

## Chapter 5

### **Novel role of cPLA<sub>2</sub>α in membrane and actin dynamics**

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# Novel role of cPLA<sub>2</sub>α in membrane and actin dynamics

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**Abstract** Actin-directed processes such as membrane ruffling and cell migration are regulated by specific signal transduction pathways that become activated by growth factor receptors. The same signaling pathways that lead to modifications in actin dynamics also activate cPLA<sub>2</sub>α. Moreover, arachidonic acid, the product of cPLA<sub>2</sub>α activity, is involved in regulation of actin dynamics. Therefore, it was investigated whether cPLA<sub>2</sub>α plays a role in actin dynamics, more specifically during growth factor-induced membrane ruffling and cell migration. Upon stimulation of ruffling and cell migration by growth factors, endogenous cPLA<sub>2</sub>α and its active phosphorylated form were shown to relocate at protrusions of the cell membrane involved in actin and membrane dynamics. Inhibition of cPLA<sub>2</sub>α activity with specific inhibitors blocked growth factor-induced membrane and actin dynamics, suggesting an important role for cPLA<sub>2</sub>α in these processes.

**Keywords** cPLA<sub>2</sub>α · Actin · Arachidonic acid · Membrane dynamics · Ruffles · Lamellae · Leading edge · Migration

## Abbreviations

PLA<sub>2</sub> Phospholipase A<sub>2</sub>  
cPLA<sub>2</sub>α Cytosolic PLA<sub>2</sub> alpha  
HUVECs Human umbilical vein endothelial cells

## Introduction

Actin plays an essential role in a wide variety of dynamic events in mammalian cells, amongst them cell motility, cytokinesis, and cell–cell and cell–matrix interactions [1–4]. Actin dynamics are determined by the activities of a large number of actin-binding proteins. Extracellular stimuli, like growth factors and extracellular matrix components, stimulate intracellular signal transduction cascades that activate actin-binding proteins resulting in changes in local actin dynamics. These changes result in morphological changes like the formation of membrane ruffles and leading edges. In addition to changed actin dynamics upon growth factor addition or cell spreading, many signal transduction components are bound to actin, indicating the mutual relationship [5–7].

An important molecule involved in the regulation of cell metabolism is the unsaturated fatty acid arachidonic acid, which serves as a precursor for leukotriene and prostaglandin synthesis. However, arachidonic acid release has also been related to the regulation of actin dynamics [8]. In HeLa cells, an increase in arachidonic acid production was shown after cell attachment. This results in both actin polymerization and actin bundling via two distinct pathways involving lipoyxygenase and cyclooxygenase [9, 10]. These enzymes metabolize arachidonic acid, resulting in the release of lipid second messengers that activate actin-binding proteins and consequently cause a change in actin

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dynamics. Additional to its role in cell spreading and immobilization, arachidonic acid was also shown to stimulate cell motility via the production of prostaglandins by cyclooxygenase. Prostaglandins play a role in actin cytoskeletal regulation and migration in a variety of ways. For example, stimulation of cells by chemokines resulted in an increase of prostaglandins and consequently in an increase of actin polymerization [11]. Moreover, several forms of stimulated migration require arachidonic acid metabolites [12, 13]. In addition, dissolution of adhesion structures like podosomes is dependent on the production of prostaglandins [14]. The role of arachidonic acid-mediated signaling in the regulation of small GTPases that modulate actin remodeling has been illustrated in several studies. In Swiss 3T3 fibroblasts, various leukotrienes induce stress fiber formation via a Rho-dependent process [15]. In Rat2 fibroblasts, arachidonic acid induces membrane ruffling and this is prevented when a dominant-negative mutant of Rac1 (Rac1N17) is expressed, indicating that Rac1 is activated downstream of arachidonic acid-mediated signaling [16]. Since Rac was also shown to act upstream of arachidonic acid release [15], it is possible that arachidonic acid and Rac1 act in a positive feedback loop.

The release of arachidonic acid in cells results from cleavage of membrane phospholipids at the sn-2 position by phospholipases A<sub>2</sub> (PLA<sub>2</sub>). The large family of PLA<sub>2</sub> consists of several sub-families like the secretory PLA<sub>2S</sub> (sPLA<sub>2</sub>), the intracellular calcium independent PLA<sub>2S</sub> (iPLA<sub>2</sub>), and the group of cytosolic PLA<sub>2S</sub> (cPLA<sub>2</sub>) including cPLA<sub>2 $\alpha$</sub>  [17]. Interestingly, cPLA<sub>2 $\alpha$</sub>  is highly specific for phospholipids carrying arachidonic acid at the sn-2 position and is therefore considered as the rate-limiting enzyme in receptor-mediated arachidonic acid release. cPLA<sub>2 $\alpha$</sub>  requires Ca<sup>2+</sup> to translocate from the cytosol to the membrane through its CaLB (calcium-lipid binding) domain, although Ca<sup>2+</sup> is not directly involved in catalysis [18]. The activation of cPLA<sub>2 $\alpha$</sub>  results from a phosphorylation by MAP kinase (for review, see [18]). This MAP kinase on its turn is activated by the same signal transduction cascades (growth factors and extracellular matrix components) that lead to modifications in actin dynamics. Therefore, it is tempting to suggest that cPLA<sub>2 $\alpha$</sub>  is one of the key enzymes involved in local actin dynamics that are involved in the formation of membrane ruffles, and in cell motility.

Therefore, we have studied the localization of cPLA<sub>2 $\alpha$</sub>  in fibroblasts during stimulation with platelet-derived growth factor (PDGF). Stimulation with PDGF introduces circular dorsal ruffles and lamellipodia. Here, we show that endogenous cPLA<sub>2 $\alpha$</sub>  and its active, phosphorylated form are localized at the cell membrane in response to PDGF stimulation. They localize specifically to areas at the cell membrane that are reshaped by actin during PDGF stimulation. Furthermore, endogenous cPLA<sub>2 $\alpha$</sub>  and its active

phosphorylated form are shown to localize at leading edges in primary human umbilical vein endothelial cells (HUVECs) and migrating mouse fibroblasts. Interestingly, inhibition of cPLA<sub>2 $\alpha$</sub>  activity with specific inhibitors blocked PDGF-induced membrane and actin dynamics, suggesting a role for cPLA<sub>2 $\alpha$</sub>  in these processes.

## Materials and methods

### Cell culture

Human umbilical vein endothelial cells were isolated according to Jaffe [19] and used from passage 1 to 4. HUVECs were cultured onto fibronectin-coated surfaces and grown in endothelial basal medium (EBM-2) supplemented with 2% fetal bovine serum (FBS) (Gibco, Paisley, UK), endothelial cell growth supplements (EGM-2) (Cambrex, NJ, USA), and 5 mM L-glutamine (Invitrogen). C3H10T1/2 mouse fibroblasts were cultured in HEPES (25 mM) buffered Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 7.5% FBS and 5 mM L-glutamine. Cells were grown at 37°C (humidified atmosphere, 5% CO<sub>2</sub>).

HUVECs were seeded on glass coverslips at 10,000 cells/cm<sup>2</sup>, coated with fibronectin and allowed to grow for 2 or more days to obtain sub-confluent monolayers for localization studies and confluent monolayers for the scratch assays.

Fibroblasts were plated on glass coverslips at 12,000 cells/cm<sup>2</sup> and allowed to grow for 24 h. For PDGF stimulation experiments, cells were subsequently growth factor-starved for 24 h at 37°C and stimulated with 20 ng/ml of PDGF-BB (Upstate, Hampshire, UK). Treatment with cPLA<sub>2 $\alpha$</sub>  inhibitors Pyrrolidine-2 (2  $\mu$ M) and Wyeth-1 (5  $\mu$ M) was performed in serum-free medium. Both inhibitors were kind gifts from Prof. Dr. M. Gelb (University of Washington, Seattle, USA).

### Immunofluorescent labeling

Cells were fixed in 3.2% formaldehyde for 15 min, washed twice with PBS, permeabilized for 5 min in PBS containing 0.2% Triton X-100, followed by two washes in PBS, and incubated for 10 min with 50 mM glycine in PBS. After washing twice with PBS containing 0.2% gelatin, cells were incubated overnight with goat anti-cPLA<sub>2</sub> (Santa Cruz; sc-1724) or for 1 h with rabbit anti-phospho-cPLA<sub>2</sub> (ser505) (Santa Cruz; sc-34391). Then, the cells were washed six times with PBS containing 0.2% gelatin and incubated for 1 h with tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-conjugated phalloidin (Sigma) combined with goat-anti-rabbit-Alexa488 or donkey-anti-

goat-Alexa488 (Molecular Probes) and washed six times PBS containing 0.2% gelatin. In some samples, this was followed by a DAPI staining for 10 min. Finally, cells were mounted in Mowiol-DABCO.

#### Transfections

Transfections with GFP-cPLA<sub>2</sub> $\alpha$  were performed using Lipofectin (Invitrogen) according to the manufacturer's instructions. This construct, kindly provided by Dr. C. Lenoir (Inserm Unit 538, Paris, France), was checked by sequencing.

#### Scratch assay

HUVECs were seeded on fibronectin-coated glass coverslips at 10,000 cells/cm<sup>2</sup> (~10–20% confluency) and allowed to grow for 7 days with regular change of medium. Cells were gently scratched from the substrate using a pipette tip. The remaining cells were washed with culture medium and allowed to reoccupy the wounded area for various time intervals. Mouse C3H10T1/2 fibroblasts were seeded at 20,000 cells/cm<sup>2</sup> and allowed to grow to confluency before performing the scratch assay as described above.

#### Acquisition of images

Samples were studied with a Leitz microscope (Orthoplan Flu 043944) equipped with Leitz oil objectives  $\times 40$  NA 1.3 and  $\times 63$  NA 1.4. Images were acquired using a Leica CCD camera (model DC350F; Leica Microsystems) using Leica Image Manager 50 software. Confocal pictures were obtained using a Zeiss CLSM (Pascal 510) equipped with Zeiss water objectives ( $\times 40$  NA 1.3 and  $\times 63$  NA 1.4). Images were processed with Adobe Photoshop 8.0.

## Results

cPLA<sub>2</sub> $\alpha$  is recruited to PDGF-induced dorsal membrane ruffles and lamellae in C3H10T1/2 fibroblasts

Mouse C3H10T1/2 fibroblasts provide an excellent model cell line to study actin dynamics. When these cells are serum-starved for 24 h, they are spread and exhibit a clear network of actin stress fibers as shown by staining of F-actin with phalloidin (Fig. 1b). Addition of PDGF-BB, (20 ng/ml for 15 min at 37°C), results in the partial disappearance of stress fibers, and the formation of large circular dorsal membrane ruffles, characterized by a ring of F-actin beneath the membrane (Fig. 1e). Successive optical sections obtained by confocal scanning light microscopy confirmed that these ruffles extend from the dorsal plasma

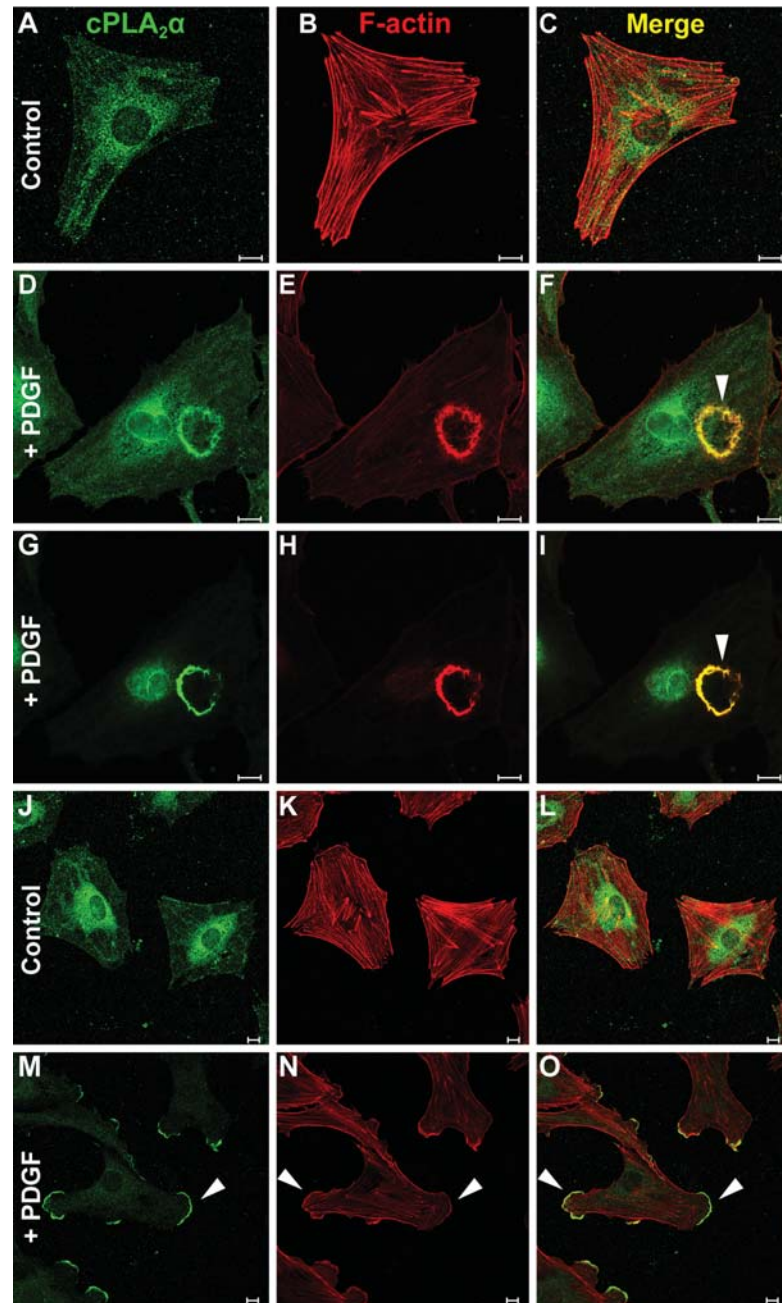
membrane (Fig. 1e, h). As reported previously, ruffle formation is dependent upon actin polymerization and is highly transient [20]. Since actin polymerization is induced by growth factors [21] and is dependent on the production of arachidonic acid metabolites [8, 9], it is tempting to suggest that cPLA<sub>2</sub> $\alpha$  plays a role in actin dynamics, because cPLA<sub>2</sub> $\alpha$  is also activated by growth factors [22–24]. Therefore, the subcellular localization of endogenous cPLA<sub>2</sub> $\alpha$  was determined upon PDGF-induced changes in actin polymerization. Cells were labeled with an antibody specific for cPLA<sub>2</sub> $\alpha$  as characterized previously [25]. As shown in Fig. 1a, in serum-starved fibroblasts, cPLA<sub>2</sub> $\alpha$  is mainly detected in the perinuclear area and throughout the cytoplasm. Upon addition of PDGF-BB to the cells, cPLA<sub>2</sub> $\alpha$  is recruited to newly formed circular dorsal ruffles (Fig. 1d, g) as demonstrated by the colocalization of cPLA<sub>2</sub> $\alpha$  and PDGF-induced F-actin structures (Fig. 1f, i). This result indicates that upon growth factor stimulation, cPLA<sub>2</sub> $\alpha$  is rapidly and specifically recruited at areas of actin polymerization at the plasma membrane.

In addition to the formation of membrane ruffles, C3H10T1/2 cells also form lateral lamellae upon addition of PDGF (Fig. 1n). Whether the cells form lamellae or circular ruffles is determined by the PDGF concentration used. At concentrations  $>20$  ng/ml, abundant circular ruffling takes place, whereas at concentrations in the range of 1–5 ng/ml, less circular dorsal ruffles occur and more lamellae are formed. The formation of lamellae is related to cell spreading and/or cell motility. As clearly shown in Fig. 1n, treatment of cells with 20 ng/ml PDGF for 10 min at 37°C results in the formation of lamellae characterized by a lateral band of newly formed F-actin (Fig. 1n) in which cPLA<sub>2</sub> $\alpha$  accumulates (Fig. 1m), while the perinuclear localization of cPLA<sub>2</sub> $\alpha$ , as observed in untreated cells (Fig. 1j), disappeared. Together these data clearly demonstrate that PDGF-induced actin remodeling is accompanied by a recruitment of cPLA<sub>2</sub> $\alpha$  to sites of new actin polymerization and membrane dynamics.

cPLA<sub>2</sub> $\alpha$  is localized at leading edges of migrating mouse fibroblasts

To determine whether the recruitment of cPLA<sub>2</sub> $\alpha$  at sites of newly formed F-actin represents a feature common to other cellular processes that depend on actin polymerization, the localization of cPLA<sub>2</sub> $\alpha$  was determined in the context of cell migration. We performed a commonly used wound-healing assay, also called scratch assay. In this assay, a confluent monolayer is gently scratched with a pipette tip to introduce a wound. Cells located at the border of the wound respond by polarizing towards the wound, emit protrusions, and start to migrate into the wounded area which is finally healed. Directly after scratching

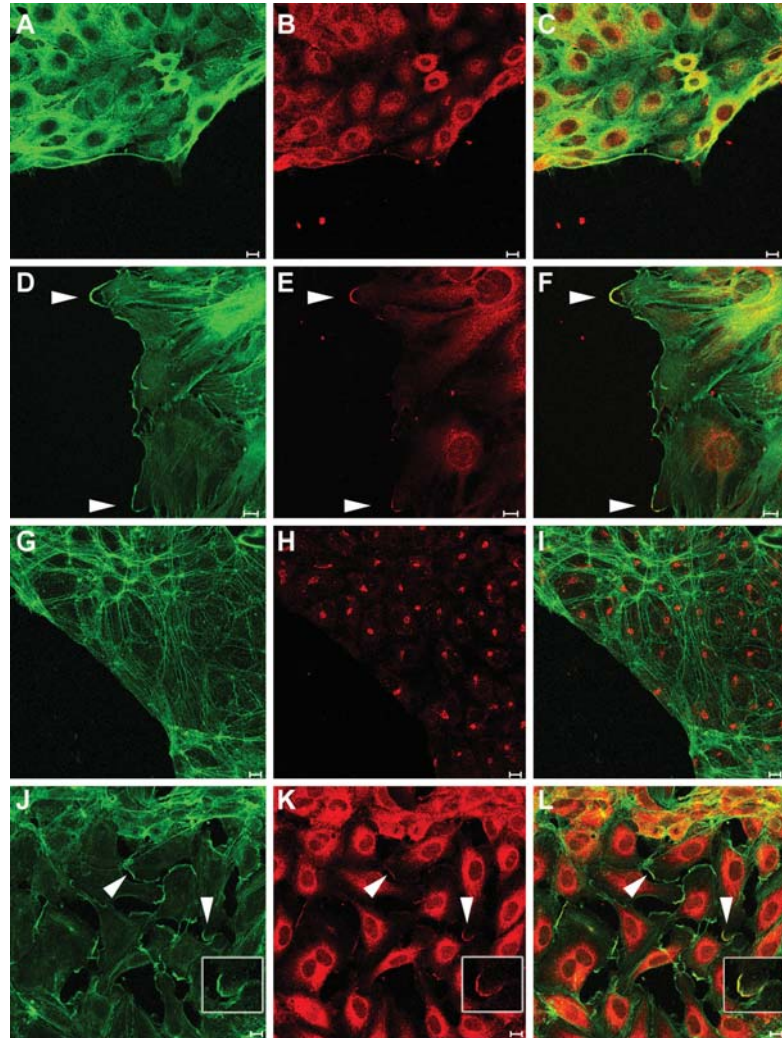
**Fig. 1** Relocalization of cPLA<sub>2</sub> $\alpha$  to dorsal membrane ruffles and to lamellae in C3H10T1/2 fibroblasts upon PDGF stimulation. Fibroblasts were serum deprived for 24 h and subsequently incubated in the presence (d–i, m–o) or absence (control: a–c, j–l) of 20 ng/ml PDGF-BB for 10 min (j–o) or 15 min (a–i) at 37°C. Cells were fixed and subjected to immunofluorescent staining (cPLA<sub>2</sub> $\alpha$  green, F-actin red) and samples were analyzed by confocal microscopy. In non-stimulated cells, cPLA<sub>2</sub> $\alpha$  is found in the perinuclear area and in the cytoplasm (a, j) and F-actin is organized in large stress fibers (b, k). Stimulation with PDGF-BB results in the partial disappearance of stress fibers (e, h, n), in the formation of circular dorsal ruffles (e, h, f, i, arrowheads) to which cPLA<sub>2</sub> $\alpha$  is recruited (d, g), and in the formation of lamellae (n, o, arrowheads) in which cPLA<sub>2</sub> $\alpha$  accumulates (m, arrowhead). Panels d–f and g–i are images taken from the same cell but d–f is a section at 0.88  $\mu$ m through the cytoplasm, the nucleus, and the base of the circular ruffle whereas g–i represents a section at 3.08  $\mu$ m through the top of the nucleus and the protrusion of the circular ruffle. Bar 10  $\mu$ m



monolayers of C3H10T1/2, cPLA<sub>2</sub> $\alpha$  was mainly cytoplasmic and perinuclear in cells lining the wound (Fig. 2b). Seven hours after the scratch, cells located at the border of

the wound displayed protrusions of the plasma membrane in which cPLA<sub>2</sub> $\alpha$  was detected (Fig. 2e, f). This membrane localization was restricted to the leading edge of the

**Fig. 2** cPLA<sub>2</sub> $\alpha$  localizes at the leading edge in migrating mouse fibroblasts and HUVECs. Confluent monolayers of C3H10T1/2 fibroblasts were subjected to the scratch assay. Cells were fixed immediately after scratch (a–c) or 7 h after scratch (d–f) and subjected to immunofluorescent staining (cPLA<sub>2</sub> $\alpha$  red, F-actin green) and samples were analyzed by confocal microscopy. In confluent mouse fibroblasts, cPLA<sub>2</sub> $\alpha$  is found mainly cytoplasmic directly after scratch (b). After 7 h, cells located at the border of the wound have migrated in the wounded area and cPLA<sub>2</sub> $\alpha$  has translocated from the cytoplasm to the newly formed leading edges (e). Confluent monolayers of HUVEC were subjected to the scratch assay and processed for immunofluorescence immediately after scratch (g,h) or 22 h after scratch (j–l). In confluent HUVECs cPLA<sub>2</sub> $\alpha$  is located at the Golgi directly after scratch (h). At 22 h after scratch, in cells that have migrated into the wounded area, cPLA<sub>2</sub> $\alpha$  is more abundant in the cytoplasm and has translocated to the newly formed leading edges. *Insert* shows a leading edge where cPLA<sub>2</sub> $\alpha$  colocalizes with F-actin. Bar 10  $\mu$ m

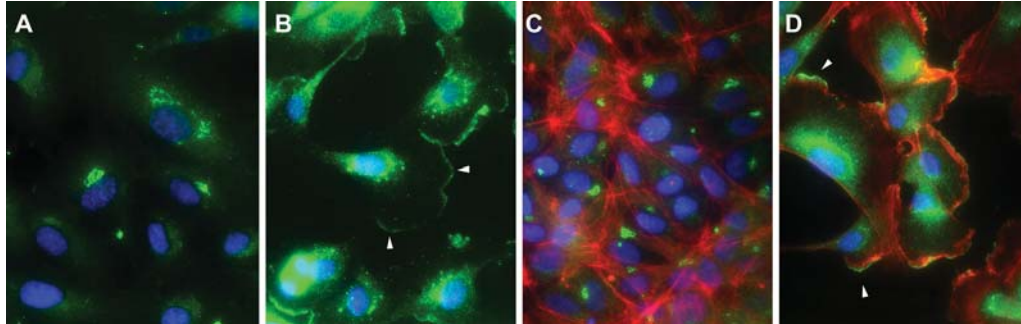


migrating cells. These edges are enriched in F-actin (Fig. 2d) and in ARP 3 indicating active actin remodeling (data not shown). At later time points, cPLA<sub>2</sub> $\alpha$  was detected at leading edges of cells that had migrated into the wound. In conclusion, these data show that in migrating cells, endogenous cPLA<sub>2</sub> $\alpha$  is recruited to the leading edges of migrating cells, i.e. at sites of active actin remodeling.

cPLA<sub>2</sub> $\alpha$  is localized at leading edges of migrating primary human endothelial cells

To determine whether the recruitment of cPLA<sub>2</sub> $\alpha$  at sites of actin polymerization as observed in fibroblasts is a

conserved phenomenon, the relationship between cPLA<sub>2</sub> $\alpha$  and F-actin was studied in primary endothelial cells of human origin, i.e. HUVECs. It has been demonstrated that cPLA<sub>2</sub> $\alpha$  translocates to the Golgi complex in response to confluency in HUVECs [25, 26]. A scratch assay was performed on a confluent monolayer of HUVECs and the localization of cPLA<sub>2</sub> $\alpha$  and F-actin was examined. Immediately after scratch, cPLA<sub>2</sub> $\alpha$  was localized at the Golgi apparatus in all cells (lining the wound or not) (Fig. 2h). Four hours after the scratch, cells located at the border of the wound displayed protrusions of the plasma membrane in which cPLA<sub>2</sub> $\alpha$  was detected and where cPLA<sub>2</sub> $\alpha$  locally co-localized with F-actin (data not



**Fig. 3** Subcellular localization of endogenous cPLA<sub>2</sub>α in non-confluent and confluent HUVEC. Non-confluent or confluent HUVECs were stained for cPLA<sub>2</sub>α (green), F-actin (red) and the nuclei were stained with DAPI (blue). In confluent HUVECs (a,c) cPLA<sub>2</sub>α is

localized at the Golgi; in non-confluent cells (b,d) cPLA<sub>2</sub>α is localized both in the perinuclear area and at specific areas of the plasma membrane (arrowheads) corresponding to sites of actin polymerization

shown). Subsequently migrating cells reoccupy the wounded area. Twenty-two hours after scratch, migrating cells filled up the wounded area (Fig. 2j, k, l) and displayed cPLA<sub>2</sub>α staining at the cell membrane (Fig. 2k) and leading edge (Fig. 2k insert), where it locally colocalizes with F-actin. In contrast to confluent cells (Fig. 2h), in the wounded area, cPLA<sub>2</sub>α is localized primarily in the cytoplasm and also at the cell periphery (Fig. 2k). This latter localization may both be related to cell spreading and cell migration.

To determine whether the recruitment of cPLA<sub>2</sub>α to leading edges of migrating HUVECs was not solely occurring in the context of the scratch assay, the localization of cPLA<sub>2</sub>α was compared in non-confluent versus confluent cultures. In contrast to confluent cultures, in which HUVECs are growth-arrested by contact inhibition and are unable to migrate, cells in non-confluent cultures do proliferate and migrate. Both migration and cell proliferation are dependent on actin polymerization, and therefore non-confluent cells display numerous and extended membrane areas where actin polymerization takes place. In confluent HUVECs, cPLA<sub>2</sub>α is located at the Golgi and does not co-localize with F-actin (Fig. 3c). In contrast, in non-confluent cells that display a migratory phenotype, cPLA<sub>2</sub>α is present at the cell periphery in restricted membrane areas which correspond to sites of actin polymerization (Fig. 3b, d).

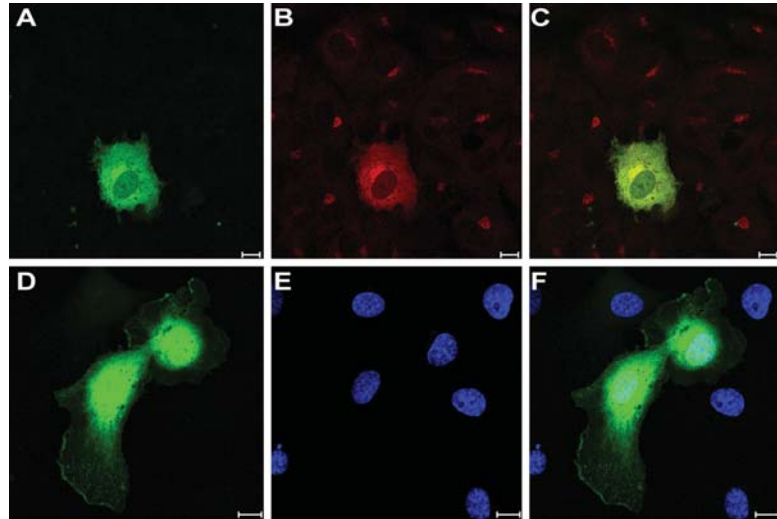
The goat anti-cPLA<sub>2</sub>α antibody used in our study is specific for cPLA<sub>2</sub>α (confirmed by western blotting of HUVEC and C3H10T1/2 lysates (data not shown and [26–28])). However, to further confirm the presence of cPLA<sub>2</sub>α at the cell membrane, we examined the subcellular localization of GFP-tagged human cPLA<sub>2</sub>α in transiently transfected HUVECs. In non-confluent cells, GFP-cPLA<sub>2</sub>α

was localized at the cell membrane, in addition to its cytoplasmic and perinuclear localization (Fig. 4d, f). This confirms our data on the localization of endogenous cPLA<sub>2</sub>α at the cell membrane in leading edges. Additionally, GFP-cPLA<sub>2</sub>α was detected at the Golgi apparatus of confluent HUVECs (Fig. 4a), showing that GFP-cPLA<sub>2</sub>α exhibits the same translocation property as endogenous cPLA<sub>2</sub>α. Finally, GFP-cPLA<sub>2</sub>α was recognized by the goat anti-cPLA<sub>2</sub> antibody (Fig. 4b, c).

Phosphorylated cPLA<sub>2</sub>α is present in circular dorsal ruffles and leading edges

In view of our data demonstrating the recruitment of cPLA<sub>2</sub>α at sites of actin remodeling and the possible involvement of the arachidonic acid pathway in actin remodeling, it is tempting to suggest that the enzymatic activity of cPLA<sub>2</sub>α is important for this process. Since phosphorylation of cPLA<sub>2</sub>α at serine residue 505 by mitogen-activated protein kinases (MAPK) is involved in cPLA<sub>2</sub>α activation [29], we studied the localization of phosphorylated cPLA<sub>2</sub>α using an antibody specific for Ser505-phosphorylated cPLA<sub>2</sub>α. In cultures of serum-starved mouse fibroblasts, phospho-S505-cPLA<sub>2</sub>α was detected in the cytoplasm and in the nucleus of most cells (Fig. 5a). After stimulation with PDGF for 15 min, phospho-S505-cPLA<sub>2</sub>α was found in circular dorsal ruffles (Fig. 5d). Additionally, phospho-S505-cPLA<sub>2</sub>α was detected at leading edges of migrating mouse fibroblasts (Fig. 5g) and of migrating HUVECs (Fig. 5h). In conclusion, cPLA<sub>2</sub>α and phospho-S505-cPLA<sub>2</sub>α were found at protrusions of the cell membrane where actin remodeling takes place, indicating that cPLA<sub>2</sub>α activity plays an active role in this process.

**Fig. 4** Subcellular localization of GFP-cPLA<sub>2</sub> $\alpha$  in non-confluent and confluent HUVECs. Confluent (a–c) and non-confluent (d–f) HUVECs were transfected with the GFP-cPLA<sub>2</sub> $\alpha$  construct. Confluent cells were fixed and stained with the goat polyclonal anti-cPLA<sub>2</sub> $\alpha$  (green GFP-cPLA<sub>2</sub> $\alpha$ , red cPLA<sub>2</sub> $\alpha$ ). a–c show that the GFP-cPLA<sub>2</sub> $\alpha$  signal (a) is recognized by the antibody against cPLA<sub>2</sub> $\alpha$  (b). Non-confluent HUVEC transfected with GFP-cPLA<sub>2</sub> $\alpha$  were stained with DAPI (blue) to visualize the nuclei. In these cells, GFP-cPLA<sub>2</sub> $\alpha$  was mainly located in the cytoplasm and in the perinuclear area and was also found at specific areas of the plasma membrane. Bar 10  $\mu$ m



Inhibition of cPLA<sub>2</sub> $\alpha$  enzymatic activity prevents the formation of PDGF-induced circular ruffles and lamellae

To determine whether the enzymatic activity of cPLA<sub>2</sub> $\alpha$  is required for actin remodeling, we used the cPLA<sub>2</sub> $\alpha$  inhibitors Pyrrolidine-2 and Wyeth-1 characterized previously [30]. These non-structurally related inhibitors do not interfere with the enzymatic activity of calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) or secreted PLA<sub>2</sub>s (sPLA<sub>2</sub>s) [30]. Serum-starved mouse fibroblasts were pre-treated with Pyrrolidine-2 or Wyeth-1 or mock-treated for 45 min. Pretreatment of cells with these inhibitors did not affect cellular morphology, nor did it affect the localization of cPLA<sub>2</sub> $\alpha$  compared to mock-treated cells (Fig. 6a, g). Subsequently, cells were treated for 15 min with 20 ng/ml PDGF-BB, they were fixed and F-actin was stained with phalloidin in parallel to cPLA<sub>2</sub> $\alpha$  immunodetection. Interestingly, when cells were pre-treated with either inhibitor, PDGF treatment did not induce circular dorsal ruffles nor lamellae (Fig. 6g–i), whereas in mock-treated cells, lamellae (data not shown) and dorsal ruffles containing cPLA<sub>2</sub> $\alpha$  were induced by PDGF (Fig. 6d–f, arrowheads). These data show that inhibition of cPLA<sub>2</sub> $\alpha$  enzymatic activity prevents PDGF-induced actin remodeling.

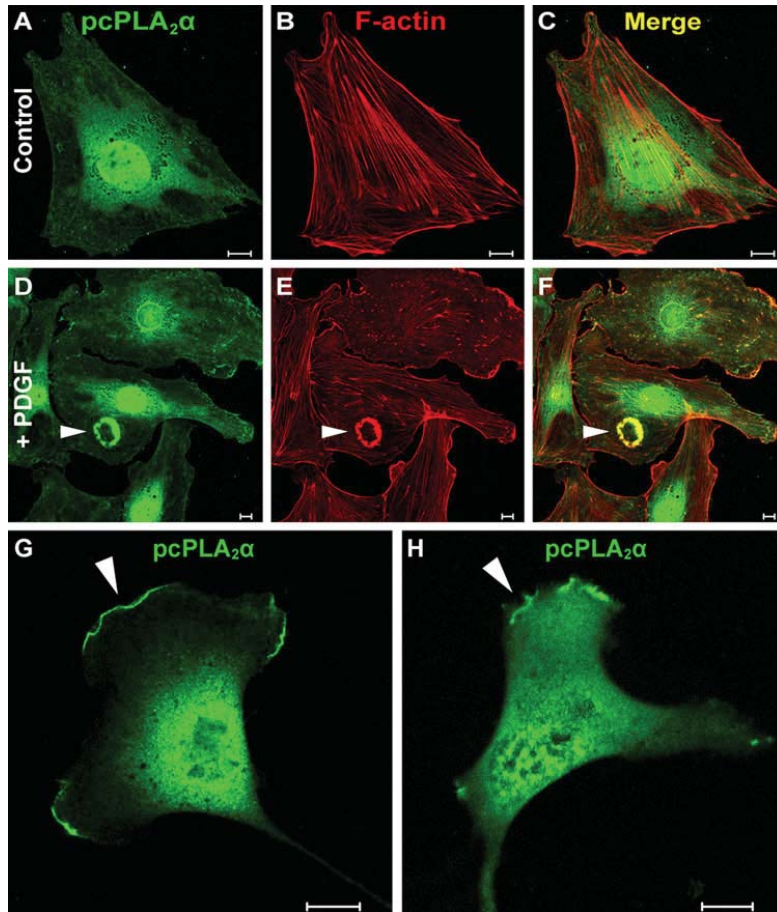
## Discussion

Arachidonic acid release has been demonstrated to play a role in actin polymerization. Since activation of cPLA<sub>2</sub> $\alpha$  results in the preferential release of arachidonic acid, it is

tempting to suggest that cPLA<sub>2</sub> $\alpha$  is a key enzyme involved in the regulation of local actin dynamics. Therefore, we have studied the localization of cPLA<sub>2</sub> $\alpha$  in fibroblasts and HUVECs in relation to induced actin dynamics. Endogenous cPLA<sub>2</sub> $\alpha$  was shown to localize at the plasma membrane in both migrating mouse fibroblasts and migrating HUVEC. Moreover, the phosphorylated form of cPLA<sub>2</sub> $\alpha$  (on Ser505) was found at the plasma membrane in both cell types. Both cPLA<sub>2</sub> $\alpha$  and phospho-S505-cPLA<sub>2</sub> $\alpha$  localize at protrusions of the cell membrane that show active actin and membrane remodeling such as circular dorsal ruffles, lamellipodia, and leading edges. Interfering with the activity of cPLA<sub>2</sub> $\alpha$  in mouse fibroblasts resulted in the blocking of PDGF-induced actin and membrane dynamics.

cPLA<sub>2</sub> $\alpha$  is present at the cell membrane in a spatio-temporal manner. Upon PDGF stimulation, cPLA<sub>2</sub> $\alpha$  docks at specific sites at the cell membrane, namely at lamellipodia and ruffles. Upon stimulation of migration, cPLA<sub>2</sub> $\alpha$  docks at the leading edge of motile cells. Also here, the localization of cPLA<sub>2</sub> $\alpha$  at the cell membrane is local and temporal. This indicates that its docking at the cell membrane is tightly regulated. It was suggested that cPLA<sub>2</sub> $\alpha$  interacts with membranes via PtdIns(4,5)P<sub>2</sub>. It was shown that PtdIns(4,5)P<sub>2</sub> stimulates cPLA<sub>2</sub> $\alpha$  activity independent of calcium suggesting an interaction between PtdIns(4,5)P<sub>2</sub> and cPLA<sub>2</sub> $\alpha$  allowing cPLA<sub>2</sub> $\alpha$  to reach its substrate at membranes [31]. PtdIns(4,5)P<sub>2</sub> is primarily located in the cytoplasmic leaflet of the plasma membrane. Interestingly, it has been demonstrated that a variety of signal transduction proteins, including growth factor receptors, reside in areas of high actin polymerization,





**Fig. 5** Localization of phospho-S505-cPLA<sub>2</sub>α to circular dorsal ruffles and at leading edges. Relocalization of phospho-S505-cPLA<sub>2</sub>α to dorsal ruffles in C3H10T1/2 fibroblasts after PDGF stimulation. Fibroblasts were serum-deprived for 24 h and subsequently incubated in the presence (d–f) or absence (control a–c) of 20 ng/ml PDGF-BB for 15 min at 37°C. Cells were fixed and subjected to immunofluorescent staining (phospho-S505-cPLA<sub>2</sub>α green, F-actin red) and samples were analyzed by confocal microscopy. In non stimulated cells, staining for phospho-S505-cPLA<sub>2</sub>α is found in the cytoplasm and frequently in the nucleus (a) and F-actin is organized in large stress fibers (b). Stimulation with PDGF-BB results in the formation

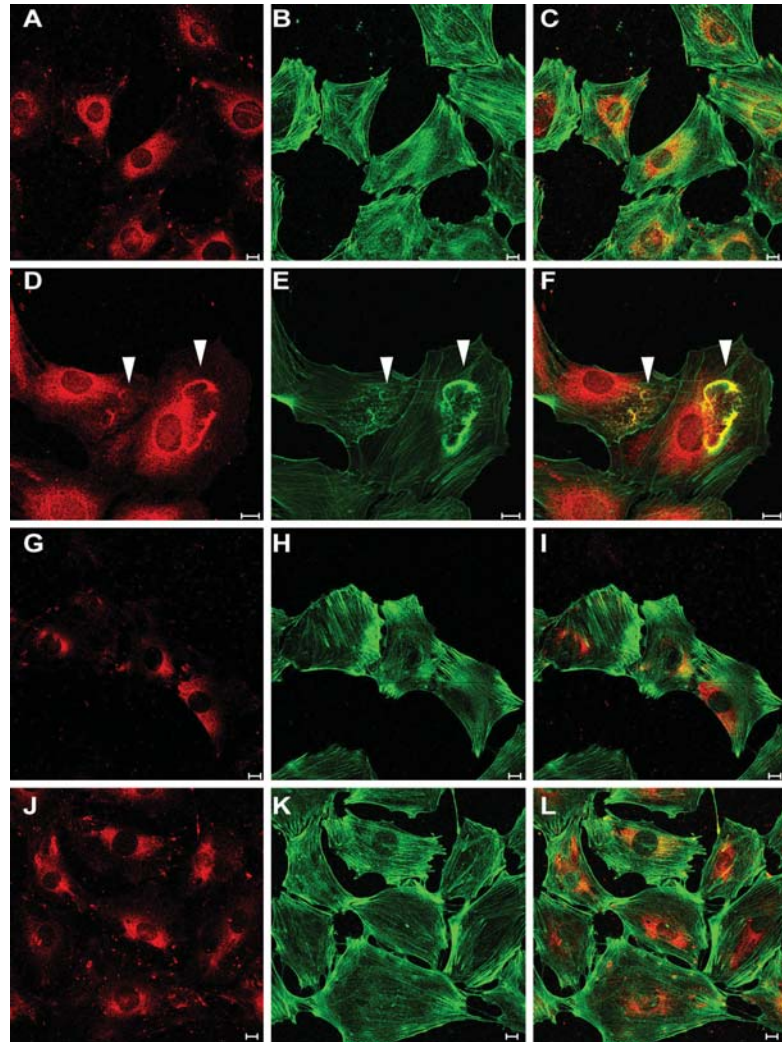
of circular dorsal ruffles (arrowhead) and the partial disappearance of stress fibers (e). Phospho-S505-cPLA<sub>2</sub>α translocates to the newly formed circular dorsal ruffles (d, arrowhead). Localization of phospho-S505-cPLA<sub>2</sub>α at the leading edges of migrating mouse fibroblasts and migrating HUVECs. Non-confluent mouse fibroblasts C3H10T1/2 (g) or HUVEC (h) were fixed and subjected to immunofluorescent staining with the anti-phospho-S505-cPLA<sub>2</sub>α antibody. Samples were analyzed by confocal microscopy. In both cell types, phospho-S505-cPLA<sub>2</sub>α is present at leading edges (arrowheads). Bar 10 μm

such as membrane ruffles [7]. Receptor activation can increase or decrease local PtdIns(4,5)P<sub>2</sub> concentrations via the local activation of kinases and phosphatases like PI5K, phospholipase C, phospholipase D, and PI3K [32]. By doing so, receptor activation might also regulate the docking of cPLA<sub>2</sub>α at the cell membrane upon growth factor stimulation. Interestingly PtdIns(4,5)P<sub>2</sub> was shown

to be enriched locally at the leading edge [33] and phosphatidylinositide metabolism was shown to be involved in ruffle formation [34–36].

The local presence of cPLA<sub>2</sub>α and its phosphorylated active form at sites of actin and membrane remodeling indicates a role for cPLA<sub>2</sub>α in regulating membrane dynamics. This is also suggested by blocking of PDGF-induced actin

**Fig. 6** Inhibition of cPLA<sub>2</sub> $\alpha$  enzymatic activity with Pyrrolidine-2 or Wyeth-1 prevents PDGF-induced ruffling in C3H10T1/2 mouse fibroblasts. Fibroblasts were serum-deprived for 24 h and subsequently incubated in the absence (a–c) or presence (d–f) of 20 ng/ml PDGF-BB for 15 min at 37°C. Cells were fixed and subjected to immunofluorescent staining (cPLA<sub>2</sub> $\alpha$  red, F-actin green) and samples were analyzed by confocal microscopy. In non-stimulated cells cPLA<sub>2</sub> $\alpha$  is found in the cytoplasm (a) and F-actin is organized in large stress fibers (b). Incubation in the presence of PDGF results in the partial disappearance of stress fibers and in the formation of membrane ruffles (e) to which cPLA<sub>2</sub> $\alpha$  translocates (d, arrowheads). Incubation of cells for 45 min with either Pyrrolidine-2 (g–i) or Wyeth-1 (j–l) before and during stimulation with PDGF prevents the formation of PDGF-induced dorsal ruffles. Bar 10  $\mu$ m



and membrane dynamics after exposure to the cPLA<sub>2</sub> $\alpha$  inhibitors Pyrrolidine-2 and Wyeth-1. Of note, these inhibitors also block the enzymatic activity of another cPLA<sub>2</sub> from group IV, namely cPLA<sub>2</sub> $\zeta$  [30]. This enzyme can release arachidonic acid from membrane phospholipids but, contrary to cPLA<sub>2</sub> $\alpha$ , it does not have specificity for arachidonic acid versus other fatty acids [30]. However, in C3H10T1/2, cPLA<sub>2</sub> $\zeta$  is not expressed, as confirmed by RT-PCR (our unpublished data). This observation strengthens our inhibitors-based results, reinforcing the idea that cPLA<sub>2</sub> $\alpha$  plays a major role in regulating actin dynamics.

The local presence of the active form of cPLA<sub>2</sub> $\alpha$  might indicate a local production of arachidonic acid by cPLA<sub>2</sub> $\alpha$ . Subsequently, arachidonic acid might affect locally the behavior of actin via arachidonic metabolites. Arachidonic acid release has been previously related to the behavior of actin. During cell spreading, actin is changing the cytoskeleton and thereby changing the cell morphology. In HeLa cells, an increase in arachidonic acid production was shown after cell attachment. This results in both actin polymerization and actin bundling via two distinct pathways involving lipoxygenase and cyclooxygenase [9, 10].

Lipoxygenase metabolizes arachidonic acid and via a cascade of lipid second messengers protein kinase C epsilon (PKC $\epsilon$ ) is subsequently activated which triggers actin polymerization leading to cell spreading. Cyclooxygenase generates prostaglandins resulting in the activation of cyclic AMP-dependent protein kinase A (PKA) that induces actin bundling. Moreover, PKA was shown to play a role in the actin cytoskeleton regulation in a variety of ways (for review, see [37]). So, an increase of arachidonic acid causes an increase in actin polymerization and bundling during cell spreading by activating actin-binding proteins like PKA and PKC $\epsilon$ . Furthermore, arachidonic acid and some of its metabolites can activate signaling pathways leading to the activation of small GTPases such as Rac1 and Rho [15, 16], ultimately resulting in actin remodeling. It has long been known that arachidonic acid and some of its metabolites can bind directly to and regulate the activity of specific GTPase-activating proteins (GAPs), which are regulators of small GTPases [38, 39]. Altogether, it is tempting to suggest that the local production of arachidonic acid and of its metabolites could trigger signaling pathways resulting in the local remodeling of actin.

In addition, cPLA $_{2\alpha}$  is able to change the local characteristics of the plasma membrane by interacting with membrane phospholipids. cPLA $_{2\alpha}$ -mediated hydrolysis of cellular membranes results in the production of lysophospholipids within the lipid bilayer and the lysophospholipids thus produced change the plasma membrane microviscosity. A change in the plasma membrane microviscosity by lysophospholipids was shown to affect cell migration [40]. Plasma membrane microviscosity has an important role on actin dynamics and thereby regulates cell motility [40, 41]. It was suggested that an increase in the plasma membrane microviscosity in lamellipodia might result in a local increase of actin-directed movement [41]. Our results show active cPLA $_{2\alpha}$  at sites of active actin remodeling, and cPLA $_{2\alpha}$  might here locally direct the behavior of actin by changing the plasma membrane microviscosity.

Altogether, our data show that cPLA $_{2\alpha}$  is specifically recruited at sites of actin and membrane remodeling in different cellular processes. Since this phenomenon occurs in mouse fibroblasts and in primary human endothelial cells, it is likely to represent a general property of cPLA $_{2\alpha}$ . However, further research is required to fully elucidate the role of cPLA $_{2\alpha}$  in the above-mentioned processes.

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