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Actin dynamics in living mammalian cells

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SUMMARY

The actin cytoskeleton maintains the cellular architecture and mediates cell movements. To explore actin cytoskeletal dynamics, the enhanced green fluorescent protein (EGFP) was fused to human β-actin. The fusion protein was incorporated into actin fibers which became depolymerized upon cytochalasin B treatment. This functional EGFP-actin construct enabled observation of the actin cytoskeleton in living cells by time lapse fluorescence microscopy. Stable expression of the construct was obtained in mammalian cell lines of different tissue origins. In stationary cells, actin rich, ring-like structured 'actin clouds' were observed in addition to stress fibers. These ruffle-like structures were found to be involved in the reorganization of the actin cytoskeleton. In migratory cells, EGFP-actin was found in the advancing lamellipodium. Immobile actin spots developed in the lamellipodium and thin actin fibers formed parallel to the leading edge. Thus EGFP-actin expressed in living cells unveiled structures involved in the dynamics of the actin cytoskeleton.

Key words: EGFP-actin, Cell migration, Cell adhesion, Actin filament

INTRODUCTION

Actin is a highly conserved and abundant cytoskeletal protein in eukaryotic cells. It is implicated in a number of cellular activities, including reorganization of cell shape and cell motility (Stossel, 1993; Small, 1994a,b; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Many of these processes require the dynamic behavior of the actin cytoskeleton which involves the polymerization and depolymerization of actin filaments (Welch et al., 1997). Monomeric actin (G-actin) polymerizes in a head to tail fashion to form helical actin filaments (F-actin) (Wegner, 1976; Pollard and Mooseker, 1981; Holmes et al., 1990). Most cells keep a large pool of G-actin to maintain the ability to quickly reorganize the cytoskeleton when subjected to environmental changes. The G-actin pool and the stability of F-actin is controlled by a large number of actin binding proteins. The amount of G-actin available for polymerization is controlled by proteins selectively binding to monomeric actin (Carlier and Pantaloni, 1994; Sun et al., 1995). Another class of G-actin binding proteins modulate the rate of assembly and disassembly of G-actin into F-actin, hence increasing the rate of actin polymerization and treadmilling significantly (Carlier et al., 1997; Theriot, 1997). Furthermore, certain actin binding proteins have actin filament severing activity allowing the creation of new nucleation sites for actin polymerization (Cunningham et al., 1991; Witke et al., 1995). Finally, existing F-actin can be bundled by bivalent F-actin binding proteins leading to the formation of thick actin cables (stress fibers) (Matsudaira, 1994).

Although many studies of actin behavior in vitro exist, the complex dynamics of the actin cytoskeleton which is required for maintaining cell shape or cellular locomotion are not well understood. Reagents such as cytochalasins or phalloidin have been widely used to perturb actin dynamics. The former inhibits actin polymerization by blocking the barbed end of actin filaments, while the latter binds selectively along the sides of actin filaments and inhibits their depolymerization (Dancker et al., 1975; MacLean-Fletcher and Pollard, 1980; Cooper, 1987; Forscher and Smith, 1988). An alternative method to study the dynamics of the actin cytoskeleton in living cells is to microinject fluorescently labeled actin, anti-actin antibodies or caged resorufin (CR)-actin (Wang, 1984; Symons and Mitchison, 1991; Theriot and Mitchison, 1991; Cao et al., 1993).

When transfected into recipient cells, green fluorescent protein (GFP)-tagged proteins can be visualized in living cells using fluorescence microscopy (Gerdes and Kaether, 1996; Ludin and Matus, 1998). In yeast cells, an actin-GFP fusion protein was used to study the movement of cortical actin patches (Doyle and Botstein, 1996). In *Dictyostelium discoideum*, GFP-actin fusion proteins were integrated into actin filaments which allowed the observation of microfilament dynamics during *Dictyostelium* movement and chemotaxis (Westphal et al., 1997). So far studies in mammalian cells were hampered due to the lack of corresponding functional GFP-actin construct.

The aim of this investigation was to study cellular processes which lead to actin reorganization in immobile and migrating mammalian cells. In order to visualize the actin cytoskeleton during these complex morphological changes, we constructed an EGFP human β-actin fusion protein which was expressed

in mammalian cells of different tissue origins. Among the EGFP β-actin transfected cells we focused on B16F1 melanoma. These cells can either assume a very well spread, stationary morphology similar to fibroblasts, or develop a lamellipodium and undergo extensive migration similar to fish keratocytes (Wang, 1984; Theriot and Mitchison, 1991; Dunlevy and Couchman, 1993; Lee et al., 1993; Small, 1994a; Small et al., 1995; Cramer et al., 1997). Therefore a possible bias of EGFP β-actin towards the formation of a particular actin structure was excluded.

In stationary spread cells we found EGFP β-actin incorporated into stress fibers. In these cells, expanding circular F-actin containing structures were found to be associated with cell shape changes and reorganization of the actin cytoskeleton. Migrating cells showed a prominent actin rich lamellipodium, followed by the lamella, a transition zone with low actin content and the cell body with the nucleus. These cells did not contain stress fibers but close inspection revealed that long thin actin fibers existed. The reorientation of those were associated with the directional change in migration. Furthermore, 'actin spots' were formed within the lamellipodium of migrating cells and remained immobile with respect to the substrate. The data presented here suggest further roles for different entities in the actin cytoskeleton which may be important for the maintenance of cell shape and the onset of cellular locomotion.

MATERIALS AND METHODS

Cell lines, plasmids and reagents

Mouse melanoma cells (B16F1) were kindly provided by G. Nicholson (Houston, TX, USA). Swiss 3T3 fibroblasts and Chinese hamster ovary cells (CHO) were from the American Type Tissue Culture Collection (ATCC). Mouse thymic endothelioma cells (t.End3) were obtained from Dr Werner Risau (Max Planck Institute, Bad Nauheim, Germany). B16F1, 3T3 fibroblasts, and t.End cells were grown in DMEM and CHO cells in F12 medium (Gibco BRL, Paisley, Scotland), each supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin (all Gibco BRL).

The mammalian expression vector for enhanced green fluorescent protein (pEGFP-1) and polyclonal anti-EGFP antibodies were obtained from Clontech (Basel, Switzerland) and pcDNA3 expression vector was from Invitrogen (Leek, Netherlands). The human β-actin promotor was amplified from the pHβ Apr-1-neo plasmid (Ng et al., 1989). Human β-actin cDNA was obtained from Dr Perriard (Zürich, Switzerland). A polyclonal anti-β-actin antibody specifically reacting with the acetylated N terminus of β-actin was kindly provided by Dr Gabbiani (Geneva, Switzerland) (Yao et al., 1995). Human fibronectin (FN) was purchased from Collaborative Biomedical Products (Bedford, MA, USA), mouse EHS-laminin (LN) was a gift from M. Chiquet (Bern, Switzerland).

Construction of EGFP β-actin fusion protein

Human β-actin promoter, EGFP and human β-actin sequences were amplified by PCR with primers containing restriction sites for cloning into pcDNA3. The cytomegalovirus (CMV) promoter of pcDNA3 was excised with *Bgl*II and *Hin*dIII and replaced by a 3 kb or 1.2 kb fragment of the human β-actin promoter (Ng et al., 1989) which were amplified using *Pfu* polymerase (Stratagen, Basel, Switzerland). The amplification of the 3 kb promoter was carried out with the following primers: forward primer (GGAAGATCTTGGCCAGCTGAATGGAG) and reverse primer (CCCAAGCTTGAGCTGCGAGAATAGCCG). The 1.2 kb human β-actin promoter was amplified with the same reverse primer and the following forward primer (GGAAGATCTGAAGTCGGCAAGGGG).

Subsequently, the EGFP sequence was amplified with *Pfu* polymerase utilizing a forward primer with a *Hin*dIII and reverse primer with *Bam*HI restriction site: forward primer (TGGAAGCTTCCACCATGGTGAGCAAGGGC) and reverse primer (CCGGATCCCTTGTACAGCTCGTCCATGC). Finally, human β-actin was amplified with *Pfu* polymerase using primers containing a *Bam*HI and *Eco*RI site respectively, and cloned into the EGFP containing pcDNA3 vector; forward primer (CCGGATCCACTAGTGGCATGGATGATGATATCGC), and reverse primer (CCGAATTCCTAGAAGCATTTGCGGTGG). The constructs were subsequently sequenced, in order to verify the integrity of the fusion protein. Final constructs were: (1) pcDNA3 with CMV promoter plus EGFP and β-actin, (2) pcDNA3 with a long version of the β-actin promoter followed by EGFP and β-actin, and (3) pcDNA3 containing the short version of the β-actin promoter followed by EGFP and β-actin.

Transient and stable protein expression

Plasmids containing the EGFP β-actin fusion construct were transfected with Lipofectamine according to the manufacturer's recommendation (Gibco BRL). Briefly, 1.5 μg of plasmid DNA and 6 μl Lipofectamine solution was incubated for 15 minutes in 200 μl of OPTIMEM (Gibco BRL) then diluted with 800 μl OPTIMEM. This solution was added to cells at 30-60% confluence in a 35 mm tissue culture plate (Falcon, Becton Dickinson, Basel, Switzerland). After 10 hours incubation at 37°C without serum, 2 ml of 10% FCS medium was added to the cells, and was changed fourteen hours later to the culture medium described for the corresponding cell lines. Two days after transfection, expression of the fusion construct was evaluated by fluorescence microscopy using a FITC filter set (Carl Zeiss, Jena, Germany). Stable expressing clones were obtained by sorting of EGFP fluorescent cells into 96-well plates using a Fluorescence Activating Cell Sorter (FACStar; Becton Dickinson, San Jose, CA, USA) and cultured in G418 (1.5 mg/ml; Geneticin, Gibco BRL) supplemented media. Colonies expressing EGFP-actin were expanded for further analysis.

Fluorescence microscopy of living cells and image analysis

Cells transfected with EGFP β-actin, were detached from plastic tissue culture plates by EDTA treatment for 5 minutes, washed 2 times in Ham's F12 medium, and plated in presence of Ham's F12 containing 10% FCS on glass coverslips coated with 5 μg/ml fibronectin or laminin. Living cells were observed under an inverted fluorescent microscope (Zeiss-Axiovert 100) equipped with Plan-Neofluar ×40, ×100 Fluar oil immersion objectives (Zeiss), and an incubation chamber for constant temperature and CO_2 regulation. EGFP fluorescence was visualized using a FITC filter set (450-490, FT 510, LP 520). Single or time lapse pictures were acquired with a Hamamatsu C4742-95-10 digital CCD camera (Hamamatsu Photonics, Japan) controlled by the Openlab software (Improvision, Oxford, UK). For time-lapse recordings, cells expressing EGFP-constructs were kept constantly at 37°C and 5% CO_2. To obtain low background fluorescence from the culture medium, we used Ham's F12 medium which has a low concentration of fluorescent media components, such as Phenol Red and riboflavin. Since prolonged exposure to intense light led to deterioration and bleaching of the observed cells, light intensity was controlled using neutral density filters, and exposure times never exceeded 600 milliseconds per 20 second interval during time lapse recording.

Fluorescence microscopy of fixed cells

Stable EGFP β-actin transfected cells were cultured on FN (5 μg/ml) coated glass coverslips at 37°C and 5% CO_2 for 12 hours. Cells were

then fixed with 2% paraformaldehyde for 30 minutes at room temperature (RT). After rinsing 3 times with PBS cells were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at RT. Samples were rinsed again with PBS containing 0.5% BSA followed by a 30 minute incubation at RT with 100 nM rhodamine-phalloidin (Fluka, Buchs, Switzerland). After final washing (3 times) with PBS, cells were embedded in 1% *N*-propyl-gallate, 80% glycerol in PBS, and analyzed using a Zeiss-Axiovert 100 microscope.

Western blotting

Western blot analysis was performed after SDS-PAGE (10% gel) according to established procedures (Winston and Fuller, 1991). EGFP β-actin expressing B16F1 cells (10^7) were washed in PBS and lysed in 0.5% Triton X-100 in PBS containing protease inhibitors (inhibitor cocktail from Boehringer Mannheim, Mannheim, Germany). The cell lysate was then mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 5 minutes. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes and non-specific binding sites were blocked with TBS containing 1% BSA and 0.1% Tween-20. After incubation with primary polyclonal anti-β-actin or anti-EGFP antibody followed by secondary peroxidase-conjugated anti-rabbit antibodies (Sigma, Basel, Switzerland), peroxidase activity was visualized by chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

RESULTS

Expression of EGFP β-actin in mammalian cells

In order to visualize the actin cytoskeleton in stationary and migrating mammalian cells, the enhanced green fluorescent protein (EGFP) was fused to the amino-terminal end of the human β-actin (Fig. 1). EGFP and β-actin sequences were separated by a flexible, hydrophilic 5 amino acid spacer. This hinge region should reduce steric hindrance by EGFP, in order to allow interaction of actin with actin binding proteins.

Since overexpression of β-actin cDNA in mammalian cells has been shown to have a severe impact on cell morphology (Mounier et al., 1997) three plasmids encoding EGFP β-actin were constructed with promoters of different strength; the CMV promoter, a long and short version of the human β-actin promoter (see Materials and Methods). Transient expression of EGFP β-actin in COS-7 cells was strong under the control of the CMV and readily detectable with the long actin promoter. Expression under the short actin promoter was barely visible. The strong expression of EGFP β-actin under the CMV promoter affected the cellular morphology of COS-7 cells. Many of these cells were round shaped, unable to spread, and green fluorescence was observed in clusters along the cell periphery or in vesicular structures within the cell (data not shown). In contrast, cell morphology of COS-7 cells expressing EGFP β-actin under the long actin promoter appeared normal, therefore this promoter was chosen for subsequent studies.

This EGFP β-actin construct was then tested for expression in cells of different tissue origins. Transfection was performed with B16 F1 melanoma cells, mouse 3T3 fibroblasts, Chinese hamster ovary cells (CHO), and thymic endothelial cells (t.End 3). All four cell lines expressed EGFP β-actin (Fig. 2). This suggests that expression and function of this construct is not cell lineage restricted. After two weeks of G418 selection, stable expression was obtained in B16, 3T3, and CHO cells demonstrating that cell proliferation was not blocked by EGFP β-actin fusion protein. Expression of EGFP β-actin was also analyzed by western blotting using stable transfected B16 cell lines (Fig. 3). Anti-GFP specific antibodies detected a single band with an apparent molecular mass of 70 kDa using cell lysate of EGFP β-actin transfected cells; non-transfected cells were negative. Normal β-actin appeared at 43 kDa and was detected with a polyclonal monospecific antibody recognizing the acetylated N terminus of β-actin (Yao et al., 1995). Apparently the epitopes of this antiserum were lost by generating the EGFP β-actin fusion protein. Both, transfected and non-transfected cells showed the 43 kDa wild-type actin band at the same level of expression. Thus coexpression of EGFP β-actin did not influence the expression level of wild-type actin. The band detected with the anti-GFP antibody at 70 kDa corresponds to the calculated molecular mass of the EGFP β-actin fusion protein (69, 45 kDa).

EGFP β-actin fusion protein integrates into actin filaments

The fiber-like structures seen in EGFP β-actin expressing cells were strongly reminiscent of stress fiber bundles in adherent cells. In order to test whether EGFP β-actin was incorporated into actin filaments, cells were fixed and then filamentous actin (F-actin) was counter-stained with rhodamine-phalloidin (Rh-Ph). All Rh-Ph labeled fibers were also positive for EGFP β-actin, indicating that the fusion protein is incorporated into actin stress fibers in living cells (Fig. 4A,B). In addition to stress fibers EGFP β-actin was also found in non-filamentous structures surrounding the nucleus. This staining disappeared after detergent treatment suggesting that it consisted of monomeric G-actin (data not shown). Thus EGFP β-actin can be visualized as monomeric G-actin and as polymerized F-actin.

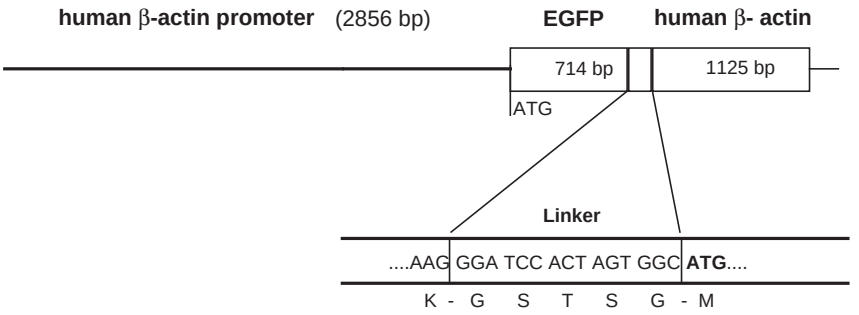

Fig. 1. Scheme of the EGFP-human β-actin DNA construct. A 2,856 bp long version of the human β-actin promoter was linked to the coding sequence of enhanced green fluorescent protein (EGFP) followed by human β-actin. A flexible linker encoding five amino acids was introduced between the EGFP and actin genes.

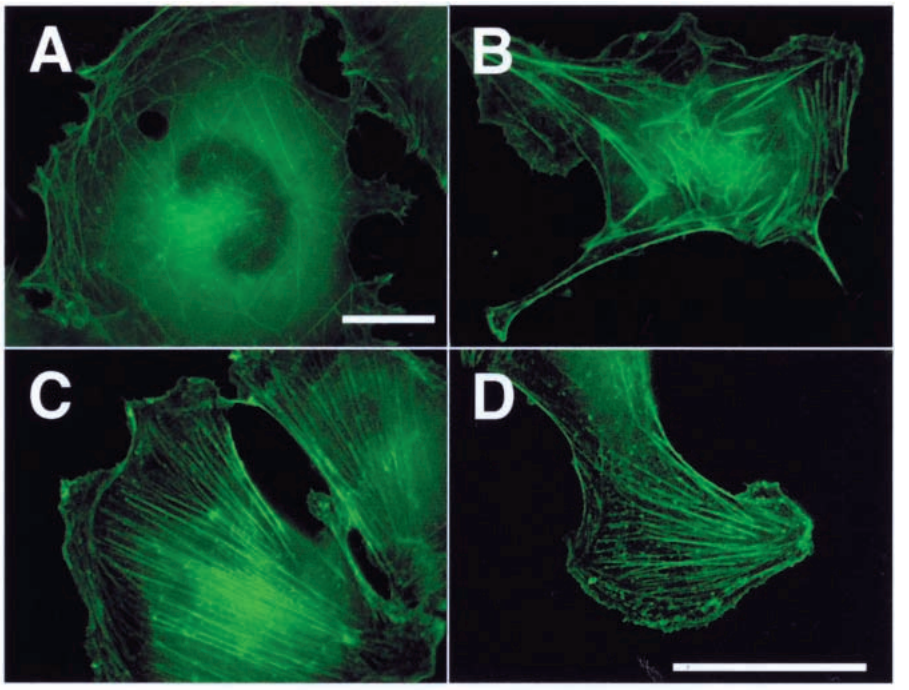

Fig. 2. Expression of EGFP β-actin in cell lines of different tissue origin. The EGFP β-actin construct driven by the long actin promoter was transfected into adherent cell lines. The EGFP β-actin expression was observed by fluorescence microscopy. (A) B16F1 mouse melanoma cells; (B) 3T3 mouse fibroblasts; (C) Chinese hamster ovary (CHO) cells; (D) thymus derived endothelioma (t-End). (A-C) Cell lines expressed the fusion protein stably; (D) transient expression. Bar, 20 μM.

Polymerization and depolymerization of EGFP β-actin in living cells

Actin depolymerization and polymerization are required for cell locomotion and reorganization of the actin cytoskeleton. To test whether these processes were altered by the presence of the EGFP-tag, EGFP β-actin depolymerization and polymerization were recorded by time lapse video fluorescence microscopy. Actin depolymerization was obtained with cytochalasin B which prevents association and dissociation of G-actin at the barbed end of actin filaments, without affecting the depolymerization at the pointed end of actin filaments. B16 cells were cultured for 24 hours on fibronectin (FN) coated glass coverslips to obtain stress fiber containing spread cells. Cells were then treated with 2 μM cytochalasin B, which resulted in rapid rupturing of fibers at the cell periphery. Subsequently, the ruptured fibers were shortened by depolymerization (Fig. 5A-C), and F-actin was lost within 10 minutes of cytochalasin B application.

Further evidence that the EGFP β-actin fusion protein did not disturb actin function was shown by recording the video images of spreading cells. As shown in Fig. 5D-F, actin polymerization readily occurred in spreading B16 cells plated on FN coated glass coverslips. Growing actin filaments showed intense fluorescence at the tip and advanced towards the cell border (arrow in Fig. 5G), indicating accumulation of G-actin at actin polymerization sites. Subsequently, these filaments were further bundled to stress fibers which appeared as thicker fluorescent lines than the de novo formed filaments (data not shown). Spreading and adhesion occurred within 15 minutes, which is the same time scale as for non-transfected B16 cells, indicating that the EGFP-actin fusion protein is a valuable tool to study the actin cytoskeleton dynamics in living mammalian cells.

These results show that the EGFP β-actin fusion protein has no severe impact upon function of the actin cytoskeleton with respect to actin-polymerization, depolymerization, and cell adhesion.

Actin dynamics in stationary cells

B16 cells were stationary when plated on FN coated at 5 μg/ml. Twelve hours after plating, immobile cells were observed using time lapse fluorescence microscopy. The cells exhibited many stress fibers and intense ruffling at the cell edges (Fig. 6A-F). Furthermore, in the presence of serum, ring-shaped actin structures with intense green fluorescence appeared spontaneously within the cell (arrow in Fig. 6A). As shown in Fig. 6A-D, these ring-like actin structures, which most likely represent actin ruffles, expanded concentrically (Ruusala et al., 1998). Because of their appearance we will refer to these structures from now on as ‘actin clouds’. When associated with

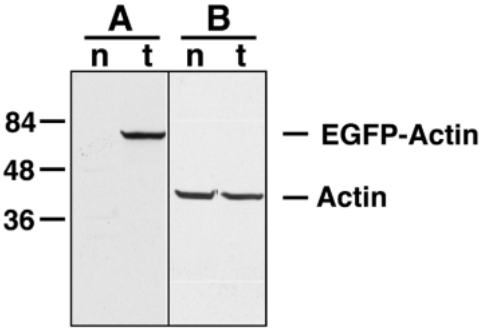

Fig. 3. Western blot of EGFP β-actin fusion protein. Whole cell lysates of nontransfected control B16 (n) and EGFP β-actin expressing B16 cells (t) were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. (A) Polyclonal anti-EGFP antibody detected a single band with an apparent molecular mass of 70 kDa in transfected but not in nontransfected control cells. (B) A polyclonal anti-actin antibody recognizing the N terminus of β-actin detected a single band with an apparent molecular mass of 43 kDa in both transfected and nontransfected cells. The 70 kDa EGFP β-actin was not detectable with this antibody, since the N terminus of β-actin was modified by the fusion to EGFP.

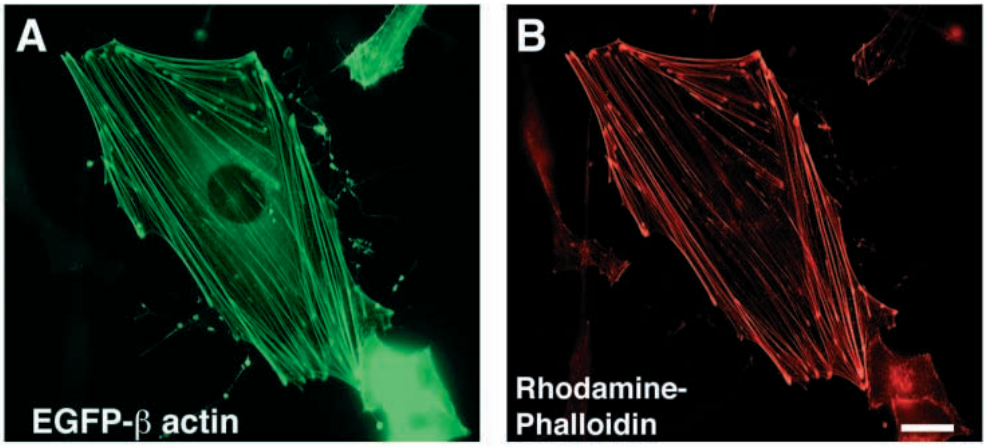

Fig. 4. Overlapping staining of EGFP β-actin organized in stress fibers with rhodamine-phalloidin. EGFP β-actin expressing B16 cells were allowed to spread on culture dishes for 14 hours. (A) The image of living cells was taken by fluorescence microscopy. (B) The cells were then fixed, lysed and stained with rhodamine-phalloidin. Note the identical pattern of F-actin in the two images. Bar, 10 μm.

the cell periphery these expanding actin clouds lead to the local protrusion of the plasma membrane with subsequent formation of new stress fibers. The actin in clouds is in its polymerized form as phalloidin binds to these structures (Fig. 6G). The clouds were stained with the anti-β-actin antibody which exclusively recognizes endogenous β-actin and can not bind to the EGFP β-actin (Fig. 6H, compare with Fig. 3). This assured that the clouds were not an artifact of the EGFP β-actin expression in B16 cells. Artifacts due to EGFP β-actin expression can be excluded by visualization of clouds by phalloidin in nontransfected B16 cells (Fig. 6I). Interestingly these clouds were always associated with the lower side of the cells (data not shown), in contrast to dorsal ruffles observed in MDCK cells (Dowrick et al., 1993).

Actin dynamics in migrating cells

Next we studied actin dynamics in migrating EGFP β-actin expressing B16 cells by time lapse microscopy. These cells moved with a velocity of 3 to 5 μm/minute on laminin (LN) coated at 5 μg/ml. Cells migrated by forming a large lamellipodium and retracting the rear part of the cell (Fig. 7). The lamellipodium was formed by an EGFP β-actin containing

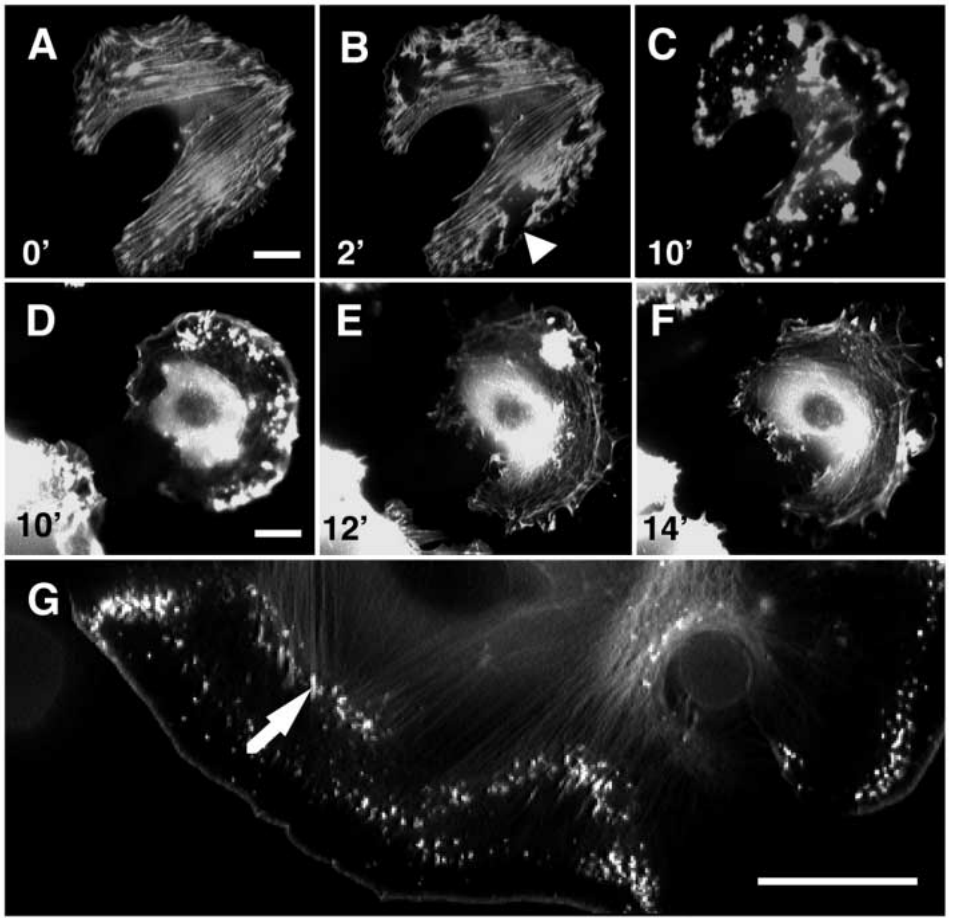

Fig. 5. EGFP β-actin does not interfere with depolymerization and polymerization of F-actin. (A-C) Spreaded, EGFP β-actin expressing B16 cells on FN were treated with 2 μM cytochalasin B at time point 0' (A), the depolymerizing actin stress fibers were then observed for 10 minutes (A-C). Note that 2 minutes after cytochalasin B treatment large zones were already free of stress fibers (arrowhead in B); depolymerization was complete after 10 minutes (C). (D-F) The formation of the actin filament network was observed in spreading B16 cells on FN. Already within 10 minutes the cell was well spread (D). Filaments were formed by 12 minutes (E), reaching a complete level at 14 minutes (F). High magnification of growing actin filaments in a different spreading cell on FN (arrow in G). Bars, 20 μm.

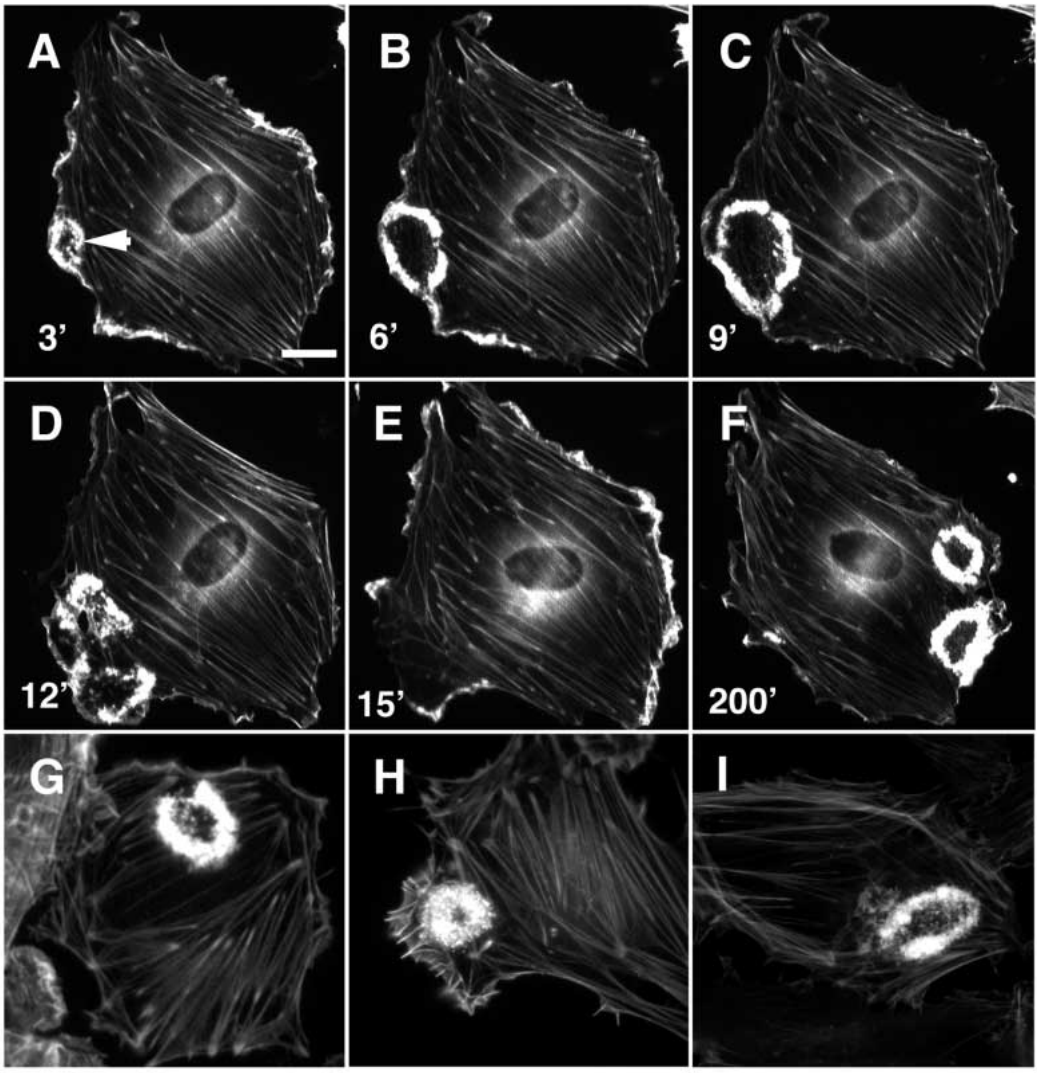

Fig. 6. Reorganization of the actin cytoskeleton in stationary cells. B16 cells were allowed to adhere for 24 hours on glass coverslips coated with 5 μg/ml of FN. (A-F) Actin stress fibers are clearly visible. (A-E) A series of images with 3 minutes intervals showed the appearance and disappearance of 'actin clouds' (arrow in A). (F) The same cell developed new 'actin clouds' at a different location by 200 minutes. (G) RH-phallodin staining of an 'actin cloud' in EGFP β-actin transfected B16 cells. (H) Visualization of an 'actin cloud' in an EGFP β-actin transfected B16 cell with an anti-β-actin specific antibody. Note that this antibody recognizes only endogenous β-actin (Fig. 3). (I) RH-phalloidin staining of an 'actin cloud' in nontransfected B16 cells. Bar, 20 μm.

rim with a constant diameter of 4-5 μm. The lamellipodium contained a ribbed pattern of intense fluorescent actin bundles (Figs 7, 8). These bundles remained associated with the protruding leading edge of the migrating cell and their length was constant while approaching a given reference point (arrowhead in Fig. 8A'-C'). Similar to the lamellipodium, the EGFP β-actin fluorescent intensity in these bundles was maximal at the cell margin and declined towards the interior of the cell (Fig. 8). In the moving lamellipodium, distinct fluorescent spots of actin were observed, which at first glance seemed to migrate against the motion of the advancing cell. Closer inspection, however, proved that they were immobile in respect to the substrate (compare circles in Fig. 8A'-C'), suggesting that they could represent anchoring points of the cell.

Within the cell body, EGFP β-actin was organized in fibers perpendicular to the direction of cell migration, parallel to the leading edge (Fig. 9A). Similar to the fluorescent actin spots most fibers were static. In all observed migrating cells the nucleus moved over this network in direction of migration (data not shown). All migrating cells changed the direction of locomotion during the time of observation. This occurred in a very characteristic way and is exemplified on the cell shown in Fig. 7 and 9. The leading edge started to split into two parts after six minutes of observation. Both fronts then migrated in different directions until the smaller edge collapsed and was retracted towards the main part of the cell body. After 27 minutes of observation the remaining leading edge divided again into two fronts and the cell movement proceeded as described before. During this change of direction the actin fibers were reorganized parallel to the leading edge. Fig. 9 shows a schematic outline of the actin fibers; the direction of the migrating cell is indicated by arrows.

DISCUSSION

EGFP β-actin integrates into actin filaments

Expression of β- and γ-actin isoforms was shown to have different impact on myoblasts morphology. Beta isoform transfectants displayed well defined filamentous organization, whereas gamma isoforms showed a more diffuse organization of actin cables (Schevzov et al., 1992). This result prompted us to choose the β-actin isoform for fusion to EGFP. Transfection experiments with the EGFP β-actin fusion protein under the strong CMV promoter showed accumulation of green

fluorescent proteins at the cell periphery and morphological changes of transfected COS-7 cells. These changes could be due to a cytotoxic effect of overexpressed EGFP alone, but this is unlikely since transfection of EGFP under the CMV promoter had no effect on the cell shape of COS cells (data not shown).

Morphological changes may be affected by altering the proportion of actin isoforms. Recent work has shown that overexpression of β-actin influences the function of cardiomyocytes (von Arx et al., 1995). Cellular malfunctions could also result from steric hindrance due to the linkage of the 26 kDa EGFP to the 43 kDa actin, as shown by in vitro experiments with *Dictyostelium* GFP-actin (Westphal et al., 1997). It was found that GFP-actin was fully functional in in vitro motility experiments when the fusion protein did not exceed 30% of native actin. In order to be within these limits the control of the transcription rate was achieved by the long form of the β-actin promoter. By testing the different constructs in mammalian cells, a correlation between increased changes in cell morphology and increased expression levels of EGFP β-actin fusion protein was observed. Therefore the selection of the appropriate promoter is crucial for successful use of the EGFP-tagged β-actin.

Polymerization and depolymerization of actin filaments

Counter-staining of actin filaments with Rh-Ph in cells expressing EGFP β-actin showed the successful incorporation of the fluorescent fusion protein into actin filaments. The time course of changes observed in cytochalasin treated cells was similar to that in control cells, indicating that actin polymerization and the binding of capping proteins to actin is not disturbed by the fusion of EGFP to actin.

The mechanism of cytochalasin B is not yet entirely clear. Besides the fact that this drug inhibits polymerization by binding to the barbed end, in vitro studies have shown that it severs F-actin (Hartwig and Stossel, 1979; Theodoropoulos et al., 1994). Fragmentation of large actin stress fibers in living cells within seconds after addition of cytochalasin B was indeed observed in the experimental setup used. After the severing of actin filaments depolymerization of the fragments followed, and within ten minutes most F-actin was

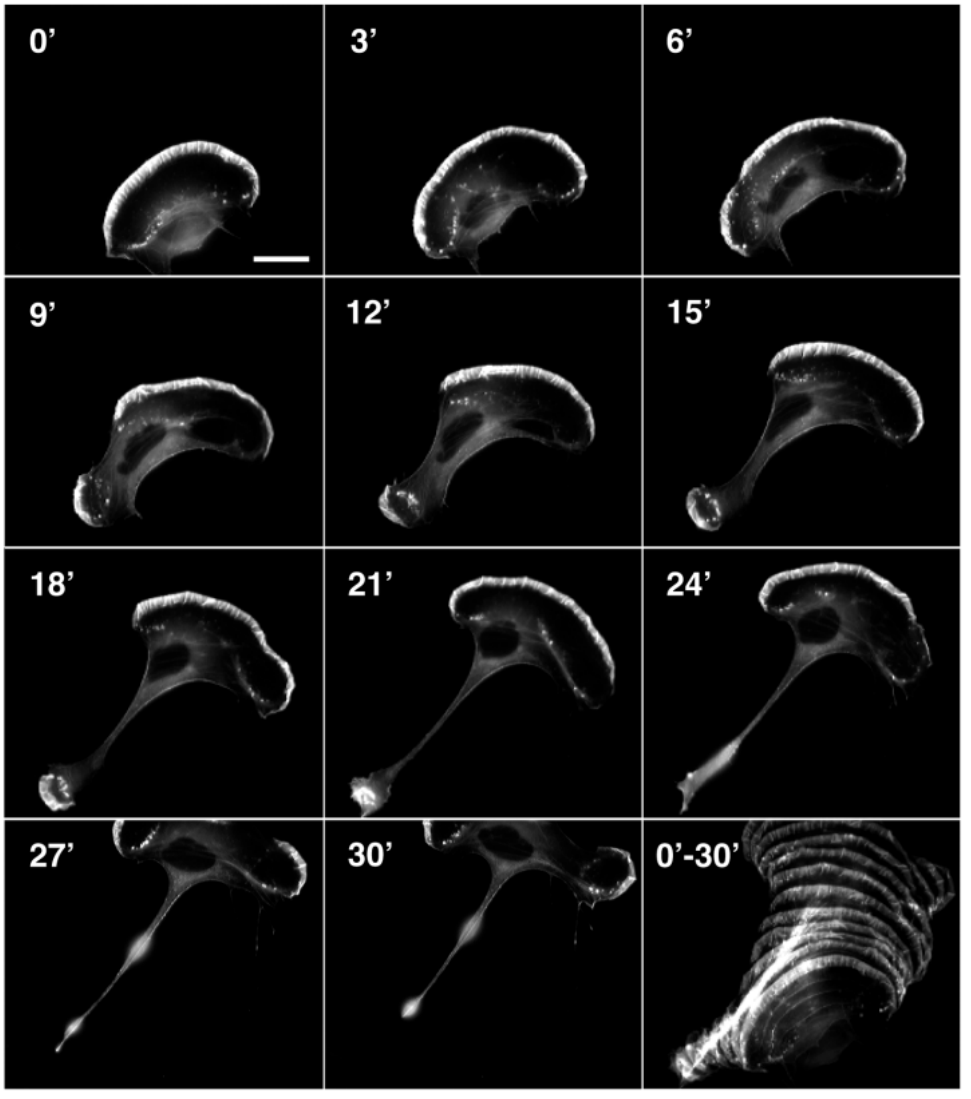

Fig. 7. EGFP β-actin in a migrating cell. EGFP β-actin transfected B16 cells were allowed to adhere for 12 hours to glass coverslips coated with 5 μg/ml of LN. The illustrated images correspond to three minute time intervals of a migrating cell. The composite at the lower right represents an overlay of images taken during the 30 minute period of migration. Bar, 20 μm.

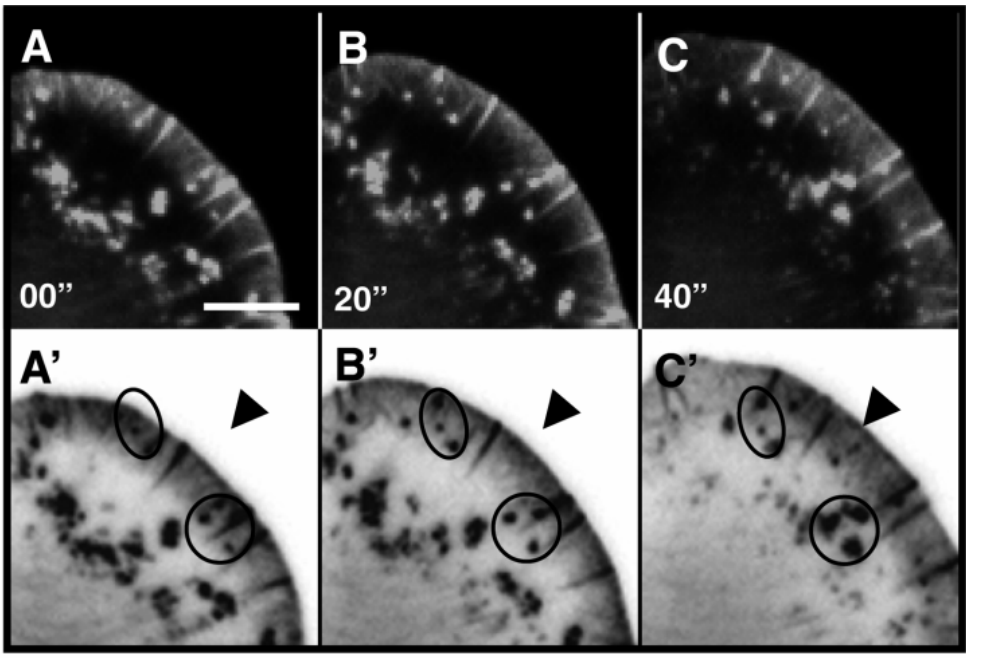

Fig. 8. Immobile actin spots in a migrating B16F1 cell. Cells were plated on LN as described in Fig. 7. Fluorescent β-actin was observed at high magnification in a migrating cell. (A-C) Images taken at intervals of 20 seconds. (A'-C') Image colors were inverted in order to obtain better visibility of EGFP β-actin structures. Migration is illustrated by a static arrowhead given as reference point. Immobile actin spots are marked by circles at identical coordinates. Bar, 10 μm.

depolymerized. In general, peripheral stress fibers in stationary cells depolymerized more rapidly than stress fibers in the center of the cell. This indicates that the turnover of actin filaments is more rapid at the periphery of the cell and demonstrates that two mechanisms are involved in the decay of F-actin by cytochalasin B; severing and depolymerization.

Dynamics of actin clouds

Observed stationary cells displayed actin stress fibers and showed prominent ruffling of the plasma membrane. These cells spontaneously formed 'actin clouds', actin rich ring-like structures most likely represent actin ruffles (Boschek et al., 1981; Mellström et al., 1988; Dowrick et al., 1993; Ruusala et al., 1998). The clouds emerged irrespective of the cell region and their ring-shaped extension coincided with local protrusions of the cell membrane. When the actin rich ring developed close to the edge of the cell a local lamellipodium was formed. The structure of this lamellipodium resembles the lamellipodium found in migrating cells. Therefore the generation of 'actin clouds' may be a ubiquitous mechanism essential for the building of lamellipodia by the actin cytoskeleton (Ruusala et al., 1998). In stationary and migrating cells the outer semicircle of the actin cloud forms an organized lamellipodium, whereas the inner semicircle persists as a diffuse actin rich area. While the leading edge in a migrating cell is constitutive, the actin cloud in a stationary cell is a temporary structure which is replaced by polymerizing fibers. A further function of actin clouds may be the reorientation of stress fibers within the stationary cell. This function may be prevalent in 'actin clouds' appearing in the center of the cell, away from the plasma membrane, possibly allowing the cell to adapt to changes of the extracellular microenvironment.

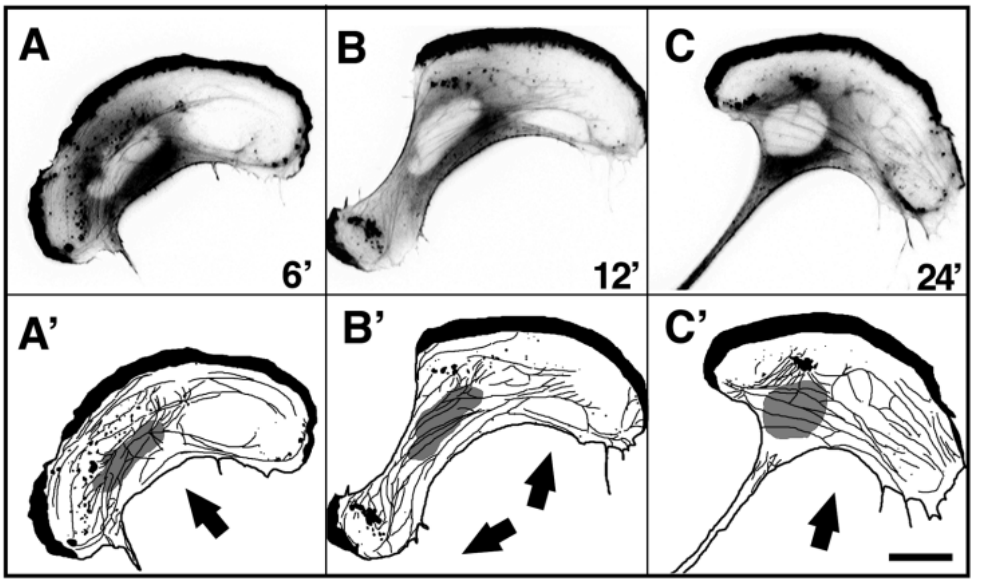

Fig. 9. Behavior of the actin cytoskeleton in cells changing the direction of migration. (A-C) Color inverted illustration of the migrating cell shown in Fig. 7 at time point 6', 12', and 24', respectively. (A'-C') The actin filaments (black lines), the lamellipodium (black) and the nucleus (gray) are outlined in the drawings derived from image overlays with A,B,C, respectively. The direction of migration is indicated by arrows. Bar, 20 μm.

Actin cytoskeleton remodeling in migrating cells

Migrating B16 cells showed an actin rich, arc-shaped lamellipodium containing microspikes, which was followed by the lamella, a zone low of actin content, and the cell body containing the nucleus. The direction of cell migration was imposed by the progression of the lamellipodium.

Studies with fish keratocytes proposed two hypothesis for the advancement of the lamellipodium. In the nucleation release model, short actin filaments are created at the cell front and then subsequently released into the cell body (Theriot and Mitchison, 1991, 1992). A particular orientation of actin filaments is not required in this model. In the treadmilling model, actin filaments are oriented with their barbed ends towards the cell margin; polymerization is expected to occur at the front of the cell edge and depolymerization towards the rear of the lamellipodium (Wang, 1985; Small, 1994b). Studies with fixed cells demonstrated that the highest density of actin filaments in the lamellipodium is at the cell edge and that the barbed ends of these filaments point towards the cell margin, thus forming an actin density gradient within the lamellipodium (Small et al., 1995; Cramer et al., 1997; Svitkina et al., 1997). In the present study, actin rich lamellipodia were observed to remain constant in width during cell migration and displayed an actin concentration gradient. This finding seemed to be more consistent with the treadmilling model. We postulate that treadmilling also occurs at the edge of spreading cells and in local lamellipodia of stationary cells. Since we also observed an actin concentration gradient in microspikes embedded in the lamellipodium, their forward movement may also be achieved by treadmilling.

Several actin structures in moving cells were reported to be stationary or follow a retrograde movement with respect to the substrate (Symons and Mitchison, 1991; Theriot and Mitchison, 1991; Cramer et al., 1997). One study with fish keratocytes suggested that myosin II is involved in the compression of actin filaments in the lamellipodium leading to arc-shaped actin bundles parallel to the leading edge. Compression of the actin network was thought to result in the forward translocation of the cell body (Svitkina et al., 1997). In B16 cells actin fibers were observed to evolve out of the lamellipodium and then oriented parallel to the leading edge. These actin fibers remained fixed relative to the substrate, suggesting that they were linked to anchoring points and participated in the forward translocation of the cell.

In migrating cells, Cao et al. (1993) observed rearward moving punctuate structures which were formed behind the lamellipodium. It is likely that these spots consist of G-actin since they were not detectable by phalloidin. The authors suggested that such spots may represent an actin reservoir from which G-actin can be released and then reincorporated into filaments of the lamellipodium. We found actin spots evolving at the cell margin which remained fixed in respect to the substrate and increased in diameter during forward movement of the cell. In our opinion these spots resembled anchoring points of the cell, which have the capacity to immobilize G-actin. Treatment of cells with phorbol esters, which increases the adhesion strength of cells by activation of integrins (Shaw et al., 1990; Gismondi et al., 1992), enhanced the appearance of actin spots. A recent study reported that actin spots which developed in the lamellipodium of fish keratocytes contained β1 integrin, and several other molecules which are normally found to be in focal adhesion contacts (Lee and Jacobson, 1997). We are currently investigating whether the actin rich spots correspond to nascent focal adhesion sites.

In summary, the EGFP β-actin fusion protein proved to be a valuable tool to study the dynamic changes of the different components of the actin cytoskeleton. Since the actin cytoskeleton is involved in a vast number of cellular functions, the EGFP β-actin fusion protein will help to provide further insights into actin function in living cells.

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REFERENCES

Boschek, C. B., Jockush, B. M., Friis, R. R., Back, R., Grundmann, E. and Bauer, H. (1981). Early changes in the distribution and organization of microfilament proteins during cell transformation. *Cell* **24**, 175-184.

Cao, L., Fishkind, D. J. and Wang, Y. (1993). Localization and dynamics of nonfilamentous actin in cultured cells. *J. Cell Biol.* **123**, 173-181.

Carlier, M. F. and Pantaloni, D. (1994). Actin assembly in response to extracellular signals: role of capping proteins, thymosin β2 and profilin. *Semin. Cell Biol.* **5**, 183-191.

Carlier, M. F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G. X., Hong, Y., Chua, N. H. and Pantaloni, D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J. Cell Biol.* **136**, 1306-1323.

Cooper, J. A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105**, 1473-1478.

Cramer, L. P., Siebert, M. and Mitchison, T. J. (1997). Identification of novel graded polarity actin filament bundles in locomoting heart fibroblasts: implications for the generation of motile force. *J. Cell Biol.* **136**, 1287-1305.

Cunningham, C. C., Stossel, T. P. and Kwiatkowski, D. J. (1991). Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. *Science* **251**, 1233-1236.

Dancker, P., Low, I., Hasselbach, W. and Wieland, T. (1975). Interaction of actin with phalloidin: polymerization and stabilization of F-actin. *Biochim. Biophys. Acta* **400**, 407-414.

Dowrick, P., Kenworthy, P., McCann, B. and Warn, R. (1993). Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. *Eur. J. Cell Biol.* **61**, 44-53.

Doyle, T. and Botstein, D. (1996). Movement of yeast cortical actin cytoskeleton visualized *in vivo*. *Proc. Nat. Acad. Sci. USA* **93**, 3886-3891.

Dunlevy, J. R. and Couchman, J. R. (1993). Controlled induction of focal adhesion disassembly and migration in primary fibroblasts. *J. Cell Sci.* **105**, 489-500.

Forscher, P. and Smith, S. J. (1988). Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* **107**, 1505-1516.

Gerdes, H. H. and Kaether, C. (1996). Green fluorescent protein: applications in cell biology. *FEBS Lett.* **389**, 44-47.

Gismondi, A., Mainiero, F., Morrone, S., Palmieri, G., Piccoli, M., Frati, L. and Santoni, A. (1992). Triggering through CD16 or phorbol esters enhances adhesion of NK cells to laminin via very late antigen 6. *J. Exp. Med.* **176**, 1251-1257.

Hartwig, J. H. and Stossel, T. P. (1979). Cytochalasin B and the structure of actin gels. *J. Mol. Biol.* **134**, 539-553.

Holmes, K. C., Popp, D., Gebhard, W. and Kabsch, W. (1990). Atomic model of the actin filament. *Nature* **347**, 44-49.

Lauffenburger, D. A. and Horwitz, A. F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Lee, J., Ishihara, A., Theriot, J. A. and Jacobson, K. (1993). Principles of locomotion for simple-shaped cells. *Nature* **362**, 167-171.

Lee, J. and Jacobson, K. (1997). The composition and dynamics of cell-substratum adhesions in locomoting fish keratocytes. *J. Cell Sci.* **110**, 2833-2844.

Ludin, B. and Matus, A. (1998). GFP illuminates the cytoskeleton. *Trends Cell Biol.* **8**, 72-77.

MacLean-Fletcher, S. and Pollard, T. D. (1980). Mechanism of action of cytochalasin B on actin. *Cell* **20**, 329-341.

Matsudaira, P. (1994). Actin crosslinking proteins at the leading edge. *Semin. Cell Biol.* **5**, 165-174.

Mellström, K., Heldin, C. H. and Westermark, B. (1988). Induction of circular membrane ruffling on human fibroblasts by platelet-derived growth factor. *Exp. Cell Res.* **177**, 347-359.

Mitchison, T. J. and Cramer, L. P. (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.

Mounier, N., Perriard, J. C., Gabbiani, G. and Chaponnier, C. (1997). Transfected muscle and non-muscle actins are differentially sorted by cultured smooth muscle and non-muscle cells. *J. Cell Sci.* **110**, 839-846.

Ng, S. Y., Gunning, P., Liu, S. H., Leavitt, J. and Kedes, L. (1989). Regulation of the human β-actin promoter by upstream and intron domains. *Nucl. Acids Res.* **17**, 601-615.

Pollard, T. D. and Mooseker, M. S. (1981). Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J. Cell Biol.* **88**, 654-659.

Ruusala, A., Sundberg, C., Arvidsson, A. K., Rupp-Thuresson, E., Heldin, C. H. and Claesson-Welsh, L. (1998). Platelet-derived growth factor (PDGF)-induced actin rearrangement is deregulated in cells expressing a mutant Y778F PDGF β-receptor. *J. Cell Sci.* **111**, 111-120.

Schevzov, G., Lloyd, C. and Gunning, P. (1992). High level expression of transfected β- and γ-actin genes differentially impacts on myoblast cytoarchitecture. *J. Cell Biol.* **117**, 775-785.

Shaw, L. M., Messier, J. M. and Mercurio, A. M. (1990). The activation dependent adhesion of macrophages to laminin involves cytoskeletal anchoring and phosphorylation of the alpha 6 beta 1 integrin. *J. Cell Biol.* **110**, 2167-2174.

Small, J. V. (1994a). Introduction: actin and cell crawling. *Semin. Cell Biol.* **5**, 137-138.

Small, J. V. (1994b). Lamellipodia architecture: actin filament turnover and the lateral flow of actin filaments during motility. *Semin. Cell Biol.* **5**, 157-163.

Small, J. V., Herzog, M. and Anderson, K. (1995). Actin filament organization in the fish keratocyte lamellipodium. *J. Cell Biol.* **129**, 1275-1286.

Stossel, T. P. (1993). On the crawling of animal cells. *Science* **260**, 1086-1094.

Sun, H. Q., Kwiatkowska, K. and Yin, H. L. (1995). Actin monomer binding proteins. *Curr. Opin. Cell Biol.* **7**, 102-110.

Svitkina, T. M., Verkhovsky, A. B., McQuade, K. M. and Borisy, G. G. (1997). Analysis of the actin-myosin II system in fish epidermal keratocytes: mechanism of cell body translocation. *J. Cell Biol.* **139**, 397-415.

Symons, M. H. and Mitchison, T. J. (1991). Control of actin polymerization in live and permeabilized fibroblasts. *J. Cell Biol.* **114**, 503-513.

Theodoropoulos, P. A., Gravanis, A., Tsapara, A., Margioris, A. N., Papadogiorgaki, E., Galanopoulos, V. and Stournaras, C. (1994). Cytochalasin B may shorten actin filaments by a mechanism independent of barbed end capping. *Biochem. Pharmacol.* **47**, 1875-1881.

Theriot, J. A. and Mitchison, T. J. (1991). Actin microfilament dynamics in locomoting cells. *Nature* **352**, 126-131.

Theriot, J. A. and Mitchison, T. J. (1992). The nucleation-release model of actin filament dynamics in cell motility. *Trends Cell Biol.* **2**, 219-222.

Theriot, J. A. (1997). Acceleration on a treadmill: ADF/cofilin promotes rapid actin filament turnover inthe dynamic cytoskeleton. *J. Cell Biol.* **136**, 1165-1168.

von Arx, P., Bantle, S., Soldati, T. and Perriard, J. C. (1995). Dominant negative effect of cytoplasmic actin isoproteins on cardiomyocyte cytoarchitecture and function. *J. Cell Biol.* **131**, 1759-1773.

Wang, Y. L. (1984). Reorganization of actin filament bundles in living fibroblasts. *J. Cell Biol.* **99**, 1478-1485.

Wang, Y. L. (1985). Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. *J. Cell Biol.* **101**, 597-602.

Wegner, A. (1976). Head to tail polymerization of actin. *J. Mol. Biol.* **108**, 139-150.

Welch, M. D., Mallavarapu, A., Rosenblatt, J. and Mitchison, T. J. (1997). Actin dynamics *in vivo*. *Curr. Opin. Cell Biol.* **9**, 54-61.

Westphal, M., Jungbluth, A., Heidecker, M., Mühlbauer, B., Heizer, C., Schwartz, J. M., Marriot, G. and Gerisch, G. (1997). Microfilament dynamics during cell movement and chemotaxis monitored using a GFP-actin fusion protein. *Curr. Biol.* **7**, 176-183.

Winston, S. E. and Fuller, S. A. (1991). Isolation and analysis of proteins. In *Current Protocols in Immunology* (ed. J. E. Coligan et al.), section 8.10. pp. 11-171. Greene Publishing Associates & Wiley-Interscience, New York.

Witke, W., Sharpe, A. H., Hartwig, J. H., Azuma, T., Stossel, T. P. and Kwiatkowski, D. J. (1995). Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell* **81**, 41-51.

Yao, X., Chaponnier, C., Gabbiani, G. and Forte, J. G. (1995). Polarized distribution of actin isoforms in gastric parietal cells. *Mol. Biol. Cell* **6**, 541-557.