabolites (Evans *et al.,* 1984; Whatley *et al.,* 1994) and stimulation of endothelial cells with the potent mitogen, basic fibroblast growth factor, induces AA release (Fafeur *et al.,* 1991; Sa *et al.,* 1995). Eicosanoids, the bioactive metabolites of AA, also play a major role in the proliferation and migration of endothelial cells (Dethlefsen *et al.,* 1994; Nie *et al.,* 2000; Dormond *et al.,* 2001; Antoniotti *et al.,* 2003).

Cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$) is an 85-kDa, Ca^{2+}-sensitive member of the phospholipase A$_2$ family of enzymes (Clark *et al.,* 1995) that includes the calcium-independent (iPLA$_2$) and secretory (sPLA$_2$) phospholipases A$_2$. These are responsible for hydrolysis of the *sn*-2 fatty-acyl bond of phospholipids to simultaneously generate free fatty acid and lysophospholipid (Dennis, 1997). There are at least four cPLA$_2$ paralogs (cPLA$_2\alpha$, cPLA$_2\beta$, cPLA$_2\gamma$, and cPLA$_2\delta$), of which cPLA$_2\alpha$ is most characterized. On cell stimulation and intracellular Ca^{2+} elevation, cPLA$_2\alpha$ translocates from the cytosol to intracellular membrane substrates utilizing an N-terminal Ca^{2+}-dependent lipid binding (CalB) domain (Channon and Leslie, 1990; Nalefski *et al.,* 1994; Schievella *et al.,* 1995; Bittova *et al.,* 1999; Evans *et al.,* 2001). cPLA$_2\alpha$ preferentially cleaves phospholipids containing AA at the *sn*-2 position (Dennis, 1997) and is thus considered the key enzyme responsible for receptor-mediated AA liberation and subsequent eicosanoid synthesis (Kramer and Sharp, 1997). As such, it is a pivotal enzyme in AA-mediated cell proliferation and migration. At confluence endothelial cells undergo contact inhibition of growth, cease proliferating, and enter the G$_0$ phase of quiescence. Correspondingly there is a significant decline in AA output and eicosanoid synthesis (Evans *et al.,* 1984; Whatley *et al.,* 1994), which has been attributed to the reduced cPLA$_2\alpha$ activity (Whatley *et al.,* 1994). Because of the importance of endothelial cell proliferation in human retinopathies and cancers, there is great emphasis on developing therapies that modulate endothelial cell growth. Despite this, the exact mechanisms by which cPLA$_2\alpha$ activity is differentially regulated in nonconfluent and confluent endothelial cells and the role of cPLA$_2\alpha$ in endothelial cell proliferation remain unclear.

In this study we demonstrate that cPLA$_2\alpha$ activity is required for endothelial cell proliferation and cell cycle control. We also describe a novel redistribution of cPLA$_2\alpha$ to the Golgi apparatus of human umbilical vein endothelial cells

(HUVECs) in a confluence-dependent manner. The subcellular localization of cPLA$_2\alpha$ is closely linked to the proliferative status of HUVECs. When associated with the Golgi apparatus, cPLA$_2\alpha$ activity is 87% inhibited. After mechanical wounding, cPLA$_2\alpha$ redistribution is linked to the induction of Ki67 expression and entry into the cell cycle. These observations shed light on the mechanisms by which cPLA$_2\alpha$ localization, activity, and function are modulated in quiescent and proliferating endothelial cells.

MATERIALS AND METHODS

Materials

Alexafluor 594–conjugated concanavalin A (ConA) and secondary anti-goat, anti-mouse, and anti-rabbit Alexafluor 488– or 594–conjugated antibodies were purchased from Molecular Probes (Eugene, OR). Rhodamine-conjugated wheat germ agglutinin (WGA) was purchased from Sigma (Poole, United Kingdom). Affinity-purified goat polyclonal anti-cPLA$_2\alpha$ antibodies were purchased from Santa Cruz Biotechnology (*sc-1724*, Santa Cruz, CA). Rabbit anti-GM130 antibodies were from M. Lowe (University of Manchester, United Kingdom). Mouse monoclonal anti-ERGIC-53 and β-1,4-galactosyltransferase (GalT) antibodies were provided by H. P. Hauri (Basel, Switzerland) and T. Suganuma (Miyazaki, Japan), respectively. Rabbit polyclonal anti-TGN46 was supplied by S. Ponnambalam (University of Leeds, United Kingdom). Rabbit polyclonal anti-mannosidase II (ManII) antibodies were purchased from Serotec Ltd (Oxford, United Kingdom). HRP-conjugated anti-goat secondary antibodies were purchased from Pierce (Tattenhall, United Kingdom). 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082) was purchased from BioMol (Plymouth Meeting, PA). Pyrrolidine-1 was supplied by M. H. Gelb (University of Washington, Seattle,WA). All other reagents were obtained from Sigma or Life Technologies (Paisley, United Kingdom) unless otherwise stated.

Cell Culture

HUVECs were isolated from human umbilical cords as previously described (Jaffe, 1984; Howell *et al.*, 2004). Cells were cultured in endothelial cell basal medium supplemented with human recombinant epidermal growth factor (EGF; 5 ng/ml), hydrocortisone (0.2 μg/ml), vascular endothelial growth factor (VEGF, 0.5 ng/ml), human recombinant basic fibroblast growth factor (bFGF, 10 ng/ml), recombinant long R3 insulin-like growth factor-1 (IGF-1, 20 ng/ml), ascorbic acid (1 μg/ml), heparin (22.5 μg/ml), amphotericin B (50 ng/ml), gentamicin (50 μg/ml), and 2% (vol/vol) fetal calf serum (PromoCell, Heidelberg, Germany). All HUVEC cultureware was coated with 0.1% (wt/vol) pigskin gelatin unless stated otherwise. HUVECs were never used in excess of three passages and expressed the characteristic endothelial markers von Willebrand factor and platelet-endothelial cell adhesion molecule-1. All cells were grown at 37°C in a humid incubator containing 5% (vol/vol) CO$_2$.

Cell Proliferation ELISA

HUVEC proliferation rates in the presence or absence of varying concentrations of ONO-RS-082 or pyrrolidine-1 were compared using a 5-bromo-2′-deoxyuridine (BrdU) incorporation-based ELISA (Roche Diagnostics, Lewes, East Sussex, United Kingdom). Cells were seeded at 1×10^3 cells per well (0.55×10^3 cells/cm²) in 96-well plates and cells, grown for 24 h, and then processed according to manufacturer's instructions. The BrdU incorporation period was fixed at 16 h.

SDS-PAGE and Immunoblotting

Samples (20 or 10 μg protein) were resolved for 60 min at 30 mA/gel on 10% SDS-PAGE mini-gels using a discontinuous buffer system (Laemmli, 1970). For immunoblotting, protein was transferred onto nitrocellulose membranes for 3 h at 300 mA (Towbin *et al.*, 1979). Membranes were blocked in 5% (wt/vol) nonfat milk in phosphate-buffered saline (PBS) for 30 min and then incubated overnight with primary antibody (1:500) at room temperature. After incubation with HRP-conjugated anti-goat IgG (1:3000) for 1 h, immunoreactive bands were visualized using a West Pico–enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). A Fuji Film Intelligent dark box II image reader using Fuji Las-1000 Prosoftware (Tokyo, Japan) was used to capture images. Band intensities were determined densitometrically using Aida (Advanced Image Data Analyzer, Raytest, Straubenhardt, Germany) 2.11 software. For comparison of nonconfluent and confluent HUVEC protein expression, cells were grown on noncoated plastic to control for misleading protein estimation due to gelatin-coated surfaces.

Immunofluorescence Microscopy

This technique was adapted from previous protocols (Heggeness *et al.*, 1977; Barwise and Walker, 1996). Cells were seeded on 0.1% (wt/vol) gelatin-coated coverslips and grown to the required level of confluence. Medium was

aspirated and cells were fixed in 10% (vol/vol) Formalin (in neutral buffered saline; Sigma) for 5 min at 37°C. Before fixation some cells were stimulated for 1 min with 5 μM A23187 as previously described (Grewal *et al.*, 2003, 2004). All ensuing steps were performed at 25°C. After permeabilization with 0.1% (vol/vol) Triton X-100 in PBS for 5 min, cells were refixed for 5 min, washed three times with PBS, and then incubated in 50 mM ammonium chloride in PBS for 10 min. After three further PBS washes, nonspecific binding sites were blocked with 5% (vol/vol) donkey serum in PBS for 3 h. Cells were incubated overnight with primary antibody followed by the appropriate Alexafluor-conjugated secondary antibodies, Alexafluor 594–conjugated ConA or rhodamine-conjugated WGA for 3 h. For antigenic adsorption, antibodies to cPLA$_2\alpha$ were incubated with blocking peptide (1:5 ratio of μg antibody to μg peptide) for 30 min before incubation, as previously described (Grewal *et al.*, 2003). Finally cells were washed eight times in PBS and mounted on microscope slides in prolong mounting medium (Molecular Probes).

Deconvolution Imaging and Quantitation

Deconvolution fluorescence microscopy was performed using an Olympus IX-70 inverted fluorescence microscope and DeltaVision deconvolution system (Applied Precision, Issaquah, WA). Individual optical sections of 0.2 μm were generated from 15 iterative cycles of deconvolution. Quantification of colocalization was determined using the IMARIS software suite (Bitplane AG, Zurich, Switzerland) on selected Golgi regions. Background was eliminated by excluding gray scale values lower than 10% of the maximum pixel intensity. Colocalized pixels were recorded and expressed as percentages of the total pixels selected. Mean pixel intensities were determined using Image J software (http://rsb.info.nih.gov/ij/). Regions of interest were selected, mean pixel intensity calculated then mean background pixel intensity subtracted from this. No images analyzed were pixel saturated.

Brefeldin A Treatment

HUVECs were grown to confluence on 0.1% (wt/vol) gelatin-coated coverslips. Cells were washed twice with prewarmed (37°C) PBS and then incubated with 5 μg/ml brefeldin A (BFA; Roche Diagnostics, Sussex, United Kingdom) in serum-free media for 30 min and processed for immunofluorescence microscopy as described above.

AA Release

This technique was adapted from previous protocols (Whatley *et al.*, 1994; Bailleux *et al.*, 2004). HUVECs were cultured to the required cell density in six-well culture dishes and labeled for 24 h with 1 μCi/ml [³H]AA. Cells were washed three times with PBS and then incubated with 10 μM bromoenol lactone for 30 min to inhibit iPLA$_2$ activity. Medium was then aspirated and cells were incubated with serum-free medium supplemented with 5 μM A23187 and 0.3% (wt/vol) fatty acid–free bovine serum albumin. Aliquots of media were removed at times indicated, centrifuged at $16,000 \times g$ for 5 min, and supernatant was counted for radioactivity by liquid scintillation. Cells were lysed in 0.1% Triton X-100 for 5 min and also counted by liquid scintillation for radioactivity.

RESULTS

cPLA$_2\alpha$ Mediates Endothelial Cell Proliferation

cPLA$_2\alpha$ activity but not sPLA$_2$ activity was essential for maximal HUVEC proliferation (Figure 1). The contribution of cPLA$_2\alpha$ to HUVEC proliferation was assessed by determining BrdU incorporation in the presence of varying concentrations of pyrrolidine-1 (Figure 1A). Pyrrolidine-1 specifically inhibits cPLA$_2\alpha$ and has no effect on iPLA$_2$ or sPLA$_2$ (type IIA, X and V) activity (Ghomashchi *et al.*, 2001). Pyrrolidine-1 acted in a dose-dependent manner, inhibiting proliferation by 28.7 ± 4.6, 49.9 ± 4.2, and $70.7 \pm 2.9\%$ at concentrations of 5, 10, and 20 μM (Figure 1A). At concentrations above 20 μM, pyrrolidine-1 was toxic, whereas at concentrations of 10 μM and below, cell viability was unaffected, as determined by trypan blue exclusion. Varying concentrations of the sPLA$_2$ inhibitor, ONO-RS-082 (Hashimoto *et al.*, 2003), had no significant effect on BrdU incorporation (Figure 1B). The inhibitory effects of pyrrolidine-1 could be significantly reversed by addition of exogenous free AA (Figure 1C). AA, 10 μM, reversed the effect of 10 μM pyrrolidine-1, reaching $75.1 \pm 2\%$ of control. We decided to investigate further the properties of cPLA$_2\alpha$ under different growth conditions.
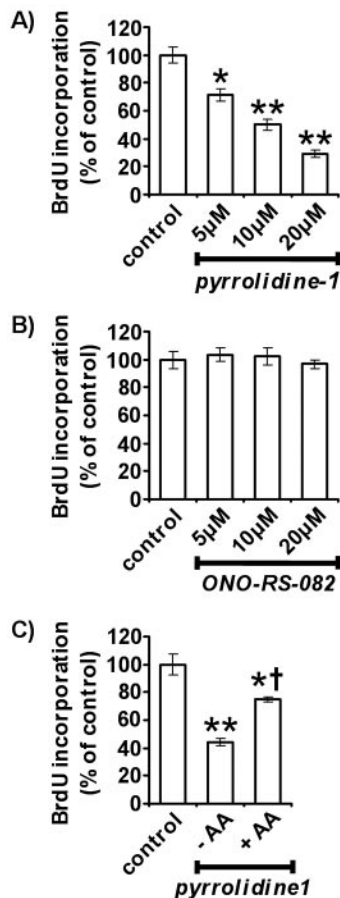
Figure 1. Inhibition of HUVEC proliferation by $cPLA_2\alpha$ inhibition. Quantitation of HUVEC growth in the presence of varying concentrations of pyrrolidine-1 (A) and ONO-RS-082 (B) for 16 h (n = 6, ±SEM). Quantitation of HUVEC growth in the presence of 10 μM pyrrolidine-1 with and without 10 μM arachidonic acid supplementation (C; n = 5, ±SEM). Proliferation was determined using a colorimetric ELISA based on BrdU incorporation, as described in *Materials and Methods.* * p < 0.02 versus control. ** p < 0.001 versus control. [+] p < 0.001 versus 10 μM pyrrolidine-1 without AA.

Figure 2. $cPLA_2\alpha$ expression and localization in nonconfluent and confluent HUVECs. $cPLA_2\alpha$ was detected in nonconfluent and confluent HUVEC total protein (10 μg) by immunoblotting using a goat polyclonal antibody (A). Relative amounts of $cPLA_2\alpha$ immunoreactivity were determined using Aida densitometry software and plotted (±SEM) (B). The localization of $cPLA_2\alpha$ in nonconfluent (C) and confluent cells (D) was determined by immunofluorescence microscopy. HUVECs were grown to nonconfluence or confluence on 0.1% gelatin coated coverslips, fixed, permeabilized, and $cPLA_2\alpha$ detected with a goat antibody. In E, the percentage of total cells with juxta-nuclear $cPLA_2\alpha$ localization was quantified for confluent, subconfluent, and nonconfluent cells (n = 3, ±SEM). The localization of $cPLA_2\alpha$ in nonconfluent cells upon A23187 stimulation was determined by immunofluorescence microscopy (F). HUVECs were grown to nonconfluence or confluence on 0.1% gelatin-coated coverslips, fixed, and permeabilized, and $cPLA_2\alpha$ was detected with a goat antibody. All images represent 0.2-μm sections through cell nuclei. Scale bar, 25 μm. * p < 0.001 versus confluent cells. All results are representative of at least 3 separate experiments.

$cPLA_2\alpha$ Expression and Localization in Confluent and Nonconfluent HUVECs

The lower levels of AA release seen with confluent endothelial cells are not due to reduced $cPLA_2\alpha$ expression (Figure 2, A and B). It is well established that nonconfluent endothelial cells release much greater levels of AA and eicosanoids than confluent endothelial cells and that the mechanical wounding of confluent monolayers results in elevated AA production (Evans *et al.,* 1984; Whatley *et al.,* 1994). Lower levels of AA release could be due to reduced $cPLA_2\alpha$ expression because it is the rate-limiting enzyme in AA liberation. The protein levels of $cPLA_2\alpha$ were determined in subconfluent proliferating and in confluent nonproliferating HUVECs (Figure 2, A and B). $cPLA_2\alpha$ levels were assessed by Western blotting of HUVEC total protein using a well-characterized affinity-purified antibody specific to the C-terminal region of $cPLA_2\alpha$ (Grewal *et al.,* 2002, 2003, 2004). Densitometric analysis demonstrated no significant difference in total $cPLA_2\alpha$ levels between subconfluent and confluent cells when using 10 μg (Figure 2B) and 20 μg (unpublished data) of total protein. The amounts of total cell protein used for Western blot detection of $cPLA_2\alpha$ gave
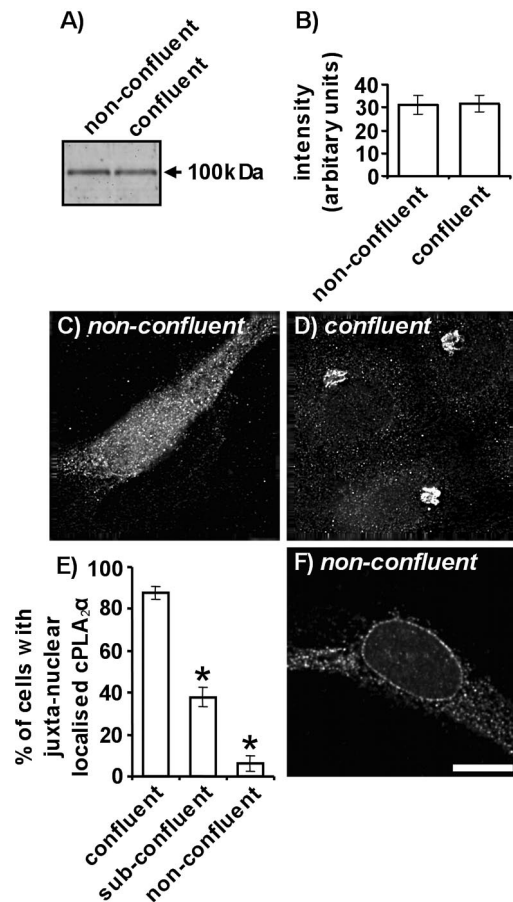
responses that reside in the linear region of a saturation curve. Antigenic absorption with a blocking peptide, corresponding to the C-terminal 20 amino acids unique to $cPLA_2\alpha$, eliminated detection by immunoblotting (unpublished data).

$cPLA_2\alpha$ redistributes to a compact juxtanuclear reticulum in confluent HUVECs (Figure 2, C–E). Indirect immunofluorescence microscopy was used to determine the subcellular localization of $cPLA_2\alpha$ in HUVECs exhibiting differing levels of confluence (Figure 2, C and D). As previously described for endothelial cells (Sierra-Honigmann *et al.,* 1996; Grewal *et al.,* 2002), nonconfluent HUVECs displayed homogeneous $cPLA_2\alpha$ staining throughout the cytoplasm and

nucleus (Figure 2C). High-resolution deconvolution microscopy shows that cPLA$_2\alpha$ was present both as diffuse and structured pools of staining in nonconfluent cells, similar to observations with fibroblasts (Bunt *et al.,* 1997). At confluence, cPLA$_2\alpha$ was redistributed to a compact juxtanuclear reticulum (Figure 2D). HUVECs of differing levels of confluence were scored for the presence of juxtanuclear cPLA$_2\alpha$ staining (Figure 2E). Our criteria defined confluent HUVECs as those completely surrounded by other cells. Cells not contacting any other were categorized as nonconfluent. Cells contacting one or more cells but not entirely surrounded were classed as subconfluent. A distinct correlation between confluence and the presence of juxtanuclear cPLA$_2\alpha$ staining was evident. A high percentage of confluent cells (87.5 ± 3.2%) displayed juxtanuclear cPLA$_2\alpha$ staining, whereas only 37.8 ± 4.7% of subconfluent and 6.2 ± 3.5% of nonconfluent cells displayed this localization pattern.

On stimulation of nonconfluent cells with the Ca^{2+} ionophore, A23187, cPLA$_2\alpha$ translocates to the peri-nuclear membrane and endoplasmic reticulum (Figure 2F). These regions are classical sites of cPLA$_2\alpha$-mediated AA release in response to Ca^{2+} elevation (Hirabayashi *et al.,* 1999). Stimulation of confluent endothelial cells with A23187 did not induce cPLA$_2\alpha$ translocation and cPLA$_2\alpha$ staining remained restricted to the juxtanuclear region (unpublished data). Antigenic absorption with a blocking peptide, corresponding to the C-terminal 20 amino acids unique to cPLA$_2\alpha$, eliminated detection by immunofluorescence microscopy (unpublished data).

cPLA$_2\alpha$ Redistributes to Membrane Components of the Distal Golgi Apparatus at HUVEC Confluence

cPLA$_2\alpha$ redistributes to the Golgi apparatus in confluent endothelial cells under standard cell culture conditions (Figure 3, A and B). Because the juxtanuclear staining pattern for cPLA$_2\alpha$ resembled the location of the Golgi apparatus, possible colocalization was investigated by counterstaining confluent HUVECs with rhodamine-conjugated WGA (Figure 3A). This lectin specifically binds N-acetyl-β-D-glucosaminyl residues found predominantly in the Golgi apparatus, with lower levels at the plasma and nuclear membranes (Virtanen *et al.,* 1980). cPLA$_2\alpha$ was seen to colocalize extensively with WGA-positive juxtanuclear structures corresponding to the Golgi apparatus (yellow indicates overlap), but no significant overlap was seen with the endoplasmic reticulum (ER) marker lectin, ConA (Virtanen *et al.,* 1980; Figure 3B). A smaller pool of cPLA$_2\alpha$ staining that did not colocalize with WGA-positive or ConA-positive structures was also evident (Figure 3, A and B).

cPLA$_2\alpha$ is associated with membrane components of the Golgi apparatus in confluent HUVECs and does not simply reside in close proximity (Figure 3, C and D). Cells were treated with BFA, a fungal metabolite that inhibits trafficking through the secretory pathway and redistributes the Golgi stack to the ER. Protein transport through the Golgi apparatus is blocked and Golgi- and *trans*-Golgi network (TGN)-resident proteins accumulate in the ER and endosomal systems, respectively (Lippincott-Schwartz *et al.,* 1990, 1991; Wood *et al.,* 1991; Klausner *et al.,* 1992). Mannosidase II (ManII), a Golgi-resident protein (Novikoff *et al.,* 1983), and cPLA$_2\alpha$-positive Golgi structures were fully dispersed (Figure 3D) by 5 μg/ml BFA compared with control cells (Figure 3C). ManII- and cPLA$_2\alpha$-positive Golgi structures reform upon BFA washout and recovery (unpublished data).

In confluent HUVECs, cPLA$_2\alpha$ colocalized with components of the distal Golgi apparatus, i.e., the *medial*-Golgi, *trans*-Golgi, and *trans*-Golgi network (Figure 4). The Golgi
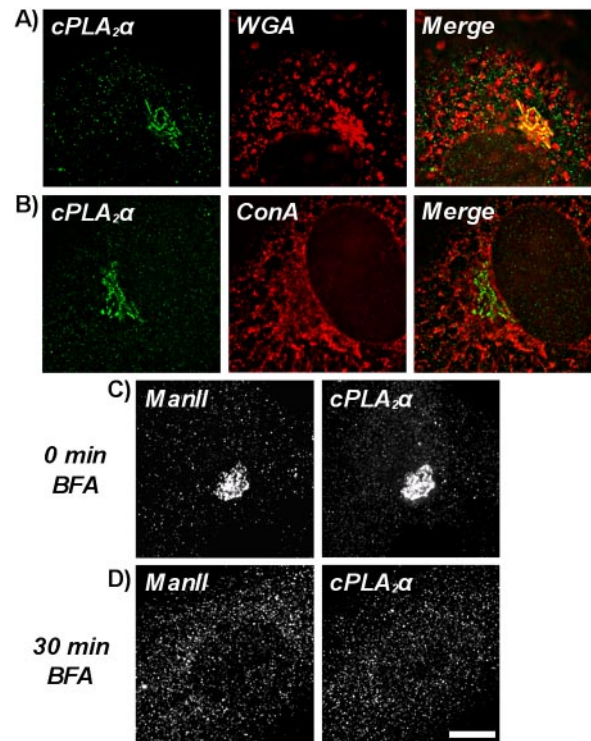

Figure 3. Association of cPLA$_2\alpha$ with membrane components of the Golgi apparatus. HUVECs were grown to confluence on 0.1% gelatin-coated coverslips, fixed, and then permeabilized, and cPLA$_2\alpha$ was detected with a goat antibody (A and B). Cells were then incubated with Alexafluor 488–conjugated anti-goat antibodies and either rhodamine-conjugated WGA (A) or Alexafluor 594–conjugated ConA (B). Images represent 0.2-μm sections through cell nuclei. Disruption of the Golgi apparatus with brefeldin A (BFA; C and D). Confluent HUVECs were grown on 0.1% gelatin-coated coverslips and directly fixed (C) or treated for 30 min with 5 μg/ml the fungal metabolite BFA (D) before being fixed and permeabilized. Cells were then incubated with goat anti-cPLA$_2\alpha$ and rabbit anti-ManII antibodies. Then cells were incubated with Alexfluor 488–conjugated anti-goat and Alexfluor 594–conjugated anti-rabbit antibodies. Images represent projected stacks of whole cells. Scale bar, 10 μm. All results are representative of three separate experiments.

complex consists of five spatially distinct subcompartments: the ER-Golgi intermediate compartment (ERGIC), *cis*-Golgi, *medial*-Golgi, *trans*-Golgi, and TGN. Antibodies specific to subcompartment resident proteins were used in costaining microscopy experiments to determine more specifically the localization of cPLA$_2\alpha$ in confluent HUVECs (Figure 4). ERGIC-53 (Figure 4A), GM130 (Figure 4B), ManII (Figure 4C), β-1,4-galactosyltransferase (GalT; Figure 4D) and TGN46 (Figure 4E) are specific markers for the ERGIC (Schweizer *et al.,* 1988, 1990), *cis*-Golgi (Nakamura *et al.,* 1995), *medial*-Golgi (Novikoff *et al.,* 1983), *trans*-Golgi (Roth *et al.,* 1985; Nilsson *et al.,* 1993), and TGN (Prescott *et al.,* 1997), respectively. cPLA$_2\alpha$ appeared to colocalize most extensively with ManII- and GalT-positive structures (yellow indicates overlap; Figure 4, C and D). Extensive overlap was also seen with TGN46 (Figure 4E) but not to the extent of ManII and GalT. cPLA$_2\alpha$ only appeared closely associated and not superimposed with ERGIC-53 (Figure 4A) and GM130 (Figure 4B). Quantification of the colocalization of cPLA$_2\alpha$ with Golgi subcompartment markers was determined (Figure 4F). Percentage colocalization of cPLA$_2\alpha$ with
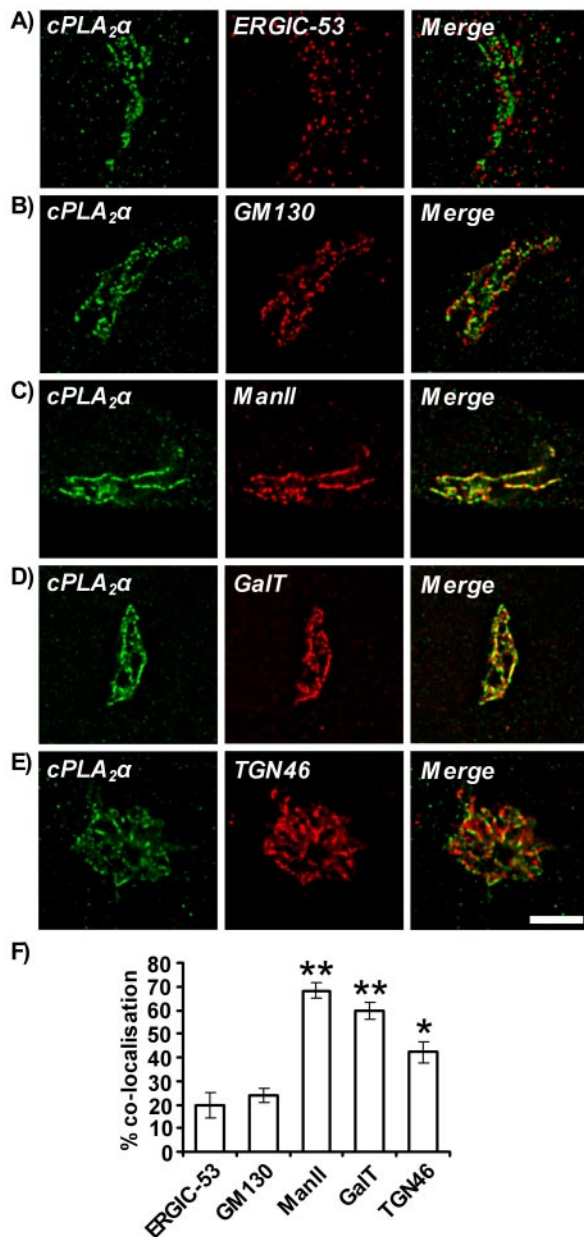
Figure 4. Colocalization of cPLA$_2\alpha$ with Golgi compartment markers. Confluent HUVECs were grown on 0.1% gelatin-coated coverslips, fixed, and permeabilized. Cells were incubated with goat anti-cPLA$_2\alpha$ antibody and either mouse anti-ERGIC-53 (A), rabbit anti-GM130 (B), rabbit anti-ManII (C), mouse anti-GalT (D), or rabbit anti-TGN46 (E) antibodies. Cells were then incubated with anti-goat Alexafluor 488–conjugated and either anti-rabbit or anti-mouse Alexafluor 594–conjugated antibodies. Images represent 0.2-μm sections through cell nuclei. Scale bar, 10 μm. Results are representative of three separate experiments. Quantification of cPLA$_2\alpha$ overlap with ERGIC-53, GM130, ManII, GalT, and TGN46 (F). Percentage colocalization was calculated using the IMARIS computer package as described in *Materials and Methods* (n = 9, ±SEM). * p < 0.02 versus GM130. ** p <0.001 versus GM130.

ManII, GalT, and TGN-46 was seen to be 68.4 ± 3.5, 59.7 ± 3.8, and 42.2 ± 4.5%, respectively, whereas ERGIC-53 and GM130 displayed only 19.8 ± 5.6 and 23.8 ± 3.1% colocalization. All secondary antibody controls gave no staining (unpublished data).

The Subcellular Localization of cPLA$_2\alpha$ Is Associated with HUVEC Proliferation

HUVEC confluence and proliferation are directly linked (Figure 5A). Subconfluent cultures of endothelial cells proliferate until they reach confluence then cell-cell contacts inhibit cellular proliferation (Chen *et al.*, 2000). HUVECs were seeded at equivalent densities (0.55 $\times$ 10^3/cm^2) and cultured for 7 d. From days 2–7, cell numbers and proliferation per cell were determined and compared (Figure 5A). Cell density rose exponentially to reach a plateau after 5 d. BrdU incorporation per cell declined in correlation to also plateau at 5 d. At this point a confluent HUVEC monolayer is formed, cell-cell contacts inhibit proliferation, cells enter the G$_0$ phase of quiescence, and cell numbers cease to increase.

cPLA$_2\alpha$ redistributed to the Golgi apparatus upon inhibition of HUVEC proliferation (Figure 5B). The subcellular localization of cPLA$_2\alpha$ is confluence-dependent (Figure 2E), and endothelial cell confluence is directly linked to proliferation (Figure 5A). As cPLA$_2\alpha$ mediates HUVEC proliferation, we hypothesized that the subcellular localization of cPLA$_2\alpha$ was also associated with HUVEC proliferation. From days 2–7, indirect immunofluorescence microscopy was used to determine the subcellular localization of cPLA$_2\alpha$. HUVECs were scored for Golgi-localized cPLA$_2\alpha$ staining, and results again plotted against proliferation per cell (Figure 5B). A direct correlation between BrdU incorporation per cell and the percentage of total cells with Golgi-localized cPLA$_2\alpha$ was observed. The percentage of cells displaying Golgi-localized staining again reached a plateau after 5 d of growth. As cPLA$_2\alpha$ mediates HUVEC proliferation, it is significant that its subcellular localization is closely linked to the proliferative status of these cells.

It was important to determine whether association of cPLA$_2\alpha$ with the Golgi apparatus affected its enzyme activity. Consequently, nonconfluent, proliferating, and confluent nonproliferating cultures of cells were assayed for AA release in response to elevation of cytosolic Ca^{2+} (Figure 5C). cPLA$_2\alpha$ activity was reduced by 87% in confluent endothelial cells (Figure 5C); therefore, Golgi associated cPLA$_2\alpha$ was inactivated.

Mechanical Wounding Induces cPLA$_2\alpha$ Relocation in HUVECs

On mechanical wounding of confluent HUVEC monolayers, cPLA$_2\alpha$ reversibly redistributes from the Golgi apparatus to the diffuse cytoplasmic and nuclear location of nonconfluent HUVECs (Figure 6A–D). Mechanical wounding of confluent endothelial monolayers induces exit from G$_0$, entry into the cell cycle, proliferation, and migration of cells to fill denuded areas (Chen *et al.*, 2000). HUVECs were mechanically wounded to investigate the effects on Golgi-localized cPLA$_2\alpha$ upon conversion from a confluent, quiescent to a subconfluent, proliferating state. Cells were fixed at various time points after initial wounding and cPLA$_2\alpha$ was detected by immunofluorescence microscopy (Figure 6A–C). Areas to the right side of the panels depict wound borders at the edge of denuded zones. At 0 h after wounding, HUVECs retained Golgi localized cPLA$_2\alpha$ (Figure 6A). However, 8 h (Figure 6B) and 24 h (Figure 6C) after wounding, HUVECs at the wound border displayed progressively less Golgi-associated cPLA$_2\alpha$. Cells invading the wound revert to the diffuse nuclear and cytoplasmic localization characteristic of nonconfluent cells. From 2 h after wounding, the percentage of HUVECs at wound borders displaying Golgi-localized cPLA$_2\alpha$ was significantly reduced (Figure 6D). After 24 h,
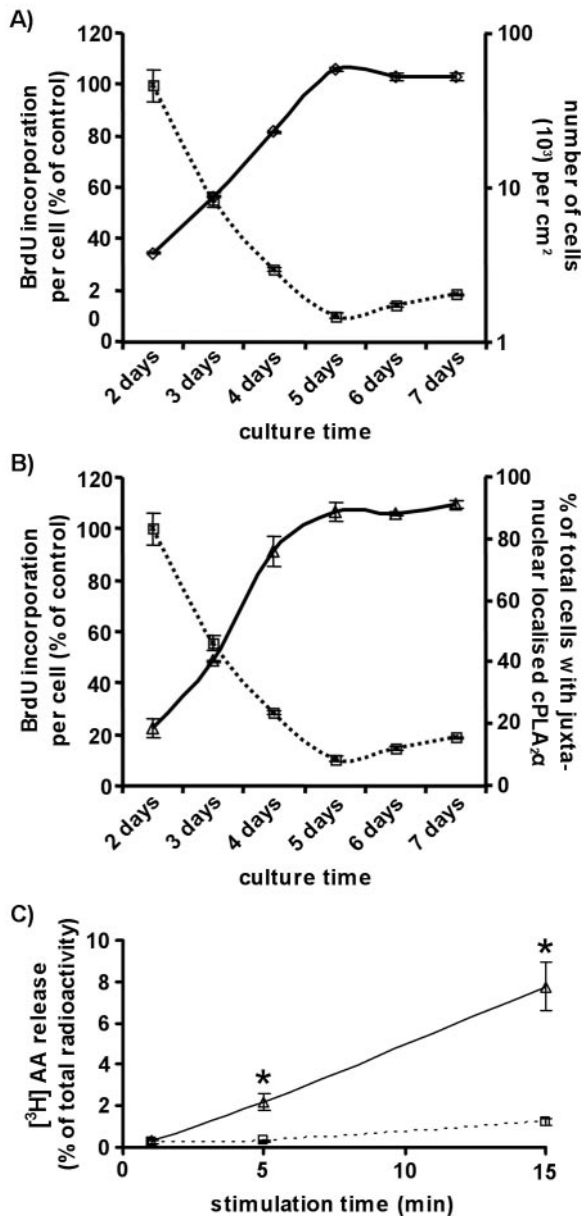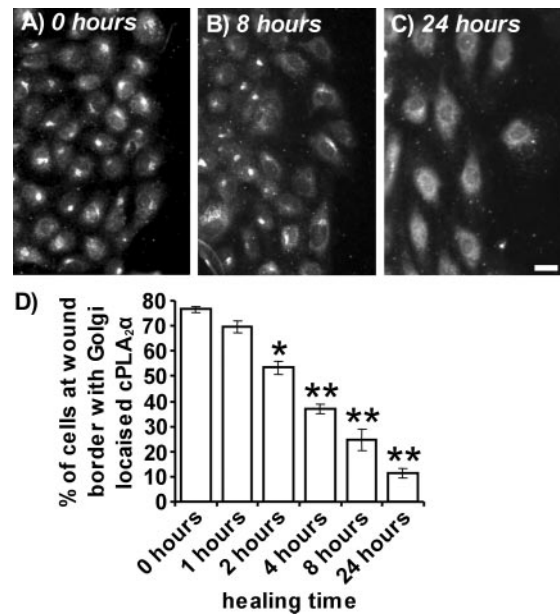
Figure 6. The Golgi association of cPLA$_2$α is reversible upon wounding of confluent HUVEC monolayers. HUVECs were grown to confluence on 0.1% gelatin-coated coverslips and subjected to mechanical wounding. HUVECs were allowed to recover for varying periods of time before fixation. The localization of cPLA$_2$α in cells at the wound border (right of panels) after 0 h (A), 8 h (B), and 24 h (C) of recovery was determined by immunofluorescence microscopy using a goat polyclonal antibody. Images represent projections of whole cells; scale bar, 100 μm. Results are representative of three separate experiments. In D, the percentage of cells at the wound border with Golgi-localized cPLA$_2$α were quantified after 0, 1, 2, 4, 8, and 24 h of recovery (n = 3, ±SEM). * p < 0.05 versus 0 h, ** p < 0.001 versus 0 h.

only 11.4 ± 1.7% of cells at the wound border retained Golgi-localized cPLA$_2$α. The Golgi apparatus remained intact (ManII immunofluorescence staining) throughout the experiment. Association of cPLA$_2$α with the Golgi apparatus is thus reversible upon conversion from confluent to subconfluent cells.

cPLA$_2$α Activity Is Required for Induction of Ki67 Expression and Entry Into the Cell Cycle

After mechanical wounding, entry into the cell cycle occurs upon cPLA$_2$α relocation (Figure 7). Ki67 is a nuclear protein vital for proliferation (Schluter *et al.,* 1993) and is expressed in the G$_1$, S, G$_2$, and mitotic phases of the cell cycle but not in the G$_0$ phase of quiescent cells (Gerdes *et al.,* 1984). Confluent monolayers of endothelial cells undergo contact inhibition of cellular proliferation and enter G$_0$. On mechanical wounding, cells migrate and proliferate to fill the denuded zone and Ki67 expression is induced upon entry into the cell cycle. By immunofluorescence microscopy, only 11 ± 0.1%

Figure 5. The subcellular localization of cPLA$_2$α is associated with HUVEC proliferation. HUVEC cell density and growth were quantified over a culture period of 7 d (A). HUVECs (0.55 × 10³/cm²) were seeded in 0.1% gelatin-coated 24-well culture dishes. They were counted after trypsinization each day from day 2 to day 7 after seeding (logarithmic scale, solid line with diamonds). Results represent the number of cells per cm² (n = 3, ±SEM). HUVEC proliferation was determined using a colorimetric ELISA based on BrdU incorporation, as described in *Materials and Methods* (dotted line with squares). HUVECs (0.55 × 10³/cm²) were seeded in 0.1% gelatin-coated 96-well culture dishes and grown for varying periods of time. Cells were labeled for 16 h with BrdU before processing. Results represent the BrdU incorporation per cell (n = 6, ±SEM). The subcellular localization of cPLA$_2$α was quantified over a culture period of 7 d and compared with the BrdU incorporation per cell (B). HUVECs were seeded in 0.1% gelatin-coated coverslips at an equivalent density to those seeded in 96-well culture dishes. From day 2 to day 7 they were fixed, permeabilized, and processed for immunofluorescence microscopy and the localization of cPLA$_2$α determined (solid line with diamonds). Results represent the percentage of total cells with juxta-nuclear localized cPLA$_2$α at each time point (n = 3, ±SEM). Results were plotted alongside the

Figure 5 (cont). quantification of BrdU incorporation per cell from Figure 5A. Arachidonic acid release in response to A23187 stimulation was determined for confluent (dotted line) and nonconfluent (solid line) HUVECs (C). Cells were loaded with 1 μCi/ml [³H]AA, stimulated with 5 μM A23187, and released AA was determined at the times indicated (n = 3, ±SEM). Results are expressed as a percentage of the total [³H]AA incorporated. * p < 0.01 versus confluent.
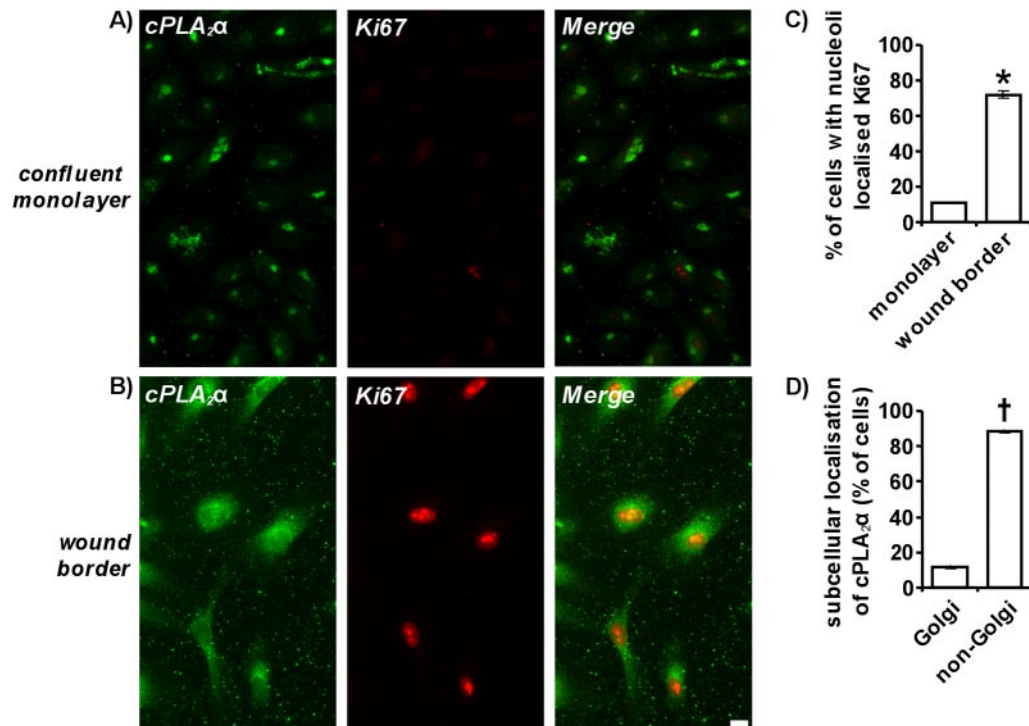
Figure 7. Ki67 upregulation induced by mechanical wounding is associated with cPLA$_2\alpha$ relocation. Confluent HUVECs were grown on 0.1% gelatin-coated coverslips and either directly fixed (A) or mechanically wounded and allowed to recover for 24 h before fixing (B). Cells were permeabilized and incubated with goat anti-cPLA$_2\alpha$ antibody and rabbit anti-Ki67 antibodies. Cells were then incubated with anti-goat Alexafluor 488–conjugated and either anti-rabbit Alexafluor 594–conjugated antibodies. Images represent a confluent monolayer (A) or wound border (B) and are projections of whole cells. Scale bar, 100 μm. In C, the percentage of total cells with nucleoli localized Ki67 was quantified in confluent monolayers of cells and at wound borders after mechanical wounding and 24 h of recovery (n = 3; ±SEM). In D, the subcellular localization of cPLA$_2\alpha$ in cells at the wound border with nucleoli localized Ki67 was determined. * p < 0.001 versus confluent monolayer. † p < 0.001 versus Golgi. All results are representative of at least three separate experiments

of HUVECs in a confluent monolayer expressed Ki67 because most cells are in the G$_0$ phase of quiescence (Figure 7, A and C). On mechanical wounding and recovery for 24 h, 72 ± 1.8% of cells at the wound border expressed Ki67 as they migrate and proliferate into the wound (Figure 7, B and C). A large proportion of HUVECs expressing Ki67 at wound borders (88.8 ± 0.7%) also displayed relocation of cPLA$_2\alpha$ from the Golgi apparatus to the nucleus and cytosol (Figure 7, B and D). In correlation, 87.8 ± 0.72% of cells that have lost their Golgi-localized cPLA$_2\alpha$ are Ki67 positive (unpublished data). Thus upon mechanical wounding, Ki67 expression, entry into the cell cycle and proliferation are induced in cells where cPLA$_2\alpha$ has relocated to the cytosol and nucleus.

cPLA$_2\alpha$ activity is vital for exit from G$_0$ and entry into the S phase of the cell cycle as pyrrolidine-1 inhibits Ki67 induction upon wounding (Figure 8). Confluent monolayers of HUVECs were mechanically wounded and recovered for 24 h in the absence or presence of 10 μM pyrrolidine-1. Cells that underwent cPLA$_2\alpha$ relocation were analyzed by immunofluorescence microscopy to assess the level of cPLA$_2\alpha$ and Ki67 expression (Figure 8, A and B). Levels of cPLA$_2\alpha$ expression do not significantly change (Figure 8C) but a 48.2% (±4.7%) reduction in Ki67 expression was observed after recovery in pyrrolidine-1 (Figure 8D). Ki67 expression is essential for cell cycle progression and proliferation. Thus, induction of HUVEC proliferation requires cPLA$_2\alpha$ activity and occurs upon relocation of cPLA$_2\alpha$ to the cytoplasm and nucleus.

DISCUSSION

Our results link cPLA$_2\alpha$ activity to the regulation of proliferation of endothelial cells and also show that cPLA$_2\alpha$ activity is required for entry into the cell cycle from G$_0$. The induction of HUVEC proliferation from G$_0$ occurs upon relocation of cPLA$_2\alpha$ from the Golgi apparatus to a diffuse cytoplasmic and nuclear location. Thus, the subcellular localization of cPLA$_2\alpha$ mediates its function.

Our report is the first to document the importance of cPLA$_2\alpha$ activity for controlling endothelial cell proliferation and entry into the cell cycle. Previously AACOCF$_3$, an inhibitor of both cPLA$_2$ and iPLA$_2$ (Ackermann *et al.*, 1995; Street *et al.*, 1993) was seen to inhibit bovine aortic endothelial cell proliferation (Antoniotti *et al.*, 2003) but that study lacked specific inhibitor studies. Here we use the specific cPLA$_2\alpha$ inhibitor, pyrrolidine-1 (Ghomashchi *et al.*, 2001), to directly implicate cPLA$_2\alpha$ in endothelial cell proliferation. Reduced AA liberation by cPLA$_2\alpha$ is at least partially responsible for the inhibitory effects of pyrrolidine-1. Despite this, exogenous AA does not augment the proliferation of nonconfluent HUVECs (unpublished data) although they may proliferate at an optimal rate in our culture conditions so exogenous AA has no effect. In addition, exogenous AA does not induce entry into the cell cycle and proliferation of confluent endothelial cells (unpublished data). This demonstrates that although cPLA$_2\alpha$ activity is essential for cell cycle entry, it is insufficient by itself to override the contact-inhibition of proliferation. Our report suggests that inhibi-
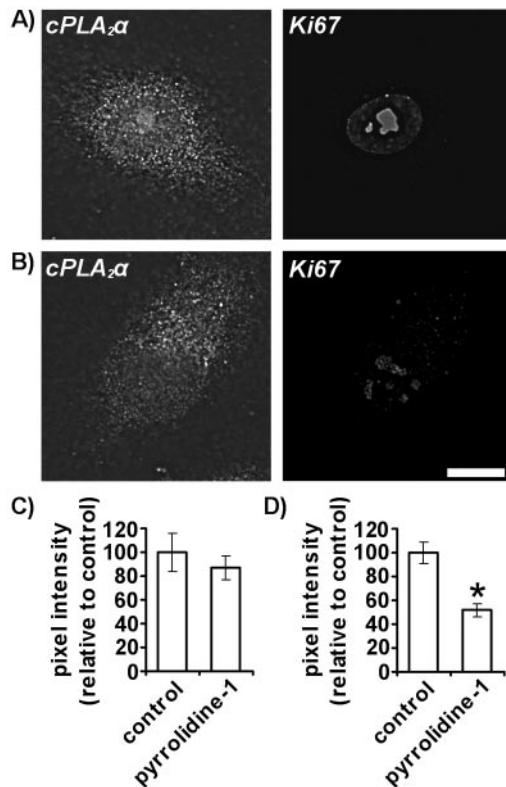
Figure 8. cPLA$_2\alpha$ inhibition prevents Ki67 induction in response to mechanical wounding. Confluent HUVECs were grown on 0.1% gelatin-coated coverslips, mechanically wounded, and recovered for 24 h in the absence (A) and presence (B) of 10 μM pyrrolidine-1. Cells were fixed, permeabilized, and incubated with goat anti-cPLA$_2\alpha$ antibody and rabbit anti-Ki67 antibodies. Cells were then incubated with anti-goat Alexafluor 488–conjugated and either anti-rabbit or anti-mouse Alexafluor 594–conjugated antibodies. Only cells displaying non-Golgi localized cPLA$_2\alpha$ at wound borders were imaged. Images represent 0.2-μm sections through cell nuclei. Scale bar, 25 μm. Mean cellular cPLA$_2\alpha$ pixel intensity (C) and nuclear Ki67 pixel intensity (D) were determined using Image J software as described in *Materials and Methods*. Results represent cells displaying non-Golgi localized cPLA$_2\alpha$ at wound borders for those recovered with and without 10 μM pyrrolidine-1 (n = 20). * p < 0.001 versus control. All results are representative of at least three separate experiments.

tion of cPLA$_2\alpha$ could be a viable antiangiogenic route because the induction of cell cycle entry and proliferation in resting endothelial cells is crucial to angiogenesis (Carmeliet, 2000) and cPLA$_2\alpha$ activity is essential to this process.

We describe the novel confluence-dependent association of cPLA$_2\alpha$ with membrane components of the distal Golgi apparatus. When associated with the Golgi apparatus, cPLA$_2\alpha$ activity is inhibited by 87%. This is in contrast to previous studies that demonstrate increased cPLA$_2\alpha$ activity upon association with the Golgi apparatus (Pettus *et al.*, 2004; Grimmer *et al.*, 2005). Association with the Golgi apparatus and cPLA$_2\alpha$ inactivation may occur to differing extents depending on cell type, cell confluence, or the ability of cells to undergo contact inhibition of proliferation. We propose that Golgi association and inactivation of cPLA$_2\alpha$ is a feature of the contact-inhibition of endothelial cells.

Our results suggest that the Golgi association and inactivation of cPLA$_2\alpha$ is important to the regulation of its function in endothelial cells. cPLA$_2\alpha$ associates with the Golgi

apparatus at HUVEC confluence when proliferation is inhibited and cells exit the cell cycle. Entry back into the cell cycle occurs upon relocation of cPLA$_2\alpha$ back to the diffuse cytoplasmic and nuclear location seen in subconfluent HUVECs. Thus, cPLA$_2\alpha$ is mediating HUVEC proliferation when it is found throughout the cytoplasm and nucleus so the localization of cPLA$_2\alpha$ in proliferating endothelial cells is essential for targeting it to areas where it functions to aid proliferation. AA is evolved in response to Ca^{2+} elevation and cPLA$_2\alpha$ relocation to membrane substrate (Hirabayashi *et al.*, 1999) as occurs in response to growth factor stimulation. In nonconfluent endothelial cells in response to Ca^{2+} elevation, cPLA$_2\alpha$ associates with perinuclear membrane substrate, a known site of cPLA$_2\alpha$-derived AA production (Hirabayashi *et al.*, 1999). This does not occur in confluent endothelial cells because cPLA$_2\alpha$ remains immobilized on the Golgi apparatus and cPLA$_2\alpha$ activity remains inhibited. Thus, we outline a novel mechanism for the inhibition of cPLA$_2\alpha$ activity. We propose that association with the Golgi apparatus is a mechanism to partition cPLA$_2\alpha$ from sites of action in quiescent endothelial cells, excluding it from substrate. This model is also consistent with the regulation of MEK/ERK by Sef, a molecular scaffold that resides in the Golgi complex. Binding to Sef allows MEK/ERK signaling to cytosolic substrates but excludes nuclear targets (Torii *et al.*, 2004).

We are the first to document the confluence-dependent relocation of cPLA$_2\alpha$ in any cell type. Association of cPLA$_2\alpha$ with distal components of the Golgi apparatus has been documented previously in epithelial cell lines but only in response to agents that elevate intracellular free Ca^{2+} (Evans *et al.*, 2001; Grewal *et al.*, 2003). Similar experiments using the Ca^{2+} ionophore, A23187, or histamine to elevate HUVEC intracellular free Ca^{2+}, does not induce cPLA$_2\alpha$ relocation to the Golgi apparatus but to the nuclear envelope (unpublished data). Stimulation-dependent relocation of cPLA$_2\alpha$ to the Golgi apparatus of epithelial cells is seen to place cPLA$_2\alpha$ in close apposition with the AA-metabolizing enzyme COX-1 (Grewal *et al.*, 2003). In HUVECs, costaining revealed no such functional association with COX-1, COX-2, or prostacyclin I synthase upon HUVEC confluence (unpublished data).

Golgi-associated cPLA$_2\alpha$ may play a role in Golgi architecture maintenance or protein trafficking. The PLA$_2$ family of enzymes are strongly implicated in these processes, specifically via membrane tubule formation (Brown *et al.*, 2003). Previous reports document disruption of the Golgi apparatus upon treatment of cells with PLA$_2$ inhibitors (de Figueiredo *et al.*, 1998, 1999; Drecktrah and Brown, 1999; Kuroiwa *et al.*, 2001). We see that in HUVECs, the sPLA$_2$ inhibitor, ONO-RS-082 (Hashimoto *et al.*, 2003), induces Golgi dispersal but pyrrolidine-1 does not (unpublished data). A previous study did report selective blocking of constitutive trafficking to the plasma membrane and retention of proteins in the endoplasmic reticulum of canine kidney cells overexpressing cPLA$_2\alpha$ (Choukroun *et al.*, 2000). But the possible role of cPLA$_2\alpha$ in specific trafficking events in confluent HUVECs has yet to be investigated.

cPLA$_2\alpha$ has previously been linked to cellular proliferation, as seen with studies in macrophages (Tashiro *et al.*, 2004). Although no studies of cPLA$_2\alpha$ activity were conducted, cPLA$_2\alpha$ was seen to regulate expression of the c-*myc* protooncogene in a B-Myb–dependent manner (Tashiro *et al.*, 2004). AA metabolites also induce endothelial cell proliferation (Dethlefsen *et al.*, 1994; Nie *et al.*, 2000; Antoniotti *et al.*, 2003), and cPLA$_2\alpha$ activity is involved in gene regulation (Pawliczak *et al.*, 2002), but little is known of how these

processes are regulated. cPLA$_2\alpha$ is the rate-limiting enzyme of AA evolution and is thus a prime candidate for controlling the synthesis of proproliferative AA metabolites in proliferating and nonproliferating cells. We find that in HUVECs, cPLA$_2\alpha$ activity is essential for proliferation and cell cycle entry and that these functions are mediated by its subcellular localization.

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