Chapter 4

Co-localization of the PDGF β-receptor and actin during PDGF stimulation in mouse fibroblasts

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Co-localization of the PDGF β-Receptor and Actin during PDGF Stimulation in Mouse Fibroblasts

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The subcellular localization of the PDGF β-receptor was investigated in relation with PDGF-induced actin and membrane dynamics in mouse C3H10T1/2 fibroblasts. Serum-starved cells exhibit a nonhomogenous distribution of PDGF β-receptors. However, the observed pattern does not resemble the localization of PDGF-induced actin structures. Interestingly, the PDGF β-receptor showed a changed subcellular distribution in relation to the formation of PDGF-BB-induced actin structures. Upon PDGF exposure, PDGF β-receptors were found to accumulate in dorsal circular ruffles. The presence of both macropinosomes and clathrin in the induced circular ruffles suggests that the accumulation of PDGF β-receptors in circular ruffles results in the efficient internalization of PDGF β-receptors.

1. Introduction

One of the most essential proteins involved in cellular dynamics is represented by actin. Actin plays a dominant role in cell motility, morphogenesis, and cell-cell and cell-matrix interactions [1–3]. In addition actin is involved in many local dynamic processes such as the formation of leading edges and membrane ruffle formation [4, 5]. These local actin-driven processes are intriguing with respect to the fact that they can be induced by extracellular signal molecules like polypeptide growth factors. Thus, for example, epidermal growth factor (EGF) has been demonstrated to induce membrane ruffle formation in A431 epidermoid carcinoma cells within 5 minutes after addition of the growth factor [6, 7]. Similarly, platelet-derived growth factor (PDGF) has been demonstrated to induce very typical circular membrane ruffles in fibroblasts [8–10].

The PDGF β-receptor is a tyrosine kinase receptor of ~180 kDa. Each receptor contains five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain. PDGF isoforms are dimeric molecules that bind two receptors simultaneously [11]. The complex of the ligand with its receptors is further stabilized by direct receptor-receptor interactions mediated by its extracellular immunoglobulin-like domains. After binding of PDGF by the receptor, the intracellular kinase domains become activated and subsequently phosphorylated, and this is the starting point for various signal transduction cascades. Eventually, these signal transduction cascades activate actin induced membrane ruffling. Other phenomena that were described to originate from PDGF-induced signal transduction cascades are, for example, the increase of intracellular calcium levels and loss of stress fibers. Ultimately, PDGF β-receptor signaling determines cellular behavior by, for example, induction of cell cycle progression, prevention of apoptosis, and motility responses such as chemotaxis and ruffling (reviewed in [11]) [12]. In the case of chemotaxis, cells start to migrate and a leading edge can be identified where actin forms a branched network that pushes the cell membrane outwards (reviewed in [4]). Similarly, induced actin dynamics cause local bulges of the cell membrane in ruffles. For example, stimulation with PDGF results in the spatiotemporal formation of both linear ruffles and dorsal circular ruffles where newly formed actin filaments push the cell membrane locally outwards [8].

The activation of the PDGF receptor is the first step of the cellular response upon PDGF stimulation. Therefore we have

investigated whether the spatial temporal localization of the PDGF β-receptor can explain the local morphological responses in cells observed after stimulation with PDGF-BB. Next to representing the first activation step of the cellular response upon PDGF exposure, growth factor receptors were described to have close links with the actin cytoskeleton. The EGF receptor was characterized as an actin-binding protein [13] with actin-binding domains in the cytoplasmic part of the receptor that enables direct interaction with F-actin [14, 15]. Such a close link between growth factors receptors and actin may facilitate dynamic ruffle formation.

Next to direct interactions, like for the EGF receptor, also indirect interactions were suggested between growth factor receptors and actin. Indirect interaction is achieved through adaptor proteins or signal transduction components [16, 17]. Adapter proteins such as Nck, Crk, and Grb2 bind to the activated receptor and regulate the tyrosine kinase cascades that originate from the activated receptor. Ultimately, these signalling routes regulate the actin cytoskeleton by influencing the activities of actin-binding proteins (ABPs). Actin-binding proteins, such as cofilin, α-actinin, and gelsolin, bind directly to actin and regulate depolymerisation, polymerization of actin, or filament stabilization, resulting in local remodelling of the actin cytoskeleton and changes in cellular behaviour. It was suggested that different adapter proteins link to different signalling cascades thereby translating the activation of receptor kinases into distinct processes such as cell proliferation and cellular movement [18]. Also the formation of new actin filaments by, for example, the Arp2/3 complex is initiated by tyrosine kinase signalling derived from the activated receptor and regulated by adapter proteins. For example, Nck was shown to be required for signalling to actin dynamics downstream of the PDGF β-receptor and to be involved in dorsal ruffle formation [19]. Nck regulates WASP and N-WASP, members of the Wiskott-Aldrich syndrome family proteins, which were shown to activate the Arp2/3 complex resulting in actin nucleation. Interestingly N-WASP was shown to be involved in dorsal ruffle formation [20]. So, Nck/N-WASP-stimulated actin nucleation is an example of the close link between growth factor receptor stimulation and actin dynamics.

Next to direct binding and binding via adapter proteins of the actin cytoskeleton to receptors, another mechanism of interactions of receptors with the actin cytoskeleton was described. During phagocytosis, a clustering of receptors takes place. This clustering of receptors during phagocytosis was related to the restriction of lateral mobility of receptors by the actin cytoskeleton that underlines the cell membrane [21].

In this study we focused on the possible relationship between PDGF-BB-induced actin dynamics in mouse C3H10T1/2 fibroblasts and the subcellular localization of the PDGF β-receptor. Serum-starved cells exhibit a nonhomogenous distribution of PDGF β-receptors. However, the observed pattern of PDGF β-receptors does not correspond to the pattern of PDGF-induced actin structures. Interestingly, the PDGF β-receptor showed a changed subcellular distribution in relation to the formation of PDGF-BB-induced actin structures. Upon PDGF-BB exposure, PDGF β-receptors were found to accumulate in dorsal circular ruffles. The presence of both macropinosomes and clathrin in the induced circular ruffles suggests that the accumulation of PDGF β-receptors in circular ruffles is related to the efficient internalization of PDGF β-receptors.

2. Materials and Methods

2.1. Materials. Tissue culture nutrients, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). PDGF-BB was purchased from Upstate (Hampshire, UK). All other chemicals used were obtained from Sigma or Merck (Darmstadt, Germany).

2.2. Cell Culture. C3H10T1/2 mouse fibroblasts were grown at 37°C in HEPES-(25 mM)-buffered DMEM supplemented with 7.5% FBS and 5 mM L-glutamine (humidified atmosphere). For IF experiments, cells were plated at 12,000 cells/cm^2 on coverslips and allow to grow for 24 hours after seeding. For PDGF stimulation experiments, cells were subsequently serum-starved for 24 hours at 37°C and stimulated with 20 ng/mL of PDGF at 37°C.

The monoclonal stable transfected cell line expressing GFP-actin was constructed according to Michiels et al. [22]. In short, Phoenix cells were transiently transfected, and subsequently the harvested virus was used to infect C3H10T1/2 fibroblasts. Next, a monoclonal cell line was selected for low expression of GFP-actin to prevent disturbing cell characteristics like cell morphology, cell cycle speed, and number of nuclei. These cells exhibit a similar duration of the cell cycle and morphology compared to the fibroblasts that did not express GFP-actin. The GFP-actin construct was kindly provided by Dr. J. C. Stam (Faculty of Science, Utrecht University).

2.3. Immunofluorescent Labeling. Cells were fixed in 3.2% formaldehyde at 37°C and subsequently washed twice with PBS and incubated for 10 min with 50 mM glycine in PBS. After washing twice with PBS containing 0.2% gelatin, cells were incubated for 1 h with the primary antibody. Subsequently the cells were washed six times with PBS containing 0.2% gelatin and incubated for 1 hour with the secondary antibody and/or tetramethylrhodamine-5-(and-6) isothiocyanate-(TRITC-) conjugated phalloidin (Sigma-Aldrich, St. Louis, USA), followed by six washes in PBS containing 0.2% gelatin and two in PBS. Finally, cells were mounted in (2.5% w/v) 4–88 Mowiol (Hoechst, Frankfurt, Germany)-DAPCO (Sigma-Aldrich, St. Louis, USA). All individual experiments were carried out at least three times. All immunofluorescence studies were performed using single labelings as well.

2.4. Antibodies

Primary Antibodies. The anti-PDGF β-receptor antibodies were purchased from Upstate (06-131; raised against a peptide corresponding to the amino acids 1013–1025 of human PDGF type B receptors) and Oncogene (Ab-1; raised against

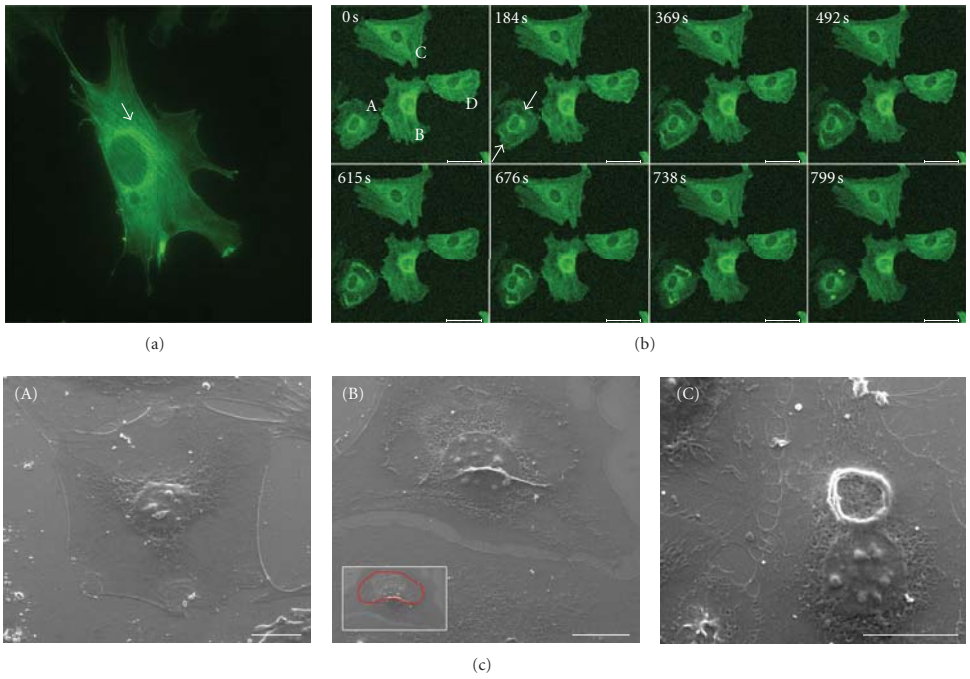


Figure 1: (a) Visualization of the stable transfected monoclonal cell line expressing GFP-actin. GFP-actin is present in the cytoplasmic pool surrounding the nucleus and in F-actin structures such as stress fibers (arrow) and the cortical skeleton. (b) Effect of PDGF-BB stimulation on GFP-actin expressing C3H10T1/2 mouse fibroblasts. Recording of images started two minutes after the addition of PDGF-BB. Dorsal circular ruffles start at the edge of cells (cell A, arrows). During the response, circular ruffles move across the surface and contract. The time from the formation of these ruffles until the full disappearance is 17 minutes. Bar represents 50 μm. (c) Effect of PDGF-BB stimulation on C3H10T1/2 mouse fibroblasts visualized with environmental scanning electron microscopy (ESEM). Serum-starved mouse C3H10T1/2 fibroblasts exhibit an extremely flat cell morphology with a nucleus bulging out (A). Five minutes after stimulation with PDGF-BB, a large circular ruffle is formed (B) stretching out over a large part of the cell surface. After 15 minutes, circular ruffles are further contracted, extend further in the dorsal direction, and exhibit a thicker rim. Imaging conditions: HV = 6 kV at 100%RH. Bar represents 20 μm.

a peptide that corresponds to amino acids 425 to 446 of murine PDGF β-receptors; extracellular domain), the antibody against the phospho-PDGF β-receptor Y1009 was purchased from LabFrontier, the antibody raised against ARP3 was purchased from Upstate (07-272), the antibody against NCK and clathrin (heavy chain) were from BD Transduction Lab, the antibody against N-WASp was from Santa Cruz.

Secondary Antibodies. GAM-Alexa488, GAR-Alexa488, and GAR-Alexa543 were purchased from Molecular Probes.

2.5. Acquisition of Fluorescent Images. The picture of Figure 1(a) was taken with a Leitz microscope (model Orthoplan Flu 043944) equipped with a Leitz oil objective 40x/1.3. The image was acquired using a Leica CCD camera (model DC350F; Leica Microsystems, Inc.) using Leica Image Manager 50 software. Confocal pictures (Figures 1(b), 2–6, 7(b), and 7(c)) were taken on a Zeiss Pascal 510 CLSM equipped with Zeiss water objectives 40x/1.3 and 63x/1.4.

2.6. Environmental Scanning Electron Microscopy (ESEM). Cells were fixed in 1.5% glutaraldehyde at 37°C during 10 minutes and subsequently stained in 1% uranyl acetate. After washing in PBS, cells were kept in PBS overnight before analysis in ESEM mode in a Quanta FEG (FEI Company, Eindhoven, The Netherlands). This microscope enables one to study cells in a fully hydrated state, avoiding all kind of drying artifacts [23, 24].

3. Results

3.1. Effect of PDGF-BB on Actin Dynamics in Mouse Fibroblasts. Various cell lines were described to respond upon growth factor stimulation with the formation of membrane ruffles [6–9]. Serum-starved C3H10T1/2 mouse fibroblasts were exposed to stimulation with PDGF-BB to investigate the formation of ruffles in this cell line. Local actin dynamics are involved in ruffle formation [25], and therefore the behavior of actin was investigated.

A stable transfected monoclonal GFP-actin cell line was constructed which allows studying the behavior of actin in living cells over a period of time (Figure 1(a)). In these cells GFP-actin was present in the cytoplasmic pool of G-actin surrounding the nucleus and in F-actin structures such as stress fibers (Figure 1(a), arrow). Next to the cytoplasmic localization, GFP-actin was also present in the nucleus as confirmed by experiments blocking nuclear export using leptomycin B (data not shown).

Time lapse microscopy was performed at 37°C with cells expressing GFP-actin that were stimulated with PDGF-BB. The time series in Figure 1(b) start two minutes after stimulation with PDGF. In Figure 1(b) cell A showed obvious reaction to PDGF stimulation at about 3-4 minutes after recording (arrows). A ruffle was formed at the edge of the cell that covered the total dorsal cell surface hereby forming a circular ruffle (Figure 1(b), 184 s and 369 s). This circular ruffle divided into two parts during the contraction of the ruffle. These two parts turned to bright dots (Figure 1(b), 799 s) and disappeared at about 17 minutes after recording. The time lapse microscopy with the GFP-actin expressing cells (Figure 1(b)) revealed that cells respond upon PDGF stimulation by forming circular ruffles repeatedly.

During the time lapse experiments, the formation of ruffles started around 3 minutes after stimulation till about 20 minutes after stimulation with PDGF. However, cells did not respond to PDGF simultaneously. Circular ruffles often started at the edge of cells and subsequently "moved" to a certain center at the dorsal surface of cells at a speed of about 2-3 μm/min and fused to form circular ruffles. Next, circular ruffles seem to contract thereby forming smaller half-open or closed circles (Figure 1(b), cell A). Most cells exhibit one or two large circular ruffles that before contraction stretched out over a large part of the plasma membrane, as described above. In other cells, a number of small circular ruffles were formed that often were located near newly formed lamella (data not shown). The response of the GFP-actin transfected cells upon stimulation with PDGF-BB, as detected by fluorescence microscopy, exhibited a similar pattern compared to cells only expressing endogenous actin that were investigated using immunofluorescence microscopy (data not shown).

The morphology of dorsal ruffles was further studied using environmental scanning electron microscopy (ESEM). Using ESEM mode, cells can be scanned with electrons resulting in high resolution without the need for dehydration of cells [23, 24]. This technique does not require the exposure of cells to high vacuum, and therefore removal of water from the sample material is not required. Dehydration of cells is well known to affect cell morphology and to disturb the cellular membrane. Imaging of cells at 100% relative humidity (RH) and low acceleration voltage revealed a smooth cellular surface of nonstimulated cells. No structures on the surface of nonstimulated cells were observed that might indicate possible starting positions for ruffle formation (Figure 1(c)-A)). Neither were these structures observed when cells were investigated using higher magnifications and lower acceleration voltage to reveal more surface structures (data not shown). Cells that were fixed 10 minutes after stimulation exhibit circular ruffles that stretched out over the dorsal surface of cells (Figure 1(c)-B). Studies of cells that were fixed at later time points confirmed the contraction of circular ruffles and revealed that ruffles become higher during contraction (Figure 1(c)-C)).

The time lapse microscopy studies using the cell line expressing GFP-actin demonstrated that these circular ruffles usually last for less than 20 minutes. We also observed the movement of linear "edge ruffles," which were formed at the cell edge. However, these "edge ruffles" did not move exclusively at the edge, but also on the dorsal cell surface. Circular and linear ruffles were formed repeatedly in cells. After the formation and disappearance of 0–3 circular ruffles, only linear ruffles were observed. Formation of circular ruffles starts at the edge of cells, and it was observed that 3 successive circular ruffles originated from the same area in a cell.

Altogether, we conclude that PDGF-BB induced a transient and local response in these cells illustrated by the formation and disappearance of dorsal circular ruffles.

3.2. PDGF β-Receptor Localization in Serum-Starved C3H10T1/2 Fibroblasts. So far, we have established that exposure of cells to PDGF in their extracellular environment results in local and temporal responses within cells. Therefore some spatiotemporal organization is required in these cells that translates the sensing of PDGF by receptors in local responses such as local ruffle formation. The first step in the signal transduction cascade is the activation of the receptor upon binding of the ligand to the receptor and the subsequent intracellular activation of signaling cascades. Investigations of the distribution of the PDGF receptor might explain the local initiation of responses in cells upon PDGF stimulation. Therefore the localization of the PDGF β-receptor was investigated in nonstimulated cells. These cells were serum-starved for 24 hours and are susceptible to PDGF stimulation as demonstrated in Figure 1(c).

The localization of the PDGF β-receptor was visualized by immunofluorescence microscopy using two different antibodies that recognize the PDGF β-receptor (Figures 2(a) and 2(d)). Two different antibodies were used to minimize the effect of the antibody selection on the outcome of this study. In Figure 2(a) cells were labeled with an antibody raised against a peptide corresponding to the amino acids 1013–1025 of the intracellular domain of the PDGF β-receptor. The morphology of cells was visualized by staining for F-actin with phalloidin-TRITC (Figures 2(b) and 2(e)). F-actin is present in the form of stress fibers as well as in the cortical skeleton. In Figure 2(d) cells were labeled with an antibody that recognizes amino acids 425–446 of the extracellular domain of the PDGF β-receptor (Figure 2(d)). Labeling with both antibodies revealed a nonhomogenous distribution of the PDGF receptor (Figures 2(a) and 2(d)). PDGF β-receptors appear to be partly organized in patches in the membrane (Figures 2(a) and 2(d), arrows). In addition to the clustered PDGF β-receptors in patches, a homogenous scattered distribution was observed with both antibodies. The antibody raised against the extracellular domain of the PDGF β-receptor (Figure 2(d)) detected this homogenous scattered distribution more clearly compared to the antibody used in

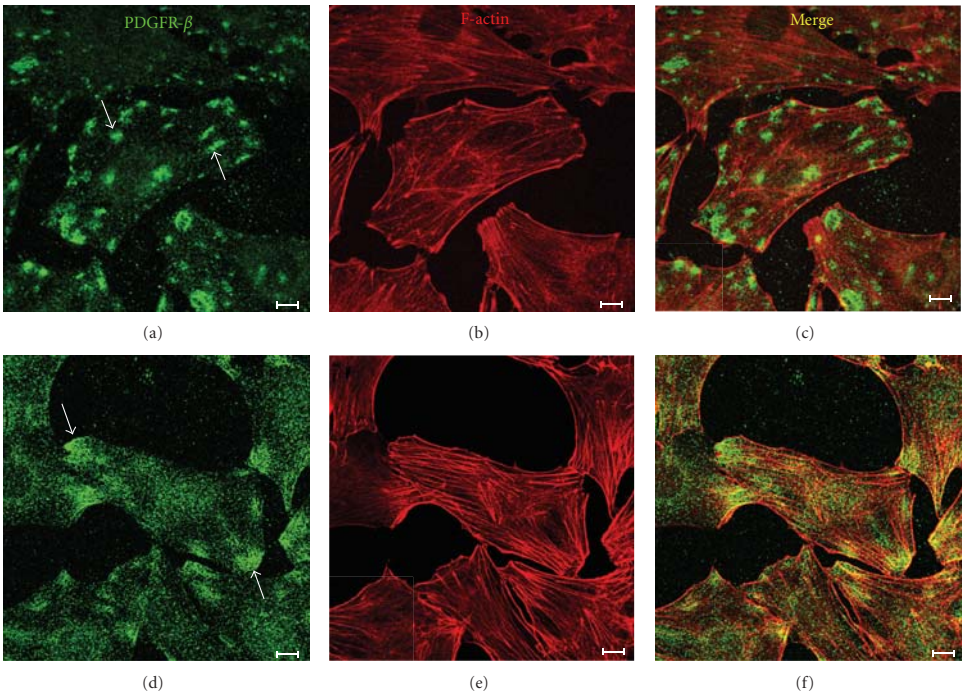


Figure 2: Immunofluorescence labeling revealing the localization of the PDGF β-receptor in serum-starved cells. Serum-starved mouse fibroblasts were stained with two different antibodies raised against the PDGF β-receptor (green) and stained for F-actin with phalloidin-TRITC (red). The antibody raised against a peptide corresponding to amino acids 1013–1025 of human PDGF β-receptors located the PDGF β-receptor in patches on the cell surface in serum-starved cells (a, arrows). Patches with receptors are often located at the edge of cells. The patches with PDGF β-receptors were also detected with the antibody raised against a peptide that corresponds to part of the extracellular domain of murine PDGF β-receptors in serum-starved cells (d, arrows). In addition a more homogenous scattered distribution of PDGF β-receptors was detected with both antibodies (a, d). Bar represents 10 μm.

Figure 2(a). The differences in labeling between the two antibodies may be related to a different accessibility of the intracellular and the extracellular antigenic sites. Upon the use of triton after chemical fixation, the patches and homogenous scattered receptors were no longer visible and a more intense cytoplasmic staining was observed (data not shown). This suggests that both the PDGF β-receptors in the observed patches and the homogenous scattered receptors are localized in the plasma membrane. In addition the labeling is partly intracellular since the nucleus can be discriminated from the staining that is present in the cytoplasm (Figures 2(a) and 2(d)).

When comparing the localization of the observed patches of PDGF β-receptors with the observed sites of circular ruffle formation, it can be concluded that the patches of PDGF β-receptors are often located near the edge of cells. The circular ruffles that are formed upon exposure to PDGF-BB often started at the edge of cells (Figure 1(b)). However, the circular ruffles that are formed upon exposure to PDGF-BB often stretch out over the entire surface of cells (Figures 1(b) and 1(c)) in contrast to the patches of PDGF β-receptors. In addition cells exhibit smaller numbers of circular ruffles compared to the number of patches that was observed in cells. In a minority of cells, exposure to PDGF-BB resulted in numerous small circular ruffles that were often localized near newly formed lamella. This type of ruffling behavior may correspond to the patches of PDGF β-receptors that were detected in Figures 2(a) and 2(d). Based on these data, it was concluded that the observed patches of receptors do not fully correspond with the newly induced circular F-actin structures that were observed after stimulation with PDGF.

3.3. Redistribution of the PDGF β-Receptor during PDGF-BB Stimulation in C3H10T1/2 Fibroblasts. Subsequently the distribution of PDGF β-receptors was investigated after stimulation with PDGF-BB with both antibodies that were used in the previous section. Serum-starved cells were stimulated with PDGF-BB at 37°C and after 15 minutes cells were chemically fixed at 37°C. Fluorescence labeling revealed that circular ruffles were induced as detected by staining for F-actin with phalloidin-TRITC (Figures 3(b), 3(e) and 3(h)). The antibody that recognizes the intracellular domain of

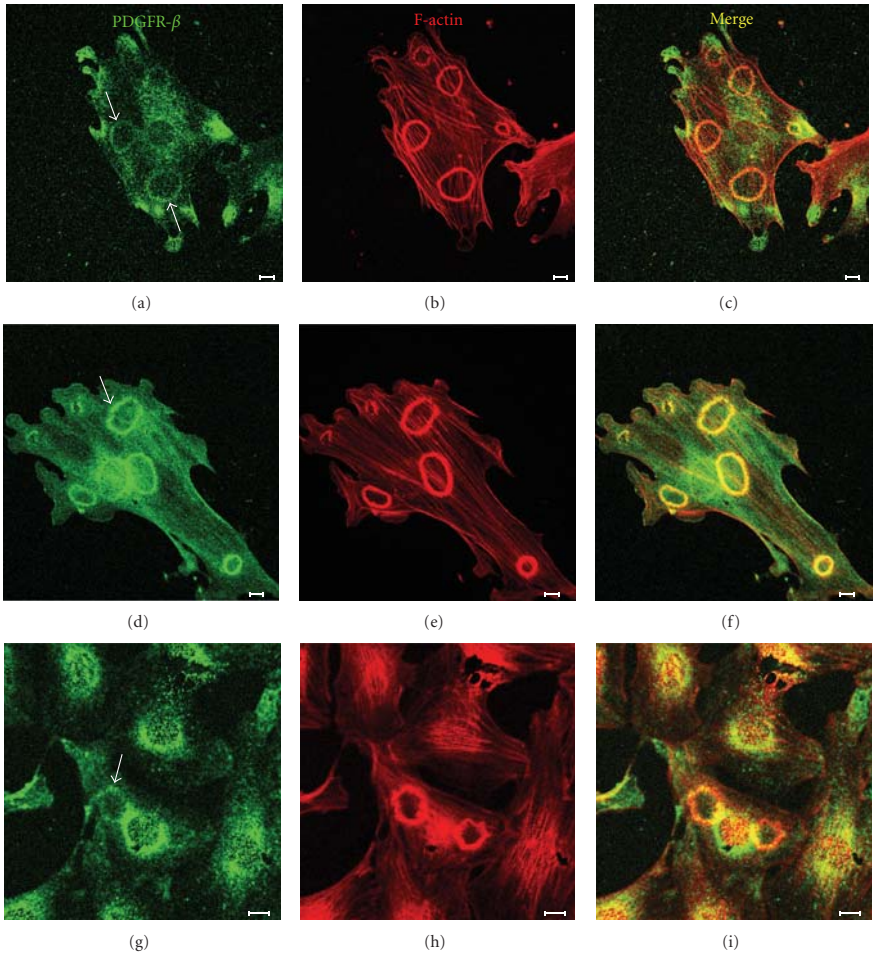


Figure 3: Immunofluorescence labeling revealing the localization of the PDGF β-receptor upon exposure to PDGF-BB. Serum-starved mouse fibroblasts were stimulated with PDGF-BB and chemically fixed after 15 minutes. Samples represented in (d), (e), and (f) were subjected to a triton treatment after chemical fixation. Subsequently cells were stained with two different antibodies raised against the PDGF β-receptor (green) and stained for F-actin with phalloidin-TRITC (red), similar to Figure 2. Upon PDGF-BB stimulation circular ruffles were induced as detected by staining for F-actin ((b), (e), and (h)). The antibody that recognizes the intracellular domain of the PDGF β-receptor co-localized with F-actin in the dorsal ruffles ((a), arrows). The number of patches with receptors decreased as compared with nonstimulated cells (Figure 3(a) versus Figure 2(a)). In samples that were subjected to a triton treatment after chemical fixation ((d), (e), and (f)), the patches were no longer visible and the intracellular cytoplasmic labeling was more intense (d). Also in these samples the PDGF β-receptor co-localized with F-actin in dorsal circular ruffles ((d), arrow). Immunofluorescence studies with the antibody that recognizes the extracellular domain of the PDGF β-receptor revealed that upon stimulation with PDGF-BB the patches of PDGF β-receptors often disappear (g). In cells that form circular ruffles, the PDGF β-receptor partly localizes in the newly formed circular ruffles ((g), arrow) that were revealed by labeling for F-actin (h). Bar represents 10 μm.

the PDGF β-receptor co-localized with F-actin in the dorsal ruffles (Figure 3(a), arrow). The number of patches with receptors decreased as compared with nonstimulated cells (Figure 3(a) versus Figure 2(a)). In samples that were subjected to a triton treatment after chemical fixation, the patches were no longer visible and the intracellular cytoplasmic labeling was more intense (Figure 3(d)). Also in these samples, the PDGF β-receptor co-localized with F-actin in dorsal circular ruffles (Figure 3(d), arrow). This suggests that the PDGF β-receptors in the patches that were observed in Figure 3(a) are localized in the plasma membrane (as described in the previous section) and that the docking of PDGF

β-receptors in circular ruffles takes place by another mechanism. Immunofluorescence studies with the antibody that recognizes the extracellular domain of the PDGF β-receptor revealed that upon stimulation with PDGF-BB the patches of PDGF β-receptors often disappear (Figure 3(g)). In cells that form circular ruffles, the PDGF β-receptor partly localizes in the newly formed circular ruffles (Figure 3(g), arrow) that were revealed by labeling for F-actin (Figure 3(h)). This co-localization of the PDGF β-receptor with newly induced ruffles was more clear in the dorsal tip of mature circular ruffles. So after PDGF stimulation, the PDGF β-receptor translocates to the ruffles and co-localizes with F-actin.

3.4. The Phosphorylated PDGF β-Receptor Is Present in Circular Ruffles after PDGF-BB Stimulation in C3H10T1/2 Fibroblasts. Next it was investigated which part of the localized receptors in Figures 2 and 3 represents activated receptors. Activation of PDGF β-receptors results in the phosphorylation of the receptor. Therefore serum-starved cells were labeled using an antibody recognizing the PDGF β-receptor when phosphorylated on Y1009. In serum-starved cells, we found some residual staining of phosphorylated PDGF β-receptors (Figure 4(a)). The presence of phosphorylated PDGF β-receptors in serum-starved cells was also observed with western blotting experiments of total cell lysates of serum-starved cells (data not shown), indicating that serum starvation did not yield fully quiescent cells. A substantial part of the labeling is present intracellularly in the cytoplasmic pool surrounding the nucleus (Figure 4(a)). The phosphorylated PDGF β-receptor was less clearly organized in patches in serum-starved cells compared to the results obtained with the two antibodies used in Figure 2. The morphology of cells was visualized by staining for F-actin with phalloidin-TRITC. Serum-starved cells exhibit numerous stress fibers and a clear cortical skeleton (Figure 4(b)). After 15 minutes of PDGF stimulation, the number of stress fibers decreases, and dorsal circular ruffles (Figures 4(e) and 4(h), arrow) and edge ruffles are formed (Figure 4(h), arrow). Upon exposure to PDGF-BB, the staining for the phosphorylated PDGF β-receptor receptor clearly increased in intensity (Figures 4(d) and 4(g)). A large part of the population of phosphorylated PDGF β-receptor co-localized with F-actin in the newly formed circular dorsal ruffles (Figures 4(d) and 4(g), arrows). The localization of the phosphorylated PDGF β-receptor in dorsal circular ruffles was more clear in mature ruffles compared to the initial stages of circular ruffles. Similar with the results in Figure 3(g), also the phosphorylated receptor translocates to circular ruffles during PDGF stimulation. Therefore we conclude that at least a part of the receptors that is present in circular ruffles represents phosphorylated, and consequently activated PDGF β-receptors.

3.5. Nck, N-WASP, and ARP3 Are Present in Circular Ruffles after PDGF-BB Stimulation in C3H10T1/2 Fibroblasts. The observed co-localization with local areas of actin remodeling suggests a link between the phosphorylated PDGF β-receptor and F-actin. This can be direct, such as in the case of the EGF receptor [13], or indirect via adapter or signaling proteins. No actin-binding domain was described for the PDGF β-receptor so far, but various pathways were described that link the activated PDGF β-receptor to actin polymerization [11, 19, 26]. Interestingly, it was described that the neural Wiskott-Aldrich syndrome protein (N-WASP) binds to the phosphorylated PDGF β-receptor via the linker protein Nck [26]. N-WASP is a member of the WASP family of proteins that regulates actin remodeling through activation of the ARP2/3 complex that is a nucleator of actin filaments.

To investigate whether this signaling pathway from the activated PDGF β-receptor to actin filament formation takes place in the mouse fibroblasts upon PDGF-BB stimulation, we investigated the localization of these proteins both before and during PDGF-BB stimulation. In serum-starved cells, Nck was found in the cytoplasm and nucleus (Figure 5(a)). Upon PDGF stimulation Nck translocated to newly formed circular ruffles (Figure 5(d)). The labeling for N-WASP was similar compared to the labeling for Nck both in serum-starved cells (Figure 5(b)) and in cells that were stimulated with PDGF-BB (Figure 5(e)). In cells that responded with the formation of circular ruffles upon PDGF-BB stimulation, both proteins were found in circular ruffles and the cytoplasmic pool around the nucleus seemed slightly reduced (Figures 5(d), 5(e), and 5(f) versus Figures 5(a), 5(b), and 5(c)). Also the localization of ARP3 was investigated using immunofluorescence microscopy. In cells that exhibit circular ruffles as detected by labeling for F-actin (Figure 5(h)), ARP3 was found to localize in PDGF-BB-induced circular ruffles and lamellae (Figure 5(g), arrow). So, once circular ruffles are induced by activated PDGF β-receptors, the phosphorylated PDGF β-receptors themselves, the linking protein Nck, the actin regulating protein N-WASP, and the member of the ARP2/3 complex ARP3 all co-localize in these structures.

3.6. The Phosphorylated PDGF β-Receptor Is Present at the Leading Edge after PDGF-BB Stimulation in C3H10T1/2 Fibroblasts. To determine if the co-localization of phosphorylated PDGF β-receptors also takes place in other areas of induced actin and membrane dynamics, the distribution of phosphorylated PDGF β-receptors was investigated in motile cells. Motile cells were selected in random cell cultures and labeled for the phosphorylated PDGF β-receptor Y1009 and F-actin. Interestingly the phosphorylated PDGF β-receptor localized at leading edges (Figure 6, arrow). At the leading edge, dynamic remodeling of actin takes place that allows a cell to move forward. The localization of phosphorylated PDGF β-receptors at the leading edges of motile cells illustrates another example of co-localization of the phosphorylated PDGF β-receptor with local actin and membrane dynamics.

3.7. Macropinocytotic Vesicles and Clathrin Localize in Circular Ruffles. In the previous sections, a local accumulation of phosphorylated PDGF β-receptors in circular ruffles was established. These phosphorylated PDGF β-receptors might be involved in the activation of downstream pathways such as actin filament formation. Alternatively they might accumulate to become efficiently internalized resulting in the down-regulation of the PDGF-induced stimulus.

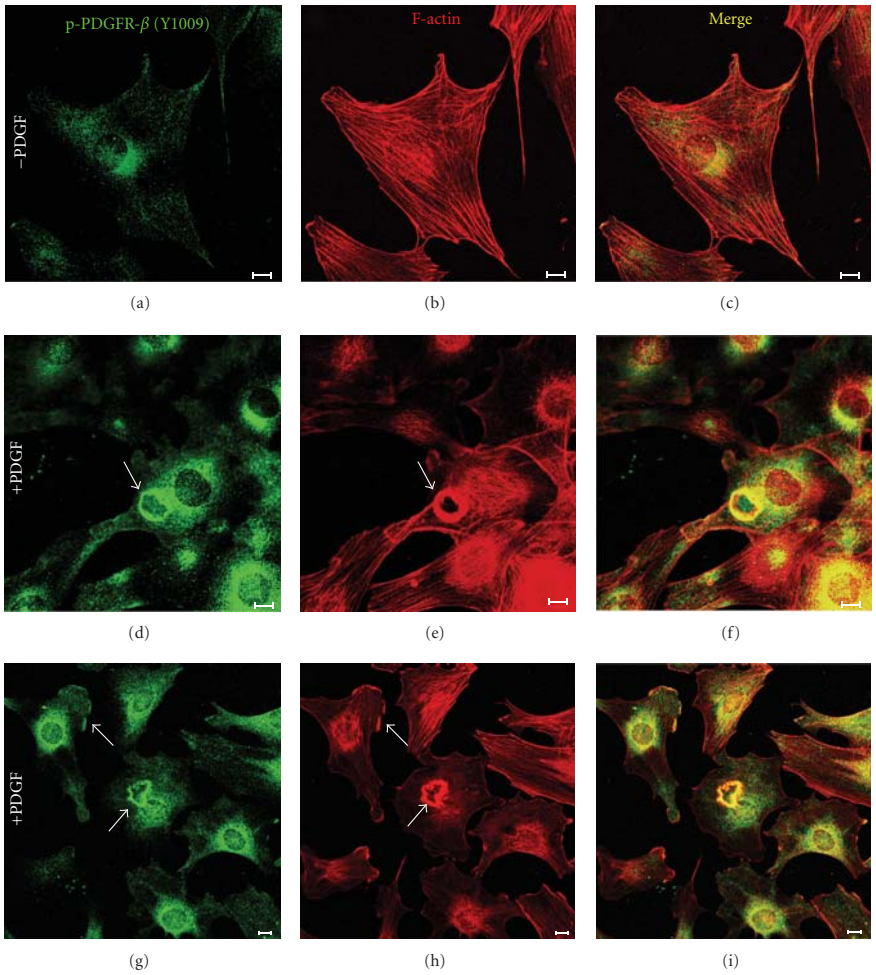


Figure 4: Induced circular ruffles are enriched with phosphorylated PDGF β-receptors. Cells were stained for phospho-PDGF β-receptors (Y1009) (green) and F-actin (red). Serum-starved cells exhibit no ruffles or lamella (b) and exhibit some staining for the phosphorylated PDGF β-receptor (a). Upon stimulation with PDGF-BB, circular ruffles are formed as detected by labeling for F-actin ((e), (h), arrow). The phosphorylated PDGF β-receptor is abundant in the newly formed circular ruffles ((d), (g), arrow). Next to circular ruffles, also edge ruffles and lamella are formed upon stimulation with PDGF-BB ((h), arrow). The phosphorylated PDGF β-receptors also co-localize in these newly formed F-actin structures ((g), (i)). Bar represents 10 μm.

Various pathways are known to be involved in the internalization of activated growth factor receptors [27]. In circular ruffles, macropinocytosis takes place [20, 28–30]. Macropinocytosis was described to result from membrane ruffling induced by actin dynamics. The subsequent folding back of ruffles and fission with the plasma membrane may result in large vesicles that are internalized and become macropinosomes [30, 31]. Circular ruffling was suggested to result in macropinosomes when protrusions in circular ruffles combine followed by membrane fission [30]. Next to actin-induced membrane ruffling also membrane blebbing was described to result in macropinocytosis [30, 31]. ESEM studies of cells that were stimulated with PDGF-BB revealed the formation of macropinocytic cups (Figures 7(a)-A and 7(a)-B) and membrane blebs on the dorsal membrane (Figures 7(a)-A and 7(a)-D). The macropinocytic cups might represent final stages of circular ruffling. Membrane blebs co-localized with circular ruffles but were also localized on other parts of the dorsal membrane (Figure 7(a)-D). The membrane blebs that did not co-localize with circular ruffles were larger and might result from the closure of dorsal circular ruffles (Figure 7(a)-D). The smaller membrane blebs that did

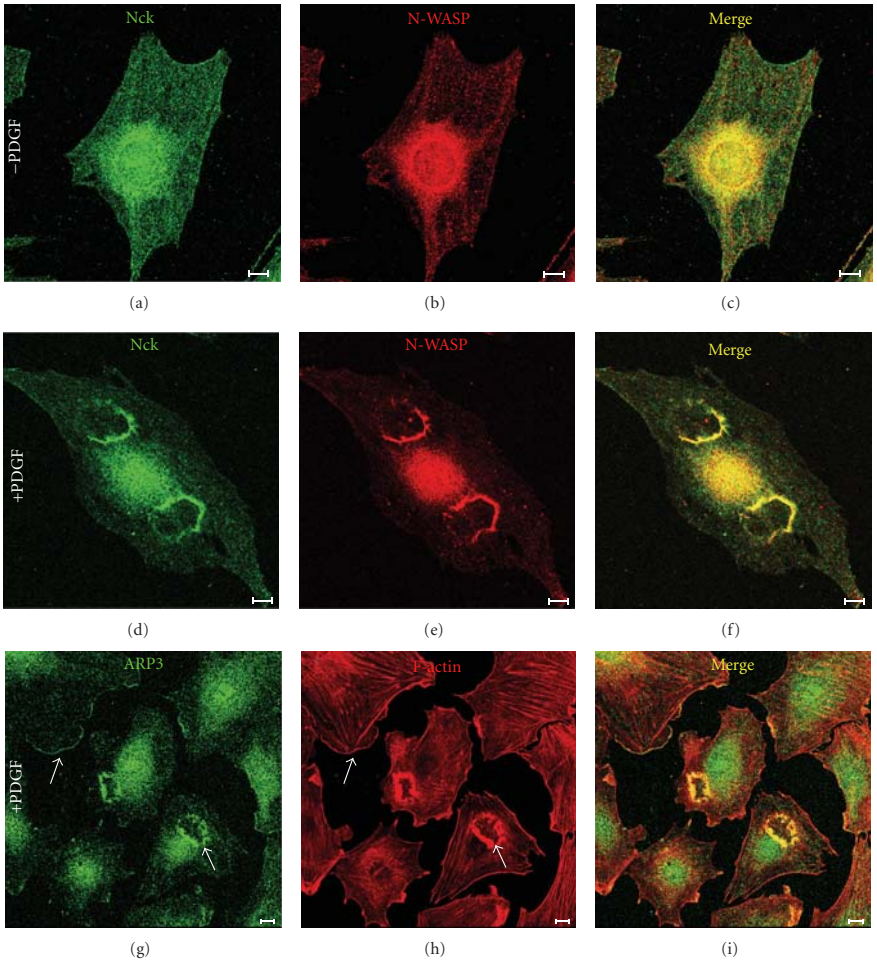
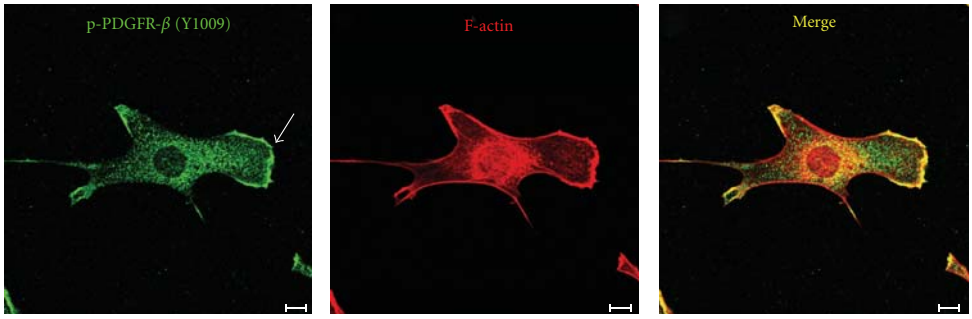


Figure 5: Nck, N-WASP and ARP3 are localized in newly formed circular ruffles upon PDGF stimulation in mouse C3H10T1/2 fibroblasts. Nck (green) and N-WASP (red) are localized in the cytoplasmic pool and in the nucleus of serum-starved cells ((a), (b), (c)). After 15 minutes of stimulation with PDGF-BB, circular ruffles are formed and Nck (green) and N-WASP (red) co-localize in circular ruffles ((d), (e), (f)). Also ARP3 (g) is localized in newly formed F-actin structures (h) such as circular ruffles and lamella (arrows). Bar represents 10 μm.

co-localize with circular ruffles (Figure 7(a)-A) may represent macropinosomes that result from small ruffles that are formed on top of circular ruffles as represented in Figure 7(a)-C. The small protrusions of a circular ruffle might combine, subsequently followed by membrane fission as described in [30] (Figure 7(a)-C). In Figure 7(b) a cell exhibits macropinocytic vesicles in a PDGF-BB-induced dorsal circular ruffle that was recognized by staining for F-actin (Figure 7(b)-B, arrow). The macropinosome is enriched with phosphorylated PDGF β-receptors (Figure 7(b)-A, arrow).

Another way of receptor internalization is via receptor-mediated endocytosis. Clathrin is a key protein in this process by forming the clathrin-coated pits resulting in invaginations of the cell membrane and subsequential formation of vesicles enriched with growth factor receptors. We investigated the localization of clathrin in serum-starved cells and in PDGF-stimulated cells by immunofluorescence microscopy. Before stimulation clathrin exhibited a cytoplasmic localization (Figure 7(c)-A). Upon PDGF stimulation clathrin co-localized with induced circular ruffles (Figure 7(c)-B). At these sides we also located the phosphorylated PDGF β-receptors. Therefore we conclude that next to the internalization of phosphorylated PDGF β-receptors by macropinocytosis (Figure 7(b)) also receptor-mediated endocytosis may take place in dorsal circular ruffles in C3H10T1/2 mouse fibroblasts.



(a) (b) (c)

Figure 6: Phosphorylated PDGF β-receptors localize at the leading edge of motile cells. Migrating mouse fibroblasts are stained for the phospho-PDGF β-receptor Y1009 (a) and F-actin (b). Phosphorylated PDGF β-receptors are mainly localized at the leading edge (a, arrow). Bar represents 10 μm.

4. Discussion

In this study the distribution of the PDGF β-receptor was investigated in relation to PDGF-BB-induced actin structures. PDGF β-receptor signaling is amongst others involved in motility responses. Motility responses such as chemotaxis and ruffling are the result of locally induced changes in cells such as actin remodeling. In case of chemotaxis, cells start to migrate, and a leading edge can be identified where actin forms a branched network that pushes the cell membrane outwards (reviewed by [4]). Next to the formation of leading edges, serum-starved mouse C3H10T1/2 fibroblasts respond upon PDGF-BB stimulation with other motility responses like membrane ruffling. For example, PDGF-BB stimulation results in the spatiotemporal formation of dorsal circular ruffles and peripheral ruffles where actin locally pushes the membrane outwards [8, 9].

In the present study, cells were exposed to an extracellular stimulation with PDGF-BB that resulted in spatiotemporal responses within cells including actin and membrane dynamics. Investigations of the outside of the dorsal membrane by performing environmental scanning electron microscopy (ESEM) at high magnifications and low acceleration voltage revealed no structures that might explain the local formation of ruffles. Therefore a spatial organization on a molecular level is required in cells.

First it was investigated whether the spatial temporal localization of the PDGF receptor can explain the local responses in cells observed after stimulation with PDGF-BB. In fibroblasts both α-receptors and β-receptors for PDGF are present. Stimulation with PDGF-BB will also activate α-receptors. However the formation of dorsal circular ruffles was demonstrated to be specific for β-receptor signaling [32]. That is why the localization of β-receptors was studied before and during PDGF-BB stimulation, and subsequently correlations with the induced circular ruffling were investigated. Interestingly we found that only a fraction of the PDGF β-receptor is randomly and homogeneously distributed in the membrane of nonstimulated mouse fibroblasts, the majority of the PDGF β-receptors were present in patches. These patches may represent caveolae since localization studies showed that the PDGF β-receptor is located in caveolae in human fibroblasts [33]. The observed pattern of receptors was compared with the observed pattern of actin-induced circular ruffling. In a minority of cells, exposure to PDGF-BB resulted in numerous small circular ruffles that may correspond to the patches of PDGF β-receptors that were detected in Figures 2(a) and 2(d). However, circular ruffles that are formed upon exposure to PDGF-BB often stretch out over the entire surface of cells in contrast to the patches of PDGF β-receptors. In addition cells exhibit smaller numbers of circular ruffles compared to the number of patches that was observed in cells. We therefore conclude that the local presence of PDGF β-receptors in patches does not explain the initial local responses of actin in mouse fibroblasts that were observed after stimulation with PDGF-BB.

Interestingly, PDGF β-receptors translocated to newly formed circular ruffles upon PDGF-BB stimulation as was demonstrated by the PDGF-BB-dependent spatiotemporal co-localization of PDGF β-receptors with F-actin in ruffles. Dorsal circular ruffles were found to be enriched with the phosphorylated PDGF β-receptor as was demonstrated by immunofluorescence microscopy using an antibody raised against phosphorylated PDGF β-receptors (Y1009). The co-localization of phosphorylated PDGF β-receptors with induced actin and membrane dynamics is not restricted to dorsal circular ruffles. The phosphorylated PDGF β-receptor was also found to localize at the leading edge in motile cells. Also in these cells the phosphorylated PDGF β-receptors co-localized with induced actin and membrane dynamics.

The translocation of PDGF β-receptors that is mentioned above might result from direct or indirect binding to the induced membrane and actin modulations such as newly formed actin filaments in circular ruffles. When receptors bind to the initiated circular ruffles and subsequently stay connected, the receptors will follow the movement of the circular ruffles that is described in Figure 1. Direct binding for the PDGF β-receptor and actin has not been demonstrated so

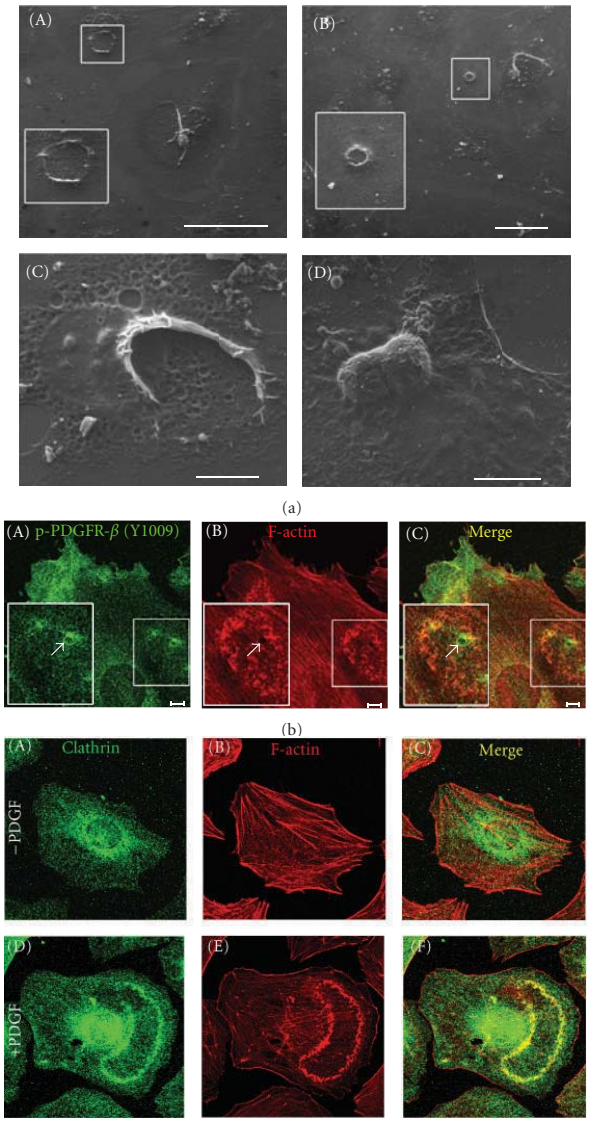


Figure 7:Macropinocytosis and clathrin localize in PDGF-BB-induced circular ruffles. (a) Visualization using ESEM of macropinocytic structures in cells that were stimulated with PDGF-BB. ESEM studies revealed the formation of macropinocytic cups ((a)-A and (a)-B) and membrane blebs on the dorsal membrane ((a)-A and (a)-D). Membrane blebs co-localized with circular ruffles (a)-A but were also localized on other parts of the dorsal membrane (a)-D. The membrane blebs that did not co-localize with circular ruffles were larger and might result from the closure of dorsal circular ruffles (a)-D. The smaller membrane blebs that did co-localize with circular ruffles (a)-A may represent macropinosomes that result from the fission of small ruffles that are formed on top of circular ruffles as represented in (a)-C. Imaging conditions of A, B, C: HV = 6 kV at 100%RH. Image D: HV = 3 kV (to reveal more surface detail) at 100%RH. A, B: bar represents 50 μm, C, D: bar represents 10 μm. (b) Cells exhibit macropinocytic vesicles in dorsal circular ruffles. Circular ruffles were identified in PDGF-BB stimulated cells by staining for F-actin (b)-B. Circular ruffles exhibit macropinocytic vesicles (arrows). The macropinosome is enriched with phosphorylated PDGF β-receptors (b)-A. Bar represents 10 μm. (c) Clathrin localizes in newly formed circular ruffles upon PDGF-BB stimulation in mouse fibroblasts. Serum-starved cells exhibit abundant stress fibers (c)-B, and clathrin is randomly distributed in the cytoplasm of serumstarved cells (c)-A. Upon stimulation with PDGF-BB, circular ruffles are induced by actin dynamics (c)-E and clathrin co-localizes with F-actin in the newly formed circular ruffles (c)-D. Bar represents 10 μm.

far. Other receptors like the EGF receptor were shown to possess an actin-binding domain [13]. Indirect binding to actin in circular ruffles might be possible via short chains of interacting proteins, for example, via Nck/N-WASP and the ARP2/3 complex. A number of studies related Nck to downstream signalling from the activated PDGF β-receptor to the actin cytoskeleton [18]. In addition Nck was related to the docking of the actin polymerization machinery to sides of local actin remodelling, including dorsal circular ruffles [19, 26, 34]. Also in the present study, the adapter Nck was demonstrated to co-localize at newly formed F-actin structures in dorsal circular ruffles. Nck is known to bind directly to the activated PDGF β-receptor [26, 35]. For example, Nckβ binds at Tyr-1009 of the phosphorylated PDGF β-receptor and subsequently acts as an adapter protein in signaling from the receptor to the actin cytoskeleton [26]. Next to Nck also the actin modulators N-WASP and Arp3 were demonstrated to co-localize at newly formed F-actin structures in dorsal circular ruffles. N-WASP is a member of the WASP family of proteins that regulates actin remodeling through activation of the ARP2/3 complex that is a nucleator of actin filaments. N-WASP was also demonstrated to be involved in dorsal ruffle formation, and furthermore, N-WASP and the ARP2/3 complex were suggested to be part of a multiprotein complex that is involved in the generation of dorsal circular ruffles [20]. Based on the observation that the PDGF β-receptor accumulates in dorsal ruffles where it co-localizes with Nck, N-WASP, Arp3, and newly formed F-actin upon PDGF-BB stimulation, we suggest that Nck physically links the activated PDGF β-receptor with the actin cytoskeleton via binding to actin-binding proteins. The subsequent contraction of circular ruffles on the dorsal cellular surface results in the accumulation of activated receptors in mature circular ruffles.

Alternatively, the local organization of the actin cytoskeleton that underlines the plasma membrane in circular ruffles might restrict the lateral mobility of receptors resulting in the accumulation of receptors, as has been suggested for the clustering of receptors during phagocytosis [21]. Finally, no interaction with the cytoskeleton might take place, and the described concentration of phosphorylated PDGF β-receptors might reflect changing membrane characteristics that move along with the circular ruffles in time. Lipid-dependent compartmentalization of the plasma membrane might result in the local confinement of receptors.

The accumulation of activated PDGF β-receptors in mature circular ruffles might affect intracellular responses to PDGF-BB. Linear ruffle formation was suggested to facilitate efficient signal transduction by forming a signaling compartment that allows the concentration of signal transduction proteins [7]. Similarly, the concentration of PDGF β-receptors in dorsal circular ruffles might reflect enhancement of PDGF-BB-induced signal transduction. Moreover, the indirect binding of activated PDGF β-receptors with the actin cytoskeleton might result in the formation of protein complexes consisting of the receptor and its substrates that is facilitated by the actin microfilaments system, similar to the way that was suggested for the direct binding of the EGF receptor to the actin cytoskeleton [13, 36]. This meshwork may increase the efficiency of signal transduction between the PDGF receptor and its substrates.

In addition, the concentration of receptors in contracting cir-cular ruffles might facilitate efficient internalization. In dorsal circular ruffles macropinocytosis was shown to take place [20, 28–30], and the accumulation of receptors in these structures could lead to effective internalization. Also in mouse C3H10T1/2 fibroblasts macropinocytic structures such as macropinocytic cups were formed upon exposure to PDGF-BB. In combination with the accumulation of PDGF β-receptors in circular ruffles, this might represent an efficient mechanism for the internalization of PDGF β-receptors. The EGF receptor was shown to accumulate within minutes in circular ruffles, and 50% of ligand-bound EGF receptors were subsequently internalized from the cell surface. It was suggested that the caveolin and clathrin-based endocytic pathways do not participate in this form of endocytosis of the EGF-receptor [37]. A similar event might take place in mouse C3H10T1/2 fibroblasts with PDGF β-receptors upon PDGF-BB stimulation. We located also clathrin in circular ruffles upon exposure to PDGF-BB. Clathrin was demonstrated to associate with Nck via interaction with ACK1 [38]. The adapter Nck was also localized in dorsal circular ruffles. ACK1 and Nck were suggested to link clathrin to tyrosine kinase receptors [38], and it is of interest to note that the endocytotic rate of a tyrosine-inactivated PDGF β-receptors was demonstrated to be slower compared to wildtype PDGF β-receptors [39]. The presence of clathrin in circular ruffles might indicate the existence of an alternative mechanism for the internalization of PDGF β-receptors by efficient receptor mediated endocytosis. Receptor internalization will reduce the sensitivity of cells for PDGF. This is also supported by the observation that fibroblasts form only a limited number of successive circular ruffles that is subsequently followed by the formation of linear ruffles.

Acknowledgments

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