Actin dynamics in microgravity

Actine-dynamiek onder micrograviteit

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Cover Launch of Soyuz spacecraft in Kazakhstan, April 2004.
Mammalian cells visualized by fluorescence microscopy

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Chapter 1

General introduction

General introduction

During the last decades a wide variety of space flight experiments have indicated that gravity has significant effects on whole organisms, organs and tissues, resulting for example in bone and muscle mass reduction, the occurrence of cardiovascular malfunctioning, as well as many other processes (Carmeliet et al., 2001; Fitts et al., 2001; Fritsch-Yelle et al., 1996). In addition, the virtual absence of gravity was demonstrated to have profound effects on the cellular and molecular level, including changes in cell morphology (Rijken et al., 1991; Hughes-Fulford et al., 2003), modification of gene expression (de Groot et al., 1991a; Hammond et al., 1999; Liu and Wang, 2008), changes in signal transduction cascades (de Groot et al., 1991b; Ullrich et al., 2008) and even changes in the self-organization of the cytoskeletal protein tubulin (Papaseit et al., 2000; Glade et al., 2006; Tabony et al., 2007). That is why the underlying mechanism for the observed physiological responses in microgravity might be on the molecular level within cells.

Previous studies of adherent A431 epithelial cells in microgravity using sounding rockets revealed that growth factor-induced signal transduction is sensitive to microgravity. EGF-induced (Epidermal Growth Factor) early gene expression of c-fos and c-jun was demonstrated to decrease under microgravity conditions (de Groot et al., 1990; de Groot et al., 1991a). Subsequently, EGF signaling was investigated upstream of this early gene expression by studying receptor clustering, ligand binding and by studying EGF-induced intracellular signaling cascades. Receptor clustering and ligand binding were demonstrated not to alter under microgravity conditions. However, selective transduction pathways were identified to be susceptible to changes in gravity conditions whereas other pathways originating from the EGF receptor were not (Rijken et al., 1992).

Furthermore, it was demonstrated during sounding rocket flights that microgravity exposure of A431 cells resulted in increased actin polymerization and cell rounding (Rijken et al., 1991). Cell rounding is dependent on actin. Interestingly, the signaling cascade that was susceptible to changes in gravity – PKC-mediated signal transduction – was also demonstrated to depend on actin. That is why the actin cytoskeleton may represent a gravity-sensitive component in cells (Boonstra, 1999).

Actin is a main component of the cytoskeleton in eukaryotic cells that provides structure and determines the shape of cells. The cytoskeleton consists of proteins that dynamically interact with one other. Next to actin, the cytoskeleton consists of tubulin and intermediate filaments. Actin is present in the form of individual actin proteins known as

globular actin (G-actin) as well as filamentous polymers (F-actin). F-actin filaments consist of multiple G-actin subunits that interact with one another and are continuously assembled and disassembled. Actin microfilaments organize themselves in dynamic structural components in cells that, for instance, determine the shape of cells, provide the infrastructure for intracellular transport and facilitate processes such as cell motility and cell cycle progression (for a review, see Boonstra and Moes, 2005).

As indicated above, it was hypothesized that actin may represent a microgravity-sensitive component in mammalian cells. Microgravity might affect the behavior of actin directly or indirectly via actin-binding proteins. This thesis describes experiments that aim to identify the role of actin in sensing microgravity.

It was reported that cell proliferation and consequently cell cycling is affected by microgravity conditions (Vassy et al., 2003). Progression through the cell cycle is dependent on the activation of signal transduction cascades that originate from growth factors as well as from the attachment of cells (Hulleman et al, 1999a, b). Both cell attachment and signal transduction were demonstrated to alter under conditions of microgravity and are known to depend on actin. That is why the actin dynamics during the attachment of cells at the early G1 phase of the cell cycle represent an interesting model for studying actin functionality under different gravity conditions. By doing so, microgravity-induced changes in actin behavior that induce modifications in cell proliferation might be identified.

As mentioned above, signal transduction that is induced by growth factors has been demonstrated to alter under conditions of microgravity. In order to focus on the relation between growth factor-induced signal transduction and the behavior of actin, the dynamics of actin were studied in mouse fibroblasts that were stimulated with PDGF (Platelet-Derived Growth Factor). PDGF induces spectacular rearrangements of actin in mouse fibroblasts within minutes. This implies that regulators of the actin metabolism become activated. Therefore, this model is suited to study actin dynamics, including the activity of regulators of the actin metabolism, in cells that are exposed to microgravity conditions for a matter of minutes. This model of PDGF-induced actin dynamics in mouse fibroblasts was not only selected for the spectacular response of the actin cytoskeleton, but also because of the ability of these cells to survive the harsh conditions that are unavoidable when doing experiments in real microgravity.

In addition to ruffle formation, actin is well-known for its role in facilitating motility responses in cells upon stimulation by growth factors. Interestingly, cell migration was

reported to be affected by changing microgravity conditions (Meloni et al., 2011). Hence, the relation between PDGF-induced motility responses and actin dynamics also represents an interesting model for studying actin functionality in microgravity.

Thus, the first part of this thesis describes these three cellular processes that display a specific role of actin in cells. The appearance of actin was studied in cell cycle progression, cellular movement and membrane dynamics and in relation with growth factor-induced signal transduction. In the future, these models may be used to investigate the influence of microgravity on specific functions of actin. The second part of this thesis describes experiments that were conducted in both real and simulated microgravity.

This thesis has the following outline:

Chapter 2 provides an overview of the actin metabolism in mammalian cells. The various roles of actin in cells are described and the key players involved in the actin metabolism in mammalian cells are listed. The focus of this review is on the role of actin in the regulation of G1-phase progression.

Chapter 3 describes the appearance of actin during cell cycle progression, in particular during the early G1 phase of the cell cycle. Though it is clear that actin plays important roles in the regulation of cell cycle progression, the underlying molecular mechanisms remain to be elucidated. The experiments in Chapter 3 focused on the local activation of signal transduction – as directed by cellular attachment and growth factors – and actin during the early G1 phase of the cell cycle. Interestingly, various key signal transduction proteins were found in blebs at the cell membrane within 30 minutes after mitosis. These membrane blebs appeared in round, mitotic-like cells and disappeared rapidly during spreading of the cells in the G1 phase.

The relation between growth factor-induced signal transduction and the behavior of actin is studied in **Chapter 4**. The growth factor PDGF induces spectacular rearrangements of actin in mouse fibroblasts within minutes. This results for instance in the formation of dorsal circular ruffles. The formation of these dorsal circular ruffles was investigated in detail. Furthermore, a mutual interaction between growth factor signaling and actin is described that might explain how cells become less sensitive towards PDGF stimulation during the stimulation itself.

In **Chapter 5**, the role of actin in membrane dynamics such as growth factor-induced membrane ruffling and motility is further investigated. A new relation between $cPLA_2\alpha$ and actin-directed cell migration is described that may eventually be studied in

(simulated) microgravity. Upon stimulation of ruffling and cell migration by growth factors, endogenous cPLA$_2$α and its active phosphorylated form relocate at protrusions of the cell membrane involved in actin and membrane dynamics. Inhibition of cPLA$_2$α activity with specific inhibitors blocked growth factor-induced membrane and actin dynamics, suggesting an important role for cPLA$_2$α in these processes.

In **Chapter 6** the models described above are studied in microgravity. First, an overview is provided of research that was conducted in the past in (simulated) microgravity in relation to the actin cytoskeleton. Based on previous microgravity experiments and current knowledge of actin behavior at 1g, it was hypothesized that the actin microfilament system is the microgravity-sensitive component in mammalian cells (Boonstra, 1999). Next, Chapter 6 describes experiments that were performed in both simulated and real microgravity. In **6.1** two different ways to simulate microgravity conditions on earth are explored: random positioning and magnetic levitation. In **6.2a** experiments that were performed in real microgravity during the Dutch Soyuz Mission are described. In **6.2b** experiments that were conducted in real microgravity during the MASER-10 mission are described.

Chapter 7 discusses the results in relation to previous research performed in (simulated) microgravity and in relation to current knowledge of the behavior of actin at 1g on earth.

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Chapter 2

An introduction to actin:

2.1 Signal transduction and actin in the regulation of G1-phase progression
Boonstra, J., Moes, M.J. (2005) Critical Reviews in Eukaryotic Gene Expression 15: 255-276

2.2 Appendix

Signal Transduction and Actin in the Regulation of G1-Phase Progression

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ABSTRACT: Regulation of cell proliferation is dependent on the integration of signal transduction systems that are activated by external signal molecules, such as growth factors and extracellular matrix components. Dependent on these signal transduction networks, the cells decide in the G1 phase to continue proliferation or, alternatively, to stop cell-cycle progression and undergo apoptosis, differentiation, or quiescence. The MAP kinase and PI-3 kinase pathways have been demonstrated to play an essential role in these G1-phase decisions. Interestingly, actin has been demonstrated to mutually interfere with signal transduction. In addition, it has been indicated that the FOXO transcription factors are involved in these decisions, as well. Actin has been demonstrated to play an important role in the regulation of G1-phase progression. Because of its properties as a structural protein, actin is essential in cytokinesis and in cell spreading and, thus, is involved in G1-phase progression. As an intermediate factor in signal transduction, actin is likely to be involved in cell-cycle regulation induced by external signal molecules. And, finally, actin has been demonstrated to play a direct role in transcription. These observations indicate a prominent role of actin in the regulation of G1-phase progression.

KEY WORDS: ongoing cell cycle, growth factors, cyclins, extracellular matrix, actin-binding proteins, FOXO transcription factors

I. INTRODUCTION

Cell-cycle research has gained enormous attention during the last decades, especially research focusing on the processes underlying the regulation of G1-phase progression. The G1 phase constitutes an important cell-cycle phase, because virtually all nonproliferating cells in an organism contain a G1-phase amount of DNA, indicating that in the G1 phase, decisions are made as to whether the cell continues progression through the cell cycle, or whether this progression is stopped and followed by differentiation programs, induction to apoptosis, or just the establishment of a quiescent status. Thus the G1 phase is characterized by several decision processes. In addition, the G1 phase has been known as a cell-cycle phase in which several checkpoints are active. In these checkpoints, the cells control whether mitosis has been finished properly and whether cells are able to pass S phase properly. Finally, the G1 phase is known for its large morphological changes. During mitosis, the cells are rounded, followed by attachment to the substratum in early G1 phase. After attachment, the cells spread until the flattened well-known morphology has been obtained in mid-G1 phase. These morphological changes are primarily due to actin and the related actin-binding proteins. In this article, we briefly describe the molecular machinery that underlies G1-phase progression, focusing on the interplay between signal transduction complexes activated by soluble signal molecules, such as growth factors, and localized signal molecules, such as attachment factors. Subsequently, we describe the possible role of actin in the processes underlying G1-phase progression, with specific emphasis on actin as a structural protein, as a mediator in cytoplasmic signal transduction, and as a regulator of transcription processes in the nucleus.

II. REGULATION OF CELL-CYCLE PROGRESSION

Progression through the cell cycle is a well-regulated process that depends upon the interplay between external and internal factors. The external factors, such as growth factors and extracellular matrix components, activate an elaborate intracellular signal transduction network. Subsequently, the signal transduction network ultimately regulates the activities of the cell-cycle engines—the cyclin-CDK complexes (Fig. 1). The precise regulation of the activities of cyclin-CDK results in an orderly sequence of events that constitutes the cell cycle. Because both the intracellular signal transduction networks and the regulation of cyclin-CDK activities have been described in detail in recent reviews,[1–8] we only briefly summarize the main characteristics in this article.

As mentioned above, the engines of the cell cycle are the cyclin-CDK complexes. In these complexes, the cyclins are the activating subunits that interact with specific CDKs to regulate their activity and substrate specificity, whereas the CDKs are serine/threonine kinases that require binding of a specific cyclin in order to be ready to become activated. In addition to binding a cyclin, CDK activity is also dependent on the phosphorylation of threonine and tyrosine residues—some of which are stimulatory, whereas others are inhibitory[9,10]—and on the interaction with specific inhibitory proteins—the CDK inhibitory proteins.[11–13] Two families of CKIs have been identified on the basis of their structures and affinities for cyclin-CDK complexes. The Cip/Kip family, including p21 and p27, influence cyclin-CDK activity by promoting their assembly and/or stabilization. The effect can be either stimulatory or inhibitory.[11] The INK family of inhibitors are specific for the G1-phase CDKs.[11]

The most important mammalian cyclin-CDK complexes known so far are the mitotic cyclins A and B in association with CDC2, and the G1 cyclins D and E in association with CDK4,6 and

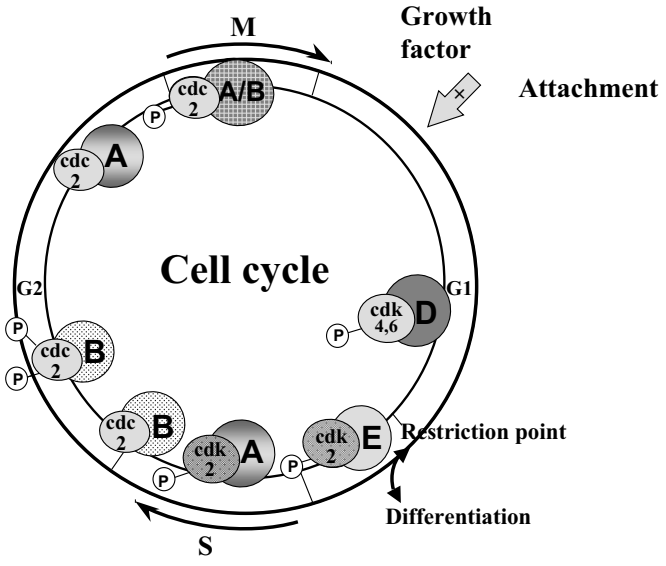

FIGURE 1. Overview of the cell cycle of mammalian cells. The mammalian cell cycle basically consists of four phases: first gap phase (G1), DNA synthesis (S), second gap phase (G2), and mitosis (M). The transition between the different phases is regulated by cyclin/CDK complexes. Different cyclins (A, B, D, E) are present during different cell cycle phases and interact with different CDKs. R is the restriction point defined as the point in the G1 phase after which the cells are independent from external factors for progression of the remainder of the cell cycle.

CDK2, respectively.[10] The first cyclin-CDK complex to be activated during the G1 phase is composed of a D-type cyclin in association with CDK4 or CDK6, depending on the cell type.[14] As cells progress through the G1 phase, cyclin E is synthesized, with a peak late in G1. Cyclin E associates with CDK2 and is important for entry into S phase.[15] Once cells enter S phase, cyclin E is degraded and CDK2 then associates with cyclin A.[16] Finally, cyclin A and the B-type cyclins associate with CDK1 to promote entry into mitosis (Fig. 1). Cyclin A binds to CDK1 with a peak of activity in G2 phase and is then suddenly degraded, whereas entry into mitosis is triggered by cyclin B-CDK1. For exit from mitosis, cyclin B destruction is required.[17]

An important strategy employed in cell-cycle regulation is that one regulatory molecule stimulates one cell-cycle phase and simultaneously inhibits another. Thus, for example, cyclin-CDK activities required for G1/S-phase transitions inhibit the G2/M-phase transition. This strategy ensures that cell-cycle progression is irreversible. This irreversible character of cell-cycle progression is even reinforced by ubiquitin-mediated proteolysis of cyclins once a checkpoint has been passed.[18] Cyclins all encode a PEST sequence, which is recognized by the appropriate F-box protein and targets them for ubiquitination and subsequent proteolytic degradation.[19,20]

One of the most important G1-phase cyclin/CDK substrates in mammalian cells is the product of the retinoblastoma tumor-suppressor gene (pRB).[21] pRB is phosphorylated in a cell-cycle–dependent manner and binds in the hypophosphorylated state to transcription factors, particularly members of the E2F family. E2F consists of at least five different isoforms that form heterodimers with a second group of proteins, known as DP-1.[21] pRB is present in this hypophosphorylated form during early G1 and becomes phosphorylated on several residues during mid- to late G1. This phosphorylation causes the release and activation of the E2F transcription factors, allowing the transcription of genes that mediate progression through S phase.[22] Initial activation of pRB is thought to occur in the G1 phase through phosphorylation by cyclin D/CDK complexes. D-type cyclins can bind directly to pRB in the absence of a kinase and, thus, might target the pRB to CDK4/CDK6 kinases. After the initial phosphorylation by cyclin D/CDK, cyclin E/CDK2 complexes are thought to subsequently phosphorylate pRB late in G1, thereby triggering the onset of S phase.[22]

Another important protein involved in cell-cycle regulation concerns p53. Under normal conditions, the levels of p53 protein are low due to the relatively short half-life of the protein. However, intracellular and extracellular stress signals can induce the stabilization and activation of p53.[23,24] This activation of p53 leads to the transcription of several genes whose products can influence cell-cycle progression, such as the CKI $p21^{Cip1/WAF1}$. Of special interest is the increase in p53 activity upon DNA damage, resulting in cell-cycle arrest and subsequent DNA repair.[24]

III. SIGNAL TRANSDUCTION AND G1-PHASE PROGRESSION

Cell-cycle progression of mammalian cells is largely determined by the action of extracellular signal molecules, such as growth factors and extracellular matrix components. These extracellular signal molecules exert their effects by interaction with specific cell surface located receptors. These receptors have in common that they, upon activation by their ligand, activate an intracellular signal transduction cascade that ultimately results in specific gene expression (Fig. 2). The signal transduction cascades activated by growth factors and extracellular matrix components have been reviewed in detail.[25–28] Therefore, we only briefly summarize these features by describing the most well-known cascades that have been indicated to play an essential role in cell-cycle progression.

A. The MAP Kinase Pathway

One of the most important signaling pathways in the complex network of signal transduction involved in cell-cycle regulation is the mitogen-activated protein kinase (MAPK) pathway (Fig. 2). The MAPK pathway is one of the best-known cascades that is activated by both growth factors and the extracellular matrix. Growth factors bind to their receptors, which are receptor tyrosine

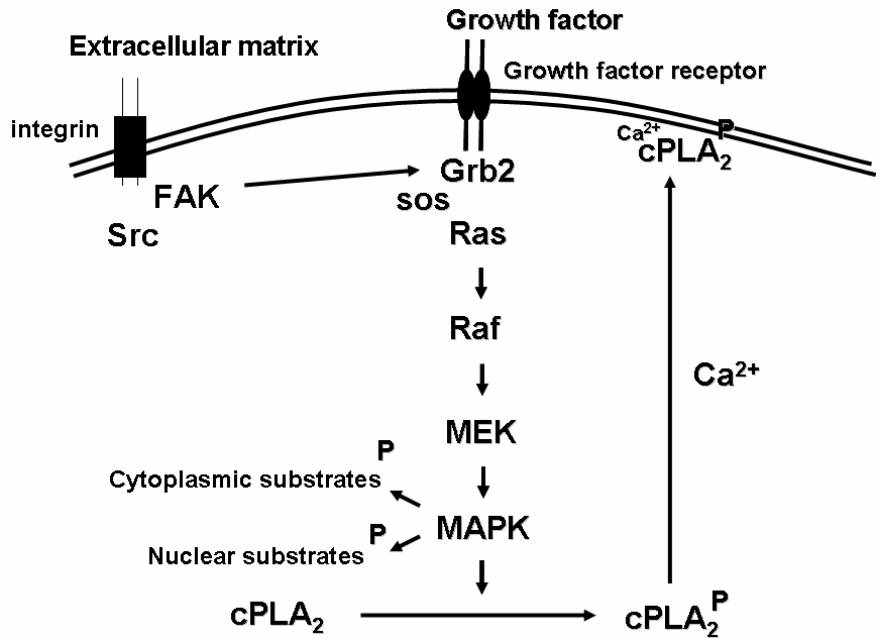

FIGURE 2. Overview of the MAP kinase (ERK1/2) signal transduction pathway. Activation of growth factor receptors or integrins by binding to their respective ligands results in the activation of the small G-protein Ras, which, in its turn, activated the serine/threonine kinase raf. Activated raf phosphorylates and activates the dual-specificity kinase MEK, which, in its turn, phosphorylates MAP kinase on serine and tyrosine, resulting in full activation. MAP kinase phosphorylates several substrates in both cytoplasm (including the cytosolic phospholipase A_2) and nucleus (including several transcription factors).

kinases. Upon binding, the tyrosine kinase receptors are activated and phosphorylated, thus creating high-affinity binding sites for their substrates. One of the substrates is the adaptor protein Grb2, which, in turn, binds and activates the guanine exchange factor Sos. Sos, in its turn, results in the activation of the G-protein ras. One of the substrates of ras is the serine/threonine kinase raf, which is activated by ras at the plasma membrane. In its turn, raf phosphorylates and activates the dual-specificity kinase MEK, which subsequently phosphorylates and activates the MAP kinase proteins ERK1 and ERK2, as reviewed previously.[29] Similarly, the MAP kinase pathway can be activated by the extracellular matrix (Fig. 2). Upon activation of the extracellular matrix receptors—the integrins, the signal is transmitted to focal adhesion kinase (FAK), also a tyrosine kinase. FAK associates with the cytoplasmic domain of integrins, and upon activation of integrin, the FAK is autophosphorylated. Activated FAK subsequently associates with c-src, a cytoplasmic tyrosine kinase, which further phosphorylates FAK on additional tyrosine residues, leading to full activation of FAK.[30] This phosphorylation results in the binding of the adaptor protein Grb2,[31] which results in the activation of the MAP kinase pathway, as described above for growth factors.[32,33]

The proteins in the MAP kinase cascade that play a central role in cell-cycle regulation are the p42 and p44 MAP kinases ERK2 and ERK1, respectively. ERK1,2 are threonine serine kinases that, upon activation, are able to translocate to the nucleus where specific transcription factors are phosphorylated and activated, such as c-myc, c-jun, Elk-1, c-Ets-1, and c-Ets-2.[34–37] In addi-

tion the activated ERKs can also phosphorylate several cytoplasmic substrates, such as cytoskeletal proteins, cytosolic phospholipase A_2, and others (Fig. 2).[38–41]

Most research done on the role of the MAP kinase pathway in the regulation of cell-cycle progression has been performed by the activation of quiescent cells by mitogens. Upon activation, ERK1/2 translocate to the nucleus where they phosphorylate and activate transcription factors and induce early gene expression.[42] In fibroblasts, activation of the ERK1/2 pathway at the G0/G1 transition has been shown to induce the expression of cyclin D.[43,44] More recently, evidence has been obtained that demonstrates that two waves of growth factor–dependent signaling events are required for progression from the G0 through G1 phase. The first one is an acute burst immediately after growth factor receptor stimulation, and the second one occurs hours after the stimulation.[45] These observations suggest that the first burst of activity is related to the activation of the G0 cells, whereas the second may well be required for G1-phase progression. This latter would be in agreement with our studies on the role of ERK1/2 in the ongoing cell cycle.[46] In these studies, it was demonstrated, using Chinese hamster ovary (CHO) cells synchronized by mitotic cell selection, that p42/44 is phosphorylated immediately after mitosis in early G1 phase. Subsequently, the phosphorylated p42/44 was translocated to the nucleus during the mid-G1 phase, several hours after the initial phosphorylation. Treatment of the cells with an inhibitor of p42/44 phosphorylation in early G1 phase caused a full inhibition of phosphorylation and also inhibition of the nuclear translocation. Furthermore, these cells were unable to progress into S phase, thus demonstrating the necessity of a functional ERK1/2 during progression through G1 phase.[46] Similar conclusions were obtained in fibroblasts in which the ERK1/2 cascade was inhibited by antisense constructs, overexpression of kinase inactive mutants, or inactivation by MAP kinase phosphatase (MKP-1).[47,48]

Although p42/44 appear to play a direct role in progression through the G1 phase of the cell cycle, the downstream pathways also play an essential role. One of the substrates of p42/44 is cytosolic phospholipase A_2 ($cPLA_2$). $cPLA_2$ activity results in the formation of arachidonic acid, and, in turn, arachidonic acid is metabolized into various eicosanoids, including prostaglandins, leukotrienes, thromboxanes, and others. Arachidonic acid and its metabolites have been proposed to play an important role in cell-cycle regulation. Thus, cyclin D1 expression and S-phase entry were induced by prostaglandin F2α in Swiss 3T3 fibroblasts, whereas other prostaglandins were able to arrest cells at the G2/M phase of the cell cycle.[49,50] We have shown that $cPLA_2$ activity increased transiently during mid-G1 phase of the ongoing cell cycle of CHO and neuroblastoma N2A cells, this activity being dependent upon the activity of p42/44.[51,52] By using different inhibitors of $cPLA_2$, it was demonstrated that the activity of $cPLA_2$ in mid-G1 phase was required for entry into S phase. The effects of $cPLA_2$ inhibition on cell-cycle progression were mediated by lipoxygenase rather than cyclooxygenase products, because G1/S transition was only inhibited when lipoxygenase activity was prevented.[52,53]In addition to the ERK1/2 pathways, MAP kinase homologs have been identified in mammalian cells such as the JNK/SAPK and the p38 HOG1 kinase. These MAP kinases respond to distinct extracellular stimuli and have different intracellular substrates. Usually the pathways are involved in specific stress conditions.[54,55]

B. The Phosphatidylinositol 3 Kinase Pathway

Another important signal transduction pathway that plays an essential role in the regulation of cell-cycle progression concerns the phosphatidylinositol 3 kinase (PI 3-kinase) pathway (Fig. 3), because this pathway has been identified as the antiapoptotic pathway. The PI 3-kinase pathway is activated by binding the p85 regulatory subunit of PI 3-kinase to the phosphorylated tyrosine residues of tyrosine kinases, both activated by growth factors and by extracellular matrix components. This recruits the catalytic subunit of PI 3-kinase, p110, to this complex, resulting in the activation of the catalytic domain. Once the p110 subunit of PI 3-kinase is activated, it will catalyze the specific phosphorylation of the inositol ring of phosphoinositides at 3D, generating primarily phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P_3) and phosphatidylinositol-3,4-bisphos-

phate (PtdIns-3,4-P_2). The mechanisms by which PI 3-kinases activate signaling pathways have been recently unraveled. Their lipid products interact with a number of signaling proteins, resulting in membrane targeting and/or modulation of enzyme activity. For example, PtdIns(3,4,5)P_3 binds to a conserved protein motif called the pleckstrin homology (PH) domain, thereby inducing the activation of the serine/threonine kinase Akt/protein kinase B (PKB) and its upstream activators, the phosphoinositide-dependent kinases (PDKs). These kinases phosphorylate a number of substrates, such as $p21^{CIP1}$, Raf, and the forkhead family of transcription factors (FKHR/ AFX/ FOX), which are involved in the control of cell proliferation and survival.[56–58]

Because the PI 3-kinase pathway has been described as the antiapoptotic pathway, the role of PI 3-kinase was determined during the ongoing cell cycle of CHO cells. CHO cells were synchronized by the mitotic shake-off and were subsequently incubated with the specific PI-3 kinase inhibitor wortmannin at several time points after mitosis. The cells were assayed for cell-cycle progression after 24 hours by measuring the [^{3}H]-thymidine incorporation. The addition of wortmannin 4, 6, or 8 hours after mitosis did not cause a significant change in thymidine incorporation as compared to control cells. In contrast, the addition of wortmannin during mitosis, or 2 hours after mitosis, caused a significant decrease of thymidine incorporation. Similar results were obtained with the PI 3-kinase inhibitor LY294002. These observations suggest that PI 3-kinase activity during mitosis and immediately after mitosis is essential for normal cell-cycle progression.[59]

C. Cross Talk Between Growth Factor Receptors and Integrins

Several studies during recent years have indicated cross talk between growth factor receptor tyrosine kinase- and integrin-induced signal transduction cascades.[60–63] Thus, activated growth factor receptors modulate integrin localization and activation, which results in changes in cell adhesion, cell spreading, and cell motility.[64–66] On the other hand, integrin signaling is required for the full activation of growth factor signaling pathways, as

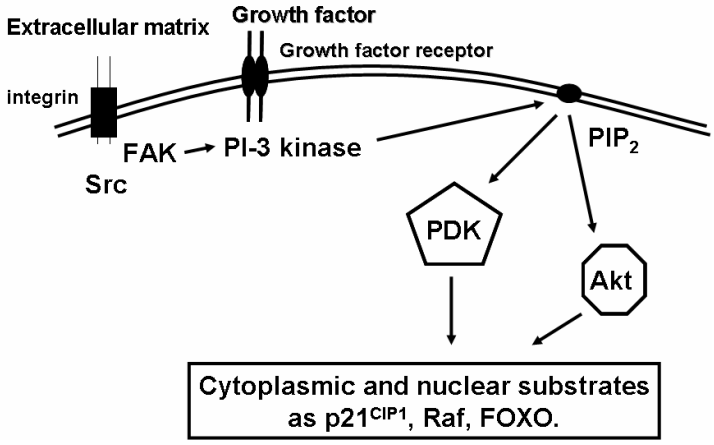

FIGURE 3. Overview of the PI 3-kinase signal transduction pathway. Activation of growth factor receptors or integrins by binding to their respective ligands results in the activation of the PI 3-kinase. This kinase phosphorylates PI on the 3 position in the plasma membrane, resulting in the generation of docking sites for PH-containing proteins, such as Akt. Upon binding the Akt kinase is activated and subsequently phosphorylates cytoplasmic and nuclear substrates. PDK are phosphoinositide-dependent kinases.

deduced from the observations that integrin activation is required for growth factor–induced expression of G1-phase cyclins.[28,67,68] Interestingly, integrins are able to activate receptor tyrosine kinases in the absence of exogenously added receptor ligands, including receptors for EGF, insulin, PDGF, hepatocyte growth factor, vascular endothelial growth factor, and RON.[69–75] This cross talk between integrins and receptor tyrosine kinase receptors is most likely due to the formation of protein complexes between integrins and receptors that allow a direct or indirect interaction.[76–78] In addition to a direct interaction between integrins and receptor tyrosine kinases, downstream interaction between the respective signal transduction cascades has also been reported. For example, in fibroblasts, the loss of integrin-mediated cell anchorage blocks the propagation of the signal from Ras to Raf-1, whereas the activation of ras was not changed.[79] This observation indicates that an anchorage-dependent step exists between Ras and Raf in the signaling cascade triggered by growth factors. A similar anchorage-dependent step has been described to exist between Raf and MEK.[80] These observations indicate the existence of a complex network of signaling proteins that interact on several levels, this interaction being required for optimal signal transmission.

IV. TRANSCRIPTION AND G1-PHASE PROGRESSION

As described briefly above, several signal transduction pathways play an essential role in cell-cycle progression. In addition to the MAP kinase pathway and PI 3-kinase, other essential pathways have also been described, including key proteins such as protein kinase A, protein kinase C, the phospholipases βγ, src kinase, and many small G proteins, such as ras and others. Altogether these observations clearly indicate the existence of an elaborate signaling network in the cell that is highly interconnected, and consequently it is very difficult to pinpoint a specific cascade as being responsible for cell-cycle regulation. However, it is also clear that the ultimate decisions about the fate of the cell are made in the nucleus by specific gene transcription. In this respect the E2F transcription factors have been demonstrated to play an essential role (for review, see Ref. 21). E2F consists of at least five different isoforms that form heterodimers with a second group of proteins known as DP-1.[21] In early G1 phase, E2F is bound to hypophosphorylated pRB. Upon phosphorylation on several residues by cyclin-CDK activity during mid- to late G1, the pRB releases the bound E2F, allowing the transcription of genes that mediate progression through S phase.[22]

However, during the G1 phase, the cells have several decisions to make, ranging from an ongoing proliferation to cell-cycle arrest. In the latter case, the cell-cycle arrest may be followed by differentiation or apoptosis (for review see Refs. 24, 81). Recently, we have demonstrated the presence of two points in the G1 phase of the cell cycle, $G0^-$ and G_R, respectively, in which different decisions are made. $G0^-$ was located very early in G1 phase—immediately after mitosis, and G_R was located at the end of G1 phase. The early restriction point appears to lead to a G0-like state, whereas the second decision point appears to correlate with the restriction point. The entry into the $G0^-$ state is restricted to only a limited period of time after mitosis, whereas entry into G_R phase occurs several hours after mitosis. $G0^-$ was indicated to be related to apoptosis, whereas G_R seems to be related to cell differentiation.[82] Since FOXO transcriptional activity in the nucleus seems to have a crucial impact on the initiation of either quiescence, apoptosis, or differentiation, suggesting that activation of FOXO transcription factors govern different cell fates, depending on whether they occur during $G0^-$ (apoptosis) or G_R phase (differentiation) of the cell cycle.

There are three members of the FOXO subgroup of FOX factors, namely, FOXO1 (FKHR), FOXO3a (FKHR-L1), and FOXO4 (AFX). FOXO regulates the expression of many genes in mammalian cells, whose expression results in markedly different cell fates. For example, activation of FOXO in Chinese Hamster Ovary (CHO) cells can result in cell-cycle arrest and entry into the G0 quiescent stage, whereas T cells and neuronal cells normally respond by the induction of apoptosis.[83] On the one hand, FOXO4 has been shown to inhibit Cyclin D expression and to upregulate p27 expression (CDK4/6 and CDK2

inhibitor), thus resulting in increased protein levels, inhibition of cell growth, and, ultimately, quiescence.[84] On the other hand, FOXO3 has been postulated to trigger apoptosis by inducing the expression of the Fas ligand gene critical for entry into apoptosis.[85] Additionally, FOXO3 has been shown to regulate the expression of pro-apoptotic Bcl-2 family member Bim in T cells, thus inducing cell death in this cell type.[86] Moreover, FOXO4 is suggested to regulate apoptosis by inducing BCL-6 transcription.[87] This transcriptional repressor, in turn, suppresses the levels of the antiapoptotic BCL-XL protein, thus inducing cell death.[86] Thus, the consequences of FOXO activation are not as clear-cut as suggested by the induction of either cell-cycle arrest (leading to quiescence) or apoptosis. In view of the above-described signal transduction cascades, it is tempting to suggest that the different effects of FOXO activation on cell fate are due to activation of FOXO in different cell-cycle phases by either the PI 3-kinase or the MAP kinase pathway. In agreement with this proposal are the observations that PKB/Akt has been shown to inhibit the transcriptional activity of FOX factors. When located in the nucleus, FOX factors may lead to either apoptosis or exit into G0 (quiescence) phase upon cell-cycle arrest.[83] They are phosphorylated in vivo in the nucleus by PKB/Akt on one threonine and two serine residues.[83] Nuclear phosphorylation of FOXO on Serine 193 in the DNA-binding domain by PKB excludes FOXO from the nucleus and prevents its transcriptional activity.[88] Because FOXO shuttling between the nucleus and the cytoplasm is constitutive and dependent upon RanGTP, Crm1, and importins, phosphorylated FOXO will leave the nucleus, where it will be sequestered by its phosphorylated sites probably by the 14-3-3 protein.[88] As a result, FOXO import into the nucleus is inhibited, its transcriptional activity ceases, and the cells continue proliferating. However, upon serum starvation and in the absence of growth factors, the PI 3-kinase pathway switches off, and, as a result, PKB/Akt can no longer phosphorylate FOXO. This, in turn, enables FOXO to remain in the nucleus and perform its transcriptional activities. Interestingly, preliminary results in our laboratory demonstrate that inhibition of PI 3-kinase during early G1 phase of the ongoing cell cycle results in inhibition of cell-cycle progression, in contrast to inhibition of PI 3-kinase during mid- and late G1 phase.[59]

V. ACTIN AND G1-PHASE PROGRESSION

Actin is an extremely abundant protein in virtually all eukaryotic cells and is involved in many cellular functions, including migration, endocytosis, intracellular transport, docking of proteins and mRNA, attachment, signal transduction, membrane ruffling, neuronal path finding, and cytokinesis. Moreover, it largely determines the cell shape and the position and shape of organelles within the cytoplasm.

The actin family consists of α-, β-, and γ-isoforms. The α-isoform is present in muscle cells, whereas the β- and γ-isoforms are present in all cells. Actin is present in cells in an unassembled, globular form and a polymerized, filamentous form, called G-actin and F-actin, respectively (Fig. 4). The F-actin filaments are composed of two linear strands of polymerized G-actin wound around each other in a helix. Within these filaments, the actin monomers are oriented in the same direction, which causes an inherent polarity of the filaments resulting in the barbed or plus end and the pointed or minus end. The barbed ends are characterized by a rapid polymerization and a slow depolymerization, and the pointed ends exhibit the opposite features. In the cells, actin continuously cycles between the polymer and monomer state, a process called treadmilling.

The actin filaments constitute a highly dynamic network in the cells, the dynamics being regulated by a large number of actin-binding proteins (ABPs).[89,90] The ABPs can be characterized by their function, including cross-linking proteins, actin severing, capping and depolymerizing proteins, monomer-binding proteins, membrane-associated proteins, and actin-regulatory proteins. Several conserved domains of actin have been identified that act as binding domains for the ABPs, including the myosin motor domain,[91] the gelsolin homology domain,[92] the calpain homology (CH) domain,[93] the actin depolymerizing factor/cofilin (ADF/cofilin) domain,[94] and the

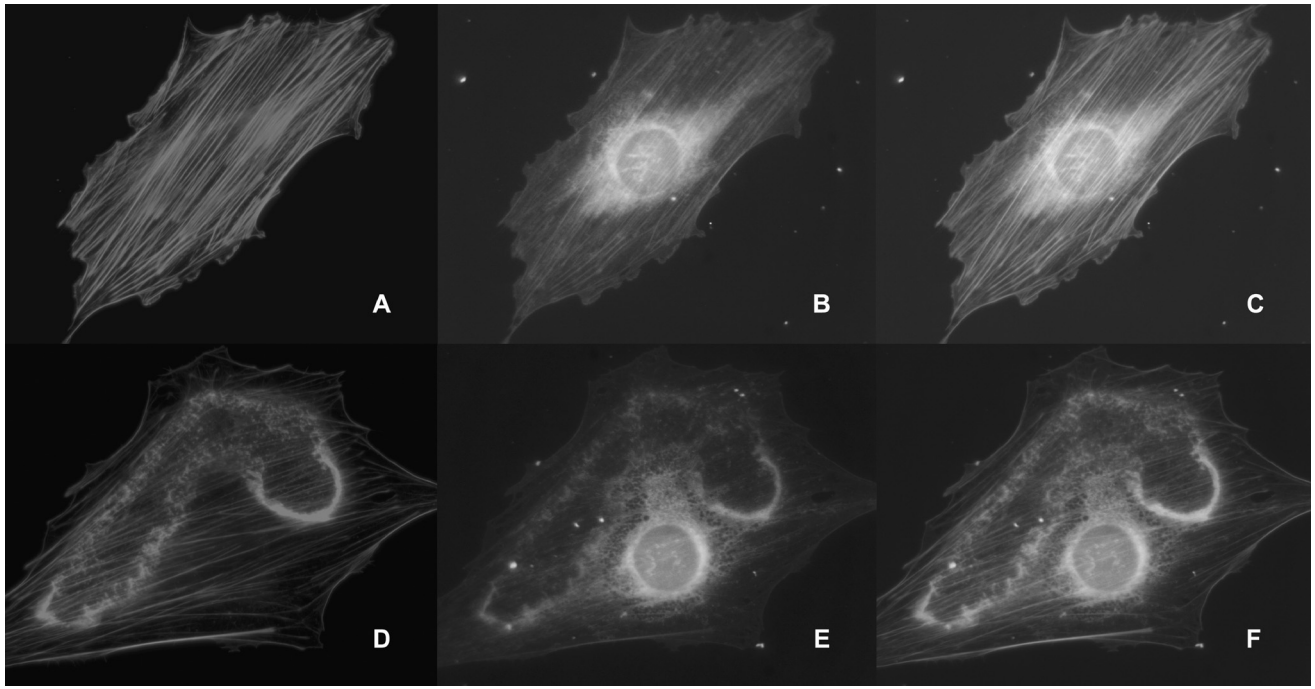

FIGURE 4. Effect of PDGF on F- and G-actin localization in fibroblasts. C3H/10T1/2 fibroblasts were serum deprived for 24 hours and subsequently incubated in the presence or absence of 20 ng/mL PDGF-BB for 10 minutes at 37°. The cells were fixed using formaldehyde and incubated with Phalloidin-Tritc to label F-actin or with DNase I-Alexa488 to label G-actin. F-actin is visible in large stress fibers (**A**), whereas G-actin is localized mainly around and in the nucleus (**B**). Incubation in the presence of PDGF-BB for 10 minutes results in the formation of membrane ruffles and the partial disappearance of stress fibers (**D**). **A** and **B**: control cells labeled for F-actin and G-actin respectively. **D** and **E**: PDGF-treated cells labeled for F-actin and G-actin, respectively. Merged images are presented in (**C**) and (**F**).

Wiskott-Aldrich syndrome protein (WASP)-homology domain-2 (WH2).[95] These observations clearly demonstrate that actin metabolism is regulated by a large number of proteins, which, in their turn, are subject to regulation, as well. This complicated network of actin and the ABPs plays an essential role in cell metabolism and, consequently, also in cell-cycle regulation.

A. Actin as a Structural Protein

Actin is the main constituent of the microfilaments and, as such, plays a dominant role in dynamic cell processes. A direct role of actin in cell-cycle progression concerns its specific activity during cytokinesis (for review see Refs. 96, 97). One of the important processes during cell division is the formation and contraction of the contractile ring. This ring consists of actin-myosin II filaments and a number of ABPs that regulate the actin rearrangements. Among the ABPs are Rho-family small GTPases to regulate the actin polymerization, profilin to regulate actin dynamics, cofilin for actin-filament severing, formin-homology proteins to link Rho signaling to profilin-mediated actin polymerization, caldesmon to regulate myosin II, radixin to cross-link actin to the plasma membrane through CD43, and a number of others.[96] Myosin II motor proteins contribute to the contractility of the cleavage furrow during cell division.[96,97] The actomyosin complex plays an essential role in cell division but is not very important for cell-cycle regulation. Many mutants have been described that lack one or more of the actin-myosin complex, resulting in incomplete or no cell division, but these mutations do not affect the nuclear division. Similarly, inhibition of cell division by actin polymerization–inhibiting compounds, such as cytochalasin, did not influence cell-cycle progression, yielding multinucleated cells.[98,99]

After completion of cytokinesis, cells attach to the substratum, followed by cell spreading in early G1 phase— the latter process again strongly dependent upon actin metabolism (Fig. 5). Cell attachment to ECM components is initiated by the binding of integrins to the ECM proteins, such as fibronectin and laminin. Integrins are heterodimers that are composed of an α and β subunit, each with a large extracellular domain, a single membrane-spanning region, and a short cytoplasmic domain.[29,76] The clustering of integrins is associated with the formation of focal adhesion complexes in cultured cells (Fig 6). These focal adhesions are complex structures containing a variety of structural proteins, such as talin, vinculin, and α-actinin; signaling molecules, such as FAK; and adaptor molecules, such as paxilin, tensin, and $p130^{cas}$.[100,101] Following the activation of focal adhesion proteins by attachment, cell spreading is accomplished with the extension of membrane protrusions, such as lamellipodia and filopodia, and the formation of actin stress fibers (Fig. 6). Activation of the integrins results in the activation of small GTPases of the Rho family of proteins, such as RhoA, Rac1, and Cdc42, the latter two acting as regulators of actin assembly,[102–105] whereas Rho induces focal adhesion and stress fiber formation. Today, on the basis of primary sequence data and known functions, the Rho proteins can be divided into five groups: the Rho-like, the Rac-like, the Cdc42-like, the Rnd, and the RhoBTB subfamilies.[105] The activation of the Rho family proteins by extracellular signal molecules, including growth factors and extracellular matrix components, as well as the downstream effects of the Rho proteins that lead to changes in actin morphology, have been described recently in detail,[105] and the reader is referred for the details and references to this review.

Here, we briefly describe an example in which activation of Rho proteins leads to changes in actin morphology (Fig. 7). In the leading edge, extracellular signal molecules bind to receptors in the plasma membrane, thereby generating intracellular signaling molecules, such as PIP_2, and activating Rho family GTPases, including Cdc42. Binding of PIP_2 and Cdc42 can subsequently activate WASp/Scar family proteins such as N-WASP. Recently, it was described that N-WASP activity is suppressed at PIP_2 levels present in quiescent cells but can be activated by increased PIP_2 levels as obtained in growth factor–stimulated cells.[106] Subsequently, WASP binds to and activates the Arp2/3 complex, which starts to nucleate branched actin filament growth, thus pushing the membrane forward. The elongation of filaments can be terminated by capping proteins such as gelsolin. ADF/Cofilin was shown to be involved in creating new

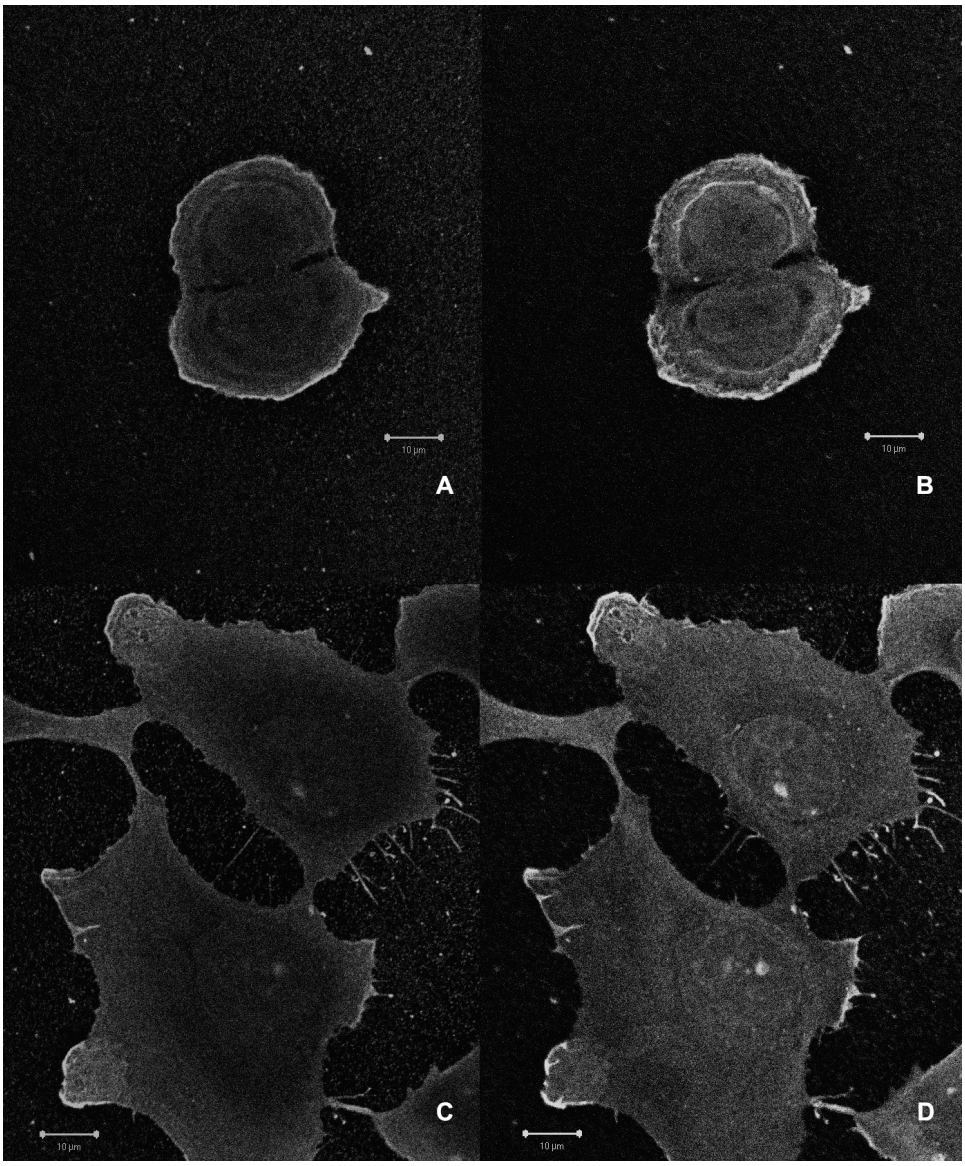

FIGURE 5. β-Actin localization during the G1 phase of HeLa cells. HeLa cells were synchronized by mitotic selection. After synchronization, the cells were plated and cultured for 1 hour (**A** and **B**) or 4 hours (**C** and **D**), fixed with formaldehyde and labeled for β-actin using a monoclonal antibody directed against β-actin (Sigma, A1978, Clone AC-15) and goat-anti-mouse-CY3 secondary antibody. The cells were studied using a confocal scanning light microscopy. Optical sections at 1.48 µm (**A** and **C**) and 2.46 µm (**B** and **D**) from the basal side of the cells, respectively. β-actin is present at the leading edge, in the cytoplasm and in the nucleus of the cells. The scalebar represents 10 µm.

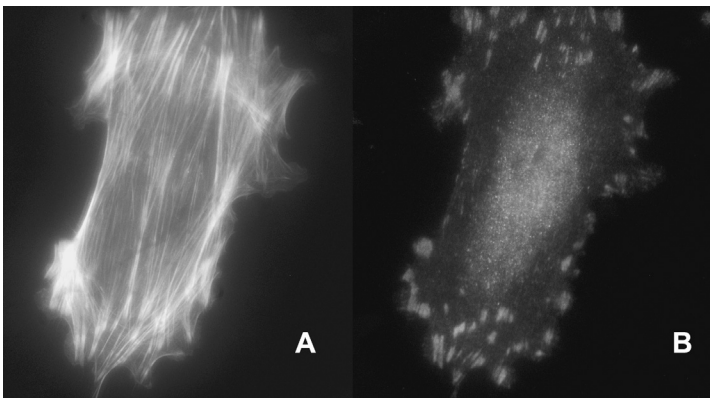

FIGURE 6. Stress fibers and focal contacts in fibroblasts. C3H/10T1/2 fibroblasts were stained for F-actin using phalloidin-Tritc (**A**) and focal adhesion kinase (FAK) phosphorylated on Tyr397 using rabbit antiFAK-pY397 (Biosource) and GARCY3 as a secondary antibody (**B**). The phosphorylated FAK is present in the focal adhesion sites and co-localizes with the F-actin stress fibers.

free barbed ends and nucleation sites for Arp2/3,[107] resulting in the formation of a branched network of filaments. In the oldest part of a filament, the ATP of each actin subunit is hydrolyzed, and the resulting ADP-actin filaments are severed by ADF/cofilin. The phosphate is released and the resulting ADP-actin dissociates from the filament-pointed ends, supplying the

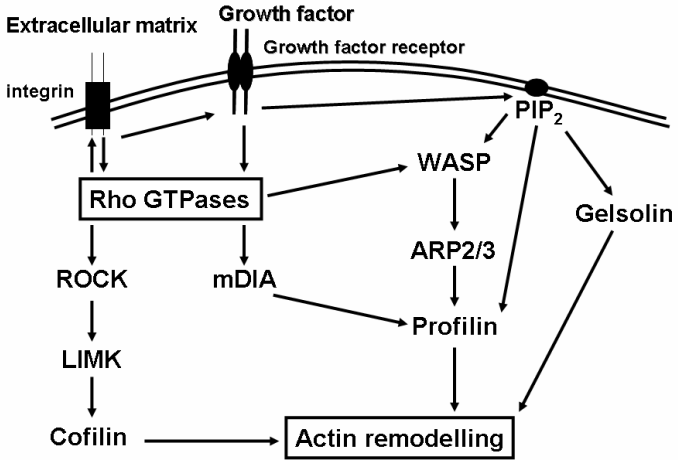

FIGURE 7. Overview of the interaction between signal transduction and actin remodeling. Activation of growth factor receptors or integrins by binding to their respective ligands results in actin remodeling through Rho GTPases. Rho GTPases subsequently activate a kinase cascade including ROCK and LIMK to activate the actin-binding protein cofilin. Alternatively, the profilin is modulated through mDIA or the WASP pathway.

cell with actin monomers that can now be recycled for new filament formation. Profilin catalyses the exchange of ADP for ATP on the actin monomers, and these can now be used to elongate the last formed filaments at the barbed ends and to form new filaments. Together with thymosin-β4, it maintains a pool of monomeric actin, thereby preventing spontaneous polymerization. Both profilin and cofilin are also under the control of various signaling molecules that result from the same extracellular signaling molecules. So the direction of filament growth is driven by ATP-hydrolysis and can be regulated by extracellular signals.

During the ongoing cell cycle of both CHO and neuroblastoma N2A cells, we have demonstrated that prevention of cell attachment after mitosis caused an arrest of G1-phase progression.[67] Mitotic cells plated on a nonadherent substrate did not attach and no cell spreading was observed. In addition, the cells were not able to progress into the S phase as deduced from thymidine incorporation studies. Interestingly, cyclin D was expressed in these cells, but no cyclin E expression was detected. Plating mitotic cells on a nonadherent substrate coated with poly-L-lysine did result in cell attachment, but no cell spreading was observed. Also, no cyclin E expression was detected in these cells, in contrast to cells plated on the same substratum coated with fibronectin in which a normal G1-phase progression was measured. The results demonstrate that cyclin E expression during the ongoing cell cycle is dependent on cell attachment and subsequent cell spreading induced by integrin activation.[67] These observations suggest that actin polymerization, which is essential for cell spreading, might play an important role in G1-phase progression.

B. Actin as Signal Transduction Mediator

Actin has been demonstrated to be closely related to signal transduction. The first indications for this relationship were obtained by studies on the effect of growth factors on cell morphology. Thus, it was demonstrated that EGF caused the formation of membrane ruffles within minutes after the addition of the growth factor.[108–110] The membrane ruffling was due to actin polymerization. In addition, it was demonstrated that EGF caused actin polymerization in the same time frame as the appearance of the membrane ruffles, whereas both features were completely inhibited by cytochalasin B.[110,111] Similar observations were made on fibroblasts treated with PDGF (Fig. 4). Interestingly, it was demonstrated that abolishment of the actin structure by cytochalasin B caused a super induction of EGF-induced c-fos expression, suggesting that EGF-induced actin polymerization was important for negative feedback regulation of signal transduction by the EGF receptor.[111]

A more close interaction between actin and signal transduction was suggested by the observations that growth factor receptors, among them the EGF receptor, were associated with the cytoskeleton.[112–114] Later, it was demonstrated that the EGF receptor was bound directly to actin.[115] In addition to the receptors, other signal transduction proteins were also found to be associated with the actin microfilaments, including phosphoinositide kinase, diacylglycerol kinase, phospholipase C, Akt/PKB, and others,[116–120] as has been reviewed by Janmey.[121] Altogether, these studies indicated that stimulation of cells with EGF caused a rapid actin polymerization, the formation of membrane ruffles, and the translocation of several of the downstream signaling proteins to these newly formed membrane ruffles, suggesting the formation of signaling complexes at the plasma membrane.[117] The observations summarized above indicate a mutual interaction between signaling cascades and the actin microfilaments, growth factor signaling-induced actin polymerization, and changes in actin morphology, whereas actin, in its turn, regulates signal transduction.

As described above, actin plays an important role in growth factor- and integrin-induced signal transduction. However, both signal transduction pathways are interacting, as well, as exemplified by the ERK pathway. ERK is recruited to focal adhesions in response to several stimuli, such as integrin activation, activation of v-Src, activation of PKCε, and activation of the FGF receptor.[122–124] PDGF and EGF induce cell migration and cause localized cell deadhesion requiring ERK signaling.[125] The effect of growth factors on cell adhesion requires the activation of calpain 2.[126,127] Of particular interest are the observations that calpain activity was decreased in

FAK-deficient cells.[128] In addition, it was demonstrated that FAK induces the formation of a complex constituting calpain 2, FAK, and ERK.[129] These data suggest that FAK is critical to the integration of migratory signals from growth factor receptors and integrins through the ERK pathway to the calpain proteolytic system, resulting in focal adhesion turnover and cell migration.[130]

Actin microfilaments have also been demonstrated to regulate integrins. Treatment of cells with cytochalasin D to cap actin filaments inhibits cell adhesion. In other cells, it was demonstrated that inhibition of actin polymerization resulted in an induction of ligand binding to integrins.[131] Activation of Cdc42 and Rac is associated with the formation of focal complexes in fibroblasts,[132] and inhibition of Rho resulted in a decrease of integrin-mediated aggregation of leukocytes and platelets (reviewed in Ref. 133).

As described above, the signal transduction cascades activated by growth factors and integrins are intimately linked to actin, and, therefore, it seems apparent that actin metabolism itself plays an important role in G1-phase progression, as well. Indeed, disruption of actin architecture with pharmacological agents leads to G1 arrest in a variety of cell types.[134–145] Although cytoskeleton-dependent G1 arrest is related to inhibition of cyclin E expression in Swiss 3T3 cells,[142] most studies report a failure to induce sustained activity of the p42/p44 MAPKs, expression of cyclin D1, and downregulation of the cdk inhibitor $p27^{KIP1}$.[138–141,144] In contrast to the cell-cycle block obtained with pharmacological inhibitors of actin polymerization, inhibition of the Rho-Rho kinase (ROCK) pathway required for stress fiber formation does not prevent the induction of cyclin D1- and G1-phase progression. In fact, inhibition of Rho revealed a cryptic pathway controlled by Rac/Cdc42, resulting in a strikingly early induction of cyclin D1 and accelerated G1-to-S phase transition independent of actin stress fibers and MAPK activation.[146–147] It was proposed that, as long as cyclin D1 is induced, cell-cycle progression is uncoupled from an organized cytoskeleton and the consequent spread cell shape.[146,147] This model is supported by observations that overexpression of cyclin D1 rescues proliferation in nonadherent cells, allowing for anchorage-independent growth as observed in many tumors.[143,148–151] Of particular interest are our observations that disruption of postmitotic actin reorganizations by cytochalasin or latrunculin did prevent cell spreading and the formation of filopodia, lamellipodia, membrane ruffles, and stress fibers but did not influence entry into S phase (unpublished observations). Mitotic cells, as selected by mitotic selection, do express cyclin D, so the results suggest that expression of cyclin D in cells exiting mitosis is sufficient to drive morphology-independent progression through the ongoing cell cycle. In addition, except for endothelial cells and wound fibroblasts, stress fiber formation is not a general feature of cells in living tissue, indicating that proliferation in vivo can and does occur in a stress fiber–independent manner.[152–155]

C. Actin Involved in Transcription

Besides its cytoplasmic localization, actin is also reported to be present in the nucleus (for review see Ref. 156) (Fig. 4). Nuclear actin was implicated to have a role in several processes, including chromatin remodeling, formation of a nucleoskeleton, transport of proteins and mRNA, and transcription. The nuclear localization of actin was demonstrated in various cell types, but often cytoplasmic contamination was seen as the most plausible explanation for the nuclear detection of actin. However, recently, actin was described as a functional component of several nuclear complexes, leaving little doubt about its nuclear presence.[157]

Actin contains two nuclear export sequences (NES) and was shown to be subjected to NES-dependent nuclear export.[158] In addition, a receptor for the export of actin/profilin complexes was identified (exportin 6).[159] Here, profilin was suggested to be a co-factor for nuclear export of actin, whereas nuclear import of actin occurs through binding to cofilin, which contains a NLS. So, it is tempting to suggest that actin is actively kept out of the nucleus to prevent spontaneous polymerization, and cofilin and profilin might play a role in maintaining a balance between the amounts of cytoplasmic and nuclear actin.

In several studies, actin was described to be involved in transcription in direct and indirect ways. Recently, β-actin was shown to have a role in the initiation and continuation of transcription

by RNA polymerase II.[160] Other isoforms of actin were shown to be inactive in transcription. Other studies suggested a functional relationship between nuclear actin and RNA polymerase II.[161,162] An actin-myosin complex associated with RNA polymerase I was described in nucleoli and functionally coupled to elongating transcripts in HeLa cells.[163] Here, an actin-based myosin motor was described to be associated with transcribing ribosomal genes in the nucleus. It was suggested that actin-myosin motors might provide a general mechanism to facilitate elongation of RNA transcripts during transcription of both ribosomal genes and protein-coding genes. Recently, it was indeed demonstrated that both actin and NMI have an essential function in the transcription of ribosomal RNA genes by interaction with the RNA polymerase I machinery.[157] Also a role for β-actin has been described in the transcription by RNA polymerase III.[164] Furthermore, all three RNA polymerase complexes, actin, and profilin were found in Cajal bodies.[165,166] Cajal bodies have been suggested to play a role in the assembly of RNA polymerases[167] and in the maturation of small nuclear ribonucleoproteins.[168]

Actin and profilin were also associated with snRNPs in nuclear speckles.[166,169,170] Interestingly, phosphatidylinositol 4,5-bisphosphate accumulates in these bodies as well as the phosphatidylinositol phosphate kinases (PIPKs).[171] Moreover, the localization of both PIPKs and Ptdlns(4,5)P_2 to speckles was described to reorganize upon inhibition of mRNA transcription, implicating a function for PIP_2 in transcription. In addition, Ptdlns(4,5)P_2 was suggested to be necessary for pre-mRNA splicing and to be present in nuclear particles, whose morphology and distribution was cell-cycle dependent.[172] So there might be a functional relationship in the co-localization of actin, profilin, and PIP_2 in speckles. The question of whether actin, profilin, and PIP_2 play together in a similar way in these bodies, as described in the cytoplasm, is not clear yet.

Besides the presence of profilin in the nucleus, many other ABPs are found in the nucleus—for example, gelsolin, cofilin, and zyxin. Here, they might function in a similar way in the actin metabolism, as occurs in the cytoplasm.

In addition to having a role in transcription, actin and actin-related proteins were implicated to have a role in chromatin remodeling. Actin was shown to be a component of the SWI/SNF-like BAF chromatin-remodeling complex. It might be Ptdlns(4,5)P_2 that couples actin to this complex. In permeabilized nuclei, Ptdlns(4,5)P_2 was able to block the exit of the SWI/SNF-like BAF complex.[173] Moreover, in vitro studies showed PIP_2 binding to the SWI/SNF-like BAF complex, allowing it to associate with actin.[174] So, PIP_2 seems to act as a signaling molecule affecting the function of actin in chromatin remodeling. At present, direct evidence for a role of nuclear actin in regulation of G1-phase progression is lacking, but it seems evident with respect to the possible role of actin in nuclear processes that future experiments will exhibit such a role.

VI. CONCLUSIONS

In this review, we have briefly described the current knowledge on the molecular basis of the regulation of G1-phase progression during the ongoing cell cycle. It is shown that both the MAP kinase pathway and the PI 3-kinase pathway play an essential role in the decisions made in the G1 phase regarding whether the cells continue to proliferate or whether they are programmed for apoptosis or differentiation, respectively. Actin, one of the most abundant proteins in the cells, appears intimately linked to cell-cycle progression, especially during the G1 phase of the cell cycle. This is due to the structural role of actin and, therefore, its role in cytokinesis, cell spreading, and motility. In addition, actin has been demonstrated to be involved in signal transduction from growth factor receptors and from integrins, and the signal transduction cascades and the actin microfilaments have been demonstrated to be mutually linked. Finally, actin is known for its regulatory role in transcription, and on this level, an involvement of actin in regulation of G1-phase progression seems possible. Altogether, these observations indicate that regulation of G1-phase progression is caused by a complex network of signal transduction cascades linked to the complex network comprising actin metabolism. This complicates an analysis of the role of actin in the molecular networks mentioned above concerning the regulation of cell

proliferation. However, a careful analysis with respect to localization and activity during the G1 phase of the ongoing cell cycle will certainly clarify the underlying molecular mechanisms.

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Appendix

The role of actin in the regulation of cell-cycle progression was reviewed in 2005 (Boonstra and Moes, 2005). The focus was on G1-phase progression. It was concluded that actin appears intimately linked to cell-cycle progression. Actin has a role as a structural protein during processes such as cell spreading, actin acts as a signal transduction mediator and nuclear actin is involved in transcription and chromatin remodelling. In this appendix some recent developments in the field of actin research are listed that are related to the roles of actin during cell-cycle progression.

1. Actin conformations

A remarkable number of versatile functions was described for actin in cells, such as endocytosis, cell motility, signal transduction, chromatin remodelling, cell adhesion, intracellular trafficking and the determination of cell shapes. It is clear that the function of actin depends on the localization within cells. In addition, it is tempting to suggest that the functioning of actin is related to the state of polymerization and the interaction with various actin binding proteins. Actin filaments are organized in at least 15 distinct structures in metazoan cells, such as lamellipodia and ruffles, filopodia and endocytic structures, that are localized in distinct areas within cells (Chhabra and Higgs, 2007). The assembly in different structures allows actin to act differently depending on the localization within the cell. However, the described variation in functions as well as the variation in the organisation of actin are not separated by compartmentalisation, i.e. they take place in common compartments in the cell, such as the cytoplasm or nucleoplasm. In these compartments different actin filaments are formed from a common pool of monomers and actin-binding proteins. Therefore the question that remains is how actin is locally organized in a distinct manner to fulfil its specific local function.

Well known explanations for the local activities of proteins are the local targeting and activation of proteins by signal transduction cascades and the local chemical environment, such as pH. These phenomena will result in the local preferential binding of actin-binding proteins to actin filament structures and result in the formation of distinct actin structures. However, recently it was demonstrated that actin filaments are structurally polymorphic (Galkin et al., 2010). It was suggested that different actin filament conformations are adopted at the time of nucleation and subsequently become stabilized by actin-binding partners (Michelot and Drubin, 2011). The variation in conformations of F-actin between actin structures has consequences for the interaction with actin-binding

proteins. Actin networks possess specific binding properties depending on the structure to which they belong. This is demonstrated by the selective decoration of different types of actin filament networks by actin-binding proteins. In addition, it was suggested that proteins that were characterized previously for example as actin-filament bundling proteins may in fact have an important role in maintaining filament identity (Michelot and Drubin, 2011; Galkin et al., 2011). The effect on the conformation of actin filaments by actin-binding proteins was reported to propagate along filaments through long-range allosteric interactions (reviewed by Hild et al., 2010). Altogether, the local activation of nucleators, such as formins, Arp2/3 complexes or spire, will determine which actin-binding proteins will associate with the actin filament network and this will determine the type of network and its local functioning. Local activation of Arp2/3 complexes will result in a branched network and local activation of formins will result in linear arrays of actin filaments. Interestingly, more and more indications are obtained that demonstrate a specific role for actin nucleators in the formation of specific actin structures, such as phagocytic structures, cell junctions, endocytic structures, membrane ruffles and lammellipodia, filopodia and cell spikes, Golgi actin and stress fibers (reviewed by Campellone and Welch, 2010). Upon exchange of actin-binding proteins a filament network may evolve in a different type of actin filament network.

Monomeric actin can also have different conformations depending on the bound molecule. For example the nucleotide-binding cleft of monomeric actin can adopt an open or a closed conformation (Chik et al., 1996). In figure 1 mouse fibroblasts are labelled with an antibody termed 2G2 that recognizes actin when bound to profilin (Gonsior et al., 1999). Labeling with this antibody reveals a distinct labelling pattern in comparison with labelling with other actin probes. In both random growing cells (data not shown) and serum-starved cells, the 2G2 antibody labels intranuclear dots (Fig 1B and 2A). Upon the addition of PDGF-BB, dorsal circular ruffles are induced in mouse fibroblasts that can be identified by labelling for F-actin with phalloidin (Fig 1D and 1G). The 2G2 antibody also labels actin in these newly formed structures (Fig 1E and 1H). Other actin structures such as the cortical skeleton and stress fibers are not labelled. In addition to circular ruffles, PDGF stimulation results in the formation of lammelipodia that are also labelled with the 2G2 antibody (Fig 2B and 2C). Other areas of the cortical skeleton are not labelled with the 2G2 antibody (Fig 2C).

In conclusion, the local organisation of actin networks with distinct conformations is determined by the nucleators of the network and maintained by actin-binding proteins.

This results in specific types of actin structures that explain the variation in the local functioning of actin in cells.

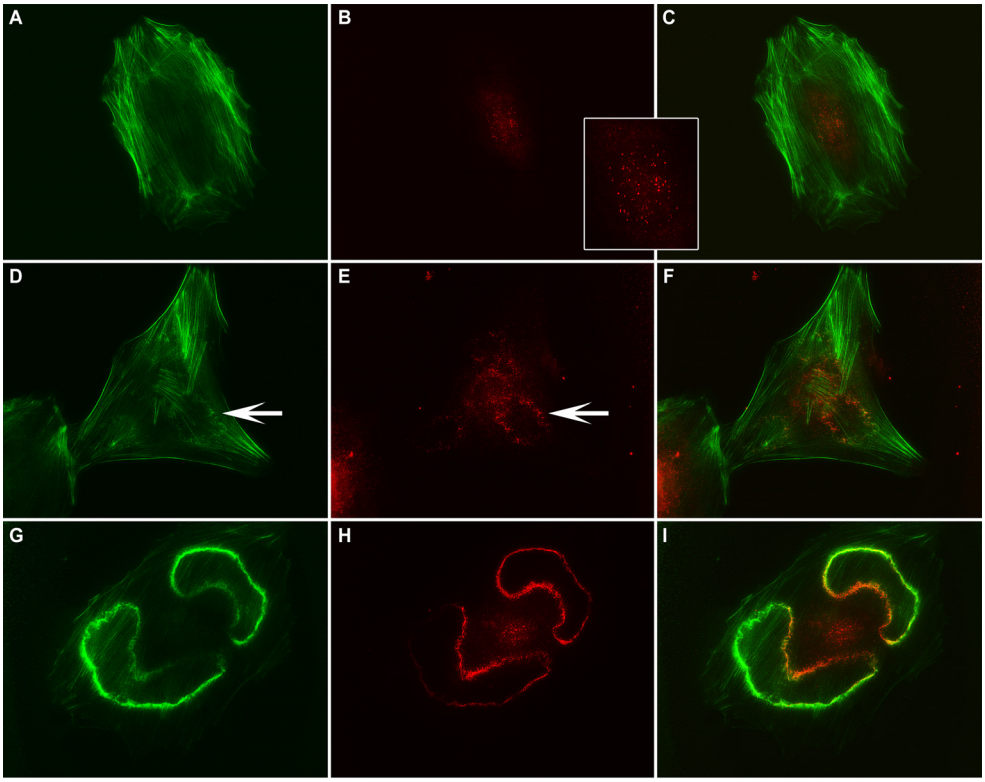

Figure 1. A conformation of actin as detected by the 2G2 antibody is exclusively localized in intranuclear dots and newly formed dorsal circular ruffles. Mouse C3H10T1/2 fibroblasts are labelled for F-actin (green) and for actin as detected by the 2G2 antibody (red). Pictures represent optical sections through cells as captured by confocal laser scanning microscopy (CLSM). Serum-starved cells exhibit abundant F-actin stress fibers (2A) and the 2G2 antibody is detected in intranuclear dots (2B, insert). After 5 minutes of stimulation with PDGF-BB, dorsal circular ruffles start to form (2D; arrow) that are also labelled with the 2G2 antibody (2E; arrow). In cells that are stimulated with PDGF-BB for 15 minutes, the circular ruffles are more pronounced and are labelled for both F-actin and actin in the conformation that is recognized by the 2G2 antibody.

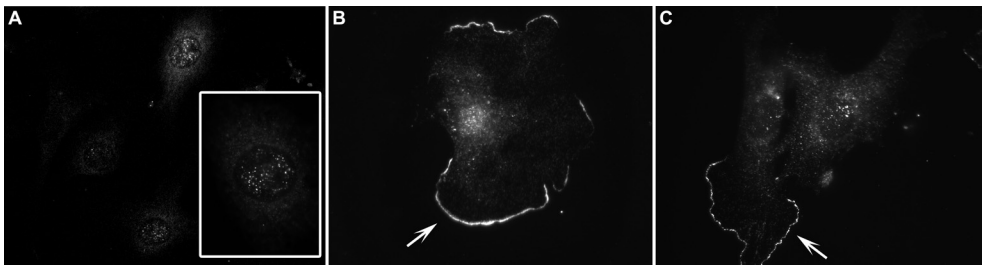

Figure 2. A conformation of actin as detected by the 2G2 antibody is exclusively localized in intranuclear dots and lammelipodia. Mouse C3H10T1/2 fibroblasts are labelled for actin with an antibody named 2G2 and images are captured by *classical immunofluorescence microscopy* of fixed cells. Serum-starved cells exhibit intranuclear dots (A, insert). In cells that are stimulated with PDGF-BB for 15 minutes, the 2G2 antibody labels newly-formed lamellipodia (B, C) in addition to the dots in the nucleus.

2. Actin involved in signalling clusters in the cell membrane

In the previous section, the interdependence of local actin conformations and local actin-binding proteins was described. The mutual dependency of actin and signal transduction was extensively discussed in Boonstra and Moes 2005. This mutual dependency is also illustrated in local signalling clusters at the cell membrane. Several types of receptors were described to be partially confined in clusters in the plasma membrane. The receptors that are present in signalling clusters diffuse less freely in the membrane compared to the receptors outside these clusters (for review see Hartman and Groves, 2011; Jaumouillé and Grinstein, 2011). Next to receptors, these signalling clusters contain specific proteins with scaffolding and catalytic activities. The specific signal transduction proteins that are present in signalling clusters, also called signalosomes, determine its functionality. Several proteins in signalling clusters, such as ERM proteins, filamins and A-kinase anchoring proteins, were described to facilitate direct or indirect binding of the membrane to the cortical actin cytoskeleton that lines the inner leaflet of the plasma membrane. Examples of signalling clusters are T-cell receptor signalosomes and signalosomes induced by β-adrenergic receptors, the PDGF β-receptor and the EGF receptor (Barda-Saad et al., 2005; Valentine and Haggie, 2011; Scarselli et al., 2012; Moes et al., 2012; Orth et al., 2006).

Several signalosomes were described to be anchored in lipid rafts. Lipid rafts are microdomains in the membrane that are enriched with sphingolipids and cholesterol (reviewed by Sengupta et al., 2007; Lingwood and Simons, 2010). It was suggested that

these lipids in the lipid rafts facilitate the formation of signalling clusters by confinement of signalling proteins (Sengupta et al., 2007; Lingwood and Simons, 2010). In addition, several studies indicate that interactions with membrane-associated proteins, including cytoskeletal proteins, play important roles in the clustering of scaffolding proteins, signalling proteins and lipids in signalosomes in the membrane (for review see Hartman and Groves, 2011; Chichili and Rodgers, 2009). For example, the cortical actin cytoskeleton was demonstrated to cluster several receptors in domains in the plasma membrane, such as β-adrenergic receptors, B cell receptors, Fcε and Fcγ receptors (Valentine and Haggie, 2011; Scarselli et al., 2012; Treanor et al., 2010; Andrews et al., 2008). Interestingly, the compartmentalization of β-adrenergic receptors in the membrane was described not to be mediated by lipids, but to be solely mediated by interactions with the actin cytoskeleton. Experiments revealed that the confinement of β-adrenergic receptors was reduced in cells upon treatment with the actin-disrupting agent Latrunculin or the actin polymerization inhibitor Cytochalasin D. Cholesterol sequestration with filipin or cholesterol removal did not affect receptor clustering (Valentine and Haggie, 2011; Scarselli et al., 2012). Actin could cluster proteins in domains in the membrane by direct or indirect protein-protein interactions. Several proteins were described to link the cortical actin cytoskeleton with the membrane, resulting in specific interactions between receptors in signalosomes and the actin cytoskeleton. Alternatively, actin might interact with lipids and alternate the local viscosity of the membrane resulting in local concentrations of proteins in the membrane. Moreover, the cortical actin network was suggested to cluster membrane proteins in a nonspecific fashion by forming a dynamic labyrinth. The mesh size of the local actin network was suggested to determine the freedom for lateral translocation of transmembrane proteins (Andrews et al., 2008; Jaumouillé and Grinstein, 2011).

Whether the actin cytoskeleton is mediating the formation of signalosomes in the membrane or not, it is clear that existing signalling clusters in the membrane are often associated with the actin cytoskeleton (reviewed by Hartman and Groves, 2011; Chichili and Rodgers, 2009). Both specific and nonspecific interactions of signalling clusters with the actin cytoskeleton bring signalling clusters under submission of dynamics of the local actin filament network. However, as mentioned before, there is often a mutual dependency of signal transduction and the actin cytoskeleton. This is illustrated by the fact that the association of signalosomes with the actin cytoskeleton can be modified upon activation of receptors in the signalosome. Activated receptors recruit molecules that

regulate the polymerization of actin to the signalosome. This affects the local polymerization of actin. An example is the local induced actin polymerization upon activation of T cell receptors (TCR) in cells. Activated TCR clusters were described to recruit WASp via the adapter protein Nck. The recruitment of WASp activates nucleation of actin filaments by the Arp2/3 complex, resulting in local actin polymerization at the TCR (Barda-Saad et al., 2005).

The association of the cortical actin cytoskeleton with signalosomes implies that the actin cytoskeleton can have a role in structuring signalling clusters in the membrane. This was indeed indicated by several studies. For example, the association with the actin cytoskeleton was suggested to facilitate lateral transport of clusters of several types of receptors (Barda-Saad et al., 2005; Moes et al., 2012; Orth et al., 2006). Moreover, the local actin polymerization facilitates the local docking of signalling components that allows signalling cascades to largely take place locally at the cell membrane. This concentration of signal transduction components might facilitate efficient signal transduction as has been suggested for linear ruffle formation (Diakonova et al., 1995). Furthermore, the local actin polymerization contributes to the spatial segregation of signal transduction components that is required to guarantee the specificity and to facilitate the regulation of signalling pathways in cells. The latter is illustrated by experiments that revealed that alteration of the actin cytoskeleton by using drugs that interfere with actin treadmilling is sufficient to induce intracellular signalling in B cells, probably by a change in diffusion of B cell receptors (Treanor et al., 2010).

3. Nuclear actin

Though traditionally both the nuclear localization of actin and the functional significance of nuclear actin were questioned, the view on the role of nuclear actin has changed during recent years. Many studies localized actin in the nucleus and the existence of actin in the nucleus has become widely accepted. Not all studies discriminate between actin isoforms but various studies indicate the presence of β-actin in the nuclear pool of actin (Hoffman et al., 2004; Boonstra and Moes, 2005; Ferrai et al., 2009; Xu et al., 2010). Actin is present in the nucleus as monomeric G-actin and in addition polymeric actin forms were reported to exist in the nucleus. Mobility studies of nuclear actin using fluorescence recovery after photobleaching (FRAP) revealed the existence of various forms of actin, namely relatively rapidly moving and relatively slowly moving forms of actin

(McDonald et al., 2006). The slowly moving form of actin was suggested to be polymeric actin based on the fact that the addition of actin depolymerising drugs resulted in an increase in the mobility of actin. This increase in mobility upon the addition of depolymerising drugs excludes the possibility that the slowly moving fraction represent monomeric actin bound to a larger complex of other proteins (McDonald et al., 2006). In addition polymeric actin was demonstrated to function in transcription (Ye et al., 2008; Taylor et al., 2010; Miyamoto et al., 2011; Huang et al., 2011).

The nuclear functions that were described for nuclear actin diverge. Nuclear actin was amongst others described to facilitate chromatin remodelling (Zhao et al., 1998), RNA processing, play a role in mRNA export, the regulation of DNase I function and gene movement, in other words the repositioning of chromosomal loci within the nucleus (Dundr et al., 2007). Furthermore actin facilitates transcription (Ye et al., 2008; Ferrai et al., 2009; Xu et al., 2010 ; Taylor et al., 2010; Miyamoto et al., 2011; Huang et al., 2011).

Actin was described to interact with all three RNA polymerases present in eukaryotic cells, i.e. RNA polymerases I, II and III (Fomproix and Percipalle, 2004; Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004; Kukalev et al., 2005; Ye et al., 2008; Ferrai et al., 2009; Xu et al., 2010). Transcription activity of RNA polymerases was inhibited using anti-actin antibodies, indicating the functional relevance of the association of actin with RNA polymerases (Hofmann et al., 2004; Philimonenko et al., 2004; Ye et al., 2008). Several studies demonstrated the involvement of polymeric actin in transcription (Ye et al., 2008; Taylor et al., 2010; Miyamoto et al., 2011; Huang et al., 2011). The interaction of polymeric actin and nuclear myosin 1 (NM1) was related to transcription of ribosomal genes by polymerase I in the nucleolus (Ye et al., 2008). Antibodies directed against actin were demonstrated to block transcription. Transcription was restored upon the addition of recombinant actin. However, only mutants that stabilize F-actin were able to rescue transcription. The addition of actin depolymerising drugs or cofilin resulted also in the inhibition of transcription. NM1 interacts with chromatin and in addition the family of myosins is known for the ability to convert chemical energy to produce movement of actin filaments. Based on these results it was suggested that actin and NM1 form an actomyosin motor that allows transcriptional elongation by pulling polymerase I forward (Ye et al., 2008). In addition, it was demonstrated that the association of actin and NMI on DNA does not require active transcription. Therefore it was suggested that actin may be involved in turning on silent genes (Ye et al., 2008). Interestingly, oligomeric nuclear actin was described to be involved in the activation of transcription by the clearance of a

complex that represses gene expression from gene promoters (Tayler et al., 2010). The involvement of polymeric actin in the process of transcription was further supported by the study of Tayler et al. (2010) that describes the involvement of both polymerized actin and the actin nucleator Wiskott-Aldrich syndrome protein (WASp) in transcription during the inflammatory response.

Next to being the building block for polymeric actin, nuclear monomeric actin was related to signal transduction, i.e. the control of gene expression. Monomeric actin regulates the localisation and activity of MAL, a coactivator of the transcription factor SRF, and therefore monomeric actin is involved in the activation of SRF regulated gene expression (Vartiainen et al., 2007). MAL binds to G-actin in both the cytoplasm and the nucleus. The ratio of cytoplasmic MAL versus nuclear MAL varies depending on the activation of serum induced signal transduction. Serum induced signal transduction results in the polymerization of actin, reducing the pool of G-actin. This decrease in G-actin concentration was described to reduce nuclear export of MAL resulting in the accumulation of MAL in the nucleus. High concentrations of G-actin retain MAL in the cytoplasm (Miralles et al., 2003; Vartiainen et al., 2007). Next to determining the localisation of MAL, actin binding to MAL regulates the activity of MAL in SRF induced gene expression. Altogether, upon serum-induced signalling nuclear G-actin regulates the subcellular localization of MAL and in addition the activity of MAL and therefore nuclear G-actin controls SRF-dependent gene expression (Miralles et al., 2003; Vartiainen et al., 2007).

In addition to mediating growth factor induced signal transduction, nuclear actin was also related to signalling induced by the extracellular matrix (Spencer et al., 2011). In mammary epithelial cells it was demonstrated that quiescence induced by growth factor withdrawal, or the addition of the extracellular matrix protein Laminin 111 (LN1), rapidly reduces the presence of β-actin in the nucleus resulting in the suppression of transcription and cell growth (Spencer et al., 2011). Furthermore, LN1 was demonstrated to destabilize RNA polymerase II and III binding to transcription sites, resulting in a reduction of transcription and DNA synthesis. Constitutive overexpression of β-actin in the nucleus abolished growth arrest by LN1. These results demonstrate that both nuclear and cytoplasmic β-actin levels can be regulated by an extracellular matrix protein. Moreover, the loss of nuclear β-actin was clearly related to quiescence in mammary epithelial cells.

Actin in the nucleus was also suggested to be involved in chromatin remodelling (Boonstra and Moes, 2005; Farrants, 2008). Actin is present in ATP-dependent chromatin

remodelling complexes from yeast, Drosophila and mammalian cells. However, not all ATP-dependent chromatin remodelling complexes were described to contain actin. From the four ATP-dependent chromatin remodelling families only the SWI/SNF complexes and the INO80 family of complexes contain actin. The mechanism by which actin functions in chromatin remodelling is not fully clear (reviewed by Farrants, 2008). It was suggested that actin, either monomeric or polymeric, bridges protein complexes that operate in the proximity of one another. By doing so, actin would provide a platform between transcription initiation, chromatin remodelling and transcription elongation (Farrants, 2008). In contrast to the role for nuclear actin in transcription, no recent findings were described that clarify the role of nuclear actin in chromatin remodelling.

In conclusion, actin was demonstrated to play important roles in the nucleus. The functioning of actin in the nucleus is influenced by cytoplasmic actin and vice versa.

4. Actin during cell cycle progression

In this appendix a selection of recent developments in the field of actin research was described. These findings further support the conclusion of the preceding review that actin appears intimately linked to cell-cycle progression. Several studies indicate that actin indeed fulfils several roles in the regulation of cell cycle progression, for example by its close cooperation with signal transduction (Margadant et al., 2007; Moes et al., 2011; Goyal et al., 2011) and the obvious structural role of actin (Figure 3). The functioning of actin near signalling centers in the plasma membrane, that was described in section 2, further indicates an important role for actin in mediating the onset of signal transduction. This role of actin is likely to also take place during the regulation of cell cycle progression since the activation of various signal transduction was demonstrated to take place during the cell cycle, especially during the G1 phase (Boonstra and Moes, 2005, Margadant et al., 2007, Moes et al., 2010).

In addition, nuclear actin was linked to the regulation of cell cycle progression (Goyal et al., 2011; Spencer et al., 2011). Moreover, as described above, actin was demonstrated to be essential for transcription in the nucleus and a properly timed transcription in turn is essential for the regulation of cell cycle progression. Interestingly, the induction of quiescence by growth-factor starvation was demonstrated to result in the depletion of nuclear β-actin and a strong reduction in transcription. Overexpression of β-actin in the nucleus restored transcription (Spencer et al., 2011). The presence of β-actin in the

nucleus might also be regulated during the cell cycle to regulate cell cycle-dependent transcription, for example during the G1 phase (Figure 4). Interestingly, several genes involved in the reorganization of the cytoskeleton were described to exhibit cell cycle-dependent regulation. For example, genes involved in motility and remodelling of the extracellular matrix are expressed in M phase (Cho et al., 2001). This illustrates again the mutual dependency between actin, signal transduction and gene expression. Moreover, the involvement of actin in the functioning of signalling centers in the plasma membrane and transcription in the nucleus also illustrate that actin is involved from the onset of signal transduction till the expression of genes.

Remarkably, post-mitotic disruption of the actin cytoskeleton did not reveal essential functions for cell cycle progression (Margadant et al., 2007). The use of actin interfering drugs resulted in both the prevention of cell spreading and the reduction of growth factor-induced MAPK activity. However, no interfere with progression through the cell cycle was observed (Margadant et al., 2007). Cells with disorganized actin cytoskeletons completed the entire cell cycle with exlusion of cytokinesis resulting in binucleated cells. Therefore, it was suggested that cytoskeletal integrity is not a prerequisite for G1-phase progression in the ongoing cell cycle (Margadant et al., 2007).

The variation in conformations of actin complicates the analysis of the role of actin in the regulation of cell progression. Local variation in the conformation of actin and local variation in binding partners indicates that the functioning of actin also varies locally and during time. See for example the detection of 2G2 actin during the formation of lamellapodia (figure 1). The involvement of 2G2 actin can be expected to also take place during the cell cycle, for example during the spreading of cells after completion of mitosis. Variations in the conformation of actin were indeed observed during the cell cycle (Hubert et al., 2011). This implies that actin in cells can not be treated as one pool of the same protein. Biochemical experiments require additional microscopy studies that reveal the spatio-temperal behaviour of actin in cells. In addition, the use of drugs that interfere with actin dynamics is complicated since these drugs interfere with the tightly regulated balance of G-actin and F-actin throughout the whole cell. For example, interference with the balance of F-actin and G-actin in cells by using drugs that mediate the treadmilling of actin, were demonstrated to result in an altered SRF mediated gene expression in the nucleus via an altered concentration of G-actin in the nucleus (Miralles et al., 2003). Moreover, the use of actin interfering drugs was demonstrated to be sufficient to induce intracellular signalling in B cells, probably by a change in diffusion of B cell receptors

(Treanor et al., 2010). The consequence of the complex and versatile functioning of actin in numerous cellular processes is that disturbances of its tightly regulated behaviour will result in a large range of effects on numerous cellular processes. This complicates an analysis of the role of actin in the regulation of cell proliferation. However, recent developments in the field of time-lapse fluorescence microscopy in single cells represent a promising approach for unravelling the role of actin in the regulation of cell-cycle progression.

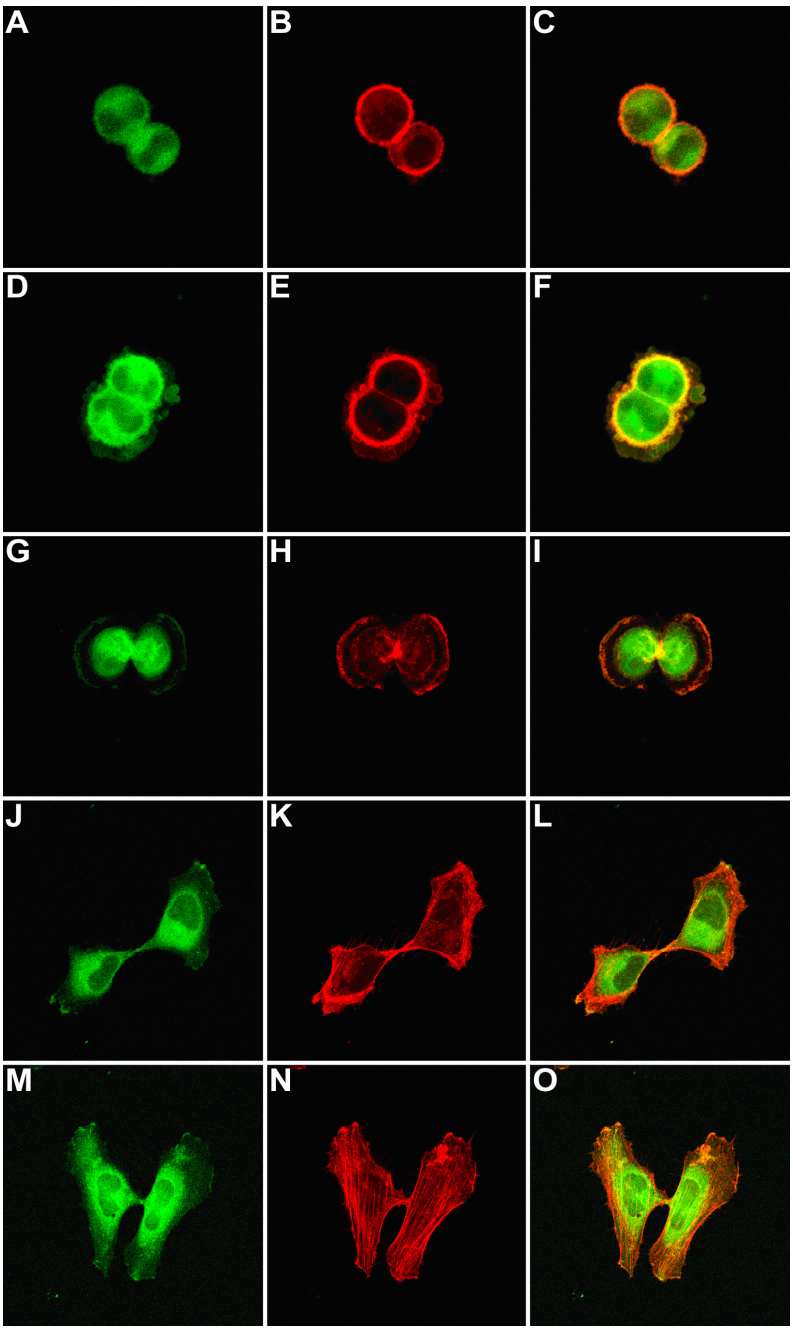

Figure 3. HeLa cells in early G1 phase of the cell-cycle. Cells were synchronized by mitotic shake off, subsequently replated and allowed to enter G1 for respectively 25 minutes (A-F) and 1 (GHI),2 (JKL), and 3 hours (MNO) before chemical fixation. Pictures represent optical sections (CLSM) through cells that were stained for G-actin (green) and F-actin (red).

A section halfway through a cell 25 minutes after mitotic shake off (ABC), displays a clear F-actin cortical skeleton and the absence of stress fibers (B). At the cortical skeleton blebs are pointing outwards. The blebs are formed by a shield of F-actin and contain G-actin. In cells that exhibit increased cell spreading (DEF), the blebs that are in contact with the substratum fuse together and increase in size (Moes et al., 2011). Together they form a ruffling edge that grows outwards resulting in cell spreading. In these cells some thin actin stress fibers were observed. Cells that were fixed 1 hour after shake off (GHI) exhibit a ruffling leading edge that is growing outwards resulting in the spreading of cells. Both F-actin and G-actin are highly enriched at these edges. Further in G1 cells spread further and this results in a flattened morphology that is also indicated by the appearance of more abundant stress fibers (J-O).

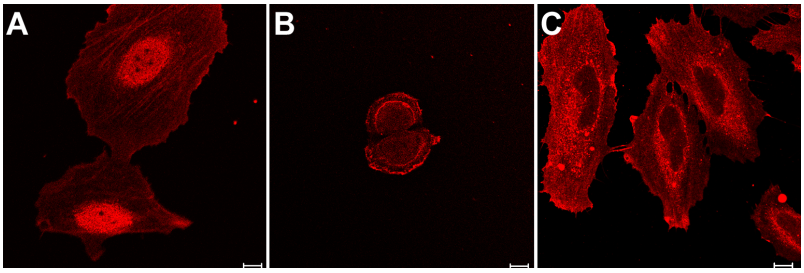

Figure 4. Optical sections (CLSM) through the nucleus of HeLa cells labelled for β-actin. β-actin is present in the cytoplasm as well as in the nucleus, indicated by the exclusion of staining in the nucleoli. In random growing cell cultures the ratio of cytoplasmic and nuclear actin varies between cells. In a large fraction of cells, abundant nuclear staining was observed (A), in other cells the extend of nuclear staining was considerably less compared to the staining in the cytoplasm (not shown). The presence of actin in the nucleus might be cell cycle-dependent. Therefore the ratio of the amount of cytoplasmic staining for β-actin versus nuclear β-actin was investigated in synchronized cells that were allowed to enter G1 after mitotic shake off for 1 hour (B), up to 6 hours (C). Cells in early G1 exhibit some nuclear staining that is indicated by the exclusion of staining in the nucleoli. In most cells the cytoplasmic pool of β-actin seems more abundant compared to the staining for β-actin in the nucleus. There was some variation in the ratio of cytoplasmic versus nuclear staining between cells. However, in the first 6 hours of the G1 phase, HeLa cells did not display a clear change in the extend of the nuclear staining for β-actin. Further investigations are required to see if cells exhibit variation in the content of nuclear β-actin during the G1.

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Chapter 3

Attachment of HeLa cells during early G1 phase

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Attachment of HeLa cells during early G1 phase

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Abstract Both growth factor directed and integrin dependent signal transduction were shown to take place directly after completion of mitosis. The local activation of these signal transduction cascades was investigated in early G1 cells. Interestingly, various key signal transduction proteins were found in blebs at the cell membrane within 30 min after mitosis. These membrane blebs appeared in round, mitotic-like cells and disappeared rapidly during spreading of the cells in G1 phase. In addition to tyrosine-phosphorylated proteins, the blebs contained also phosphorylated FAK and phosphorylated MAP kinase. The formation of membrane blebs in round, mitotic cells before cell spreading is not specific for mitotic cells, because similar features were observed in trypsinized cells. Just before cell spreading also these cells exhibited membrane blebs containing active signal transduction proteins. Inhibition of signal transduction did not affect membrane bleb formation, suggesting that the membrane blebs were formed independent of signal transduction.

Keywords Actin · Membrane blebs · G1 · Signal transduction · Attachment · Focal adhesion · FAK · cPLA$_2\alpha$

Abbreviations

FAK	Focal adhesion kinase
EGF	Epidermal growth factor
SIC	Spreading initiation center
cPLA$_2$	Cytotosolic phospholipase A$_2$

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Introduction

The transition of mitosis to early G1 phase during cell cycle progression is characterized by dramatic changes in cell morphology. The round mitotic cells attach to the substratum and flatten subsequently to the morphology that is well known from interphase cells. In addition to these morphological changes, also the onset of signal transduction cascades was described in early G1 cells (Hulleman et al. 1999a, b). Integrin dependent as well as growth factor dependent signal transduction become activated within half an hour after completion of mitosis. It has been demonstrated during the on-going cell cycle of Chinese Hamster Ovary (CHO) and neuroblastoma N2A cells that serum induces the activation of the MAP kinase pathway within 15 min after mitosis, and that inhibition of this activation causes an inhibition of cell cycle progression (Hulleman et al. 1999a). Similarly, prevention of cell attachment also resulted in cell cycle arrest (Hulleman et al. 1999b).

In addition to growth factors and adhesion, organization of the actin cytoskeleton is also implicated in G1 phase progression since disruption of actin architecture with pharmacological agents leads to G1 arrest in a variety of cell types (Bohmer et al. 1996; Bottazzi et al. 2001; Fasshauer et al. 1998; Huang et al. 1998, Huang and Ingber 2002; Iwig et al. 1995; Lohez et al. 2003; Maness and Walsh 1982; Ohta et al. 1985; Reshetnikova et al. 2000; Takasuka et al. 1987; Tsakiridis et al. 1998). In this respect it is of interest that the actin cytoskeleton has been demonstrated to play an essential role in signal transduction (Boonstra and Moes 2005; Heng and Koh 2010). The first indications for the relationship between actin and signal transduction were obtained by studies on the effects of growth factors on cell morphology. For example, addition of EGF caused the formation of membrane ruffles within

minutes after addition of the growth factor (Diakonova et al. 1995). It was demonstrated that exposure of cells to EGF caused a rapid actin polymerization. The formation of membrane ruffles and the translocation of several of the downstream signaling molecules to these newly formed membrane ruffles suggested the formation of signaling complexes at the plasma membrane in the membrane ruffles (Diakonova et al. 1995; Payrastre et al. 1991). Interestingly, treatment of the cells with F-actin disrupting agents like cytochalasin caused a severe reduction in growth factor induced signaling (Margadant et al. 2007), demonstrating the mutual interaction between signaling cascades and the actin microfilaments. Furthermore, a wide variety of signaling proteins, amongst them the epidermal growth factor receptor, were demonstrated to associate directly to actin (den Hartigh et al. 1992; Diakonova et al. 1995; Payrastre et al. 1991). These observations suggest strongly that the drastic changes in the actin microfilament system during the transition from M to early G1 phase may be related to the onset of growth factor and integrin mediated signal transduction.

To establish the possible role of actin in signal transduction in the early G1 phase of the cell cycle, we have studied the localization of these signal transduction molecules and of F-actin using immunofluorescence microscopy in the early G1 phase of the ongoing cell cycle. It was shown that various key signal transduction proteins, such as phosphorylated FAK and MAPK, were present in blebs of the cell membrane in cells in early G1 phase. Cells that progressed further into G1 phase and that were spread further, exhibit small focal adhesions and less membrane blebs. Both the presence of phosphorylated FAK within these blebs and the temporal presence of the membrane blebs during the spreading of cells directly after mitosis, suggest a role in facilitating attachment. Interestingly, inhibition of the FAK-mediated or MAPK-mediated signal transduction by specific inhibitors did not influence the formation of the membrane blebs. This suggests that membrane bleb formation is independent from signal transduction. Membrane blebs may facilitate plasma membrane enlargement and efficient integrin and growth-factor induced signalling that is required during early G1 phase.

Materials and methods

Cell culture synchronization and trypsinization

HeLa cells and CHO cells were cultured in CO_2 independent Dulbecco's modified Eagle's medium (DMEM) containing 25 mM HEPES (Gibco/Invitrogen, Paisley, UK), supplemented with 7.5% fetal calf serum (Gibco/Invitrogen, Paisley, UK). Cell cultures were synchronized by mitotic shake off (Boonstra et al. 1981). After shake off, mitotic cells were plated on glass coverslips and allowed to progress in G1 phase for various time intervals before chemical fixation.

Cells were trypsinized according general procedures and allowed to spread for 30 or 60 min after replating. Subsequently cells were chemically fixed and labelled for proteins that are phosphorylated on tyrosine residues (PY100) and F-actin as described under Fluorescence microscopy. Cells were trypsinized and plated on fibronectin and poly-L-lysine, respectively, in the absence or presence of serum for 10 or 20 min. Subsequently the fraction of the initial plated cells was calculated that displayed a spread or round morphology and the fraction of initial plated cells that contained membrane blebs. Cells were counted before replating and non-attached cells are not included in the calculation because the cells were allowed to attach for only 10 or 20 min. The presented data of the trypsinization assay represent two individual experiments.

Fluorescence microscopy

Cells were fixed in 3.7% (w/v) formaldehyde in PBS, washed with PBS and permeabilized for 5 min in PBS containing 0.2% Triton X-100. After washing with PBS, cells were incubated with 50 mM glycine in PBS for 10 minutes. Subsequently samples were washed twice with PBS containing 0.2% gelatin and incubated with the primary antibody diluted in PBS containing 0.2% gelatine during 60 min, with the exception of the anti-cPLA$_2\alpha$ antibody that was incubated overnight. After several washings with PBS containing 0.2% gelatin, cells were incubated with a secondary antibody and TRITC-conjugated phalloidin diluted in PBS containing 0.2% gelatine and washed again with PBS containing 0.2% gelatin. Finally, cells were mounted in (2.5% w/v) mowiol 4-88 (Hoechst, Frankfurt, Germany)-DAPCO (Sigma-Aldrich St. Louis, USA). All individual experiments were carried out at least three times. Experiments with double labelling were also performed with single labels, among others to control for bleedthrough of the fluorophores. Controls with incubations of solitary secondary antibodies were carried out routinely with identical settings of the microscope.

Acquisition of immunofluorescence images

Images were acquired with a Zeiss confocal laser scanning microscope (CLSM) (Pascal 510) fitted with Zeiss objective lenses (40× N.A. 1.3 and 63× N.A. 1.4). Images in Figs. 1, 2, 3, 4, 6, 7, 8 and 9 represent single optical slices along the z-axis. The optical section thickness was kept identical for both the red and the green channels. Images in

Fig. 5 represent projections along the *z*-axis. The lay-out of the pictures was created with Adobe Photoshop® 8.0.

Antibodies

The monoclonal antibody raised against PY100 was purchased from Cell Signalling, the antibody recognizing phospho-FAK^{397} was purchased from Biosource/Invitrogen (Paisley, UK), the antibody raised against cPLA$_2\alpha$ (sc-1724; concentration used: 4 µg/ml) and phospho-cPLA$_2\alpha$ (Ser 505) (sc-34391; concentration used: 1 µg/ml) were purchased from Santa Cruz, the antibody raised against MAPK (concentration used: 2 µg/ml) was from Upstate and the phopho-p44/42 MAPK antibody was purchased from Cell Signaling. Secondary antibodies (GAR Alexa 488, GAM Alexa 488 and DAG Alexa 488; all used at concentrations of 1 µg/ml) were purchased from Molecular Probes/ Invitrogen (Paisley, UK), tetramethylrhodamine-5-(and-6)isothiocyanate (TRITC) conjugated Phalloidin (concentration used: 67 ng/ml) was purchased from Sigma-Aldrich (St. Louis, USA).

Results

Signal transduction is induced at the cell membrane directly after mitosis

In order to establish the localization of signalling proteins during the transition from mitosis to G1 phase, mitotic cells synchronized by shake-off, were labelled with antibodies directed against tyrosine phosphorylated proteins and F-actin as described under "Materials and methods". Both growth factor- and integrin-directed signalling activate signal transduction cascades that involve tyrosine phosphorylation of various proteins and as such this parameter is well suited to establish signal transduction. Synchronized mitotic cells were cultured and allowed to progress into G1 phase for 30 min before chemical fixation as indicated. Other samples were allowed to progress into G1 phase for 1, 2, 3, 4, 5 and 6 h before chemical fixation. Subsequently cells were labelled with an antibody directed against proteins that are phosphorylated on tyrosine residues (PY100). In the same samples, cells were also labelled for F-actin. Immunofluorescence microscopy revealed the local presence of phosphorylated proteins at the cell membrane 30 min after replating (Fig. 1a). These local concentrated spots with phosphorylated proteins are encased in a sheath of F-actin (Fig 1b) that seems to induce blebs of the cell membrane. Other cells in the same sample were spread a bit more (Fig. 1d–f). These cells showed a broader region of F-actin at the basal side where the cells grow outwards (Fig. 1e). The spreading of cells was further illustrated by the presence of stress fibers (Fig. 1e). Here, the individual blebs were no longer recognizable. Interestingly, these cells exhibit small focal adhesions both detected with the probe directed against F-actin (Fig. 1e) and the antibody directed against phosphotyrosine proteins (Fig. 1d). The tyrosine phosphorylated proteins and F-actin clearly localize at the same sites, as shown by the merged images (Fig. 1c, f). The presence of tyrosine phophorylated proteins indicates active signal transduction localized in spots at the cell membrane within 30 min after mitosis.

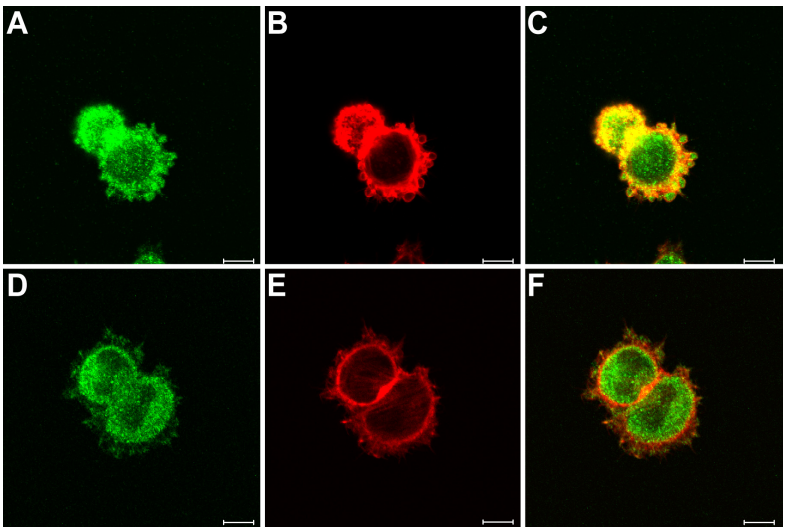

Fig. 1 Localization of tyrosine phosphorylated proteins and F-actin in early G1 phase HeLa cells. HeLa cells were chemically fixed 30 min after mitotic shake off and stained for PY100 (*green*) and F-actin (*red*) as described in "Materials and methods". The *upper panel* (**a–c**) represents the initial stage after mitosis. Cells exhibit blebs of the cell membrane that contain F-actin and are enriched with proteins that are phosphorylated at tyrosine residues. Cells that are spread further exhibit small focal adhesions and thin actin stress fibers (**d–f**). Cells that show small focal adhesions contain no blebs of the cell membrane. *Bar* represents 10 µm

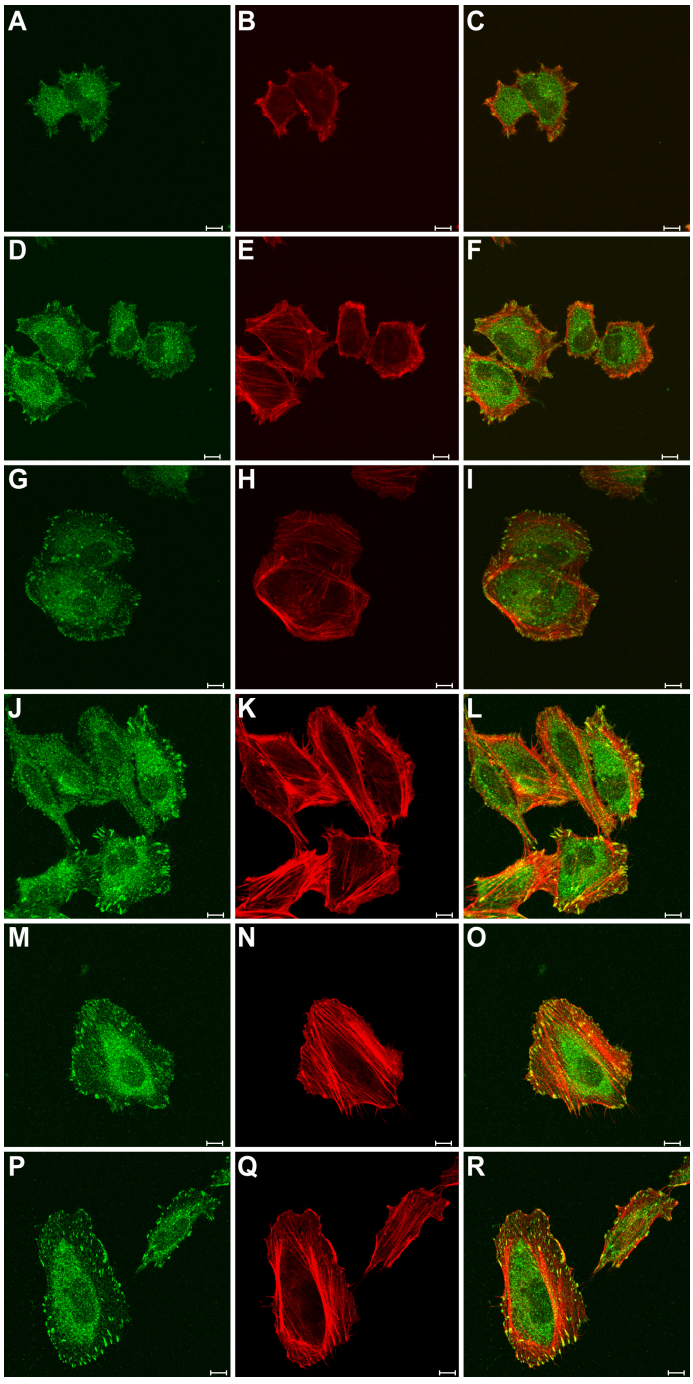

Fig. 2 Localization of tyrosine phosphorylated proteins and F-actin in G1 phase of HeLa cells. HeLa cells were synchronized by mitotic shake-off and replated for 1, 2, 3, 4, 5 and 6 h, respectively, as indicated. Labelling of tyrosine phosphorylated proteins [PY100 labelling (*green*)] is localized throughout the cytoplasm and in focal adhesions. F-actin [Phalloidin labelling (*red*)] is present in focal adhesions and stress fibers. Focal adhesions increase both in size and in number (**a**, **d**, **g**, **j**, **m**, **p**). During time, the actin stress fibers become more apparent (**b**, **e**, **h**, **k**, **n**, **q**). *Bar* represents 10 μm

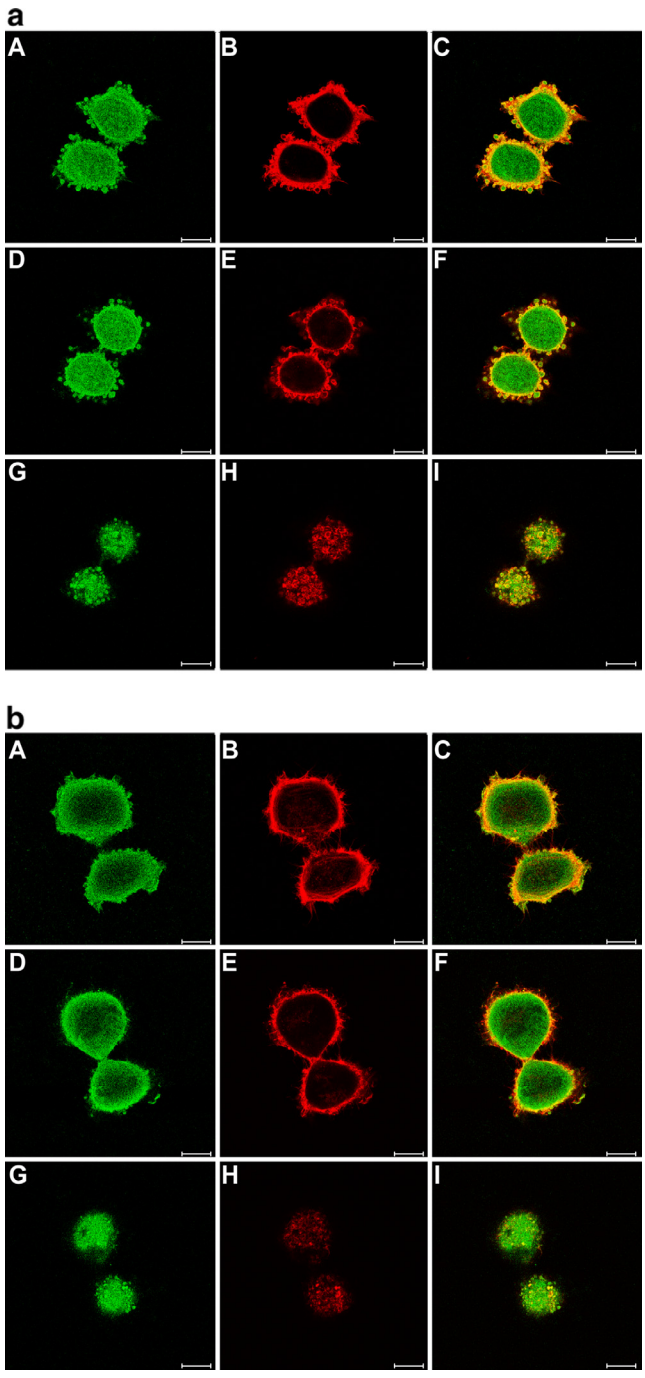

Fig. 3 Localization of phosphorylated FAK during early G1 phase in HeLa cells. **a** HeLa cells were synchronized by mitotic shake-off and replated for 40 min followed by chemical fixation as described under "Materials and methods". Phospho-FAK^{397} (*green*) and F-actin (*red*) were labeled as described under "Materials and methods". Three optical sections of the same cells are represented: *A* basal side, *B* middle part and *C* apical side. P-FAK^{397} was detected in membrane blebs at the cell surface cells. The blebs contained F-actin, and P-FAK^{397} localized in membrane blebs was surrounded with a coat of F-actin (**a** *C*, *F*). **a** *D, E, F* represents an optical section through the center of the cell that clearly exhibits blebs at the edge of the cell. The blebs of the membrane were present on the whole cell surface, from the basal side (a *A*, *B*, *C*) to the apical side (**a** *G*, *H*, *I*). Bar represents 10 μm. **b** Synchronized HeLa cells that were chemically fixed 1 h after replating were stained for Phospho-FAK^{397} (*green*) and F-actin (*red*). Three optical sections of the same cells are represented: *A* basal side, *B* middle part and *C* apical. After 60 min, the number of blebs decreased at the apical side of cells (**b** *G*, *H*, *I* vs. **a** *G*, *H*, *I*). Optical sections through the center of the cell revealed no blebs of the membrane (**b** *D*, *E*, *F*) in contrast to sections 40 min after mitosis (**a** *D*, *E*, *F*). However, in the cells that were fixed after 60 min, some blebs were observed that contain P-FAK^{397} at the basal side of cells (**b** *A*, *B*, *C*). *Bar* represents 10 μm

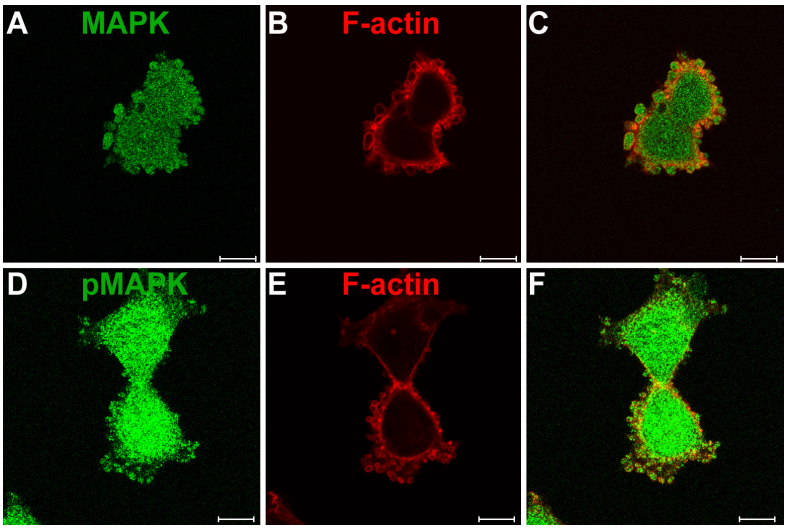

Fig. 4 Localization of MAP kinase and phosphorylated MAP kinase during early G1 phase in HeLa cells. HeLa cells were synchronized, replated and chemically fixed as described under "Material and methods" and Legends of Fig. 3. Subsequently cells were labelled for MAPK or phospho-MAPK labelling (*green*) and F-actin labeling (*red*). MAPkinase (**a**) and phospho-MAPkinase (**d**) are localized in blebs of the cell membrane that can be distinguished from the rest of the cell by the packaging in F-actin (**b, e**). *Bar* represents 10 μm

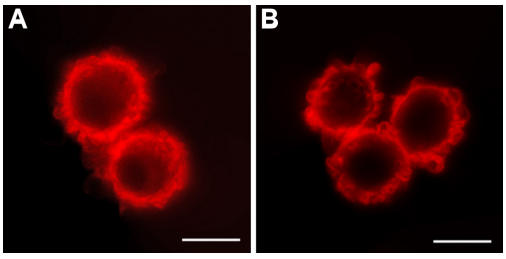

Fig. 5 Blebs of the cell membrane in post-mitotic cells in a random growing cell cultures of CHO (**a**) and HeLa cells (**b**). Blebs of the cell membrane were detected by labelling for F-actin. *Bar* represents 10 μm

During progression through the G1 phase, the tyrosine phosphorylated proteins were present at the cell membrane, especially in spots 1 h after mitosis (Fig. 2a) and onwards in G1 phase (Fig. 2d, g, j, m, p), these spots most likely represent focal adhesions. Stress fibers became clearly visible after 2 h after mitosis (Fig. 2e). The focal adhesions often are located at the tips of stress fibers, as is known from interphase cells (Badley et al. 1980). Focal adhesions increase both in size and in number (Fig. 2a, d, g, j, m, p). The merged images clearly demonstrate a co-localization of tyrosine phosphorylated proteins and F-actin in the apparent focal adhesions. In addition, a band of F-actin was clearly visible beneath the plasma membrane which did not co-localize with tyrosine phosphorylated proteins (Fig. 2). During progression into the G1 phase, cells occupy increased surface area, illustrating the flattening of cells. Both at the basal side (Fig. 2) and the apical side (data not shown), cells no longer exhibit membrane blebs as was detected with CLSM by making optical sections through the cell. Figures 1 and 2 demonstrate that active signal transduction is localized in spots at the cell membrane, first in blebs of the cell membrane and later in newly formed focal adhesions.

Phosphorylated FAK is present in blebs of the cell membrane

The local presence of tyrosine phosphorylated proteins in the membrane blebs illustrates activated signal transduction and might result both from activation by soluble growth factors and from activation by integrins. The appearance of small focal adhesions suggests the activation of integrin-induced signal transduction. Therefore attachment induced signal transduction was further investigated by measuring the local presence of phosphorylated Focal Adhesion Kinase (FAK), since the phosphorylation has been demonstrated to be prerequisite for activation of this kinase (Schaller 1996, 2010).

Cells were synchronized by mitotic shake-off and subsequently replated for 40 or 60 min as indicated. After fixation the cells were labelled with an antibody directed against FAK phosphorylated on tyrosine residue 397 (P-FAK^{397}), and with phalloidin to stain F-actin as described under "Materials and methods". The cells were studied using confocal laser scanning microscopy (CLSM). As shown in Fig. 3a, P-FAK^{397} was detected in membrane blebs at the cell surface of these cells, in addition to a cytoplasmic and nuclear localization. The blebs contained F-actin, and from the merged image it was observed that

P-FAK^{397} localized in membrane blebs was surrounded with a coat of F-actin (Fig. 3a CF). Figure 3a DEF represents an optical section through the center of the cell that clearly exhibits blebs at the edge of the cell. The blebs of the membrane were present on the whole cell surface, from the basal side (Fig. 3a ABC) to the apical side (Fig. 3a GHI).

After 60 min, the number of blebs decreased at the apical side of cells (Fig. 3b, GHI vs. Fig. 3a GHI). Optical sections through the center of the cell revealed no blebs of the membrane (Fig. 3b DEF) in contrast to sections 40 min after mitosis (Fig. 3a DEF). However, in the cells that were fixed after 60 min, some blebs were observed that contain P-FAK^{397} at the basal side of cells (Fig. 3b ABC). Therefore, P-FAK^{397} labeling is temporally present in blebs at the cell membrane in post-mitotic cells. Furthermore, membrane blebs, including P-FAK^{397} labelling, that are in contact with the extracellular matrix exist for a longer period of time compared to blebs that are not in contact with the extracellular matrix.

Previously, we have studied integrin-mediated FAK autophosphorylation in lysates of mitotic and post-mitotic cells by western blotting, using the same antibody (Margadant et al. 2007). It was shown that the antibody recognized FAK only in attached cells, that is under conditions that FAK is active and phosphorylated, and not in mitotic cells or cells that were detached from the substratum, in contrast to an antibody that recognized un-phosphorylated FAK (Margadant et al. 2007), indicating antibody specificity. Moreover, labelling with various primary antibodies in combination with the secondary antibodies that were presented in this study resulted in no staining in the blebs of the membrane. For example cells that were labelled with antibodies raised against GM130 (mouse IgG1 antibody) and Lamin B (goat polyclonal IgG antibody) revealed no staining in membrane blebs (data not shown). Therefore, we conclude that specific staining was detected in the membrane blebs.

MAPK and phosphorylated MAPK are present in blebs of the cell membrane

It has been demonstrated that serum induces the phosphorylation of MAP kinase (MAPK) immediately after mitosis during the ongoing cell cycle of CHO and of neuroblastoma N2A cells (Hulleman et al. 1999a). This activation of MAPK during early G1 was shown to be independent of integrin signalling but to require the presence of serum (Hulleman et al. 1999b). The antibodies used in these and other studies have been demonstrated to be highly specific for phosphorylated MAPK in various cell lines (Hulleman et al. 1999b; Margadant et al. 2007). In synchronized CHO cells, we demonstrated that the antibody directed against phosphorylated MAPK recognized only p42^{MAPK} and p44^{MAPK} by western blotting and that the amount of phosphorylated MAPK increased significantly upon progression of the cells from M to early G1 phase while the amount of total MAPK did not increase (Hulleman et al. 1999b). Similar results were obtained in HeLa cells (data not shown).

In order to establish the potential role of serum in the appearance of tyrosine phosphorylated proteins in the membrane blebs in early G1 phase (Fig. 1), we have studied the localization of phosphorylated MAPK in early G1 phase cells. HeLa cells were synchronized as described above and after 60 minutes cells were fixed and labelled with an antibody directed against MAPK (Fig. 4a), an antibody directed against phosphorylated MAPK (pMAPK) (Fig. 4d) or phalloidin to stain F-actin (Fig. 4b, e). Both MAPK and pMAPK were detected throughout the cytoplasm and both MAPK (Fig. 4a) and pMAPK (Fig. 4d) were present in blebs of the cell membrane, recognizable by the sheath of F-actin (Fig. 4b, e), as has been observed for tyrosine phosporylated proteins (Fig. 1). So, apparently the phosphorylation of proteins in early G1 phase that is present in membrane blebs that was demonstrated in Fig. 1, is caused by the activation of both growth factor- and integrin-induced signal transduction.

Post-mitotic membrane blebs are present in random growing cell cultures

The studies presented so far have been performed with mitotic cells synchronized by mitotic shake-off. To exclude possible artefacts due to the synchronization method, exponentially growing HeLa cells were labelled for F-actin. In these cultures, the cells in mitosis are recognized by their morphology, being round versus the flat morphology of spread interphase cells. As shown in Fig. 5, cells immediately after cytokinesis exhibit membrane blebs comparable to the membrane blebs observed in synchronized cells (Figs. 1, 3a, 4). Similar results were obtained in early G1 phase cells in an exponential growing CHO culture (Fig. 5a). This suggests that the presence of membrane blebs in post-mitotic cells is a general phenomenon and is not induced by the shake off.

Membrane blebs during the spreading of trypsinized HeLa cells

The presence of phosphorylated FAK in membrane blebs during early G1 phase in round cells suggests that the membrane blebs are involved in the attachment during spreading of these cells. This is supported by the observation of the appearance of small focal adhesions simultaneously with the disappearance of membrane blebs

during the spreading of cells that suggests a role in attachment by creating the focal adhesions in the basal membrane. Alternatively or complementary, the disappearance of membrane blebs during the spreading of round early G1 cells, might deliver the extra membrane that is required due to the increased surface volume ratio that takes place during the spreading of round cells. This implicates that the formation of membrane blebs is related to attaching and spreading of cells, rather than to a specific cell cycle phase, and as such this phenomenon may be expected to occur in trypsinized cells that are allowed to attach and spread regardless the cell cycle phase. Therefore, exponentially growing HeLa and CHO cells were trypsinized and subsequently allowed to attach and spread for 30 or 60 min, respectively. The cells were fixed and labelled using an antibody directed against tyrosine phosphorylated proteins and using phalloidin to visualize F-actin. As shown in Fig. 6a A and C 30 min after replating both HeLa and CHO cells exhibit blebs of the cell membrane that were shielded with F-actin and contained proteins that are phosphorylated on tyrosine residues. These blebs were very similar as compared to the blebs that were observed during early G1 in HeLa cells that were described in the previous figures. Similar results were obtained with synchronized CHO cells during early G1 (data not shown). 30 min later, i.e. 1 h after replating, cells occupied a larger surface area indicating the spreading of cells and most of the membrane blebs disappeared (Fig. 6aB, D). The fast disappearance of the blebs of the membrane is again comparable with the results obtained with early G1 cells. These observations indeed suggest that membrane blebbing in early G1 phase is related to cell attachment and not specifically to the cell cycle phase.

In order to determine the role of integrin-induced signaling in this process, cells were trypsinized and subsequently incubated on petridishes coated with either fibronectin or poly-L-lysine. We have shown previously that this culture strategy can be used to determine the role of integrins in cell attachment and spreading (Hulleman et al. 1999b). After 10 or 20 min, the cells were fixed and their morphology established by light microscopy. As shown in Fig. 6b, the fraction of cells that is spread increases in time from 31% after 10 min to 87% after 20 min when the cells were plated on fibronectin. In contrast, the fraction that is rounded and exhibits blebs decreases rapidly from 35% after 10 min to 7% after 20 min. In case cells were plated on poly-L-lysine, no spreading of cells was observed. Interestingly, the fraction of cells that exhibits blebs remained constant being 41% after 10 min and 39% after 20 min (Fig. 6b). Culturing the cells on fibronectin in the absence of serum resulted in an increase of spreading from 24% after 10 min to 31% after 20 min. In addition, a decrease was observed in cells containing blebs, from 15% after 10 min to 6% after 20 min. These results clearly demonstrate that cell spreading is dependent upon integrin activation. Furthermore, also the formation of membrane blebs occurs on rounded cells under all conditions tested, but the disappearance of the blebs is dependent upon the ability of the cells to spread.

Active cytosolic phospholipase $A_2\alpha$ is present in membrane blebs

Recently, we found an interesting new role for $cPLA_2\alpha$ in facilitating local actin and cell membrane dynamics (Moes et al. 2010). Blocking of $cPLA_2\alpha$ activity prevented the formation of PDGF induced local actin and membrane dynamics, such as lamellae and circular ruffles. These structures are induced by actin that locally pushes the membrane outwards. The membrane blebs that are described in this manuscript, are coated with F-actin that might reflect a role for actin in either inducing or maintaining the shape of the membrane blebs. $cPLA_2\alpha$ might facilitate the formation of membrane blebs in a similar way as in the case of PDGF induced actin and membrane dynamics (Moes et al. 2010). Therefore it was investigated if $cPLA_2\alpha$ also plays a role in facilitating the formation of membrane blebs during early G1.

First the local presence of $cPLA_2\alpha$ in the membrane blebs was investigated using an antibody specific for $cPLA_2\alpha$ (Grewal et al. 2002; Herbert et al. 2005, 2007). $cPLA_2\alpha$ was localized in the cytoplasm of cells and in the blebs of the membrane (Fig. 7a–c). In later stages, actin had formed a ruffling leading edge where cells expand lateral and less blebs shielded with F-actin were visible (Fig. 7d–f). In these cells, $cPLA_2\alpha$ was present at the ruffling edge of the cells. At this edge, $cPLA_2\alpha$ colocalizes with newly formed F-actin structures. In cells that progressed into the G1 phase for more than 2 h, these sites of co-localization of $cPLA_2\alpha$ and F-actin became less abundant and were most clear at protrusions of cells (Fig. 7g–i).

Subsequently, the activity of $cPLA_2\alpha$ that was present within the blebs and at the ruffling edge of cells was investigated by using an antibody directed against phosphorylated $cPLA_2\alpha$ (Ser 505). P-$cPLA_2\alpha$ was detected in the cytoplasm and in the nucleus. Interestingly, p-$cPLA_2\alpha$ was localized also in the cell membrane blebs (Fig. 8a ABC). Half an hour after replating of mitotic cells, p-$cPLA_2\alpha$ was present in membrane blebs both at the basal side and at the apical side of cells (Fig. 8a GHI). In later stages, the blebs of the membrane were absent at the apical side of cells (Fig. 8b GHI) and at the basal side p-$cPLA_2\alpha$ was localized at the ruffling edge of cells (Fig. 8b ABC). The localization of p-$cPLA_2\alpha$ at the edge of post-mitotic cells corresponds to the observed localization for $cPLA_2\alpha$.

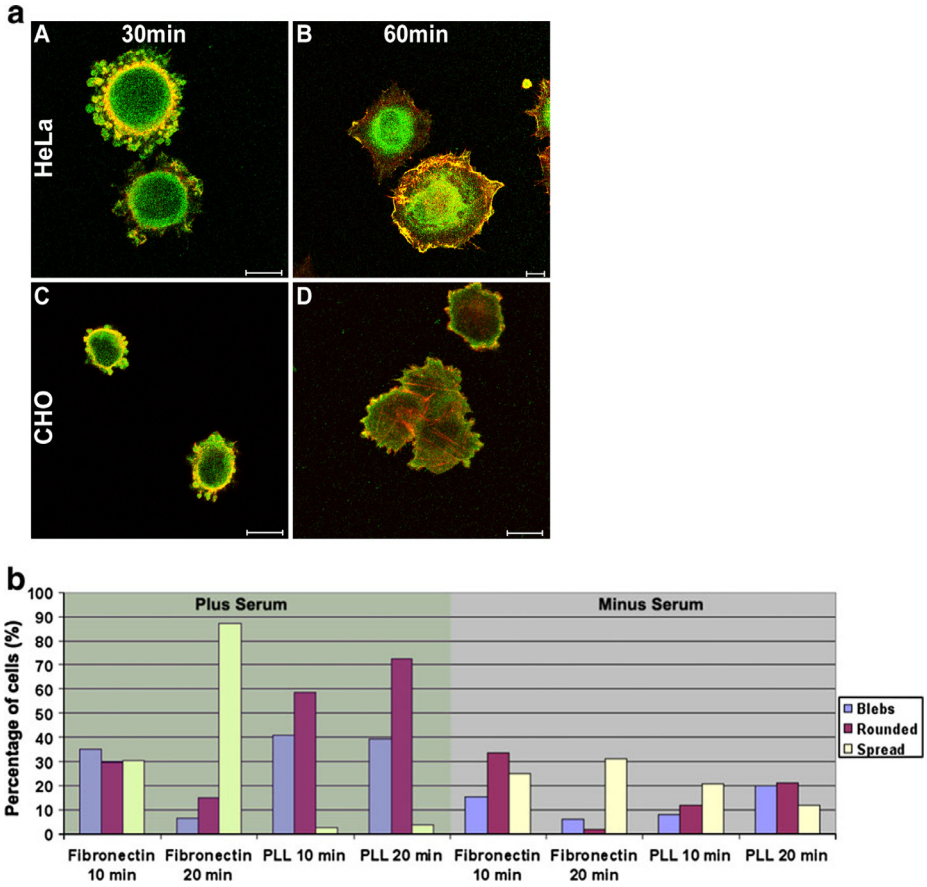

Fig. 6 Spreading of trypsinized HeLa and CHO cells. **a** Cells were trypsinized according general procedures and allowed to spread for 30 or 60 min after replating. Subsequently cells were chemically fixed and labelled for PY100 (*green*) and F-actin (*red*) as described under "Materials and methods". *Bar* represents 10 μm. **b** Effect of cell substratum on cell spreading and membrane bleb formation in CHO cells. CHO cells were trypsinized and plated on fibronectin and poly-L-lysine, respectively, in the absence or presence of serum for 10 or 20 min as indicated. Subsequently the fraction of the initial plated cells was calculated that had a spread or round morphology and the fraction of initial plated cells that contained membrane blebs. Because the cells were allowed to attach for only 10 or 20 min, non-attached cells are not included in the calculation

Formation of membrane blebs is not related to signal transduction

It is demonstrated above, that round cells (both mitotic and trypsinized) that are allowed to attach and spread to the substratum exhibit membrane blebs and active signal transduction proteins. In order to determine the relationship between these two phenomena, we have inhibited integrin-induced signaling by a specific FAK inhibitor, 1,2,4,5-Benzenetetramine tetrahydrochloride, and the MAPK-induced signaling by the specific MEK1 and MEK2 inhibitor PD98059. Subsequently membrane blebs were identified by staining for F-actin similar as for the previous experiments. The inhibitors caused an inhibition of FAK and MEK1 and MEK2 phosphorylation respectively (data not shown). Under both conditions, membrane blebs were present in the cells as shown for the MEK inhibitor in Fig. 9, indicating that membrane bleb formation is independent of signal transduction.

Discussion

Here, we describe the morphological changes of HeLa cells that occur during the transition from the M-phase to the early G1 phase. Immediately following the M-phase,

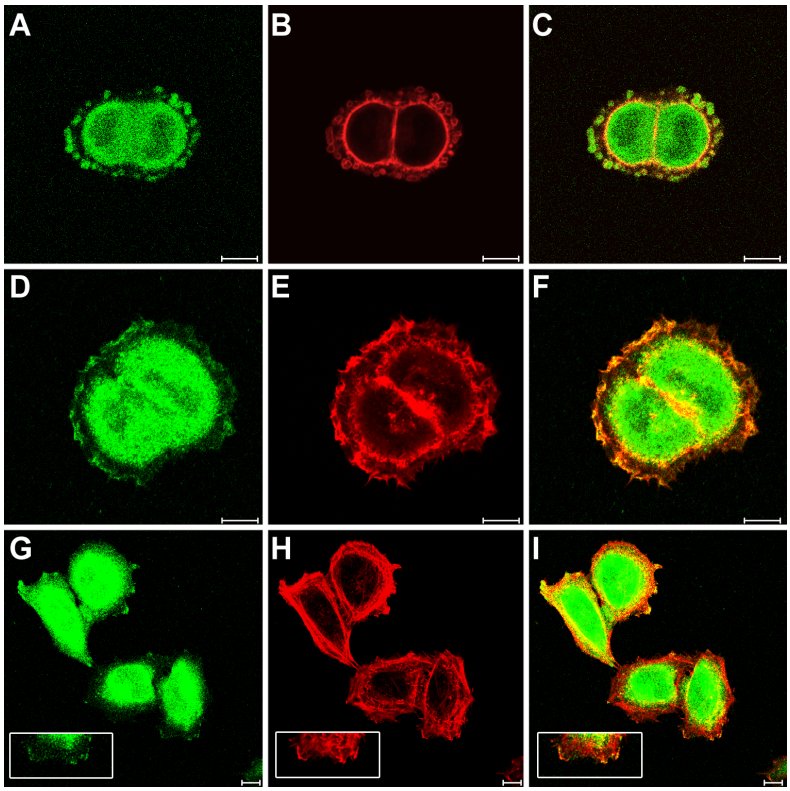

Fig. 7 Localization of cPLA$_2\alpha$ in G1 phase HeLa cells. HeLa cells were synchronized, replated and chemically fixed as described under "Materials and methods" and Legends of Fig. 3. Subsequently cells were labelled for cPLA$_2\alpha$ (*green*) and F-actin (*red*). Chemical fixation was performed 30 min (**a–c**), 1 h (**d–f**), and 4 h (**g–i**) after replating of mitotic cells. *Bar* represents 10 μm

before cell spreading, the cells exhibit numerous blebs at their cell surface. These blebs are shielded with F-actin and it is tempting to suggest that actin polymerization is responsible for the bleb formation as has been demonstrated for other membrane remodelling features as growth factor-induced membrane ruffles and leading edges (Moes et al. 2010). Of particular interest are the observations that the membrane blebs contained tyrosine phosphorylated proteins, but were devoid of lamin B and GM130, a Golgi specific protein (data not shown), suggesting that these blebs exhibit high levels of signal transduction activity. These observations are in line with previously published observations that both growth factor-induced and integrin-induced signal transduction cascades are induced within 30 min after M-phase (Hulleman et al. 1999a; Margadant et al. 2007). Furthermore, it has been demonstrated that membrane extrusions, such as membrane ruffles are enriched in signal transduction proteins (Diakonova et al. 1995). Analysis of the presence of both pFAK and of pMAPK indicates the activity of integrin- and of growth factor-induced signaling in these blebs. Similar blebs were also observed if exponentially growing cells were trypsinized and subsequently allowed to adhere and spread. Together these observations suggest that the bleb formation represents a feature related to cell spreading, and apparently not specifically to the M to G1 phase transition.

The observation that small focal adhesions appear directly after the disappearance of the membrane blebs suggests a role in focal adhesion formation and attachment. At the same time that the focal adhesions appear, half an hour after mitosis, thin actin stress fibers appear. The actin stress fibers increase in size during time. Actin has a role in attachment that is demonstrated by the presence of focal adhesions at the tips of actin stress fibers in asynchronously growing cell cultures. However, during mitosis cells are rounded and are less attached to their substratum. After mitosis, cells reattach and flatten during early G1. The observed changes in both the actin cytoskeleton and focal adhesions facilitate the spreading of post-mitotic cells. The blebs of the membrane that were detected before the appearance of small focal adhesions may act as local signalling centers to facilitate the formation of new focal adhesions, similar as described (de Hoog et al. 2004). The temporally existence of Spreading Initiation Centers (SICs)

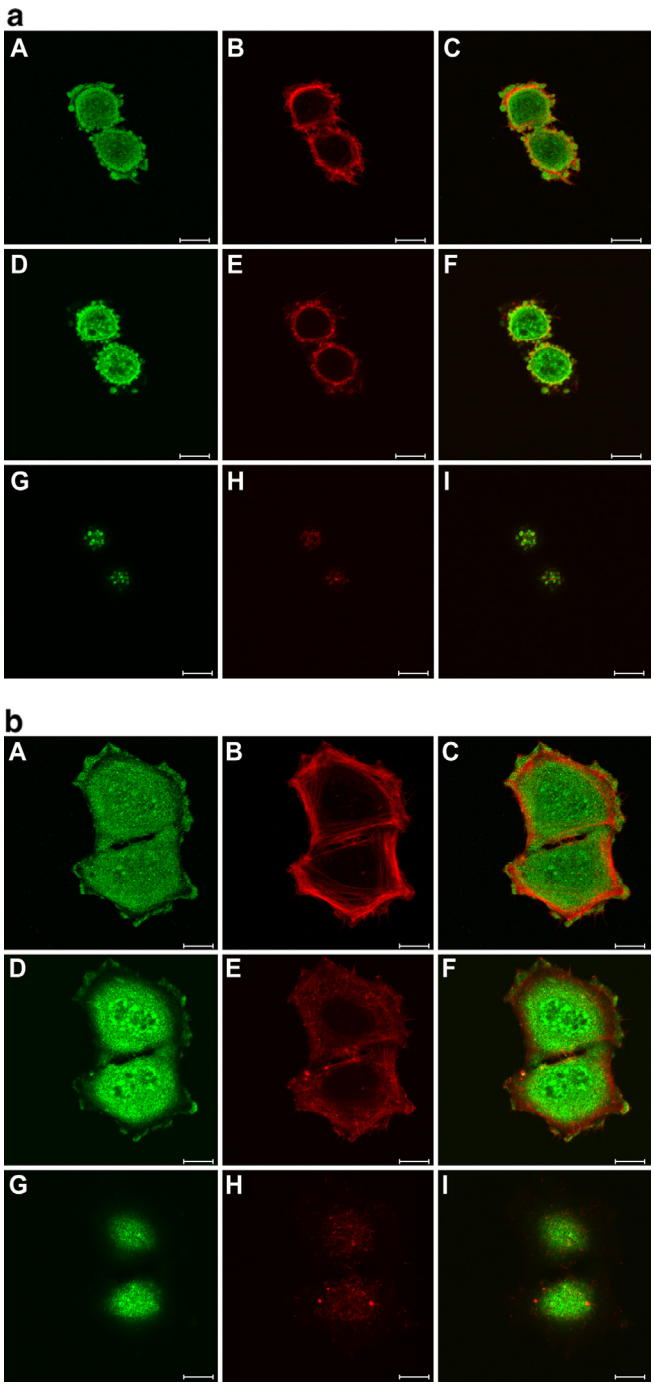

Fig. 8 Localization of phospho-cPLA$_2\alpha$ in G1 phase HeLa cells. HeLa cells were synchronized, replated and chemically fixed as described under "Materials and methods" and Legends of Fig. 3. Subsequently cells were labelled for phospho-cPLA$_2\alpha$ (*green*) and F-actin (*red*). **a** 30 min after replating, blebs of the cell membrane containing labelling for phospho-cPLA$_2\alpha$ are localized at the basal side (*A*, *B*, *C*) as well as at the apical side of cells (*G*, *H*, *I*). **b** 2 h after replating, cells exhibit no blebs of the cell membrane at the apical side of cells (*G*, *H*, *I*) and blebs have increased in size or fused together at the basal side of cells (*A*, *B*, *C*). *Bar* represents 10 μm

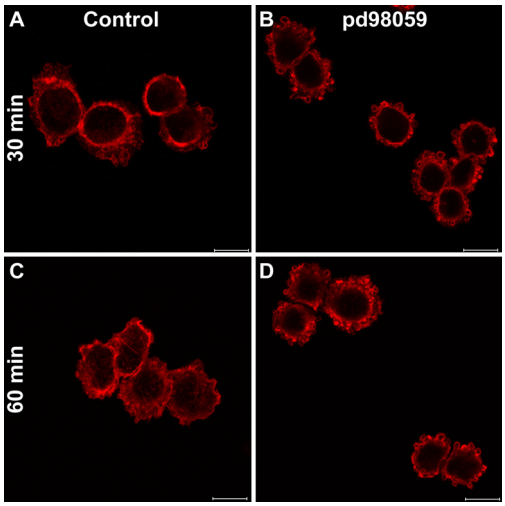

Fig. 9 Effect of inhibition of the MAPkinase pathway on membrane bleb formation. HeLa cells were synchronized, replated for 30 or 60 min as indicated and chemically fixed as described under "Materials and methods". Subsequently cells were labelled for F-actin (*red*) as described above. The MEK1 and MEK2 inhibitor pd98059 was added immediately after mitotic shake-off. In the presence of MEK1 and MEK2 inhibitor pd98059 (**b**, **d**), blebs of the membrane are present comparable to non-treated cells (**a**, **c**). *Bar* represents 10 μm

was reported during early stages of cell spreading. SICs were described to contain focal adhesion markers, to be enclosed by an F-actin sheath and were suggested to be precursors of focal adhesions (de Hoog et al. 2004).

Interestingly, the blebs of the membrane increased in size only when in contact with the substratum. This further indicates a role for integrin signalling. Moreover, the blebs disappeared faster at the apical side of cells compared to the basal side of cells. The presence of membrane blebs at the apical side may be related to the required increase in membrane area. If cells spread, the surface volume ratio increases dramatically, and this can be achieved by membrane growth. During mitosis, cells were shown to change volume (Boucrot and Kirchhausen 2008) and the area of the plasma membrane is controlled (Boucrot and Kirchhausen 2007). In this respect, it is of particular interest that the membrane blebs contain phosphorylated $cPLA_2\alpha$. $cPLA_2\alpha$ is usually activated by MAPK (van Rossum et al. 2001) and we demonstrate here that the membrane blebs indeed contain phosphorylated and consequently activated MAPK. Although a role of membrane blebs in cell attachment and signal transduction seems apparent, inhibition of both integrin-induced and MAPK-induced signaling has no effect on the formation of membrane blebs. Thus, it is tempting to suggest that the membrane blebs are required for membrane growth in order to allow cell spreading and in addition facilitate the spreading process to constitute sites of actin metabolism connected to integrin- and growth factor-induced signalling.

Alternatively, the restrictions of our cell culture conditions may mask the function of the blebs of the membrane that were observed. Membrane blebbing was related to cellular movement in rounded cells that lack mature focal adhesions and stress fibers (Friedl et al. 2001). It was described how blebs are generated during amoeboid migration in three-dimensional matrices (Charras 2008; Friedl et al. 2001; Friedl and Wolf 2010). In our experiments, cells were cultured in a two-dimensional environment. Hence, the blebs on the apical side of cells were not in contact with extracellular matrix. It will be interesting to see how the membrane blebs that are formed during early G1 behave when fully surrounded by extracellular matrix.

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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

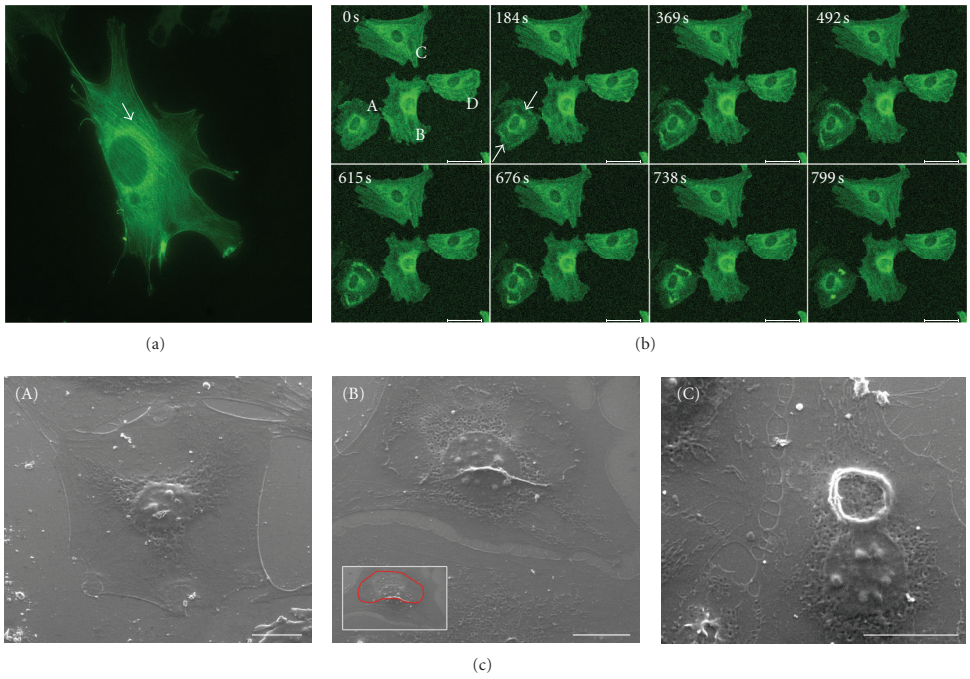


Figure 1: (a) Visualization of the stable transfected monoclonal cell line expressing GFP-actin. GFP-actin is present in the cytoplasmic pool surrounding the nucleus and in F-actin structures such as stress fibers (arrow) and the cortical skeleton. (b) Effect of PDGF-BB stimulation on GFP-actin expressing 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

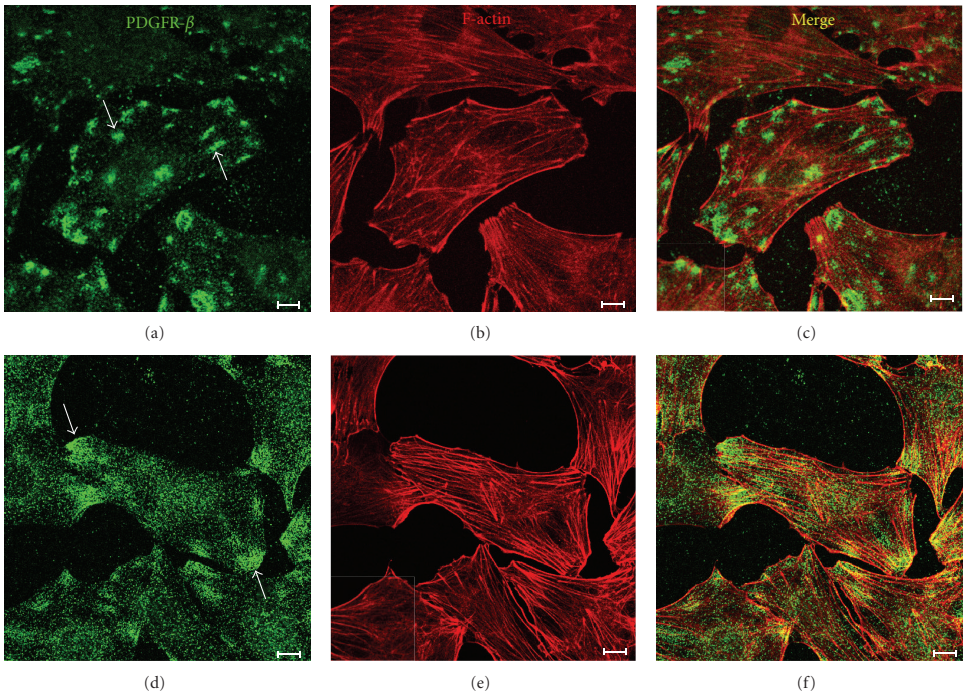


Figure 2: Immunofluorescence labeling revealing the localization of the PDGF β-receptor in serum-starved cells. Serum-starved mouse fibroblasts were stained with two different antibodies raised against the PDGF β-receptor (green) and stained for F-actin with phalloidin-TRITC (red). The antibody raised 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

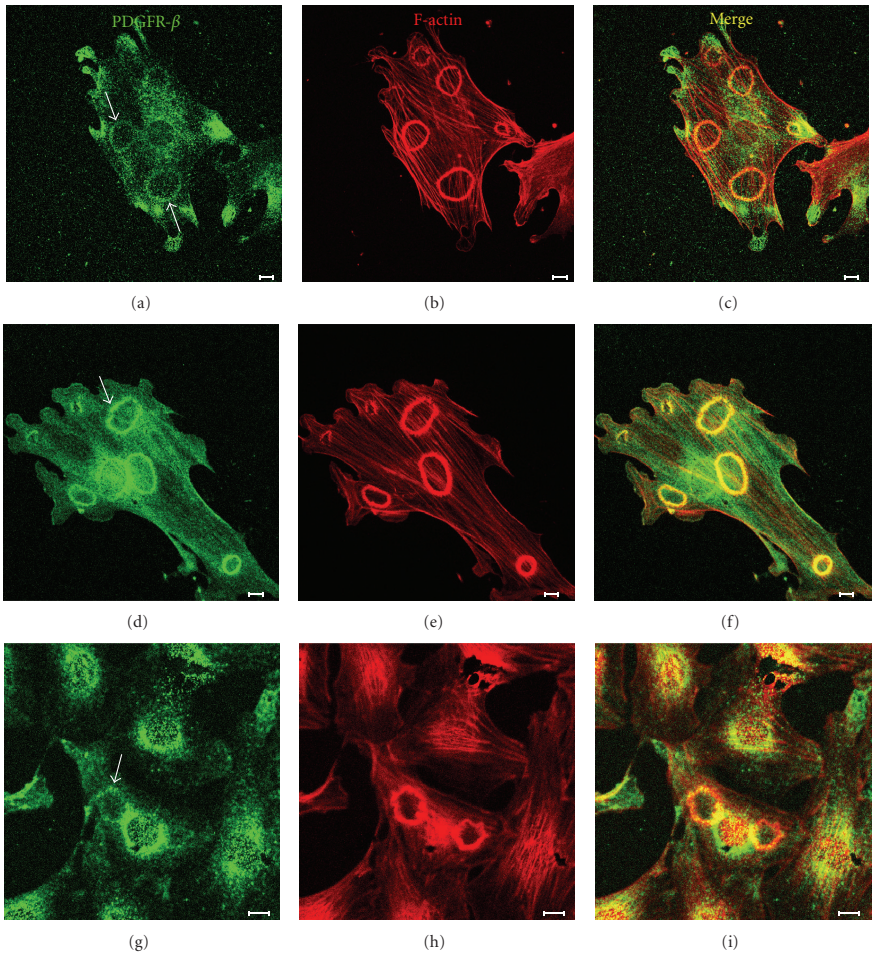


Figure 3: Immunofluorescence labeling revealing the localization of the PDGF β-receptor upon exposure to PDGF-BB. Serum-starved mouse fibroblasts were stimulated with PDGF-BB and chemically fixed after 15 minutes. Samples represented in (d), (e), and (f) were subjected to a triton treatment after chemical 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.

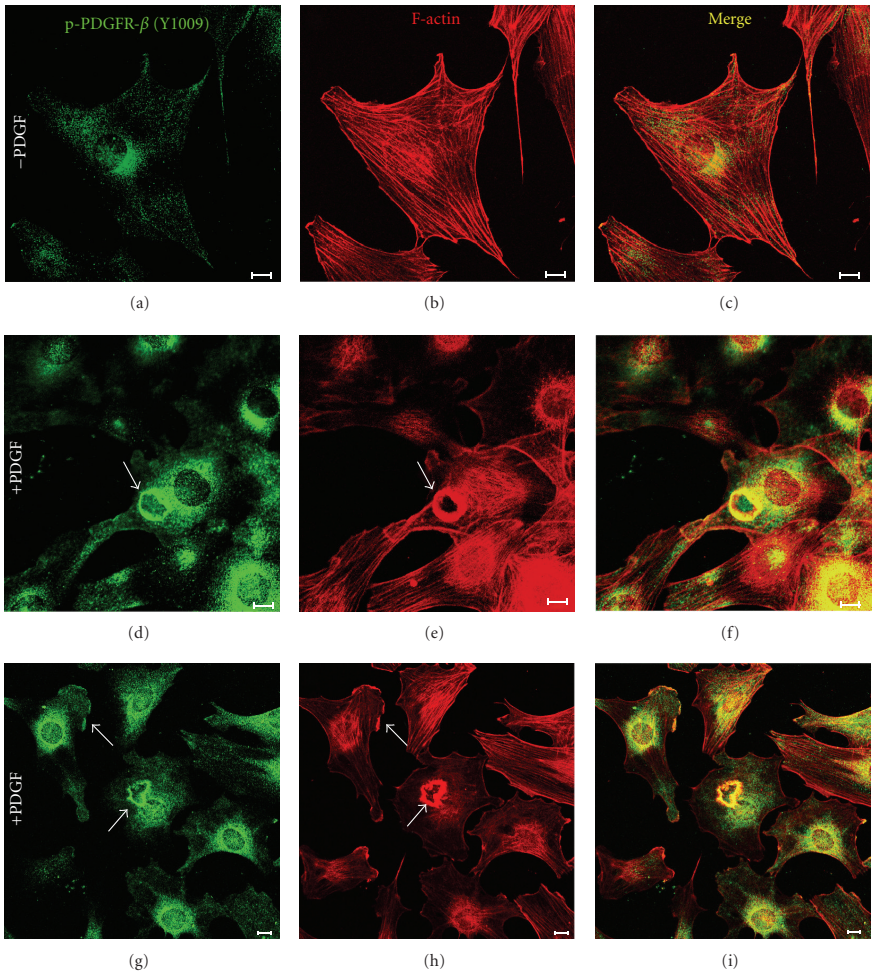


Figure 4: Induced circular ruffles are enriched with phosphorylated PDGF β-receptors. Cells were stained for phospho-PDGF β-receptors (Y1009) (green) and F-actin (red). Serum-starved cells exhibit no ruffles or lamella (b) and exhibit some staining for the phosphorylated PDGF β-receptor (a). Upon stimulation 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

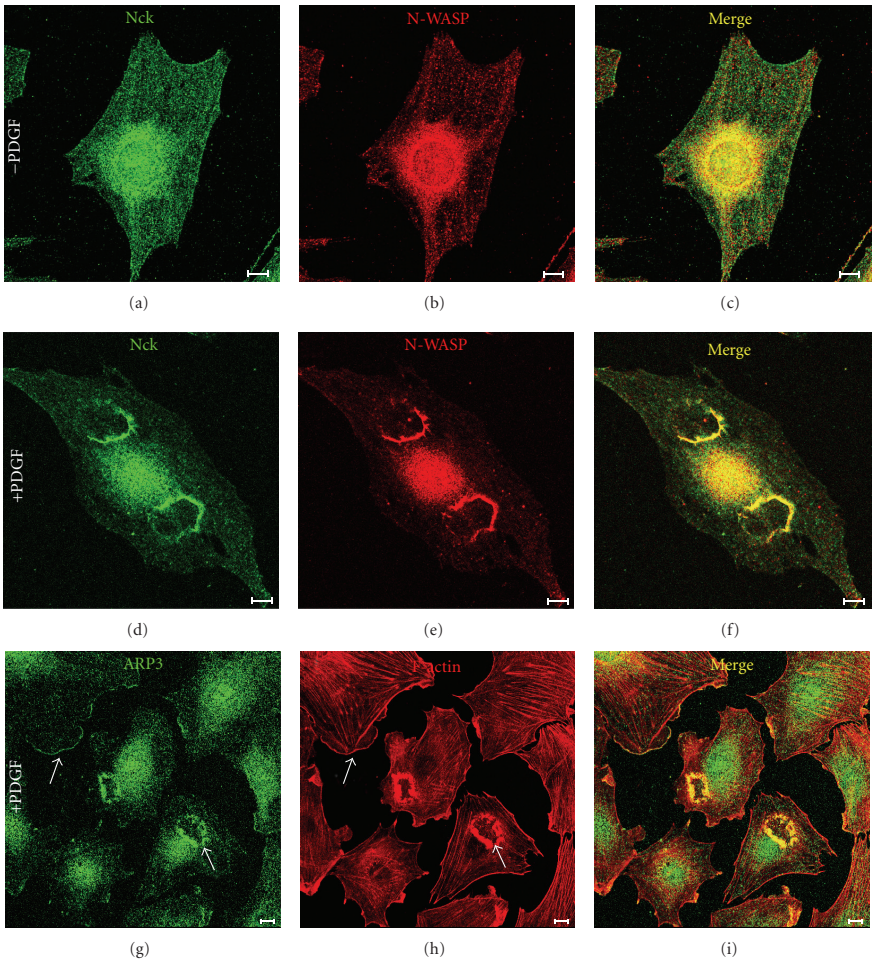
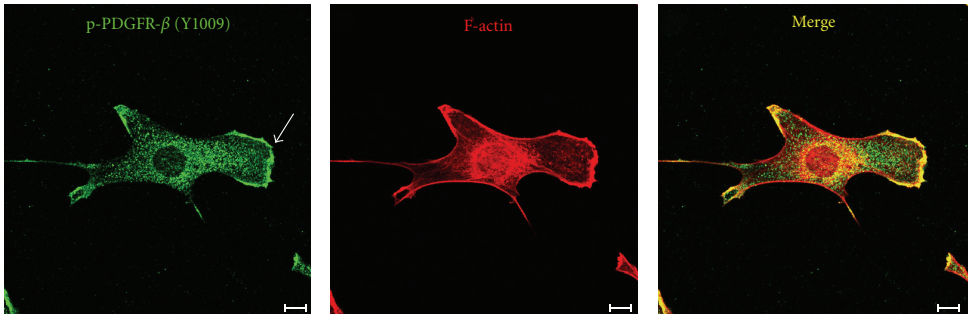


Figure 5: Nck, N-WASP and ARP3 are localized in newly formed circular ruffles upon PDGF stimulation in mouse C3H10T1/2 fibroblasts. Nck (green) and N-WASP (red) are localized in the cytoplasmic pool and in the nucleus of serum-starved cells ((a), (b), (c)). After 15 minutes of stimulation with PDGF-BB, 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

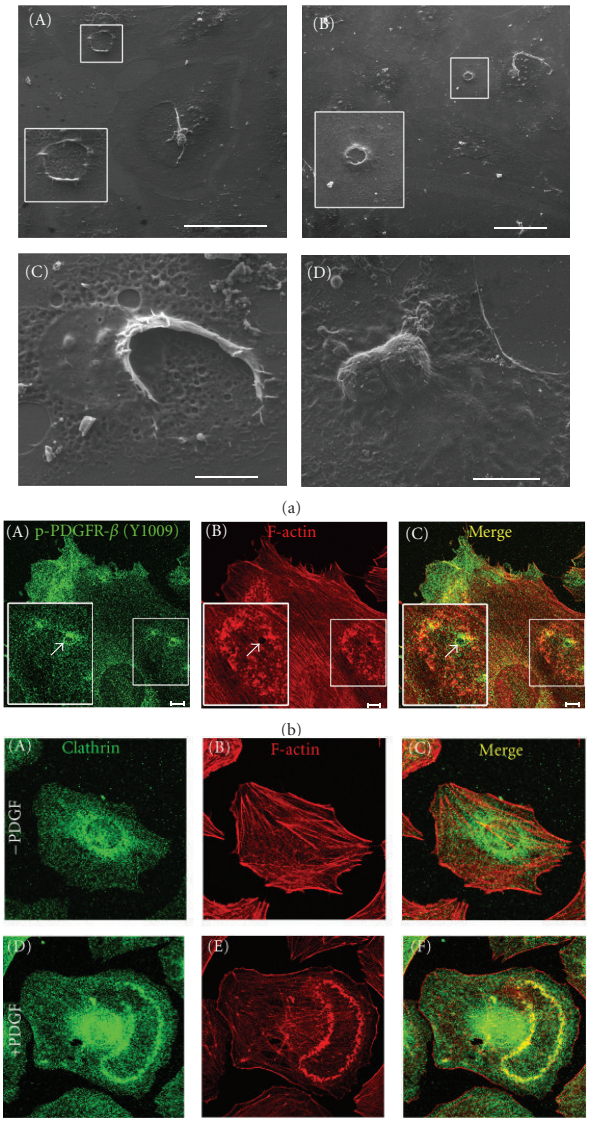


Figure 7:Macropinocytosis and clathrin localize in PDGF-BB-induced circular ruffles. (a) Visualization using ESEM of macropinocytic structures in cells that were stimulated with PDGF-BB. ESEM studies revealed the formation of macropinocytic cups ((a)-A and (a)-B) and membrane blebs on the dorsal membrane ((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.

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Chapter 5

Novel role of cPLA$_2$α in membrane and actin dynamics

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Novel role of cPLA$_2\alpha$ 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$_2\alpha$. Moreover, arachidonic acid, the product of cPLA$_2\alpha$ activity, is involved in regulation of actin dynamics. Therefore, it was investigated whether cPLA$_2\alpha$ 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$_2\alpha$ and its active phosphorylated form were shown to relocate at protrusions of the cell membrane involved in actin and membrane dynamics. Inhibition of cPLA$_2\alpha$ activity with specific inhibitors blocked growth factor-induced membrane and actin dynamics, suggesting an important role for cPLA$_2\alpha$ in these processes.

Keywords cPLA$_2\alpha$ · Actin · Arachidonic acid · Membrane dynamics · Ruffles · Lamellae · Leading edge · Migration

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Abbreviations

PLA$_2$	Phospholipase A$_2$
cPLA$_2\alpha$	Cytosolic PLA$_2$ 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 lipoxygenase 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

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_2 (PLA_2). The large family of PLA_2 consists of several sub-families like the secretory PLA_2s ($sPLA_2$), the intracellular calcium independent PLA_2s ($iPLA_2$), and the group of cytosolic PLA_2s ($cPLA_2$) including $cPLA_2\alpha$ [17]. Interestingly, $cPLA_2\alpha$ 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. $cPLA_2\alpha$ requires Ca^{2+} to translocate from the cytosol to the membrane through its CaLB (calcium-lipid binding) domain, although Ca^{2+} is not directly involved in catalysis [18]. The activation of $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ in fibroblasts during stimulation with platelet-derived growth factor (PDGF). Stimulation with PDGF introduces circular dorsal ruffles and lamellipodia. Here, we show that endogenous $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ activity with specific inhibitors blocked PDGF-induced membrane and actin dynamics, suggesting a role for $cPLA_2\alpha$ 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_2).

HUVECs were seeded on glass coverslips at 10,000 cells/cm^2, 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^2 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 cPLA2α inhibitors Pyrrolidine-2 (2 μM) and Wyeth-1 (5 μM) 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-$cPLA_2$ (Santa Cruz; sc-1724) or for 1 h with rabbit anti-phospho-$cPLA_2$ (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$_2\alpha$ 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^2 ($\sim$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^2 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$_2\alpha$ 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$_2\alpha$ plays a role in actin dynamics, because cPLA$_2\alpha$ is also activated by growth factors [22–24]. Therefore, the subcellular localization of endogenous cPLA2α was determined upon PDGF-induced changes in actin polymerization. Cells were labeled with an antibody specific for cPLA$_2\alpha$ as characterized previously [25]. As shown in Fig. 1a, in serum-starved fibroblasts, cPLA$_2\alpha$ is mainly detected in the perinuclear area and throughout the cytoplasm. Upon addition of PDGF-BB to the cells, cPLA$_2\alpha$ is recruited to newly formed circular dorsal ruffles (Fig. 1d, g) as demonstrated by the colocalization of cPLA$_2\alpha$ and PDGF-induced F-actin structures (Fig. 1f, i). This result indicates that upon growth factor stimulation, cPLA$_2\alpha$ 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$_2\alpha$ accumulates (Fig. 1m), while the perinuclear localization of cPLA$_2\alpha$, 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$_2\alpha$ to sites of new actin polymerization and membrane dynamics.

cPLA$_2\alpha$ is localized at leading edges of migrating mouse fibroblasts

To determine whether the recruitment of cPLA$_2\alpha$ at sites of newly formed F-actin represents a feature common to other cellular processes that depend on actin polymerization, the localization of cPLA$_2\alpha$ 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

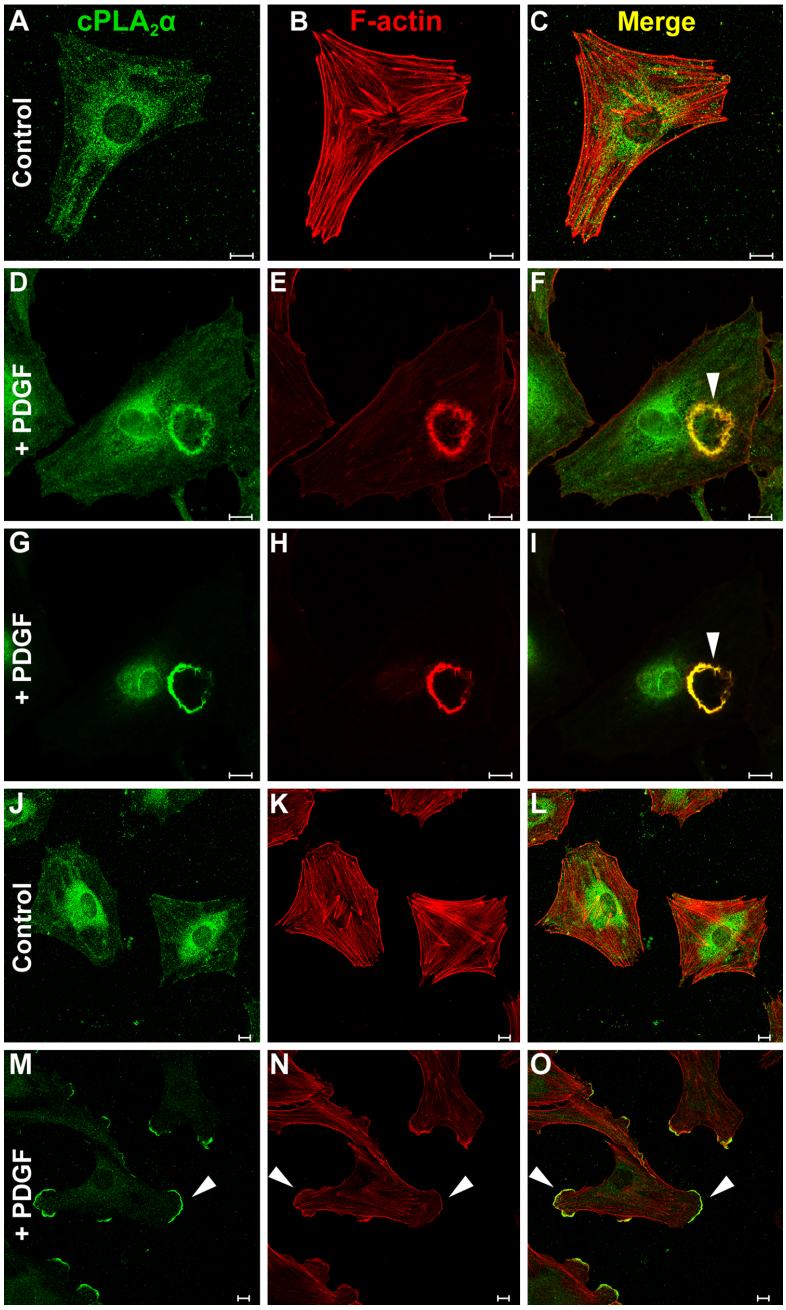

Fig. 1 Relocalization of cPLA$_2\alpha$ to dorsal membrane ruffles and to lamellae in C3H10T1/2 fibroblasts upon PDGF stimulation. Fibroblasts were serum deprived for 24 h and subsequently incubated in the presence (**d–i**, **m–o**) or absence (control: **a–c**, **j–l**) of 20 ng/ml PDGF-BB for 10 min (**j–o**) or 15 min (**a–i**) at 37°C. Cells were fixed and subjected to immunofluorescent staining (cPLA2α *green*, F-actin *red*) and samples were analyzed by confocal microscopy. In non-stimulated cells, cPLA$_2\alpha$ 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$_2\alpha$ is recruited (**d**,**g**), and in the formation of lamellae (**n**,**o**, *arrowheads*) in which cPLA$_2\alpha$ 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 μm through the cytoplasm, the nucleus, and the base of the circular ruffle whereas **g–i** represents a section at 3.08 μm through the top of the nucleus and the protrusion of the circular ruffle. *Bar* 10 μm

monolayers of C3H10T1/2, cPLA$_2\alpha$ 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$_2\alpha$ was detected (Fig. 2e, f). This membrane localization was restricted to the leading edge of the

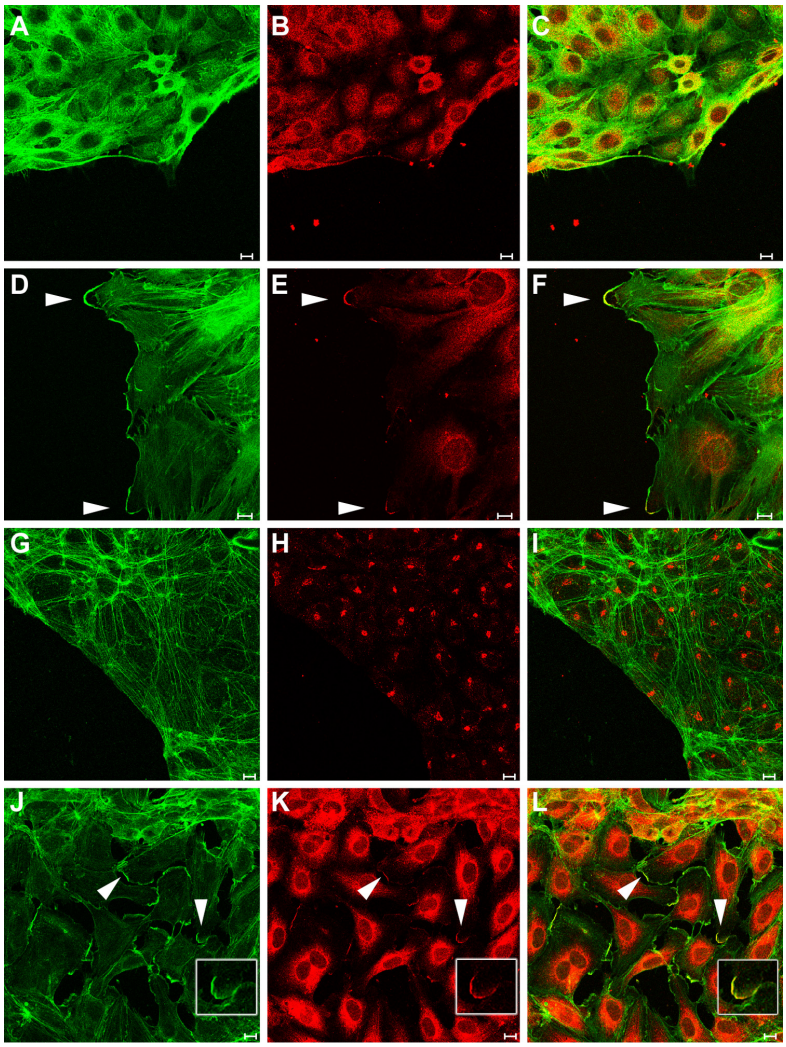

Fig. 2 $cPLA_2\alpha$ localizes at the leading edge in migrating mouse fibroblasts and HUVECs. Confluent monolayers of C3H10T1/2 fibroblasts were subjected to the scratch assay. Cells were fixed immediately after scratch (**a–c**) or 7 h after scratch (**d–f**) and subjected to immunofluorescent staining ($cPLA_2\alpha$ *red*, F-actin *green*) and samples were analyzed by confocal microscopy. In confluent mouse fibroblasts, $cPLA_2\alpha$ 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 $cPLA_2\alpha$ 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 $cPLA_2\alpha$ is located at the Golgi directly after scratch (**h**). At 22 h after scratch, in cells that have migrated into the wounded area, $cPLA_2\alpha$ is more abundant in the cytoplasm and has translocated to the newly formed leading edges. *Insert* shows a leading edge where $cPLA_2\alpha$ colocalizes with F-actin. *Bar* 10 μm

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, $cPLA_2\alpha$ was detected at leading edges of cells that had migrated into the wound. In conclusion, these data show that in migrating cells, endogenous $cPLA_2\alpha$ is recruited to the leading edges of migrating cells, i.e. at sites of active actin remodeling.

$cPLA_2\alpha$ is localized at leading edges of migrating primary human endothelial cells

To determine whether the recruitment of $cPLA_2\alpha$ at sites of actin polymerization as observed in fibroblasts is a conserved phenomenon, the relationship between $cPLA_2\alpha$ and F-actin was studied in primary endothelial cells of human origin, i.e. HUVECs. It has been demonstrated that $cPLA_2\alpha$ 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 $cPLA_2\alpha$ and F-actin was examined. Immediately after scratch, $cPLA_2\alpha$ 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 $cPLA_2\alpha$ was detected and where $cPLA_2\alpha$ locally co-localized with F-actin (data not

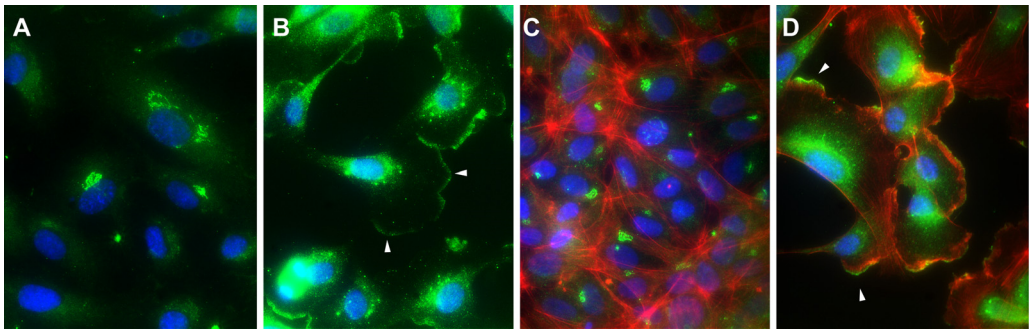

Fig. 3 Subcellular localization of endogenous cPLA$_2\alpha$ in non-confluent and confluent HUVEC. Non-confluent or confluent HUVECs were stained for cPLA$_2\alpha$ (*green*), F-actin (*red*) and the nuclei were stained with DAPI (*blue*). In confluent HUVECs (**a**,**c**) cPLA$_2\alpha$ is localized at the Golgi; in non-confluent cells (**b**,**d**) cPLA$_2\alpha$ 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$_2\alpha$ 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$_2\alpha$ 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$_2\alpha$ to leading edges of migrating HUVECs was not solely occurring in the context of the scratch assay, the localization of cPLA$_2\alpha$ 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$_2\alpha$ 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$_2\alpha$ is present at the cell periphery in restricted membrane areas which correspond to sites of actin polymerization (Fig. 3b, d).

The goat anti-cPLA$_2\alpha$ antibody used in our study is specific for cPLA$_2\alpha$ (confirmed by western blotting of HUVEC and C3H10T1/2 lysates (data not shown and [26–28]). However, to further confirm the presence of cPLA$_2\alpha$ at the cell membrane, we examined the subcellular localization of GFP-tagged human cPLA$_2\alpha$ in transiently transfected HUVECs. In non-confluent cells, GFP-cPLA$_2\alpha$ 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$_2\alpha$ at the cell membrane in leading edges. Additionally, GFP-cPLA$_2\alpha$ was detected at the Golgi apparatus of confluent HUVECs (Fig. 4a), showing that GFP-cPLA$_2\alpha$ exhibits the same translocation property as endogenous cPLA$_2\alpha$. Finally, GFP-cPLA$_2\alpha$ was recognized by the goat anti-cPLA$_2$ antibody (Fig. 4b, c).

Phosphorylated cPLA$_2\alpha$ is present in circular dorsal ruffles and leading edges

In view of our data demonstrating the recruitment of cPLA$_2\alpha$ 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$_2\alpha$ is important for this process. Since phosphorylation of cPLA$_2\alpha$ at serine residue 505 by mitogen-activated protein kinases (MAPK) is involved in cPLA$_2\alpha$ activation [29], we studied the localization of phosphorylated cPLA$_2\alpha$ using an antibody specific for Ser505-phosphorylated cPLA$_2\alpha$. In cultures of serum-starved mouse fibroblasts, phospho-S505-cPLA$_2\alpha$ was detected in the cytoplasm and in the nucleus of most cells (Fig. 5a). After stimulation with PDGF for 15 min, phospho-S505-cPLA$_2\alpha$ was found in circular dorsal ruffles (Fig. 5d). Additionally, phospho-S505-cPLA$_2\alpha$ was detected at leading edges of migrating mouse fibroblasts (Fig. 5g) and of migrating HUVECs (Fig. 5h). In conclusion, cPLA$_2\alpha$ and phospho-S505-cPLA$_2\alpha$ were found at protrusions of the cell membrane where actin remodeling takes place, indicating that cPLA$_2\alpha$ activity plays an active role in this process.

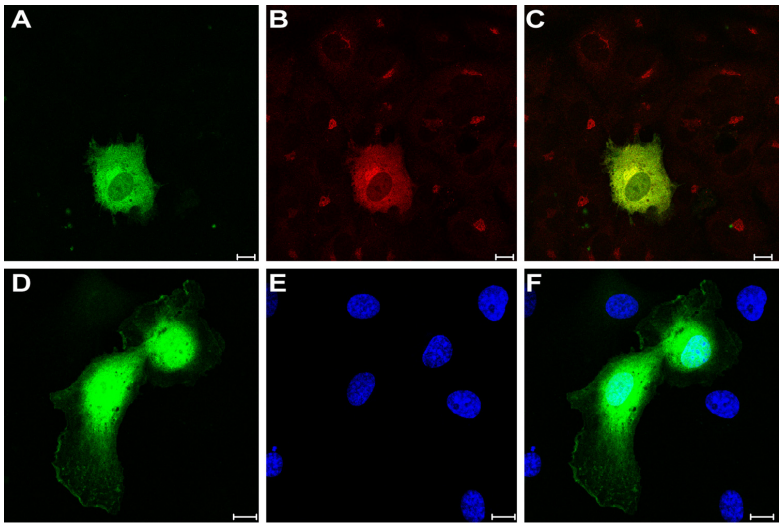

Fig. 4 Subcellular localization of GFP-cPLA$_2\alpha$ in non-confluent and confluent HUVECs. Confluent (**a**–**c**) and non-confluent (**d**–**f**) HUVECs were transfected with the GFP-cPLA$_2\alpha$ construct. Confluent cells were fixed and stained with the goat polyclonal anti-cPLA$_2\alpha$ (*green* GFP-cPLA$_2\alpha$, *red* cPLA$_2\alpha$). **a**–**c** show that the GFP-cPLA$_2\alpha$ signal (**a**) is recognized by the antibody against cPLA$_2\alpha$ (**b**). Non-confluent HUVEC transfected with GFP-cPLA$_2\alpha$ were stained with DAPI (*blue*) to visualize the nuclei. In these cells, GFP-cPLA$_2\alpha$ was mainly located in the cytoplasm and in the perinuclear area and was also found at specific areas of the plasma membrane. *Bar* 10 μm

Inhibition of cPLA$_2\alpha$ enzymatic activity prevents the formation of PDGF-induced circular ruffles and lamellae

To determine whether the enzymatic activity of cPLA$_2\alpha$ is required for actin remodeling, we used the cPLA$_2\alpha$ 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$_2$ (iPLA$_2$) or secreted PLA$_2$s (sPLA$_2$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$_2\alpha$ 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$_2\alpha$ immunodetection. Interestingly, when cells were pre-treated with either inhibitor, PDGF treatment did not induce circular dorsal ruffles nor lamellae (Fig. 6g–l), whereas in mock-treated cells, lamellae (data not shown) and dorsal ruffles containing cPLA$_2\alpha$ were induced by PDGF (Fig. 6d–f, arrowheads). These data show that inhibition of cPLA$_2\alpha$ 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$_2\alpha$ results in the preferential release of arachidonic acid, it is tempting to suggest that cPLA$_2\alpha$ is a key enzyme involved in the regulation of local actin dynamics. Therefore, we have studied the localization of cPLA$_2\alpha$ in fibroblasts and HUVECs in relation to induced actin dynamics. Endogenous cPLA$_2\alpha$ was shown to localize at the plasma membrane in both migrating mouse fibroblasts and migrating HUVEC. Moreover, the phosphorylated form of cPLA$_2\alpha$ (on Ser505) was found at the plasma membrane in both cell types. Both cPLA$_2\alpha$ and phospho-S505-cPLA$_2\alpha$ 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$_2\alpha$ in mouse fibroblasts resulted in the blocking of PDGF-induced actin and membrane dynamics.

cPLA$_2\alpha$ is present at the cell membrane in a spatio-temporal manner. Upon PDGF stimulation, cPLA$_2\alpha$ docks at specific sites at the cell membrane, namely at lamellipodia and ruffles. Upon stimulation of migration, cPLA$_2\alpha$ docks at the leading edge of motile cells. Also here, the localization of cPLA$_2\alpha$ 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$_2\alpha$ interacts with membranes via PtdIns(4,5)P$_2$. It was shown that PtdIns(4,5)P$_2$ stimulates cPLA$_2\alpha$ activity independent of calcium suggesting an interaction between PtdIns(4,5)P$_2$ and cPLA$_2\alpha$ allowing cPLA$_2\alpha$ to reach its substrate at membranes [31]. PtdIns(4,5)P$_2$ 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,

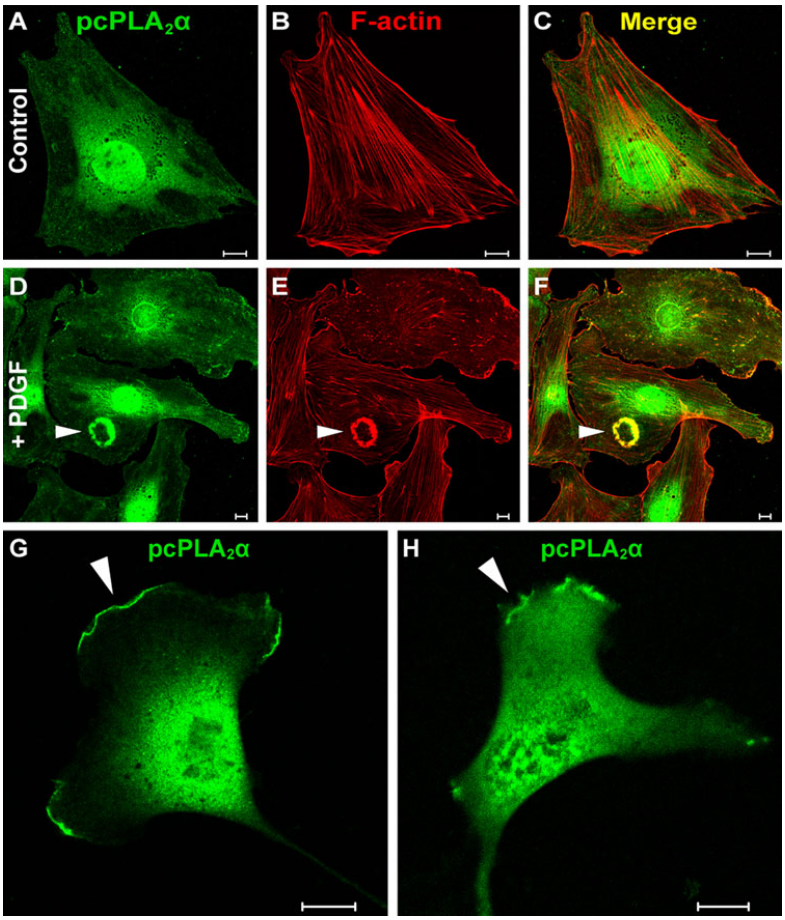

Fig. 5 Localization of phospho-S505-cPLA$_2\alpha$ to circular dorsal ruffles and at leading edges. Relocalization of phospho-S505-cPLA$_2\alpha$ to dorsal ruffles in C3H10T1/2 fibroblasts after PDGF stimulation. Fibroblasts were serum-deprived for 24 h and subsequently incubated in the presence (**d**–**f**) 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$_2\alpha$ *green*, F-actin *red*) and samples were analyzed by confocal microscopy. In non stimulated cells, staining for phospho-S505-cPLA$_2\alpha$ 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$_2\alpha$ translocates to the newly formed circular dorsal ruffles (**d**, *arrowhead*). Localization of phospho-S505-cPLA$_2\alpha$ 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$_2\alpha$ antibody. Samples were analyzed by confocal microscopy. In both cell types, phospho-S505-cPLA$_2\alpha$ 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_2 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$_2\alpha$ at the cell membrane upon growth factor stimulation. Interestingly PtdIns(4,5)P_2 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$_2\alpha$ and its phosphorylated active form at sites of actin and membrane remodeling indicates a role for cPLA$_2\alpha$ in regulating membrane dynamics. This is also suggested by blocking of PDGF-induced actin

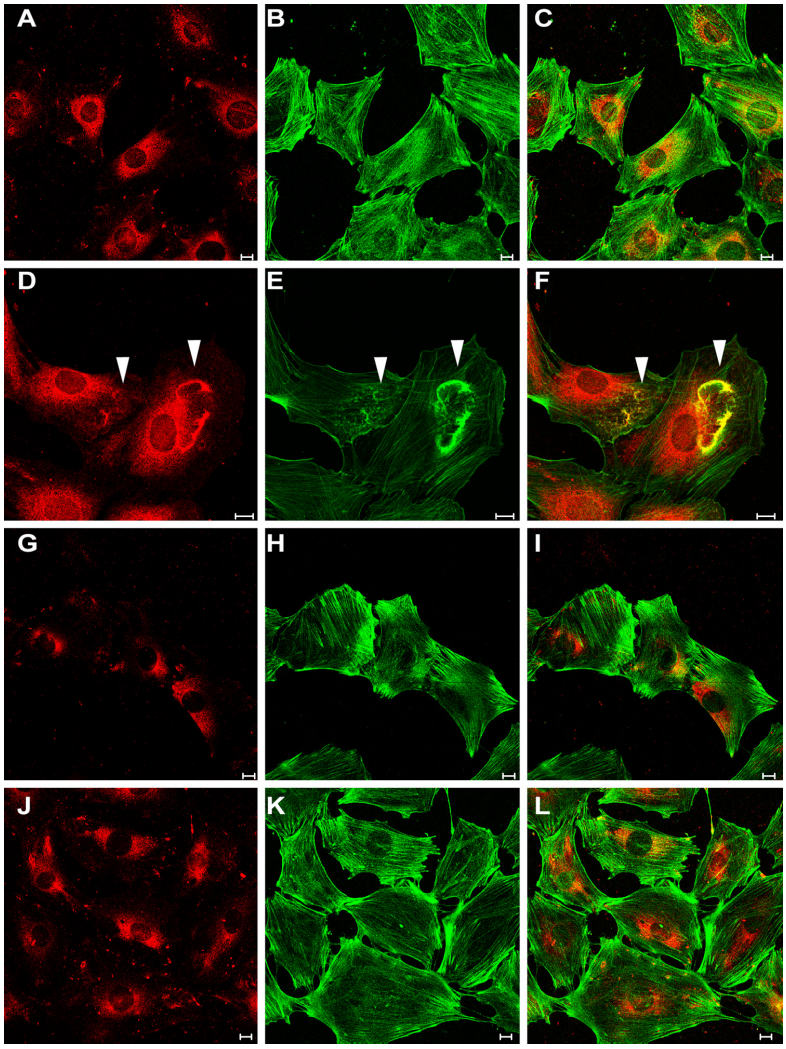

Fig. 6 Inhibition of cPLA$_2\alpha$ enzymatic activity with Pyrrolidine-2 or Wyeth-1 prevents PDGF-induced ruffling in C3H10T1/2 mouse fibroblasts. Fibroblasts were serum-deprived for 24 h and subsequently incubated in the absence (**a–c**) or presence (**d–f**) of 20 ng/ml PDGF-BB for 15 min at 37°C. Cells were fixed and subjected to immunofluorescent staining (cPLA2α *red*, F-actin *green*) and samples were analyzed by confocal microscopy. In non-stimulated cells cPLA$_2\alpha$ is found in the cytoplasm (**a**) and F-actin is organized in large stress fibers (**b**). Incubation in the presence of PDGF results the partial disappearance of stress fibers and in the formation of membrane ruffles (**e**) to which cPLA$_2\alpha$ 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 μm

and membrane dynamics after exposure to the cPLA$_2\alpha$ inhibitors Pyrrolidine-2 and Wyeth-1. Of note, these inhibitors also block the enzymatic activity of another cPLA$_2$ from group IV, namely cPLA$_2\zeta$ [30]. This enzyme can release arachidonic acid from membrane phospholipids but, contrary to cPLA$_2\alpha$, it does not have specificity for arachidonic acid versus other fatty acids [30]. However, in C3H10T1/2, cPLA$_2\zeta$ is not expressed, as confirmed by RT-PCR (our unpublished data). This observation strengthens our inhibitors-based results, reinforcing the idea that cPLA$_2\alpha$ plays a major role in regulating actin dynamics.

The local presence of the active form of cPLA$_2\alpha$ might indicate a local production of arachidonic acid by cPLA$_2\alpha$. 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 (PKCε) 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 PKCε. 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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Chapter 6

Actin dynamics in microgravity

6.1 Actin in simulated microgravity

Simulation of microgravity by magnetic levitation and random positioning: effect on human A431 cell morphology
Moes, M.J.A., Gielen, J.C., Bleichrodt, R.J., van Loon, J.J.W.A., Christianen, P.C.M., Boonstra, J. (2010) Microgravity Science and Technology 23: 249-261

6.2 Actin in real microgravity

Actin dynamics in mouse fibroblasts in microgravity:

a. Dutch Soyuz Mission
   Moes, M.J.A., Bijvelt, J.J., Boonstra, J. (2007) Microgravity Science and Technology 19: 180-183

b. MASER-10 Mission

Simulation of Microgravity by Magnetic Levitation and Random Positioning: Effect on Human A431 Cell Morphology

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Abstract Simulation of weightlessness is a desired replenishment for research in microgravity since access to space flights is limited. In real microgravity conditions, the human epidermoid cell line A431 exhibits specific changes in the actin cytoskeleton resulting ultimately in the rounding-up of cells. This rounding of A431 cells was studied in detail during exposure to Random Positioning Machine (RPM) rotation and magnetic levitation. Random rotation and magnetic levitation induced similar changes in the actin morphology of A431 cells that were also described in real microgravity. A transient process of cell rounding and renewed spreading was observed in time, illustrated by a changing actin cytoskeleton and variation in the presence of focal adhesions. However, side effects of both methods easily can lead to false linking of cellular responses to simulated microgravity. Therefore further characterization of both methods is required.

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Keywords Actin · Magnetic levitation · RPM · Simulated microgravity · Weightlessness · Focal adhesion · FAK

Abbreviations

RPM	random positioning machine
FAK	focal adhesion kinase
EGF	epidermal growth factor

Introduction

During the last decades a wide variety of space flight experiments have demonstrated that gravity has profound effects on whole organisms, organs and tissues, resulting for example in bone and muscle mass reduction as well as in the occurrence of cardiovascular malfunctioning and many other processes (Carmeliet et al. 2001; Fitts et al. 2001; Fritsch-Yelle et al. 1996). Interestingly, the virtual absence of gravity also had profound effects on the cellular and molecular level, including changes in cell morphology (Rijken et al. 1991a; Hughes-Fulford et al. 2003), modification of gene expression (de Groot et al. 1991a; Hammond et al. 1999; Liu and Wang 2008), changes in signal transduction cascades (de Groot et al. 1991b; Ullrich et al. 2008) and even changes in the self-organisation of tubulin (Papaseit et al. 2000; Glade et al. 2006; Tabony et al. 2007).

Thus it was demonstrated that exposure of epidermoid A431 cells to microgravity conditions during a sounding rocket flight, resulted in a significant decrease of EGF-induced *c-fos* and *c-jun* expression (de Groot et al. 1990). Subsequent experiments demonstrated that the effect of microgravity on gene expression was a

specific effect, as for example the EGF and phorbol ester-induced *c-fos* expression were sensitive to microgravity, but the forskolin and A23187-induced *c-fos* were insensitive, suggesting that in particular the protein kinase C (PKC)-mediated signal transduction cascades were sensitive to gravity (de Groot et al. 1991b). In addition it was demonstrated during sounding rocket flights that microgravity exposure of A431 cells resulted in increased actin polymerization and cell rounding (Rijken et al. 1991a), a process that is dependent on actin. Since PKC activation is dependent upon actin polymerization, these findings suggested that the actin microfilament system may represent a gravity sensitive component in cells (Boonstra 1999).

Actin is a major component of the cytoskeleton and has important functions, amongst others in signal transduction, motility, attachment, and cell morphology, (for review see Boonstra and Moes 2005). Actin is present both in a non-polymerized form (G-actin) and a polymerized form (F-actin). Polymerized actin can form various structures in cells such as stress fibers and the cortical skeleton that together determine the morphology of cells. The state of actin is tightly regulated by actin binding proteins (ABPs) as described in detail in several recent review papers (dos Remedios et al. 2003; Boonstra and Moes 2005).

Microgravity based research is hampered by a very limited access to space flights, both short lasting conditions (sounding rockets) and long duration missions (Space Shuttle, or International Space Station, ISS), making routine laboratory research impossible. Therefore attempts have been made to develop conditions in which microgravity was simulated. One of the first successful approaches for single cell studies concerns the fast rotating clinostat, a device that enables the rotation of a cell culture around an axis perpendicular to the gravity vector (Briegleb 1992). Thus it was demonstrated that both the reduction in gene expression and the increase in actin polymerization, as measured during sounding rocket experiments, were also observed under simulated microgravity using a fast rotating clinostat (Rijken et al. 1991a). Improvements of the principles of the fast rotating clinostat resulted in the development of the random positioning machine (RPM; Hoson et al. 1992, 1997; van Loon 2007). In the RPM, samples are mounted on a platform that randomly changes position in three dimensions by driving two independent frames that rotate independently in random directions and at random speeds. The random rotation in all directions results in a net force of zero. Simulation of weightlessness with the RPM was reported to result in a wide variety of cellular responses which have also been found partly under real microgravity conditions (Hoson et al. 1997; Schwarzenberg et al. 1998; Grimm et al. 2002; Uva et al. 2002; Infanger et al. 2006; Ulbrich et al. 2008; Versari et al. 2007a, b).

Both the fast rotating clinostat and the RPM are based on the principle that the direction of gravity is randomized. This randomization requires time and therefore only processes can be studied that experience a certain time lag phase. Since the randomization time is dependent on the rotation speed, the processes studied may require also different rotation speeds, dependent on their intrinsic time lag phase. For this reason the clinostat and RPM are not suitable for relatively fast occurring molecular and cellular processes. Perception of residual levels of gravity will depend on the time lag phase, speed of rotation and position of the sample within the system. Increasing the speed of rotation will lower residual gravity perception. On the other hand, increasing the speed of rotation might increase cellular responses introduced by other parameters, such as centrifugal forces.

An alternative to this end may be provided by magnetic levitation (Beaugnon and Tournier 1991a, b). In this technique magnetic forces are exerted on diamagnetic objects by positioning them in a strong gradient magnetic field. Since diamagnetic objects are repelled by magnetic fields this results in a magnetic force, towards regions of low field strength, that can be used to counterbalance the gravitational force, resulting in stable levitation and the simulation of microgravity. Since the vast majority of materials is diamagnetic, it is possible to magnetically levitate a wide variety of substances, such as water, organic solvents and Bismuth (Beaugnon and Tournier 1991a, b). Surprisingly, also more complex biological objects can be levitated, including frog eggs, cells and living frogs and grasshoppers (Berry and Geim 1997; Valles et al. 1997; Geim 1998; Glade et al. 2006), despite the fact that they consist of components with slightly different magnetic susceptibility. Because the gravitational force is compensated on the level of individual molecules, the condition for magnetic levitation depends on the *quotient* of the magnetic susceptibility of a material and its density. This quotient is remarkably constant for the typical constituents of biological systems, such as water, blood, tissues and bones, with mutual deviations of only a few percent (Schenck 1992). Complex biological systems are therefore relatively homogeneous with respect to diamagnetic levitation. Additional advantages of magnetic levitation are the tuneability of the effective gravity by varying the applied magnetic field strength (Heijna et al. 2007) and the fact that it provides simulated microgravity almost instantly, allowing the study of relatively fast processes.

The aim of the present research was to use random positioning and magnetic levitation to study the effect of these microgravity simulation paradigms on the actin cytoskeleton in human A431 cells. We compare the results with data found in the past in real microgravity and in simulated microgravity using the fast rotating clinostat. However, during magnetic levitation cells are exposed to high magnetic fields. Therefore we studied also the effect of such a magnetic field on the cells without levitation.

Human A431 cells were exposed to random positioning and magnetic levitation for different time intervals and chemically fixed while rotation or levitation was ongoing. Subsequently the actin morphology and behaviour of focal adhesions were investigated using fluorescence microscopy. The presence of focal adhesions is an indicator for attachment and rounding or spreading of cells. Interestingly, identical results were obtained in the RPM studies and magnetic levitation studies. However, controls for the effect of the magnetic field raised concern about the potential of magnetically simulated microgravity to measure the effects of microgravity on cell morphology and also indicated the importance of this control. Also simulation with the RPM raised concerns about side-effects. Though with both methods results obtained in real microgravity were repeated, the further characterization of both methods is required.

Materials and Methods

Materials

Tissue culture nutrients, CO_2 independent Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen (Paisley, UK). All other chemicals used were obtained from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany) and were of the highest purity available. The anti-phospho-FAK^{397} and anti-phospho-FAK^{576} antibodies were purchased from Biosource/ Invitrogen (Paisley, UK). The Alexa 488 conjugated anti rabbit antibody was obtained from Molecular Probes/ Invitrogen (Paisley, UK).

Cell Culture

Human A431 cells were grown at 37°C in CO_2 independent medium supplemented with 7.5% FBS and 5 mM L-glutamine. Cells were plated on glass coverslips and cultured for 1 or 2 days at respectively 15.000 or 30.000 cells per cm^2. Cells used for EGF stimulation (80 ng/ml EGF) and RPM experiments were serum starved for 24h.

Random Positioning Machine

The random positioning machine was manufactured by Dutch Space B.V. (Leiden, The Netherlands). The RPM consists of an inner and an outer frame that can rotate independently in random directions and random speeds. The inner frame rotates within the outer frame. For RPM modes; random direction, random interval and random speed was set and the maximum random speed was chosen as 360°/s.

In the middle of the inner rotating frame, a device was placed that allows automated refreshment of fluids while the RPM is rotating. This COmpact BioReactor Assembly or COBRA (Dutch Space, Leiden, NL; Borst and van Loon 2009) can hold a 12 well plate as a sample chamber. One well of a 12 wells plate was used and placed in the center of the rotating axes. A coverslip with cells was fixed to the bottom of this well and the well was subsequently filled with medium without gas bubbles. Cells were fixed with 4% formaldehyde at various time intervals while rotation of the RPM was ongoing. The maximum distance of the sample to the center of rotation was less than 1 cm. In combination with a speed of rotation of 360°/s this results in a residual g between 10^{-3} and 10^{-2} g, for 360°/s (van Loon 2007). Experiments were performed in a humidified room at 37°C.

Magnetic Levitation

For performing experiments in high gradient magnetic fields, a dedicated culture chamber was constructed that holds a coverslip with cells surrounded by culture medium (Fig. 1). An entrance in the chamber allowed washing and fixing, while the cells were exposed to magnetic levitation.

After insertion of the coverslip in the experimental culture chamber, the cells remained untreated for 14 min during which the temperature was brought to 36.9 ± 0.1°C. The strength of the magnetic field was reached in approximately 1 min, using a 20 T, water-cooled Bitter magnet (Perenboom et al. 2003). The levitation conditions of water were used, for which the magnetic field strength times the field gradient is equal to 1,401 T^2/m. In practice the magnetic field was set to 17 T in the center of the magnet, resulting in a field strength of 11.5 T at the position of levitation, 7.2 cm above the field center. For magnetic field controls the samples were placed in the center of the magnet using a spatially homogeneous field of 11.5 T. The samples

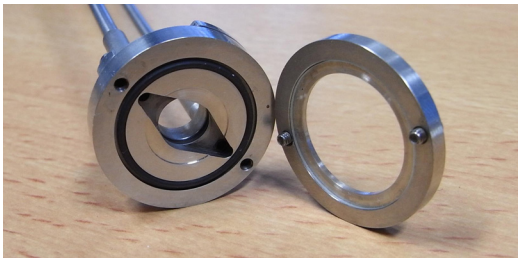

Fig. 1 Opened culture chamber that was used for experiments in the magnet. The *central circle* provides space for a 12 mm coverslip. After insertion of a coverslip, a glass lid was placed on top of the chamber. The two tubes at the other side of the chamber were used for fluid refreshment, allowing chemical fixation while the experiment was ongoing

were exposed to the magnetic fields for various time intervals and chemically fixed by flushing the sample chamber with 4% formaldehyde while levitation was ongoing. For these experiments the duration of levitation was 0, 6, 30, 60 and 120 min. The data for 6 and 30 min represent three or four independent experiments, the later time points two or three independent experiments.

Immunofluorescence Labeling

EGF stimulated samples were fixed for 30 min with 4% formaldehyde. Chemically fixed samples of magnetic levitation experiments were transported back to the laboratory at room temperature and kept overnight at 4°C. RPM samples were also kept overnight at 4°C before further processing the next day. Samples were washed extensively with PBS to remove formaldehyde, 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% gelatine, cells were incubated for 60 min at room temperature with the primary antibody raised against phospho-FAK^{397} or phospho-FAK^{576}. This was followed by washing six times in PBS containing 0.2% gelatin. Subsequently, cells were incubated for 60 min with (TRITC) or FITC-conjugated phalloidin and the GAR Alexa 488 or GARCy3 antibody. Finally, cells were mounted in Mowiol-DABCO.

Acquisition of Images

Results represented in Fig. 2 were visualized with a Leica microscope (Orthoplan) fitted with a ×40 N.A. 1.3 Leica oil objective. Images were acquired with a Leica CCD camera (model DC350F) using Leica Image Manager 50 software. Results represented in Fig. 3 were visualized with an Olympus microscope (AX70) fitted with a ×60 N.A. 1.25 Olympus Uplan Fl objective. Images were acquired with a Nikon CCD camera (DXM1200) using Nikon ACT1 software. Results represented in Figs. 4 and 5 were visualized with a Zeiss microscope (Axioskop) equipped with a Zeiss objective ×40, N.A. 0.75 oil. This microscope was coupled to a Leica CCD camera (DFC420C) and images were acquired using Leica Image Manager software. Subsequently pictures were processed with Abobe Photoshop 8.0.

Results

Rounding of A431 Cells

Under normal, 1 g conditions human epidermoid carcinoma A431 cells grow in clusters and are not fully flattened but semi rounded. This is indicated by the clear presence of a cortical F-actin skeleton (Fig. 2a). The degree of rounding of cells can be further established by the visualization of the focal adhesions. A cell that is spread exhibits clear focal adhesions whereas a cell that is fully rounded will show little or no focal adhesions.

A431 showed reduced adhesion upon stimulation with epidermal growth factor (EGF; Nelson and Fry 1997; Genersch et al. 1998; Rijken et al. 1991b). This EGF induced modification in adhesion of A431 cells was used as a model to find markers for cell rounding in RPM and magnetic levitation studies. Within 5 min of EGF stimulation (80 ng/ml) phospho-FAK^{397} labelling disappears in focal adhesions (Fig. 2d). Furthermore the cortical F-actin skeleton becomes more pronounced and stress fibres disappear (Fig. 2c). Cells retract thereby covering less surface area and retraction spikes indicate this process (Fig. 2d insert). The rounding of cells is followed by spreading, indicating the transient nature of this process. Thirty minutes after initial stimulation with EGF the cortical skeleton becomes less pronounced, cells show renewed labelling for phospho-FAK^{397} in focal adhesions (Fig. 2f) and newly formed stress fibers (Fig. 2e) indicating a more flattened morphology. Together, the behavior of F-actin and phospho-FAK^{397} labelling of focal adhesions, give a clear indication of the degree of rounding of A431 cells.

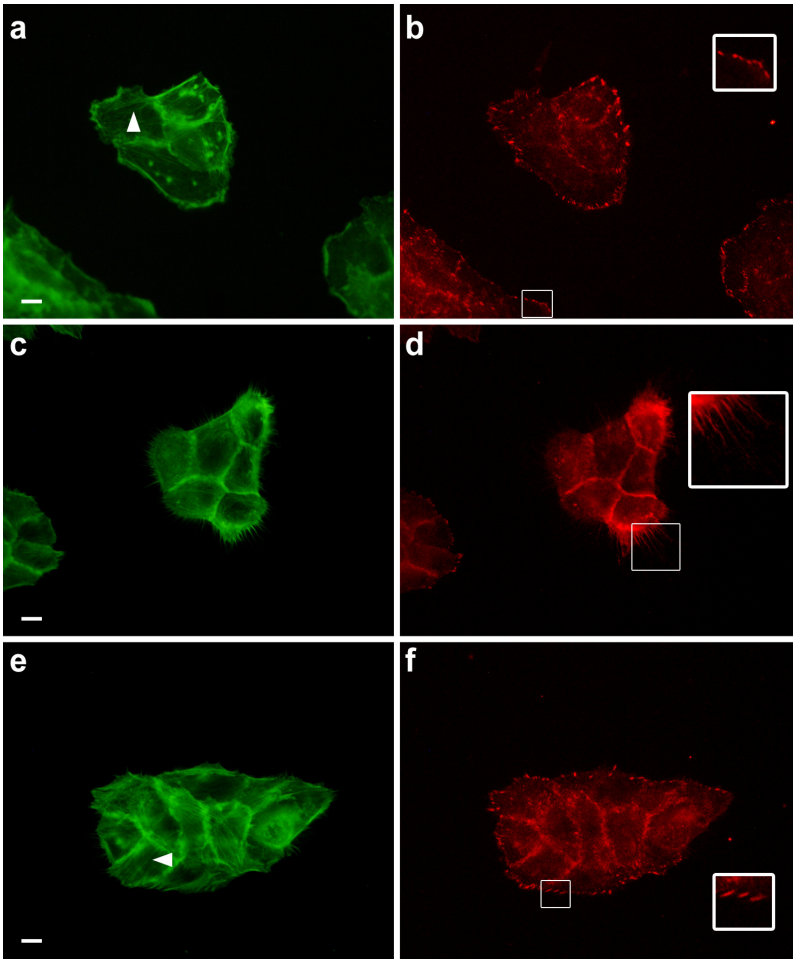

Fig. 2 EGF induced cell rounding of A431 cells. A431 cells were serum starved for 24 h and subsequently incubated in the presence of 80 ng/ml EGF for 0, 5 and 30 min at 37°C, respectively (**a**, **b**), (**c**, **d**) and (**e**, **f**). After fixation, immunofluorescence labelling was performed using Phalloidin-FITC (*green*) to stain F-actin (**a**, **c**, **e**) and anti-phospho-FAK^{397} (*red*) to stain focal adhesions (**b**, **d**, **f**). In non-stimulated cells F-actin is visible both in stress fibers (*arrow*) and in the cortical skeleton (**a**). Phospho-FAK^{397} labelling is present in focal adhesions (**b**) (*insert*). Five minutes after EGF stimulation the number of stress fibers is reduced and cells are more rounded (**c**), the number of focal adhesions that are labelled with phospho-FAK^{397} is strongly reduced (**d**) (*insert*). Thirty minutes after EGF stimulation cells flatten again indicated by the increase in the number of stress fibers (E, *arrow*) and the labelling for phospho-FAK^{397} in the focal adhesions (**f**) (*insert*). *Bar* represents 10 μm, all *inserts* are enlarged twice

Effect of Simulated Microgravity on A431 Cell Morphology Using the RPM

In order to study the effect of simulated microgravity on A431 cell morphology, the cells were placed in the RPM as described under "Materials and Methods". As shown in Fig. 3c, the cortical actin skeleton became more pronounced after 6 min of simulated microgravity. After 30 min of rotation the cells showed increased rounding (Fig. 3e), followed by spreading of cells after 60 min of rotation (Fig. 3g). This pattern of rounding and spreading was supported by the visualization of phospho-FAK^{576} labelling in focal adhesions. The phosphorylation of FAK at pY576 in focal adhesions during cell rounding is comparable with that of pY397 (data not shown). After 6 min of RPM rotation, the number of focal adhesions stained for phospho-FAK^{576} decreased but remaining focal adhesions showed intense labelling for phospho-FAK^{576} (Fig. 3d). After 30 min of rotation (Fig. 3f), the number of focal adhesions stained for phospho-FAK^{576} was clearly further decreased as compared to cells after 0 (Fig. 3b) and 6 min (Fig. 3d) of rotation while after 60 min of rotation (Fig. 3h) the number of focal adhesions detected with the anti-phospho-FAK^{576} antibody increased again. This indicates renewed attachment and spreading of cells.

These experiments clearly demonstrate that A431 cell morphology showed a transient process of rounding and subsequent reattachment during rotation in the

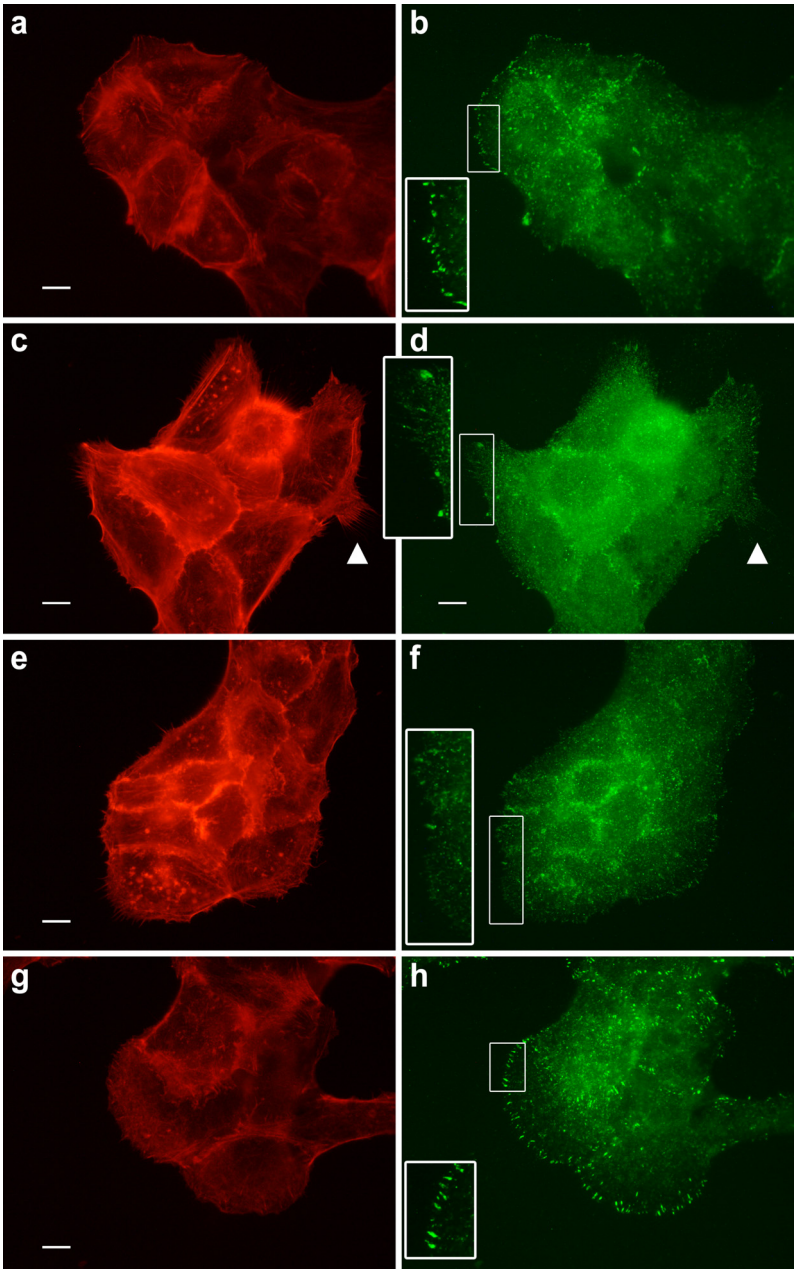

Fig. 3 A431 cells exposed to 3D RPM rotation. Cells were rotated with random speed, direction and interval (Borst and van Loon 2009) for 0, 6, 30 and 60 min, respectively (**a**, **b**), (**c**, **d**), (**e**, **f**) and (**g**, **h**). After 6 min cells started to round (**c**) and the number of focal adhesions labelled with anti-phospho-FAK^{576} decreased (**d**) but remaining focal adhesions increased in size and labelled for both F-actin (*red*) and phospho-FAK (*green*). Also retraction spikes appeared (*arrows* in **c**, **d**). After 30 min of rotation the number of detected focal adhesions is further decreased (**f**) and cells occupied less surface area indicating cell rounding (**e**). At 60 min cells were spread again (**g**) and a large number of focal adhesions was stained with anti-phospho-FAK^{576} (**h**). *Bar* represents 10 μm, all *inserts* are enlarged twice

RPM. The rounding-up of cells after 6 min of rotation is comparable to the results obtained in real microgravity using sounding rockets and in the fast rotating clinostat (Rijken et al. 1991a).

Effect of Magnetic Levitation on A431 Cell Morphology

To induce magnetic levitation, cells were subjected to a gradient magnetic field of 1,401 T^2/m, 7.2 cm above

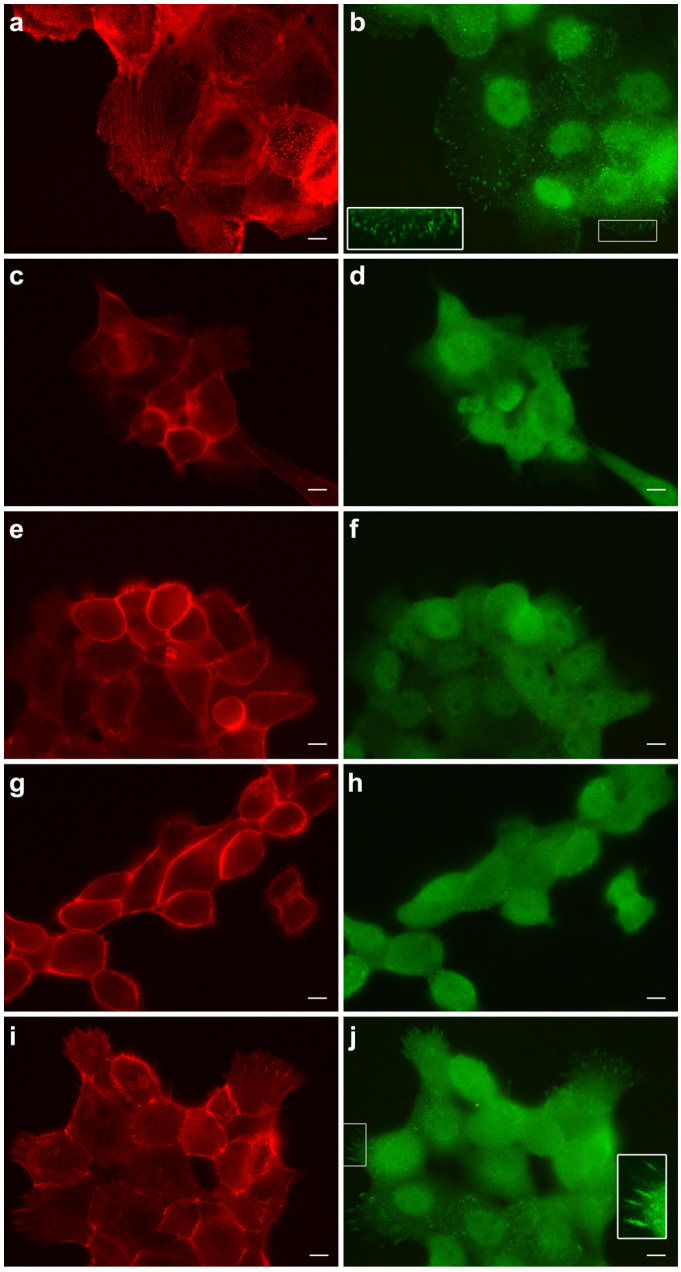

Fig. 4 Magnetic levitation of A431 cells for 0, 6, 30, 60 and 120 min. Magnetic levitation results in the rounding and subsequent spreading of cells, indicated by the increased cortical F-actin skeleton (*red*) and disappearance of phospho-FAK^{397} labelling (*green*) in focal adhesions during rounding. Only after 6 min of levitation cells are already rounding (**c**, **d**). After 30 min (**e**, **f**) and 60 min (**g**, **h**) cells round even more. The cells occupied less surface area, showed no stress fibers, had a pronounced cortical skeleton and no phospho-FAK staining in focal adhesions. Spreading is indicated by the reappearance of stress fibers (**i**) and renewed phospho-FAK^{397} labelling in focal adhesions (**j**). *Bar* represents 10 μm, all *inserts* are enlarged twice

the centre of the magnet used. These settings result in the levitation of water, the major component of cells. Results from magnetic levitation were compared with data from cells exposed to a magnetic field of similar strengths, but without the gradient required for levitation, that is at 11.5 T in the centre of the magnetic field. This creates the possibility to discriminate between effects caused specifically by magnetic levita-

tion or effects caused by the exposure of cells to the magnetic field. Finally, cells were also exposed to 0 T in the same experimental set up to control for any possible induced effects caused by other sources than the magnetic field or the magnetic levitation. Samples were evaluated using fluorescence microscopy and labelled for F-actin and phospho-FAK^{397} in focal adhesions as described under Materials and Methods. Phospho-FAK^{397} labelling was also observed in the nucleus of some cells. This is probably unspecific labelling and due to increased duration of fixation.

As shown in Fig. 4, levitation results in rounding of cells. Six minutes after the start of magnetic levitation a more pronounced cortical F-actin skeleton is visible,

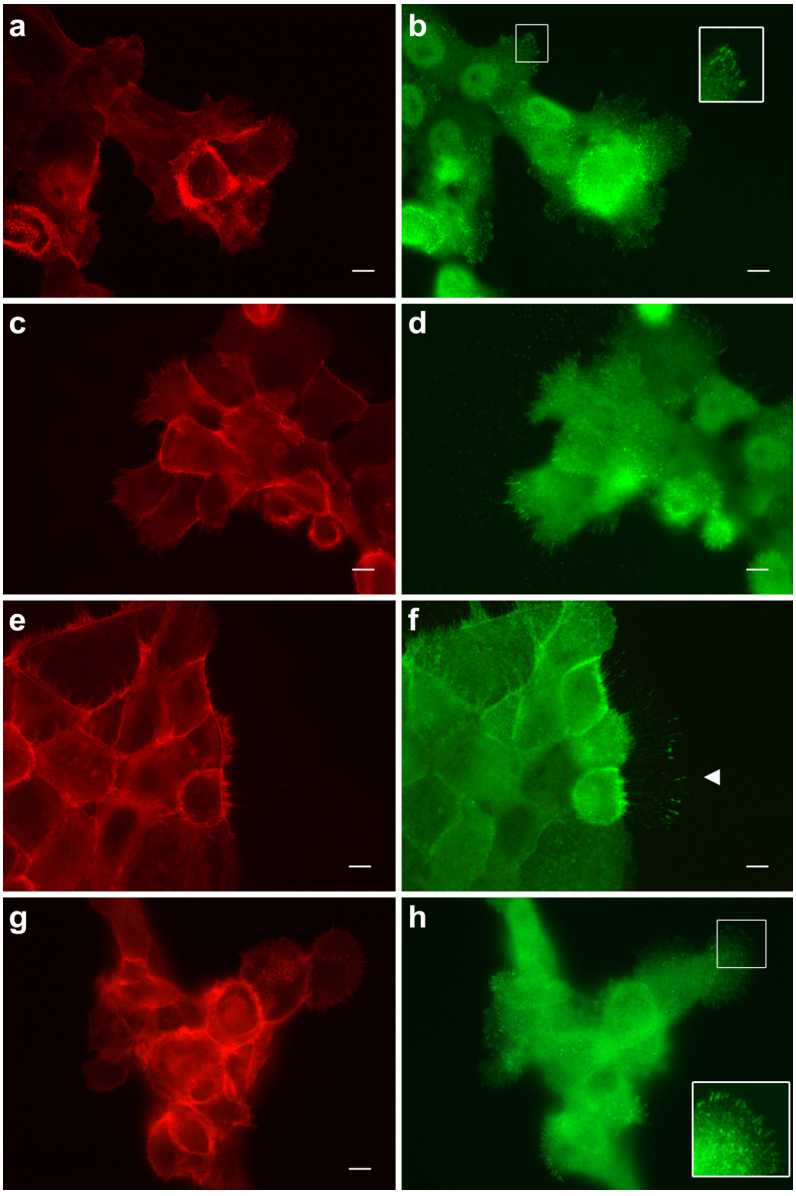

Fig. 5 A431 cells exposed to a high magnetic field without levitation (11.5 T in the center of the magnetic field) for 6 min (**a**, **b**), 30 min (**c**, **d**), 60 min (**e**, **f**) and 120 min (**g**, **h**). Cells were fixed and labelled for F-actin (*red*) and phospho-FAK^{397} (*green*). Cells exposed to a magnetic field without levitation also responded with the rounding-up and subsequent spreading of cells. During rounding the cortical F-actin skeleton became more pronounced, retraction spikes became visible (*arrow* in Fig. 4f) and phospho-FAK^{397} labelling in focal adhesions disappeared. After 120 min phospho-FAK^{397} labelling in focal adhesions reappeared again indicating the spreading of cells (**h**). *Bar* represents 10 µm, all *inserts* are enlarged twice

stress fibers (Fig. 4c) and phospho-FAK^{397} labelling in focal adhesions disappear (Fig. 4d) and the cells occupy less surface area. These effects become even more clear after 30 (Fig. 4e, f) and 60 min (Fig. 4g, h). In these samples the majority of cells show a rounded morphology. Starting from 60 min and continuing at 120 min, cells start to spread and flatten again (Fig. 4i, j). New labelling of phospho-FAK^{397} in focal adhesions is visible (Fig. 4j) and cells occupy a larger surface area. The number of stress fibers increases and the cortical F-actin skeleton becomes thinner (Fig. 4i).

Exposure to a Magnetic Field of Identical Strength Compared to Magnetic Levitation

During magnetic levitation cells are exposed to a gradient magnetic field. To control for effects caused by this magnetic field, cells were exposed to a field of identical strength without levitation, namely 11.5 T in the centre of the field. A similar pattern of rounding and spreading was observed as described for magnetic levitation (Fig. 4 vs. 5). After 6 min of exposure to the magnetic field, many peripheral focal adhesions were still stained for phospho-FAK^{397} (Fig. 5b). However, the number of labelled focal adhesions decreased after 30 and 60 min (Fig. 5d, f). Also retraction spikes became visible (arrow in Fig. 5f), indicating the rounding of cells. During the process of rounding the cortical actin skeleton became more pronounced and the number of stress fibers decreased (Fig. 5e). Both the level and rate of rounding of A431 cells seemed slightly less compared to cells exposed to magnetic levitation. This is indicated by the number of labelled focal adhesions present after 6 min of exposure (Fig. 4d vs. 5b) and also by the morphology of cells after 60 min of exposure (Fig. 4g vs. 5e). After 60 min of exposure to magnetic levitation, the cells are more rounded as compared to 60 min of exposure in the centre of the magnetic field indicated by the more pronounced cortical skeleton and absence of internal F-actin structures such as stress fibers. So the pattern of rounding and renewed spreading was comparable for both magnetic levitation and exposure to a field of identical strength.

To confirm that the described effects are indeed induced by the activated magnet and not by other sources, cells were also exposed to the same experimental set up without activation of the magnet (0 T). Control samples inside the magnet but without a magnetic field showed normal actin morphology and attachment as compared to regular lab controls (Fig. 6). Both after 30 min (Fig. 6a, b) and 60 min (Fig. 6c, d) cells exhibit abundant focal adhesions and showed a spread cell morphology. At these time points hardly any focal adhesions were observed in the samples that were subjected to the activated magnet (Figs. 4e–h and 5c–f). Moreover, cells exposed to the activated magnet were clearly rounded at these time points in contrast to the cells that were subjected to identical treatment

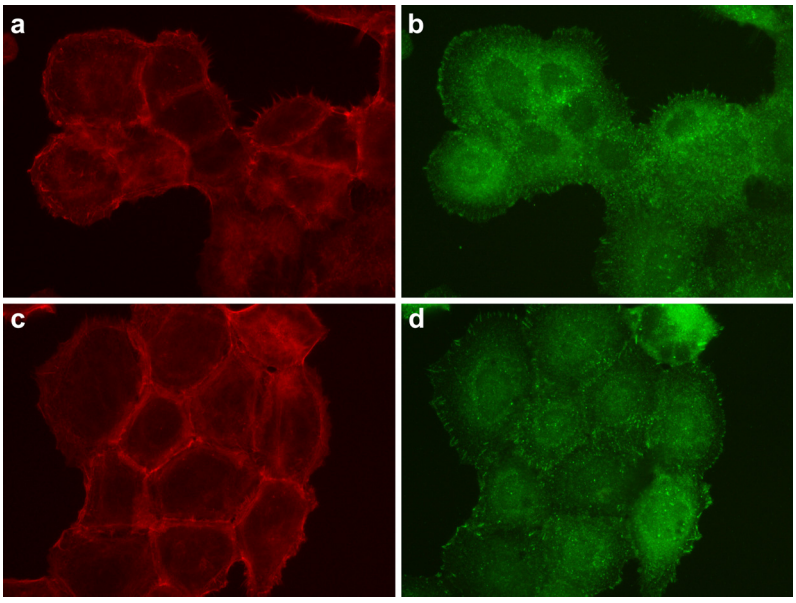

Fig. 6 Control samples in the magnet without activation of the magnet (0 T). Cells were exposed to identical sheer stress, temperature profile and vibrations as compared to cells in the activated magnet, respectively during 30 (**a**, **b**) and 60 min (**c**, **d**). Cells were fixed and labelled for F-actin (*red*) and phospho-FAK^{397} (*green*). Note the differences with the cells exposed to the activated magnet in Figs. 4e–h and 5c–f

without activation of the magnet. This indicates that other factors like temperature profile, sheer stress due to medium refreshments or residual vibrations by the magnet cooling system in the experimental set up did not cause any additional effects. Therefore the described effects in Figs. 4 and 5 are caused by the magnetic field or the magnetic levitation.

Discussion

Experiments in space revealed gravity dependent cellular responses such as a changing gene expression, cytoskeletal changes and induction of apoptosis. Exposing cells to microgravity conditions in space provides insight in the sensitivity and responsiveness of cells to mechanical forces, such as gravity. However, access to perform experiments in space is limited and therefore there is a need for simulators allowing ground based simulated microgravity research. Two methods, rotation with the Random Positioning Machine and magnetic levitation, are used in this study to investigate induced morphological changes of human A431 cells. The focus was on the morphological changes of the F-actin cytoskeleton and its relation with attachment by focal adhesions. Both methods induced a transient process of cell rounding and subsequent reattachment and flattening of A431 cells. The results after 6 min are comparable to data obtained in real microgravity and in simulated microgravity using the fast rotating clinostat (Rijken et al. 1991a). This indicates that RPM rotation and magnetic levitation induce similar effects that are known to be caused by real microgravity.

Exposure A431 cells to 3D rotation in the RPM resulted in a transient process of rounding and subsequent reattachment and flattening of cells. Rounded cells were visible after 6 min of rotation illustrated by an abundant actin cortical skeleton and the disappearance of actin stress fibers. This was also observed in real microgravity during sounding rocket missions and in simulated microgravity using the fast rotating clinostat (Rijken et al. 1991a). The rounding of cells was further illustrated by the disappearance of focal adhesions. After this rounding up of cells, the cells adapt to their new environment and reattach and flatten. This is illustrated by the reappearance of focal adhesions, the cells occupy more surface, stress fibers reappear and the cortical skeleton becomes less abundant.

The Random Positioning Machine (Hoson et al. 1992, 1997; van Loon 2007; Borst and van Loon 2009) is based on the principle that the direction of gravity is randomized in three dimensions. Rotation in three dimensions with varying speeds is required to provide truly three dimensional random motion (Borst and van Loon 2009). This variation in velocity might result in a complicated pattern of induced effects in cells, since the speed of rotation was described to be an important variable in the fast rotating clinostat that ideally rotates in one dimension (Briegleb 1992).

Increasing the speed of rotation will reduce residual gravity perception, but will increase centrifugal forces (Briegleb 1992). The perception of gravity might be dependent on the process under investigation and reflects the time lag phase of the gravity sensitive element of the process under investigation. This implies that the speed of rotation should be optimized for each different process studied, avoiding both residual levels of gravity perception and perception of centrifugal forces. For A431 cells exposed to two dimensional rotation, both the level and the speed of cell rounding and renewed spreading indeed varied with the selected speed of rotation (data not shown).

Recently, the RPM is used as a simulator for microgravity in various studies investigating responses in animal cells (for example Grimm et al. 2002; Uva et al. 2002; Infanger et al. 2006; Pardo et al. 2005; Patel et al. 2009; Meloni et al. 2006). However, the mode of operation of the RPM that was used in these studies varies. Both 10 and 60 rpm are used frequently, sometimes random speed was used and often the mode of operation and the location of the sample within the system are not mentioned. Since for the fast rotating clinostat the mode of operation, specifically the speed of rotation, was related to variation in cellular responses (Briegleb 1992; own unpublished data), it is critical to clarify the chosen mode of operation for the RPM more precise. In this study the microgravity target that was selected was the actin cytoskeleton and its relation to attachment via focal adhesions. Other microgravity sensitive components in cells might possess different sensitivity and responsiveness towards their mechanical environment, such as gravity. Therefore, it is important to validate the mode of rotation for each experiment involving simulation of microgravity by the RPM.

Another limitation of simulation by randomization of the direction of gravity in the RPM is that the randomization requires time to statistically nullify gravity in all directions. Therefore only processes can be studied that experience a certain time lag phase. For example fast occurring processes might not be suitable for studying in the RPM. So, on the one hand the specific microgravity sensitive target in the cell requires a specific mode of operation of the RPM to simulate microgravity. On the other hand there are limitations in the applicability of the RPM that are associated with its design and mode of operation. More awareness of

the importance and consequences of the selection of the mode of operation of the RPM is required. The selection of a certain mode of operation and maximum speed is no guarantee for simulating real microgravity. Moreover, the physical characterization of the RPM, such as fluid dynamics, should be investigated in more detail (van Loon 2007) as was done for the fast rotating clinostat (Briegleb 1992; Albrecht-Buehler 1992). Most importantly, results measured under conditions of simulated microgravity should be confirmed in real microgravity.

Interestingly, exposing A431 cells to magnetic levitation resulted in a similar pattern of rounding followed by spreading as was observed in the case of RPM rotation. The responses that were observed after 6 min of levitation were very similar to responses observed in cells exposed during 6 min to real microgravity using sounding rockets. This indicates that magnetic levitation induces effects that are known to be caused by microgravity.

However, exposing cells to a magnetic field of identical strength but without the levitating gradient resulted in similar responses. This raises concerns about the relevance of the data obtained with magnetic levitation experiments. For the rounding and spreading of A431 cells no clear discrimination was possible between effects caused by the high magnetic field itself and by magnetic simulation of microgravity using a gradient magnetic field of identical strength.

The use of high gradient magnetic fields allows the simulation of microgravity almost instantly and allows one to study fast occurring biological processes in contrast to other existing simulators, such as the clinostat and RPM where more time might be required to establish an averaged gravity vector randomization. For this reason magnetic levitation is an interesting additional device for simulating microgravity conditions.

The responses of cells exposed to high magnetic fields give rise to concerns about the side effects introduced by magnetic levitation. The rounding of A431 cells caused by exposure to a high gradient magnetic field without levitation appears undistinguishable from the reported rounding of A431 cells in real microgravity (Rijken et al. 1991a). This indicates the importance of this control when performing magnetic levitation studies. Studies claiming the usefulness of magnetic levitation in simulating microgravity might easily draw wrong conclusions when not performing such controls at the same field strength.

Other studies also reported responses in cells exposed to magnetic fields (Denegre et al. 1998; Valiron et al. 2005). Also the influence of high magnetic fields on the behaviour of actin was reported, both in vitro (Torbet and Dickens 1984) and in vivo (Valiron et al. 2005). This effect is most probably caused by magnetic orientation of actin. Long biomolecules, like actin and DNA, exhibit an anisotropic magnetic susceptibility that tends to align them in a magnetic field (Torbet and Dickens 1984; Maret and Dransfeld 1985; Christianen et al. 2004).This suggests that side effects are unavoidable when using magnetic levitation as a simulator for microgravity. However, when there is a clear additional microgravity sensitive response in cells exposed to magnetic levitation, it might still be possible to recognize specific cellular responses to simulated microgravity by such studies.

For the described pattern of rounding and spreading of A431 cells, the discrimination between responses to simulated microgravity and exposure to a high magnetic field seems not possible. Therefore, it will be interesting to study other cell lines and cellular responses under conditions of magnetic simulation of microgravity.

In conclusion, RPM rotation and magnetic levitation of A431 cells resulted in a very similar response of cell rounding and subsequent flattening. The induction of cell rounding is known from experiments in real microgravity. However, both methods also have disadvantages that can easily lead to misinterpretation of results. Therefore, it is required to confirm that responses are induced by the simulation of microgravity and not caused by side effects. The further characterization of both methods is required and most importantly, results measured under conditions of simulated microgravity should be confirmed in real microgravity.

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Actin dynamics in mouse fibroblasts in microgravity

After stimulating with the growth factor PDGF, cells exhibit abundant membrane ruffling and other morphological changes under normal gravity conditions. These morphological changes are largely determined by the actin microfilament system. Now these actin dynamics were studied under microgravity conditions in mouse fibroblasts during the DELTA mission. The aim of the present study was to describe the actin morphology in detail, to establish the effect of PDGF on actin morphology and to study the role of several actin-interacting proteins involved in introduced actin dynamics in microgravity. Identical experiments were conducted at 1G on earth as a reference. No results in microgravity were obtained due to a combination of malfunctioning hardware and unfulfilled temperature requirements.

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Introduction

Humans exposed to microgravity conditions experience various physiological changes like reduced immunodeficiency, loss of bone mass and muscle deterioration. The underlying mechanism for these phenomena might be within cells on the molecular level. This is supported by a large number of observations in mammalian cells. Gravity effects mammalian cell growth and differentiation and cells were shown to have altered gene expression in microgravity [1, 2, 3, 4, 5]. The presented research fits in an ongoing project that studies the effect of microgravity on mammalian cells.

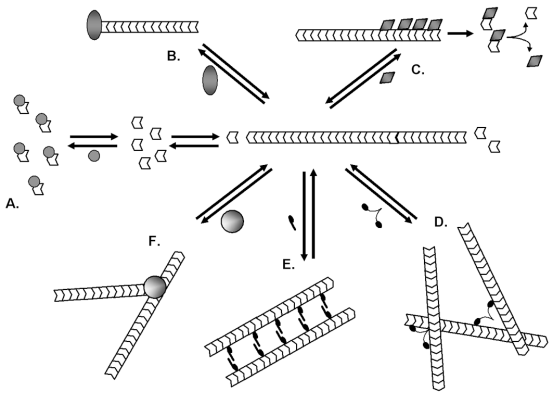

Fig. 1: Actin-binding proteins affect the behavior of actin in various ways. Some examples are illustrated in this figure: (A) Monomer-binding proteins keep the amount of monomeric actin subunits available for polymerization low, to prevent the formation of F-actin filaments. (B) When a fiber of actin is formed it can be capped by a capping protein that prevents further elongation resulting in short capped filaments. (C) Severing proteins increase the depolymerization of filaments. Cross-linking proteins can organize the filaments into gel-like networks (D) or bundles (E). Branching proteins (F) initiate the growth of daughter filaments by serving as nucleation sites on existing filaments resulting in branched actin networks.

During previous studies in microgravity it was found that growth factor-induced signal transduction is sensitive to microgravity. Epidermal growth factor (EGF) induced early gene expression of *c-fos* and *c-jun* was decreased under microgravity conditions [1, 6]. Upstream of this early gene expression, EGF signaling was investigated starting at the receptor by investigating receptor clustering, ligand binding and subsequently by investigating EGF induced signaling cascades from membrane to the nucleus. Selective transduction pathways were identified to be susceptible to changes in gravity conditions whereas other pathways originating from the EGF receptor were not [7]. This was shown in several studies in sounding rocket experiments in adherent A431 epithelial cells. Moreover, cells showed increased cell rounding under microgravity conditions [8]. Cell rounding is largely determined by the actin filament system. Interestingly, the signaling cascade that was susceptible to changes in gravity, the PKC-mediated signal transduction, was demonstrated to be linked to actin. That is why the actin cytoskeleton might well be the microgravity sensitive component in cells [9]. Microgravity might affect the behavior of actin directly or indirectly via actin binding proteins. Actin on its turn could affect the PKC-mediated signaling cascades. The experiments on the DELTA mission were part of a series of experiments that aim at determining whether actin is the microgravity sensitive component in mammalian cells.

Actin is an abundant protein in cells with important functions like migration, cell cycle progression, intracellular transport, signal transduction, transcription, membrane ruffling and cell attachment. It also largely determines the morphology of cells and the shape and position of organelles within cells (for review see [10]). Actin is present both in a polymerized form (F-actin) and a non-polymerized form (G-actin) and cycles between those states in cells thereby forming a highly dynamic network. Many proteins interact with actin and locally determine the behavior of actin. One can characterize these proteins by their function, including cross-linking proteins like α-actinin and filamin, severing proteins like actin-depolymerization factor (ADF) and cofilin, capping proteins like gelsolin, monomer-binding proteins like profilin and membrane-associated proteins like the EGF-receptor. Together these actin binding proteins (ABPs) regulate the dynamics of the actin cytoskeleton (Fig 1.). So actin and actin-binding proteins form a complicated network and are important for many cellular processes.

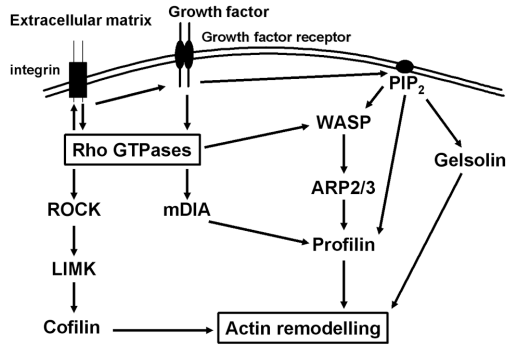

Fig. 2: Overview of the interaction between signal transduction and actin remodeling. Activation of growth factor receptors or integrins by binding to their respective ligands results in actin remodeling through Rho GTPases. Rho GTPases subsequently activate a kinase cascade including ROCK and LIMK to activate the actin-binding protein cofilin. Alternatively, the profilin is modulated through mDIA or the WASP pathway.

Actin-binding proteins on their turn are subject of regulation as well (Fig 2.). Various signaling cascades originating from both integrins and growth factor receptors stimulate or repress ABPs resulting in remodeling of the actin cytoskeleton. Integrins link the actin cytoskeleton to the extracellular matrix and by doing so play a role in cell attachment. Growth factor receptors sense the environment of a cell by binding growth factors from the outer world. Both integrins and growth factor receptors transport these extracellular signals over the membrane into the cell where cells respond by reshaping its cytoskeleton and ultimately altering gene expression.

Central players in these signaling cascades in relation to alterations of the cytoskeleton are the Rho GTPases such as RhoA, Rac1 and cdc42 (for review see [11]). Rho induces stress fiber formation and Rac1 and cdc42 play a role in the assembly of actin filaments.

Starting from the binding of a growth factor to its receptor, one can recognize the following events. The activated receptor generates intracellular signaling peptides like PIP2 and activates Rho GTPases via other peptides. Rho GTPases on their turn start signaling cascades involving proteins such as ROCK, LIMK, mDIA and WASP. Subsequently the activities of actin-binding proteins like cofilin, gelsolin and profilin are modified reshaping the actin cytoskeleton.

As described above, studies in microgravity gave rise to the hypothesis that actin might be the microgravity sensitive component in cells. Actin was indeed shown to behave differently in microgravity [4, 9, 13]. The relative F-actin content was shown to increase during microgravity conditions [14]. So, microgravity seems to either induce actin polymerization or to reduce its depolymerization. Moreover, several studies describe morphological changes of cells in simulated and real microgravity. This indicates a role for actin, since actin largely determines the cell morphology. However, only a few studies did partly investigate the role of actin in simulated or real microgravity [2, 4, 12, 15]. The present study aimed at investigating the behavior of actin in cells in real microgravity, and to investigate possible local differences in behavior of actin in cells. These differences might either be directly induced by the physical environment of a cell or indirectly by modifying the interactions with actin-binding proteins.

To study the behavior of actin in detail we developed a model allowing the study of actin dynamics in cells exposed to micro-

gravity. Actin dynamics is triggered by stimulating growth-factor starved cells with a growth factor called Platelet Derived Growth Factor (PDGF) (Fig 3.). This growth factor binds to the PDGF receptor that on its turn stimulates intracellular signaling cascades. Via RhoGTPases these signaling cascades regulate a number of actin binding proteins resulting in a response of the actin cytoskeleton. After stimulation with PDGF, the number of stress fibers and focal adhesions are reduced in cells. Moreover, circular ruffles are formed on the upper side of these cells. These circular ruffles are dynamic structures formed by actin starting from the periphery of cells and moving towards the middle of cells [16, 17]. Actin pushes the membrane outwards thereby changing the cell morphology.

Using automated flight hardware we planned to stimulate mouse fibroblasts with PDGF and subsequently chemically fix the cells in microgravity during the DELTA mission.

Material and Methods

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) and were of the highest purity available. PBUs were manufactured by CCM (Nuenen, the Netherlands). Coverslips sized 9x25 mm or 9x12 mm were purchased from Menzel (Braunschweig, Germany).

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, 5% CO_2). Cells were plated at 12,000 cells/cm^2 on coverslips and allowed to grow for 24 hours after seeding. Cells were growth factor-starved for 24 hours at 37 °C and subsequently growth factor starved for another 8 days at 20 °C during transport from the laboratory to the launch area in a temperature regulated box.

Flight hardware

Plunger Box Units (PBUs) were filled four days before launch except from cells that transported separately to keep them in good condition. Cells on coverslips were inserted one day before launch into the PBUs and kept in fresh serum-free medium.

Flight experiment

Cells were to be kept at 20 °C for the duration of 2 days during flight. 6 hours before stimulation, experimental units were to be transferred to 36.5± 0.5 °C. Plungers were to be activated to stimulate cells with PDGF and to fix them afterwards. Experimental units were to be cooled down to 4 °C and to be transported at 4 °C back to the laboratory.

Immunofluorescent labeling

Cells were fixed by adding 5.3% formaldehyde directly to the culture medium. The refreshment rate of the PBUs is ~70%, resulting in a final concentration of fixative of 3.7%. After fixation, cells were kept at 4 °C during transport to the laboratory. Here coverslips were taken out of the PBUs and 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 for 60 min at room temperature with tetramethylrhodamine-5- (and-6) isothiocyanate (TRITC)-conjugated phalloidin and Alexa 488 conjugated DNase I. Subsequently they were washed six times with PBS containing 0.2% gelatin. Finally, cells were mounted in Mowiol-DABCO.

Acquisition of images

Pictures were taken with a Leitz microscope (model Orthoplan Flu 043944) equipped with Leitz objectives 40x/1.3 oil and 63x/1.4 oil, using immersion oil Immersol 518F (Carl Zeiss MicroImaging Inc.). Images were acquired using a Leica CCD camera (model DC350F; Leica Microsystems, Inc.) using Leica Image Manager 50 software, subsequently pictures were processed with Adobe Photoshop 8.0.

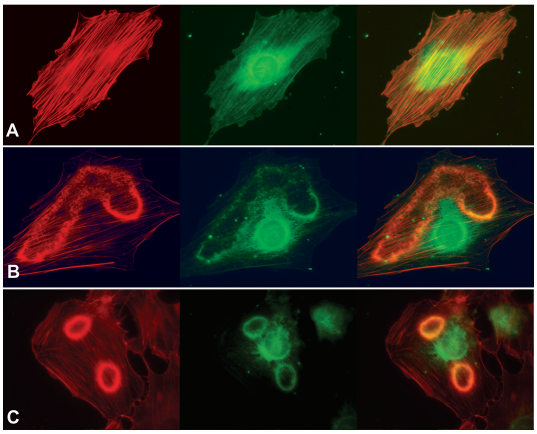

Fig 3: Effect of PDGF on F- and G-actin localization in C3H/10T1/2 mouse fibroblasts. Cells were serum deprived for 24 hours and subsequently incubated in the presence or absence of 20 ng/mL PDGF-BB for 0, 10 and 30 minutes at 37 °C, respectively (A), (B) and (C). Subsequently cells were fixed using formaldehyde and incubated with Phalloidin-Tritc to label F-actin (red) or with DNase I-Alexa488 to label G-actin (green). In non-stimulated cells F-actin is visible in large stress fibers, whereas G-actin is localized mainly around and in the nucleus. Incubation in the presence of PDGF-BB for 10 (B) and 30 minutes (C) results in the formation of membrane ruffles and the partial disappearance of stress fibers. (photo: Critical Reviews™ in Eukaryotic Gene Expression, 15(3):255–275 (2005))

Results

Ground controls were carried out simultaneously with the flight experiments using the same batch of cells. They were successful and gave similar results as normally obtained (Fig 3.)[16, 17]. Cells showed normal actin morphology and number of ruffles indicating that the cells survived the long period of starvation and exposure to room temperature during the experiment. Cells that were not stimulated showed abundant stress fibers and a pool of G-actin mainly localized around the nucleus. After stimulation with PDGF the number of stress fibers decreases and both F-actin and G-actin were found in circular ruffles indicating an extensive reorganization of actin.

No flight results were obtained due to a combination of malfunctioning hardware and unfulfilled temperature requirements.

Conclusion/ Discussion

Growth factor induced actin dynamics in serum starved cells is an interesting model for studying actin dynamics in microgravity. Because of the highly dynamic behavior of actin small differences in actin metabolism in microgravity will be enlarged compared to the situation in a more static cell. Moreover, the mouse fibroblasts were shown to stand the harsh conditions unavoidable when doing experiments in real microgravity. Because there were no results obtained in microgravity for this experiment during the DELTA mission, new flight opportunities will have to provide answers to the questions regarding the dynamics of actin in microgravity listed in this paper.

Acknowledgements

Because of space constraints we have been unable to cite all original papers. We, therefore, apologize to authors whose work in this subject area may have been omitted. This work in our laboratory is subsidized by the Dutch Space Organization (SRON, grant MG-059).

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Actin dynamics in mouse fibroblasts in microgravity: MASER-10 mission

Maarten J. A. Moes • Jose J. Bijvelt • Johannes Boonstra

Abstract

Under normal gravity conditions, cells exhibit distinct morphological changes upon stimulation with the growth factor PDGF (Platelet-Derived Growth Factor), such as the formation of linear and circular membrane ruffles and the formation of lamellae. These morphological changes are largely determined by the actin microfilament system. During the MASER-10 experiment, PDGF-induced actin dynamics were studied under microgravity conditions in mouse fibroblasts. The aim of the experiment was to describe the actin morphology in detail, to establish the effect of PDGF on the actin morphology and to study the role of several actin-interacting proteins in microgravity. Identical experiments were conducted at 1g in space and at 1g on earth as a reference.

Introduction

The ACTIN experiment on the MASER-10 was part of an ongoing program that aims at identifying the microgravity sensitive component of the actin metabolism in mammalian cells. Previous observations gave rise to the hypothesis that the target of gravity in a cell is focused on the regulation of actin polymerisation, being an increased polymerisation rate or a decreased depolymerisation rate. A detailed description is provided in Boonstra, 1999 and Moes et al., 2007.

The experiments on the MASER-10 sounding rocket aimed at studying the actin dynamics that are induced in mouse fibroblasts upon stimulation with the growth factor PDGF. Stimulation with PDGF results in the formation of spectacular actin rearrangements within minutes. During the MASER-10 mission, cells are exposed to microgravity conditions for six minutes. Therefore this model is suited to study induced actin dynamics in this relatively short time frame. The actin dynamics that are induced by PDGF require a change in the activities of actin regulating proteins, such as actin binding proteins. The examination of the role of actin regulating proteins in microgravity was another aim of the present investigation.

The model of mouse fibroblasts stimulated with PDGF was studied previously in real microgravity during a long duration space mission in the International Space Station (ISS)

(Moes et al., 2007). During this experiment cells were subjected to microgravity for several days before stimulating the cells with PDGF. The experiment that was conducted during the MASER-10 mission made use of the same experimental model but the exposure to microgravity was different. The MASER-10 experiment was conducted with a sounding rocket and cells were exposed to microgravity for 6 minutes. This implies there is little time for cells to adapt to their changed environment, i.e. microgravity. This in contrast to long term space missions, where cells may have ample time to adapt to conditions of microgravity. In other words, during short duration space missions cells are investigated that are adapting to conditions of microgravity and during long duration space missions cells are investigated that may have adapted to microgravity. Ground based simulation experiments revealed that cells indeed adapt to conditions of simulated microgravity (Moes et al., 2010). Upon exposure to conditions of simulated microgravity cells round up within minutes (Rijken et al., 1991, Moes et al., 2010) similar as was demonstrated during experiments in real microgravity (Boonstra et al., 1997). However, after exposure to simulated microgravity for more than 1 hour, cells start to spread again, become flattened and exhibit a morphology that is comparably to cells that were not exposed to simulated microgravity (Moes et al., 2010). Because cells exhibit a transient response, one must be rather careful in comparing the results of experiments that expose cells to microgravity conditions for different durations.

During the MASER-10 experiment PDGF induced actin dynamics were studied in mouse fibroblasts that were adapting to conditions of microgravity. The aim of the experiment was to describe the actin morphology in detail, to establish the effect of PDGF on the actin morphology and to study the role of several actin-interacting proteins in microgravity. Identical experiments were conducted at 1g in space and 1g on earth as a reference.

Materials and Methods

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). The MAL antibody was kindly provided by dr. Treisman (Cancer Research UK London Research Institute, UK), the monoclonal antibody directed against β-actin (A1978, clone AC-15) was purchased from Sigma. The goat-anti-mouse-Alexa488 secondary antibody, DNaseI-Alexa594 and Palloidin-Alexa633 were purchased

from Molecular Probes. All other chemicals used were obtained from Sigma or Merck (Darmstadt, Germany). Plungerbox units (PBUs) were manufactured by CCM (Nuenen, the Netherlands). Coverslips sized 9x25 mm or 9x12 mm were purchased from Menzel (Braunschweig, Germany). The experiment system BIM (Biology In Microgravity) was developed by Swedish Space Corporation (Solna, Sweden), Dutch Space (Leiden, the Netherlands) and CCM (Nuenen, the Netherlands).

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). Cells were plated at 12,000 cells/cm^2 on coverslips and allow to grow for 24 hours after seeding. Subsequently, cells were growth factor-starved for 24 hours at 37 °C.

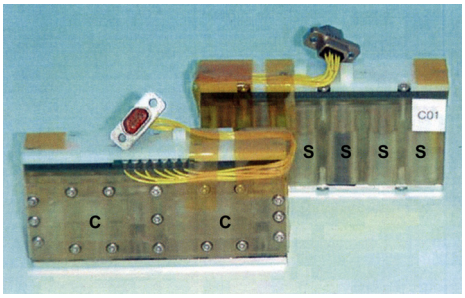

Figure 1. Plungerbox units were used as experiment containers. A plungerbox unit (80x40x20 mm^3) contains two culture compartments (C; 1 ml each) that can be processed independently. The other side of the PBU contains six cylindrical storage compartments (S; 1 ml each) that can be flushed into the culture compartment when a spring-loaded plunger is released. An electrically operated thermal cutter releases the plungers.

Flight hardware

Experiment containers (PBUs) contained two culture chambers (1 ml each) that can be processed independently (Figure 1). Each culture chamber was connected to three liquid storage reservoirs (1 ml each) that can be operated automatically. PBUs were filled one day before launch at 37 °C. Cells on coverslips were inserted into the PBUs 8 up to 18 hours before launch and kept in fresh serum-free medium. Subsequently, experimental containers for the flight experiment were transferred to the BIM (Biology In Microgravity) experiment module (Holm et al., 2005). The BIM facilitated the operation of the PBUs and consisted of two parts, namely the Late Access Insert (LAI; Figure 2) that contained the flight experiments and the system for the ground reference experiments. The LAI facilitated the flight experiments that comprehended both the microgravity set of experiments and the 1g reference set of experiments on the centrifuge. The LAI thermal

control was set to operate at 36.5± 0.5 °C with a tolerance of ± 0.2 °C between PBUs. Four hours before launch, the LAI was placed in the MASER-10 sounding rocket to ensure that cells were recovered from any stress resulting from human handling. The experiment containers for the reference on-ground were placed in an incubator at 36.5± 0.5 °C. During the experiment the temperature was constantly monitored with several temperature sensors (Holm et al., 2005).

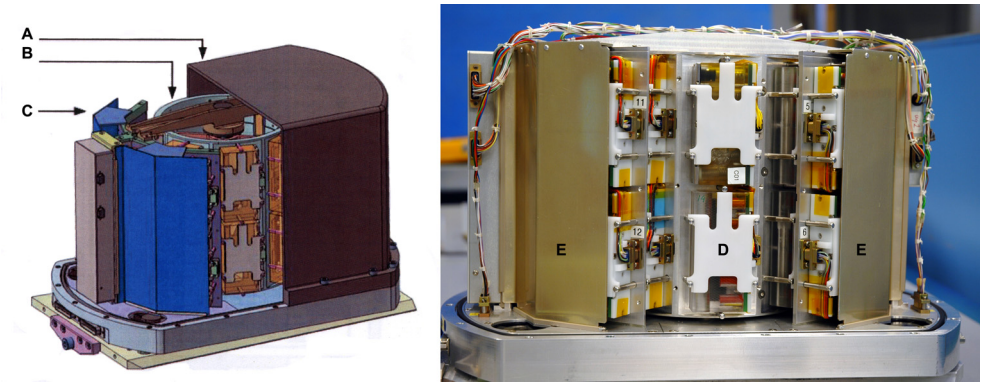

Figure 2. The Late Access Insert of the BIM that facilitates operation of the experiments in space. The drawing of the LAI (Dutch Space) with the cover (A) half-open indicates the position of the centrifuge (B) and the static racks (C). The photo on the right displays the LAI with the PBUs mounted on the centrifuge (D) and the static racks (E). The static racks facilitate the microgravity experiments and the in-flight reference experiments are mounted on the centrifuge.

Flight experiment

Cells were kept at 36.5± 0.5 °C. The launch of MASER 10 was on May 2, 2005 at the launching facility at Esrange outside Kiruna. The experiment was performed simultaneously under microgravity conditions, on a 1g reference centrifuge on board and a 1g reference set-up on-ground. At the onset of microgravity, cells were either directly stimulated and fixed, or stimulated with serum-free medium with PDGF and fixed after 6 minutes, or serum-free medium was added and cells were fixed after 6 minutes. Stimulations and fixations were performed by activating the plungers of the PBUs. All experiments were executed in triplicate. Cells were fixed by adding either 5.3% formaldehyde or 2.1% glutaraldehyde directly to the culture medium. The refreshment rate of the PBUs is approximately 70%, resulting in a final concentration of fixative of

approximately 3.7% formaldehyde and 1.5% glutaraldehyde. Subsequently experimental units were passively cooled down.

Immunofluorescent microscopy

Coverslips were taken out of the PBUs and 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 for 60 min at room temperature with the primary antibody. Subsequently cells were washed six times PBS containing 0.2% gelatine. Then, cells were incubated with the secondary antibody and/or Alexa633-conjugated phalloidin and/or DNaseI-Alexa594 and washed six times PBS containing 0.2% gelatin. Finally, cells were mounted in Mowiol-DABCO. Images were acquired with a Zeiss CSLM (Pascal 510) fitted with Zeiss objective lenses (40x N.A. 1.3 and 63x N.A. 1.4). All immunofluorescence studies were performed using single labelings as well.

Environmental Scanning Electron Microscopy (ESEM)

Coverslips were taken out of the PBUs and cells were 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 to study cells in a fully hydrated state, avoiding all kind of drying artifacts.

Results

The aim of the Actin experiment was to investigate the behaviour of actin in detail. For this purpose, the intracellular distribution of both G-actin and F-actin can be determined. However, to gain insight in the degree of actin polymerization additional information is required. Investigations of cell lysates can provide such information but the limited number and small sizes of samples that were available for this experiment did not allow both microscopy and biochemistry studies. It was decided to focus on investigating the actin morphology by various methods of microscopy. For studying the degree of actin polymerization in cells exclusively using microscopy techniques, the localization of actin-interacting proteins may provide additional information. In this respect the subcellular localization of the actin binding protein MAL is of particular interest. In serum-starved cells, MAL was described to be mainly present in the cytoplasm and in addition a fraction is present in the nucleus. Upon stimulation with serum, MAL was described to translocate

from the cytoplasm to the nucleus (Miralles et al. 2003). Stimulation of cells with serum results in an increase of actin polymerization and a depletion of the cytoplasmic pool of G-actin. MAL binds to G-actin and upon depletion of the cytoplasmic pool of G-actin, MAL translocates from the cytoplasm to the nucleus (Miralles et al., 2003). Therefore, the localisation of MAL provides information of the degree of actin polymerization in cells. The ratio of cyoplasmic MAL and nuclear MAL within single cells can be measured and subsequently the average ratio of a cell population can be determined. It was investigated if this model could be applied with the experimental set up of the Actin experiment in the sounding rocket MASER-10. For this experiment, fibroblasts were stimulated for only 6 minutes with PDGF-BB before chemical fixation. Control experiments on ground revealed that this is ample time for observing a clear translocation of MAL in mouse fibroblasts (Figure 3).

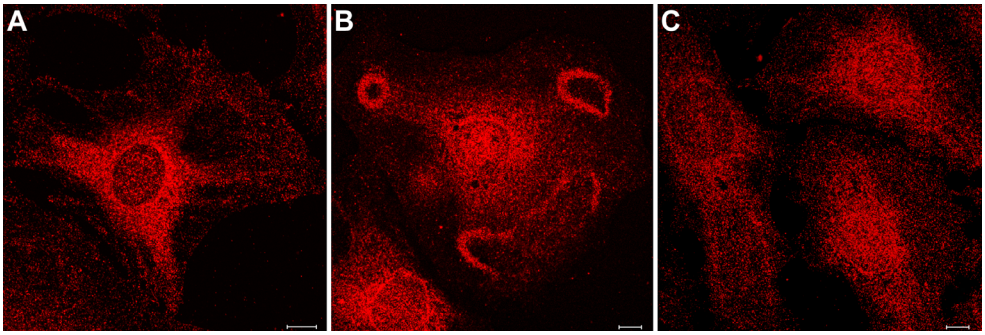

Figure 3. A: In serum-starved mouse C3H10T1/2 fibroblasts, MAL is mainly present in the cytoplasmic pool around the nucleus. B: Upon stimulation with PDGF-BB for 6 minutes, MAL translocates partly to the nucleus. In addition, MAL accumulates in newly formed circular ruffles.
C: 30 minutes after addition of PDGF-BB, MAL is less enriched in the nucleus of cells and the cytoplasmic pool of MAL becomes more abundant.

As mentioned before, previous observations in the lab gave rise to the hypothesis that the target of gravity in a cell is focused on the regulation of actin polymerisation. It was hypothesized that cells exhibit either an increased polymerisation rate or a decreased depolymerisation rate in microgravity (Boonstra, 1999). In view of this, the subcellular localisation of MAL of cells exposed to microgravity might provide interesting information about the degree of actin polymerization in cells. Similar to the translocation upon stimulation with growth factors, the localisation of MAL might change upon

exposure to microgravity conditions as a consequence of a changed polymerization rate or depolymerisation rate of actin.

During flight the Actin experiment in the BIM module was successfully performed. All temperature requirements were fulfilled before flight and during flight and all but one plunger were activated successfully (Figure 4). The recorded speed of the centrifuge during the experiment as well as the start and stop of rotation of the centrifuge were as programmed (Figure 4). After the experiment the MASER 10 sounding rocket experienced a hard landing because of failure of the landing parachute. Due to this hard landing a few glass coverslips were broken and these samples were lost. All samples were examined using phase contrast microscopy and all samples were documented by taking representative pictures of each sample. The quality of the chemical fixation was determined based on the observed morphology and in addition the number of cells that was present on the coverslips and the number of traces left by detached cells were investigated. In the samples of the flight experiments, an increase was found in the number of cells that detached during the experiment. Therefore it was concluded that part of the cells were detached from the substratum due to the hard landing. In previous microgravity studies it was demonstrated that cells round up in microgravity. In addition, cells round up upon stimulation with PDGF-BB. Hence, we assume that especially the cells that responded most to PDGF-BB and cells that responded most to conditions of microgravity did round up during the experiment. Cells that round up are less strongly attached to the substratum. Therefore it can be expected that cells that responded most to the stimulation with PDGF-BB and cells that responded most to microgravity have detached more frequently than other cells due to the impact of the uncontrolled landing. In conclusion, there are strong indications that the cells that were still attached on the glass coverslips might not be representative for the experiment and therefore we cannot draw any conclusion from the experiment with respect to possible effects of microgravity on actin.

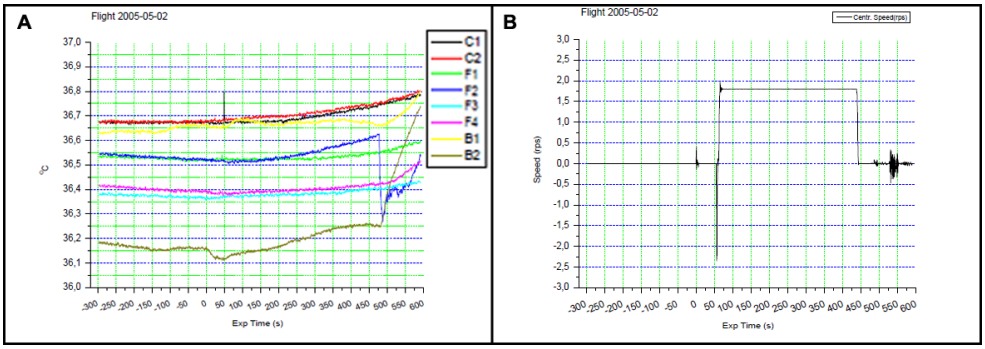

Figure 4. Recorded temperature profile (A) and recorded speed of the centrifuge (B). The onset of microgravity is at t=0 and lasts till t=360, the recording of data ends after the hard landing of the rocket. The temperature sensors on the centrifuge (C) and the sensors on the static racks (F) indicate that temperatures were within 36.5± 0.5 °C with a tolerance of ± 0.2 °C between PBUs during the period of microgravity (B1 is the regulating sensor and B2 is located at the heat exchanger of the LAI). Due to the impact of the rough landing, the thermal control and data logging were not working as planned after the landing. The recorded increase in temperature at the end is probably due to the impact of the landing. The centrifuge moved at spin-up directly after the lift-off of the rocket and at de-spin at about +60 seconds (B). The recorded speed during the experiment as well as the start and stop of rotation of the centrifuge are as programmed.

In addition, unexpected variations in cell morphology were observed in all samples, including the ground samples. These variations could not be explained by a different treatment, the use of different chemicals or differences in cell population. We have not been able to identify the precise cause for the differences that were observed. However, the experiments performed during MASER 10 have been performed many times in hand-operated PBUs. Hand-operated PBUs can be used repeatedly and during the use of hand-operated PBUs the differences in results as indicated above were never observed. The automatic operated flight PBUs can only be used once.

Although no conclusions can be drawn from the Actin experiment of MASER 10, all samples were studied extensively. Except for the observed variation between identical samples, the results from these studies again indicate that the experiment was conducted successfully (Figure 5).

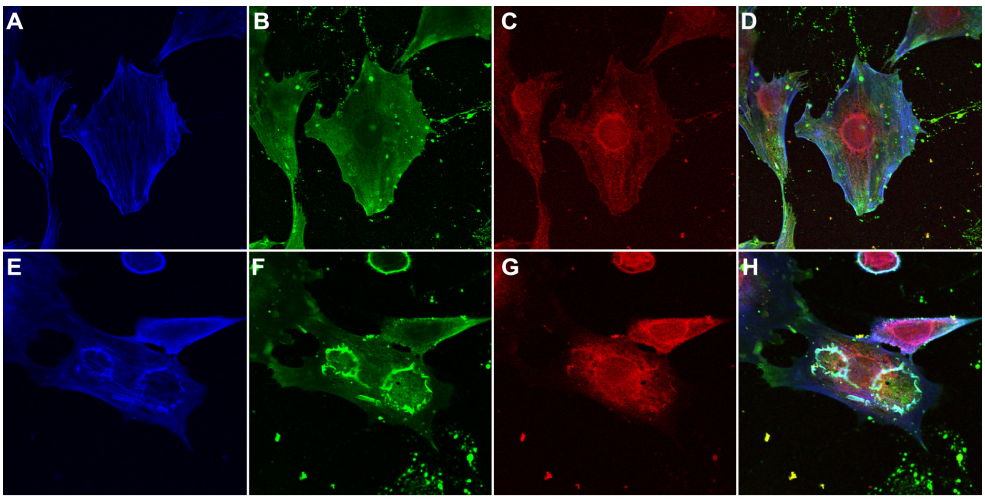

Figure 5. Mouse C3H10T1/2 fibroblasts that are exposed to 1g in the centrifuge of the BIM experiment system in the MASER-10 sounding rocket. Cells are labelled for F-actin (blue), β-actin (green) and G-actin (red). A-D: Serum-starved mouse C3H10T1/2 fibroblasts that are fixed at the onset of microgravity. E-H: Upon stimulation with PDGF-BB for 6 minutes, dorsal circular ruffles are induced by newly formed F-actin (E). These ruffles contain β-actin (F) and are enriched with G-actin (G). The induced ruffle formation in the cells of the in-flight 1g reference experiment seems comparable with the ruffle formation that is observed on earth.

Some single observations suggests some interesting starting points for future research. For example, it was observed that in the remaining cell populations that were still present on the coverslips, dorsal circular ruffles are formed under microgravity conditions. Circular ruffles were observed in various samples exposed to both PDGF-BB and microgravity using phase contrast microscopy (Figure 6), ESEM (Figure 7) and (immuno)fluorescent microscopy using Confocal Scanning Fluorescence Microscopy (CLSM; data not shown). However, since cells that were most influenced by microgravity were lost at the landing, it is not known whether this observation is representative. It can not be excluded that cells that are more susceptible to microgravity exhibit a disturbed ruffle formation.

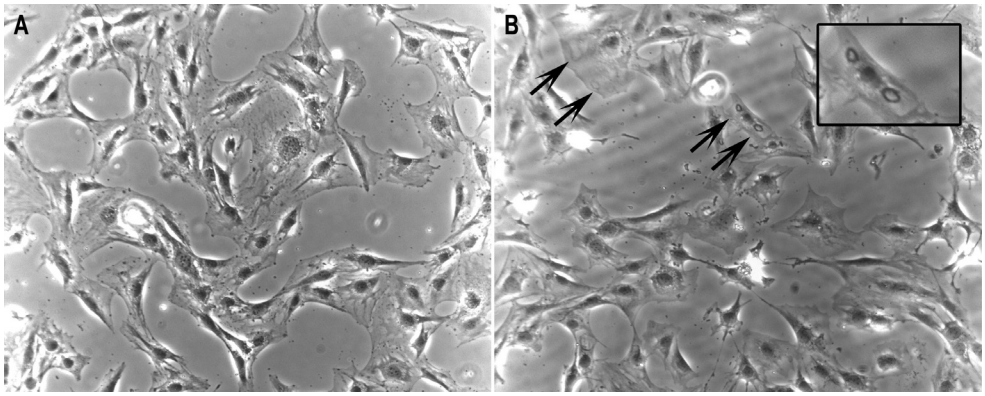

Figure 6. Mouse C3H10T1/2 fibroblasts exposed to microgravity and stimulated with PDGF-BB. Cells are fixed with glutaraldehyde and visualized by phase contrast microscopy. A: Serum-starved cells that are chemically fixed directly upon the start of microgravity. B: Cells that exposed to microgravity and stimulated with PDGF-BB for 6 minutes. Upon stimulation with PDGF-BB, dorsal circular ruffles are formed that are indicated by arrows. Insert is enlarged twice.

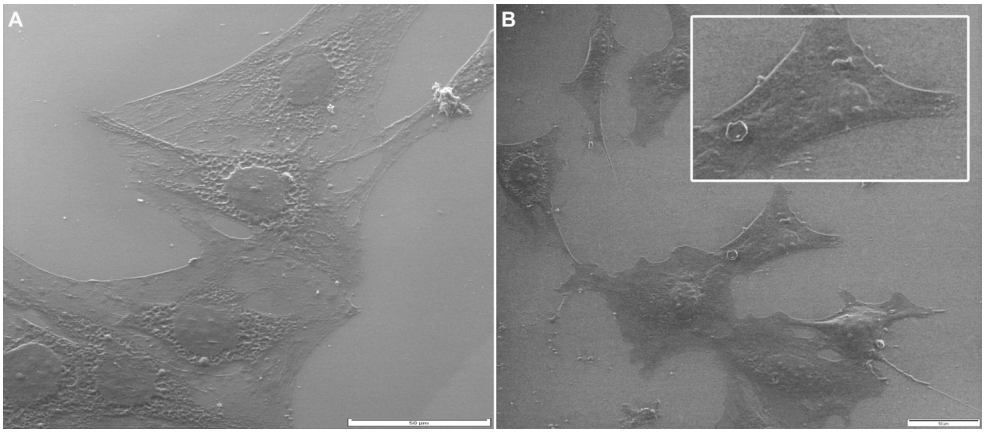

Figure 7. Mouse C3H10T1/2 fibroblasts exposed to microgravity and stimulated with PDGF-BB. Cells are fixed with glutaraldehyde and visualized by ESEM. A: Serum-starved cells that are chemically fixed directly upon the start of microgravity exhibit a flat cell morphology. B: Cells exposed to microgravity and stimulated with PDGF-BB for 6 minutes. Upon stimulation with PDGF-BB, dorsal circular ruffles are formed (insert). Insert is enlarged twice; bar represents 50 μm.

Discussion

No results in microgravity were obtained due to the hard landing of the sounding rocket MASER-10. It was concluded that part of the cells detached from the substrate because of the impact of the uncontrolled landing. We assume that especially the cells that

responded most to microgravity and PDGF-BB have detached. Therefore the samples did no longer represent a representative population of cells and no conclusions can be drawn from the results.

Fibroblasts that are stimulated with a growth factor form an interesting model for studying actin dynamics in microgravity. The actin dynamics that are induced upon exposure to growth factors will enlarge differences in the actin metabolism compared to the situation in more static cells. Moreover, these cells were demonstrated to stand the less ideal conditions unavoidable when doing experiments in real microgravity.

It should be stressed that the Actin experiment is probably the first experiment carried out in a sounding rocket in which all experiments were executed in triplicate. In addition, it is the first experiment in which cell morphology was studied in detail. Our experiment demonstrated the value of doing experiments in triplicate. The variation in results between experimental containers might otherwise not have been detected and results might be interpreted differently. To avoid the problems that were encountered with the variation between samples, re-usable experiment containers should be developed. Re-usable experiment containers would allow to execute the experiment in the lab exactly as it is conducted in the sounding rocket using exactly the same materials, both before and after the space experiment. Any influences of the experimental units can then be excluded.

Sounding rockets provide a unique method for studying cells that are not fully adapted to microgravity conditions. In addition, the facilities for doing biological research are excellent on these unmanned flights. The BIM experiment module did perform excellent and should be rebuilt. The experimental model of mouse fibroblasts that are stimulated with PDGF represents a well described and useful model for future studies under microgravity conditions using sounding rockets. New flight opportunities will provide answers to the questions regarding how cells sense microgravity and the role of the actin cytoskeleton.

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Chapter 7

Summarizing discussion

Summarizing discussion

A number of studies have indicated that gravity affects mammalian cell growth and cell differentiation. Altered gravity values were described to result in alterations in signaling cascades, ranging from the activation of cells – such as human lymphocytes – to the expression of genes. Furthermore, gravity was demonstrated to affect both cellular morphology and the morphology of the actin cytoskeleton. For example, it was demonstrated that the amount of F-actin increased under microgravity conditions. This indicates that either the polymerization of actin is increased or the depolymerization of actin is inhibited under microgravity conditions. The mechanisms that induce these effects in microgravity in cells are unknown. However, based on several observations made in (simulated) microgravity, it was hypothesized that actin might be the microgravity-sensitive component in mammalian cells (Boonstra, 1999).

Interestingly, it was demonstrated that not all intracellular signal transduction cascades are sensitive to conditions of microgravity. The activity of specific signaling routes – such as protein kinase C signal transduction – was demonstrated to be affected by microgravity (De Groot et al., 1991). Other signaling molecules were demonstrated to behave not significantly different under microgravity conditions compared to the situation at 1g. This rules out the idea that all molecular interactions in cells are affected in microgravity. Thus, the polymerization dynamics of actin might represent a specific cellular response to microgravity conditions. Interestingly, the self-organization of microtubules was demonstrated to depend on gravity (Papaseit et al., 2000; Glade et al., 2006; Tabony et al., 2007). The self-organization of actin might be sensitive to altered gravity conditions accordingly. In addition, the mechanism that underlies the sensitivity of actin polymerization in cells to microgravity conditions might result from an altered interaction with actin-binding proteins that regulate the dynamics of actin in cells. In this thesis, experiments were described that were aimed at studying the behavior of actin in microgravity.

The overview of the actin metabolism in mammalian cells that was listed in Chapter 2 illustrated the versatile and complex role of actin in cells. Actin is localized throughout the whole cell and is present in various conformations that interact with numerous actin-binding proteins. The local and dynamic interplay with actin-binding proteins enables actin to fulfill functions in cells that vary locally and over time. The metabolism of actin is tightly regulated to control this dynamic and versatile functionality of actin. Thus, the network comprising actin metabolism is linked to a complex network of signal

transduction cascades that regulate numerous cellular functions. In fact, many of the observed changes in cellular behavior in microgravity can, in theory, be related to an altered behavior of actin in cells. For example, the observed alterations in gene expression, signal transduction, attachment, motility and cell cycle progression could all result from alterations in actin dynamics in cells exposed to altered gravity conditions.

The versatile and complicated interactions of actin with other molecules in cells, and consequently the versatile functioning of actin in cells, makes it difficult to interpret any observed changes in actin behavior. That is why experimental models were studied extensively at 1g before exposing cells to altered gravity conditions. In Chapter 3-5 of this thesis, three cellular processes were characterized that display a specific role of actin in cells. The appearance of actin was studied in cell cycle progression, membrane ruffling and cellular movement.

Actin appears intimately linked to cell-cycle progression (see Chapter 2). In Chapter 3 we focused on the appearance of actin after completion of mitosis during the early G1 phase of the cell cycle. This phase of the cell cycle is of particular interest since during the G1 phase, cells are susceptible to external signals that regulate cell proliferation, such as external matrix components and growth factors. Actin acts as a structural protein in facilitating the spreading of post-mitotic rounded cells. In addition to determining alterations in cellular morphology, actin is involved in signal transduction and attachment (see Chapter 2 and 3). Furthermore, actin appears involved in the formation of membrane blebs that are temporarily present at the cell surface during the spreading of rounded post-mitotic cells. These blebs are shielded with F-actin and contain activated $cPLA_2\alpha$. Therefore it is tempting to suggest that actin interacts with $cPLA_2\alpha$ to form these membrane blebs, similar to the interaction that was demonstrated for other local actin and cell membrane dynamics (see Chapter 5). The blebs may act as temporal storage for membrane that is required during the growth of cells during the G1 phase. In addition, the blebs contain tyrosine-phosphorylated proteins, such as phosphorylated FAK and phosphorylated MAPK. This is indicative of the activity of integrin-induced as well as growth factor-induced signaling in these blebs. Newly-formed focal adhesions appear directly after the disappearance of the membrane blebs. Therefore, these blebs may act as temporal local signaling centers that facilitate the formation of new focal adhesions.

In order to study the interaction between growth factor-induced signal transduction and actin, the formation of membrane ruffles was investigated in Chapters 4 and 5. In mouse fibroblasts, stimulation with the growth factor PDGF induces spectacular

rearrangements of actin within minutes. Newly-formed actin structures locally push the cell membrane outwards, resulting in the formation of ruffles such as circular dorsal ruffles. In Chapter 4, the initiation of this response was investigated by studying the localization of the PDGF β-receptor. A mutual interaction between the PDGF β-receptor and actin was found that might explain how cells become less sensitive to PDGF during stimulation. Activated PDGF β-receptors were demonstrated to co-localize with newly-formed actin structures in dorsal circular ruffles that were induced upon stimulation with PDGF. These circular ruffles contract and move across the cellular surface. The activated PDGF β-receptors follow the movement of the circular ruffles and stay connected to the induced actin and membrane dynamics. The concentration of activated PDGF β-receptors in circular ruffles may facilitate efficient internalization of receptors.

The formation of membrane ruffles was further studied in Chapter 5. It was demonstrated that $cPLA_2\alpha$ plays an important role in facilitating actin-directed membrane ruffling. Active $cPLA_2\alpha$ is recruited at circular ruffles, and the exposure to $cPLA_2\alpha$ inhibitors blocked the formation of ruffles. The interplay between actin and $cPLA_2\alpha$ may have a broader significance, as both $cPLA_2\alpha$ and phosphorylated $cPLA_2\alpha$ were localized at several other protrusions of the cell membrane that exhibit active actin and membrane dynamics, such as lammelipodia, linear ruffles and leading edges. The leading edge represents a region of intense actin and membrane dynamics that promotes motility in cells (Ridley, 2011). Thus, $cPLA_2\alpha$ appears involved in facilitating actin and membrane dynamics in cells, such as membrane ruffling and cell migration.

The suitability of the experimental models for studying actin in microgravity depends on several factors. In all experimental models, i.e. at early G1, after stimulation with PDGF and during cell migration, the actin cytoskeleton is highly dynamic. The presence of a highly dynamic actin cytoskeleton ensures that regulators of the actin metabolism are locally activated. Therefore, these models are suited to study actin dynamics including the activity of regulators of the actin metabolism in cells. But the suitability of the model is also determined by the duration of experiments in microgravity. For short-duration experiments, such as conducted on a sounding rocket, the induction of circular ruffles upon the addition of the growth factor PDGF form a good model. To study the effects of actin in cell cycle progression and cellular movement, longer-duration missions are required.

In addition to experiments in real microgravity, experiments were conducted in simulated microgravity (Chapter 6.1). Simulation of microgravity conditions is a much-

needed addition to research in real microgravity due to the limited access to space flights. Two methods that had been described to simulate weightlessness were studied, i.e. random positioning and magnetic levitation. The use of both methods was demonstrated to result in effects in cells that have previously been described in real microgravity (Chapter 6.1). However, further analysis of both random positioning and magnetic levitation is required, as side effects were observed that complicate relating the cellular responses observed to simulation of weightlessness. Simulation experiments revealed a transient response in cells. Cells initially responded by cell rounding that was followed by renewed spreading and attachment. This transient response is facilitated by a dynamic actin cytoskeleton in combination with the disappearance and reappearance of focal adhesions. Because cells exhibit a transient response, one must be rather careful in comparing the results of experiments that expose cells to microgravity conditions for different durations.

For a first series of experiments in real microgravity, during the Dutch Soyuz Mission, the formation of ruffles induced by actin upon stimulation with the growth factor PDGF was investigated (Chapter 6.2). Mouse fibroblasts were exposed to conditions of microgravity for several days and subsequently stimulated with PDGF. Based on the results of the simulation experiments (Chapter 6.1), the duration of exposure to microgravity conditions was sufficient for the adaptation of cells. The results of the ground control experiments demonstrated that the experimental set-up worked properly. Unfortunately, there were no results in microgravity due to a combination of malfunctioning hardware and temperature requirements that were not met. In a subsequent series of experiments in real microgravity during the MASER-10 mission, cells were stimulated with PDGF directly upon exposure to microgravity, meaning cells that had not yet adapted to microgravity conditions were stimulated with PDGF to induce a response of actin in microgravity. Subsequently cells were chemically fixed after six minutes. Analysis of recorded critical parameters indicated that the experiment was successful and that the developed hardware functioned properly. Unfortunately, there were no results in microgravity due to an uncontrolled landing of the rocket.

Since experiments in space are both scarce and not without risks, it is essential to further develop methods that allow the routine performance of experiments in simulated microgravity. Combined with new experiments in real microgravity, this would certainly clarify the mechanisms that induce effects in cells that are exposed to conditions of altered gravity.

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Summary

Organisms on earth develop in the presence of gravity. A good opportunity to study the effects of gravity on organisms is to expose organisms or cells to conditions of altered gravity, such as microgravity in space. Microgravity has been described to affect numerous processes that take place in organisms. Effects of exposure to microgravity conditions were also described on a cellular level, such as changes in signal transduction cascades, modification of gene expression, and changes in cell morphology. On the basis of results from previous research, it has been suggested that many of these changes observed in cells result from a changing actin cytoskeleton. This thesis further explores the effects on the actin cytoskeleton in cells that are exposed to microgravity conditions.

In cells, a dynamic network of filaments is formed by several types of proteins. This results in the formation of a kind of skeleton that is referred to as the cytoskeleton of cells. Actin is a major component of this cytoskeleton, in addition to tubulin and intermediate filaments. Actin is present in cells as polymerized actin (F-actin) and non-polymerized actin (G-actin). Actin filaments are continuously formed and degraded, a process that is regulated by actin-binding proteins and specific signal transduction routes. This results in the local formation of several subcellular actin structures that play specific roles in cells. Actin has various functions, such as determining the shape of cells, facilitating cellular motility, supporting the compartmentalization of cellular processes, as well as playing a role in communication and transport in cells and conducting several other functions (Chapter 2).

This thesis describes experiments that exposed cells to microgravity conditions for both short and long duration. Microgravity was achieved during rocket missions in space and was simulated on-ground. Initially, the aim of these experiments was to study the behavior of actin in cells upon exposure to conditions of microgravity. To this end, several experimental models were selected in which actin is very dynamic, i.e. cell cycle progression, membrane ruffling, and cellular movement. The presence of a dynamic actin cytoskeleton implies that regulating signal transduction cascades are activated. This provides an opportunity to study the behavior of actin as well as the regulating signal transduction routes. To be able to compare results obtained in microgravity with the situation at 1g, these models were first extensively studied at 1g on-ground. In the first part of this thesis, the models are described (Chapters 3, 4, and 5). Subsequently, experiments are described that were conducted both in simulated microgravity and in conditions of real microgravity (Chapter 6).

Microgravity has been described to affect actin dynamics as well as cellular proliferation. Actin was suggested to play a wide variety of roles during cellular proliferation, such as involvement in signal transduction and transcription and as a structural protein, for example during cytokinesis and during the rounding and flattening of cells. The regulation of cellular proliferation is dependent on signal transduction routes that are activated by external signal molecules, such as growth factors and extracellular matrix components. Depending on the activation of these signal transduction networks, cells continue proliferation or, alternatively, stop cell-cycle progression and undergo differentiation, apoptosis, or quiescence. This decision is taken during the G1 phase of the cell cycle. To study the relation between the behavior of actin and the proliferation of cells, cells were synchronized in mitosis and subsequently studied in the early G1 phase of the cell cycle. Subsequently, the behavior of actin in relation to the activation of signal transduction cascades at the early G1 phase of the cell cycle was studied (Chapter 3). Results indicate that actin and activated signal transduction are intimately linked to each other at the early G1 phase. Actin is involved in the formation of blebs at the cell membrane that are temporally present directly after completion of mitosis in the early G1 phase. These blebs contain several active key signal transduction proteins, such as phosphorylated FAK and phosphorylated MAP kinase, which are known to regulate progression through the cell cycle. The formation of membrane blebs filled with active signal transduction proteins is not specific to rounded, mitotic-like cells before cell spreading at the early G1 phase of the cell cycle. Similar features were observed in rounded trypsinized cells, just before renewed cell spreading. Inhibition of signal transduction did not affect membrane bleb formation, suggesting that the membrane blebs were formed independent of signal transduction.

The interaction between growth factor-induced signaling and actin was further studied in serum-starved mouse fibroblasts (Chapters 4 and 5). These cells induce spectacular actin structures upon stimulation with the growth factor PDGF (platelet-derived growth factor). In these structures, polymerized actin is pushing the cell membrane outwards locally, thereby forming dorsal circular ruffles. This process is regulated by specific signal transduction routes that start at the activated PDGF receptor. Though the subcellular localization of PDGF β-receptors in serum-starved cells is non-homogenous, the observed pattern of receptors does not explain the local formation of actin structures. That is why the initiation of local rearrangements of the actin cytoskeleton cannot be explained by the subcellular distribution of PDGF β-receptors.

Upon PDGF exposure, cells exhibit a changing subcellular distribution of PDGF β-receptors. PDGF β-receptors stay connected to the induced actin structures and accumulate in the newly formed dorsal circular ruffles as a result. 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.

In addition to the translocation of PDGF β-receptors, stimulation with PDGF induces a relocation of cPLA$_2$α (Chapter 5). cPLA$_2$α is a member of a family of enzymes that catalyzes the cleavage of fatty acids from the sn-2 position of membrane phospholipids that results, amongst other things, in the release of arachidonic acid. Arachidonic acid is involved in several important physiological processes; for instance, it is a precursor of molecules that are involved in inflammatory responses. In mouse fibroblasts that are stimulated with PDGF, cPLA$_2$α translocates from the cytoplasm to protrusions of the cell membrane involved in actin and membrane dynamics, such as ruffles and lamellae. In migrating mouse fibroblasts as well as migrating human umbilical vein endothelial cells (HUVEC), cPLA$_2$α was also localized at the leading edge, a zone of intense actin and membrane dynamics that facilitates cellular motility. In addition, the active phosphorylated form of cPLA$_2$α was localized in ruffles, lamellae, and leading edges. Inhibition of cPLA$_2$α activity with specific inhibitors blocked growth factor-induced membrane and actin dynamics, such as the formation of ruffles. This suggests an important role for cPLA$_2$α in these processes. Since cPLA$_2$α was also localized at the membrane blebs of rounded cells that were in the process of spreading (Chapter 3), cPLA$_2$α seems to play an important role in various forms of local actin and membrane dynamics.

Simulation of microgravity conditions is a much-needed addition to research in real microgravity due to the limited access to space flights. Previously, it had been described that in real microgravity conditions, the human epidermoid cell line A431 exhibits specific changes in the actin cytoskeleton, ultimately resulting in the rounding of cells. This rounding of A431 cells was studied in detail during exposure to two methods that have been described to simulate microgravity, i.e. Random Positioning Machine (RPM) rotation and magnetic levitation (Chapter 6.1). In the RPM, samples are mounted on a platform that randomly changes position in three dimensions by driving two independent frames that rotate independently in random directions and at random speed. The random rotation in all directions results in a net force of zero. So the RPM is based on the principle

that the direction of gravity is randomized. In case of magnetic levitation, magnetic forces are exerted on cells by positioning them in a strong gradient magnetic field. Since diamagnetic objects, such as cells, are repelled by magnetic fields, this results in a magnetic force that can be used to counterbalance the gravitational force, resulting in stable levitation and the stimulation of microgravity. The use of both methods was demonstrated to result in effects on the actin morphology that had previously been described in real microgravity. Upon exposure to conditions of simulated microgravity, a transient process of cell rounding and renewed spreading was observed over time. The rounding and flattening of cells was illustrated by a changing actin cytoskeleton and variations in the presence of focal adhesions. Actin and focal adhesions are key players in facilitating the attachment of cells. Despite the fact that the results in simulated microgravity matched the effects that were observed in real microgravity, further characterization of both methods is required. Side effects of both methods can easily lead to false linking of cellular responses to simulated microgravity.

The reorganization of the actin cytoskeleton that takes place upon stimulation with the growth factor PDGF was studied during an initial series of experiments in real microgravity (Chapter 6.2). During the DELTA Soyuz mission of 2004, cells were exposed to microgravity for several days, in contrast to the MASER-10 mission of 2005 that subjected cells to microgravity conditions for just six minutes. Both experiments aimed at studying the behavior of actin upon stimulation with PDGF. However, the simulation experiments indicated that cells transiently respond to microgravity conditions. Short-duration experiments, such as the MASER missions, allow the study of cells that are adapting to microgravity conditions, whereas long-duration experiments allow the study of cells that might have adapted to the changed environment. Neither experiment in real microgravity provided any results. In case of the MASER-10 mission, the experiment was successful but the results were lost as a result of the uncontrolled landing of the rocket. In case of the DELTA mission, there were no results in microgravity due to a combination of malfunctioning hardware and temperature requirements that were not met.

Microgravity based research is hampered by a very limited access to space flights making routine laboratory research impossible. In addition, it can be concluded that the opportunities are not without risks. That is why it is essential to further develop methods that allow the routine performance of experiments in simulated microgravity. In combination with new experiments in real microgravity in space – for example with the

models described in this thesis – this would certainly shed light on the mechanisms that induce effects in cells exposed to microgravity conditions.

Samenvatting

Organismen op aarde ontwikkelen zich in de aanwezigheid van zwaartekracht. Een goede mogelijkheid om onderzoek te doen naar de invloed van zwaartekracht op organismen is het blootstellen van organismen of cellen aan een omgeving met een zwaartekracht van een andere grootte: bijvoorbeeld microgravitéit in de ruimte. Het is gebleken dat blootstelling aan condities van microgravitéit vele processen in organismen beïnvloedt. Ook op het niveau van de cel zijn veranderingen waargenomen na blootstelling aan microgravitéit, zoals veranderde signaaltransductie, expressie van genen, proliferatie van cellen en een veranderde vorm van cellen. Op basis van eerder onderzoek is gesuggereerd dat veel van deze waargenomen veranderingen in cellen wel eens het gevolg zouden kunnen zijn van een veranderend actine cytoskelet. In dit proefschrift zijn de veranderingen van het actine cytoskelet in cellen onder microgravitéit verder onderzocht.

In cellen organiseren een aantal eiwitten zich in een dynamisch netwerk van filamenten. Hierdoor wordt een soort skelet in cellen gevormd dat ook wel het cytoskelet genoemd wordt. Actine is een belangrijke component van het cytoskelet, naast tubuline en intermediaire filamenten. Actine is aanwezig in cellen in een gepolymeriseerde vorm (F-actine) en een niet gepolymeriseerde vorm (G-actine). Voortdurend worden actine filamenten gevormd en afgebroken onder invloed van met actine interacterende eiwitten. Deze processen worden door specifieke signaaltransductieroutes gereguleerd. Uiteindelijk organiseren actine microfilamenten zich lokaal in verschillende subcellulaire structuren die specifieke functies hebben in cellen. Zo bepaalt het actine cytoskelet de vorm van cellen en speelt een rol bij de beweging van cellen, draagt het er toe bij dat verschillende processen in cellen van elkaar gescheiden kunnen plaatsvinden, speelt het een rol bij de deling van cellen en bij de communicatie en het transport in cellen en heeft het nog vele andere functies (hoofdstuk 2).

In dit proefschrift zijn experimenten beschreven waarbij cellen zowel gedurende kortere als langere tijd aan condities van microgravitéit werden blootgesteld. Het ging hierbij zowel om microgravitéit tijdens raketmissies in de ruimte als om gesimuleerde microgravitéit op aarde. Doel van deze experimenten was in eerste instantie om het gedrag van actine in cellen te beschrijven na blootstelling aan microgravitéit. Hiervoor is eerst een aantal modellen geselecteerd waarbij actine in cellen (lokaal) sterk veranderd, namelijk cell cyclus progressie, het ontstaan van ruffles in de celmembraan en het bewegen van cellen. De aanwezigheid van een dynamisch actine cytoskelet impliceert dat er regulerende

signaaltransductie cascades geactiveerd worden. Dit biedt de mogelijkheid om zowel het gedrag van actine als de regulerende signaaltransductieroutes te bestuderen. Om een vergelijking met de situatie bij 1g te kunnen maken zijn deze modellen eerst bij 1g op aarde bestudeerd. Deze modellen zijn beschreven in het eerste deel van dit proefschrift (hoofdstuk 3, 4 en 5). Vervolgens zijn experimenten beschreven die zowel in gesimuleerde microgravitеit als in condities van echte microgravitеit zijn uitgevoerd (hoofdstuk 6).

Het is eerder beschreven dat zowel het gedrag van actine als de proliferatie van cellen veranderen onder condities van microgravitеit. Actine is verondersteld een veelzijdige rol te spelen tijdens de proliferatie van cellen. Te denken valt hierbij aan een rol bij signaaltransductie, transcriptie en als structuurbepalend eiwit, bijvoorbeeld bij de cytokinese en tijdens het opbollen en afplatten van cellen. De regulatie van de proliferatie van cellen is afhankelijk van signaaltransductieroutes die geactiveerd worden door externe signaal moleculen, zoals groeifactoren en componenten van de extracellulaire matrix. Op basis van het al dan niet geactiveerd worden van deze signaaltransductieroutes continueren cellen de cel cyclus, of stoppen de cel cyclus en ondergaan differentiatie, apoptose of quiescence. Deze beslissing wordt tijdens de G1 fase van de cel cyclus genomen. Voor het bestuderen van de relatie tussen het gedrag van actine en de proliferatie van cellen, zijn cellen gesynchroniseerd die zich in mitose bevinden. Vervolgens is het gedrag van actine in relatie tot de activatie van signaaltransductie cascades tijdens het begin van de G1 fase van de cel cyclus bestudeerd (hoofdstuk 3). Gebleken is dat actine en geactiveerde signaaltransductie nauw met elkaar verweven zijn aan het begin van de G1 fase. Actine is betrokken bij de vorming van blaasjes op de celmembraan die uitsluitend waarneembaar zijn aan het begin van de G1 fase, direct na de voltooiing van de mitose. Deze blaasjes bevatten diverse geactiveerde signaaltransductie eiwitten waarvan bekend is dat ze een sleutelrol spelen bij de progressie door de cel cyclus, zoals gefosforyleerd FAK en gefosforyleerd MAP kinase. Gebleken is dat de vorming van deze blaasjes gevuld met geactiveerde signaaltransductie eiwitten niet uniek is voor ronde cellen aan het begin van de G1 fase van de cel cyclus. Ook in getrypsiniseerde ronde cellen die zich opnieuw hechten en spreiden zijn deze membraan blaasjes waargenomen. De formatie van de membraanblaasjes lijkt onafhankelijk te zijn van de activatie van signaaltransductie aangezien de inhibitie van signaaltransductieroutes niet resulteerde in een inhibitie van de formatie van de membraan blaasjes.

De relatie tussen groeifactor geïnduceerde signaaltransductie en actine is verder bestudeerd in serum-gestarveerde muizenfibroblasten (hoofdstuk 4 en 5). Deze cellen

induceren spectaculaire actine structuren na stimulering met de groeifactor PDGF (platelet-derived growth factor). Gepolymeriseerd actine duwt hier lokaal de celmembraan naar buiten en hierdoor worden dorsale circulaire ruffles gevormd. Dit gebeurt onder invloed van specifieke signaaltransductie routes die beginnen bij de geactiveerde PDGF receptor. Hoewel de subcellulaire lokalisatie van PDGF β-receptoren in serum-gestarveerde cellen niet homogeen is, komt het waargenomen patroon van receptoren niet overeen met de lokale vorming van actine structuren. Daarom lijkt de distributie van PDGF β-receptoren geen verklaring te bieden voor de inductie van lokale veranderingen van het actine cytoskelet. Gebleken is dat de distributie van PDGF β-receptoren tijdens de stimulatie met PDGF verandert. PDGF β-receptoren blijven verbonden aan de geïnduceerde actinestructuren en accumuleren op deze wijze in de nieuw gevormde dorsale circulaire ruffles. De aanwezigheid van zowel macropinosomen als clathrine in de geïnduceerde circulaire ruffles suggereert dat de accumulatie van PDGF β-receptor in circulaire ruffles resulteert in de efficiënte internalisatie van PDGF β-receptoren.

Naast de translocatie van PDGF β-receptoren, resulteert de stimulatie met PDGF ook in een opmerkelijke relocatie van cPLA$_2$α (hoofdstuk 5). cPLA$_2$α maakt deel uit van een familie van enzymen die het vetzuur van de 2-plaats van membraan fosfolipiden vrijmaken waarbij onder andere arachidonzuur vrijkomt. Arachidonzuur is bij veel belangrijke processen betrokken, zo kan het bijvoorbeeld worden omgezet in componenten die een actieve rol spelen bij ontstekingen. In muizenfibroblasten die met PDGF gestimuleerd zijn, verplaatst cPLA$_2$α zich vanuit het cytoplasma naar specifieke delen van de celmembraan waar protrusies van de cel worden geïnduceerd door lokale actine en membraan dynamiek. Het gaat hier bijvoorbeeld om ruffles en lamellae. In zowel muizenfibroblasten als humane navelstreng endotheel cellen (HUVEC) is cPLA$_2$α ook gelokaliseerd in de leading edge van migrerende cellen, een zone met intense actine en membraan dynamiek die de beweging van cellen faciliteert. Ook de actieve gefosforyleerde vorm van cPLA$_2$α is in ruffles, lamellae en leading edges gelokaliseerd. De inhibitie van de activiteit van cPLA$_2$α door specifieke inhibitoren blokkeert groeifactor geïnduceerde membraan en actine dynamiek, zoals de vorming van ruffles. cPLA$_2$α lijkt dus een belangrijke rol te hebben bij deze processen. Aangezien cPLA$_2$α ook bij de waargenomen membraanblaasjes van ronde zich spreidende cellen is gelokaliseerd (hoofdstuk 3), lijkt cPLA$_2$α een belangrijke rol te spelen bij veel vormen van lokale actine en membraan dynamiek.

Aangezien de mogelijkheden voor het doen van onderzoek tijdens ruimtemissies beperkt zijn, zou het zeer wenselijk zijn om condities van microgravitеit te kunnen simuleren. Het was eerder beschreven dat onder condities van microgravitеit in de ruimte er in humane epidermale carcinoma A431 cellen specifieke veranderingen van het actine cytoskelet optreden die uiteindelijk resulteren in het opbollen van cellen. Deze opbolling van A431 cellen is gedetailleerd bestudeerd met twee methoden waarvan beschreven was dat deze methoden condities van microgravitеit kunnen simuleren: rotatie met de Random Positioning Machine (RPM) en magnetische levitatie (hoofdstuk 6.1). In de RPM worden samples op een platform geplaatst dat in drie dimensies willekeurig van positie verandert doordat twee frames onafhankelijk van elkaar en met een willekeurige snelheid in willekeurige richting bewegen. De willekeurige rotatie in alle richtingen resulteert in een netto kracht van nul. Dus de RPM is gebaseerd op het principe dat de richting van gravitеit willekeurig wordt gemaakt. In het geval van magnetische levitatie worden er magnetische krachten uitgeoefend op cellen door ze in een sterk magnetisch veld te plaatsen met een gradiënt. Doordat diamagnetische objecten, zoals bijvoorbeeld cellen, worden afgestoten door magnetische velden ontstaat hierdoor een magnetische kracht die kan worden gebruikt om de zwaartekracht te compenseren en dit resulteert in stabiele levitatie en de simulatie van microgravitеit. Zowel rotatie met de RPM als magnetische levitatie induceerde vergelijkbare veranderingen van de actine morfologie als beschreven voor cellen die aan condities van echte microgravitеit blootgesteld waren. Simulatie van condities van microgravitеit leidde tot een transiënte respons van opbolling van cellen en hernieuwde spreiding van cellen. De opbolling van cellen en spreiding van cellen ging gepaard met veranderingen van het actine cytoskelet en variatie in de aanwezigheid van focal adhesions. Actine en focal adhesions hebben een belangrijke rol bij de hechting van cellen. Ondanks het behalen van resultaten die goed overeenkomen met de resultaten zoals die waargenomen werden onder condities van echte microgravitеit is verdere karakterisering van beide methoden noodzakelijk. Het bleek namelijk dat bijeffecten van beide methoden gemakkelijk kunnen leiden tot het onterecht relateren van geïnduceerde cellulaire responsen aan het simuleren van condities van microgravitеit.

De reorganisatie van het actine cytoskelet die optreedt tijdens de stimulering met de groeifactor PDGF in muizenfibroblasten is als eerste bestudeerd onder echt condities van microgravitеit (hoofdstuk 6.2). Tijdens de Soyuz missie “DELTA” van 2004 zijn cellen gedurende langere tijd blootgesteld aan microgravitеit en tijdens de MASER-10 missie van 2005 zijn cellen slechts zes minuten blootgesteld aan microgravitеit. Alhoewel bij beide

experimenten het gedrag van actine tijdens stimulatie met PDGF bestudeerd werd, verschilde de experimenten in opzet. De simulatie-experimenten hebben laten zien dat cellen waarschijnlijk een transiënte respons laten zien na blootstelling aan microgravitéit. Bij kortdurende experimenten, zoals MASER missies, kunnen cellen bestudeerd worden die zich aan het aanpassen zijn aan de condities van microgravitéit en bij experimenten die langer duren kunnen cellen bestudeerd worden die zich aangepast hebben aan de veranderde omstandigheden. Beide experimenten hebben geen resultaten opgeleverd van cellen die aan microgravitéit zijn blootgesteld. In het geval van de MASER-10 is het experiment goed verlopen maar zijn de resultaten verloren gegaan door de ongecontroleerde landing van de raket. Bij de DELTA missie zijn geen resultaten verkregen door niet functionerende hardware en niet vervulde temperatuur vereisten.

Het onderzoek naar de effecten van microgravitéit wordt gehinderd door de beperkte toegang tot ruimtevluchten. Dit maakt het routinematig uitvoeren van laboratoriumonderzoek onmogelijk. Daarnaast kan worden geconcludeerd dat de weinige mogelijkheden die er zijn, niet gevrijwaard zijn van enig risico. Daarom is het essentieel om de methoden verder te ontwikkelen die het mogelijk maken om routinematig experimenten in gesimuleerde microgravitéit uit te voeren. Gecombineerd met nieuwe experimenten onder echte microgravitéit in de ruimte, bijvoorbeeld met de in deze thesis beschreven modellen, zal dit zeker inzicht verschaffen in de mechanismen die effecten induceren in cellen die blootgesteld worden aan condities van microgravitéit.

Illustrations

Transport of the Soyuz spacecraft to the launch platform in Baikonur (Kazakhstan, 2004).

European Space Agency astronaut André Kuipers conducting the Actin experiment in space during mission DELTA (2004). The experiment units of the Actin experiment are labeled pink (copyright photo: ESA).

Launch of the Soyuz rocket (left) in Baikonur (Kazakhstan, 2004) and the Maser-10 rocket (right) at Esrange in Kiruna (Sweden, 2005; photo Swedish Space Corporation).

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Curriculum vitae

Maarten Moes werd geboren op 30 juni 1974 in Leiderdorp. In 1992 werd het VWO diploma behaald aan het Katholiek Gelders Lyceum in Arnhem. In datzelfde jaar begon hij met de studie biologie aan de Universiteit Utrecht. De studie werd enige tijd onderbroken om te werken en te reizen in India en Zuidoost Azië. In 2002 studeerde hij af als fundamenteel biomedisch wetenschapper. Tijdens de doctoraalfase van de studie werd een stageonderzoek verricht bij de vakgroep Vergelijkende Endocrinologie onder leiding van dr. Rüdiger Schulz. Dit onderzoek betrof het identificeren van moleculaire markers voor verschillende stadia van de spermatogenese in de zebravis (*Danio rerio*). Vervolgens heeft hij onderzoek verricht bij de vakgroep Moleculaire Celbiologie van het Karolinska Instituut in Stockholm onder leiding van dr. Volker Cordes. Bij dit onderzoek werd het gedrag en de functie van het eiwit Tpr in de celkern bestudeerd. Na terugkeer in Nederland startte hij zijn promotieonderzoek onder leiding van prof. dr. Johannes Boonstra verbonden aan de vakgroep Celbiologie van de Universiteit Utrecht. Hierbij werd het gedrag van actine in cellen bestudeerd zoals beschreven in dit proefschrift.