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Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover

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SUMMARY

Focal adhesion kinase (FAK) is activated and localized at focal adhesions upon cell adhesion to extracellular matrices. Cells lacking FAK show increased focal adhesion number and decreased cell migration, functions that are regulated by the small GTPase Rho. We now report that fibroblasts from FAK–/– mice failed to transiently inhibit Rho activity when plated on fibronectin. Re-expression of FAK restored normal Rho regulation. Turnover of focal adhesions correlated inversely with Rho activity. The presence or absence of FAK was mimicked by inhibiting or activating Rho, respectively. These data suggest that loss of FAK resulting in constitutive activation of Rho and inhibition of focal adhesion turnover can account for deficiencies in cell migration and embryonic lethality of the FAK knockout.

Key words: α-Actinin, Integrin, Cytoskeleton, Cell adhesion

INTRODUCTION

Focal adhesions are sites where cells contact the extracellular matrix (ECM) (Burridge and Chrzanowska-Wodnicka, 1996; Yamada and Miyamoto, 1995). They are enriched in integrins and in cytoskeletal and signaling proteins including talin, α-actinin, vinculin, zyxin, paxillin and focal adhesion kinase (FAK). Focal adhesions are thought to function as connections between the cytoskeleton and the ECM that confer structural integrity as well as being signaling organelles that enable cells to sense their environment. Formation of focal adhesions is under the control of the small GTPase Rho (Ridley and Hall, 1992). Dynamic regulation of Rho activity and focal adhesion formation is also critical for cell migration, as either activating or inactivating Rho diminishes migration (Nobes and Hall, 1999; Allen et al., 1997; Takaishi et al., 1993).

Using a novel assay for Rho activation based on specific binding of GTP-bound Rho to the Rho-binding domain from the effector Rhotekin, we previously demonstrated complex regulation of Rho by both soluble mitogens and adhesion to the ECM protein fibronectin (FN) (Ren et al., 1999). In particular, we observed a rapid decrease of Rho activity when Swiss 3T3 cells were plated on fibronectin (FN), followed by return to higher levels at later times when large focal adhesions were forming. This transient Rho inhibition correlated with the period of active cell spreading, a process that in some respects is analogous to cell migration.

FAK is a tyrosine kinase that is localized at focal adhesions and whose activity is controlled by cell adhesion (Schaller and Parsons, 1994; Schlaepfer et al., 1999). Fibroblasts from FAK null mice show a marked overabundance of focal adhesions and a decrease in migration (Ilic et al., 1995), which were restored to the normal phenotype by re-expression with FAK (Sieg et al., 1999). Conversely, FAK overexpression in CHO cells increased migration rate (Cary et al., 1996). These considerations, together with the known involvement of Rho in regulating focal adhesions, suggested that changes in Rho activity might underlie the abnormal behavior of FAK-null cells. We now show that Rho regulation is indeed altered when FAK is absent, and that changes in Rho activity may account for some of the effects of FAK on the cytoskeleton.

MATERIALS AND METHODS

Cells and cell culture

Polyclonal fibroblasts established from FAK+/+ or FAK–/– embryos (Ilic et al., 1995; a gift from Dr Dusko Ilic, UC San Francisco) were used at passages 10-13. Cells were maintained in DMEM (Life Technologies) containing 10% fetal bovine serum. FAK–/– clones expressing HA-tagged wild-type FAK (FAK–/–(FAK) cells) or vector only (FAK–/–(vector) cells) were established by DNA transfection followed by selection with hygromycin (Sieg et al., 1999). Cells were cultured in DMEM with 10% fetal bovine serum, 500 μg/ml G418 and 200 μg/ml hygromycin on dishes coated with

1% gelatin. Microinjection was carried out as described (Meredith et al., 1995).

DNA transfection

Transient transfections were performed using Effectene reagents (Qiagen) according to the manufacturer's instructions. cDNA plasmids used in transfection were purified using Maxi-prep kit from Qiagen. The ratio of DNA:Effectene:Enhancer was optimized for FAK–/– and FAK+/+ cells to achieve a transfection efficiency of 30-50%.

Measurement of Rho activity

Rho activity was measured in a pull-down assay using the Rho-binding domain from Rhotekin (Ren et al., 1999), except that instead of RIPA buffer cells were extracted in Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF. The change prevented lysis of the nuclei in these cells but did not significantly affect the sensitivity of the assay (data not shown). Rho activity (percentage of GTP-bound Rho) is determined as the amount of RBD-bound Rho versus the total Rho in the lysate (Ren et al., 1999).

Fluorescence microscopy

Cells were fixed for 10 minutes in 3% paraformaldehyde in phosphate buffer saline (PBS), and permeabilized for 10 minutes with 0.01% Triton X-100/PBS. Coverslips were blocked in 10% goat serum at room temperature for 40 minutes, then stained for 60 minutes in a 1:10 dilution of rhodamine-phalloidin (ICN Immunobiologicals, Costa Mesa, CA). Coverslips were then stained with anti-vinculin at 1:100 (Sigma) followed by CY5-conjugated goat anti-mouse F (ab′)2 fragment (Sigma) at 1:100 for 60 minutes. Coverslips were mounted in Immunofluore mounting medium (ICN Immunobiologicals) and viewed using a Bio-Rad 1024 MRC Scanning Confocal Microscope.

Analysis of GFP-α-actinin

Cells transiently transfected with pGFP-α-actinin were incubated in growth medium for 32 hours, serum-starved for 16 hours, then replated on FN-coated glass coverslips in 35 mm dishes. Cells on the microscope stage were maintained at 37°C with a humid CO_2 atmosphere as described (Schwartz, 1993). Cells were monitored under fluorescence optics on an inverted Nikon microscope using a ×20 oil immersion lens. Time lapse sequences were captured using a SenSys cooled CCD video camera linked to a Silicon Graphics workstation running the Inovision ISEE software program. The V14-Rho transfected cells were fixed after the experiments and confirmed by immunofluorescence staining with an anti-HA antibody (data not shown). Focal adhesions in at least five cells (10 focal adhesions from each cell) from each cell type were assessed for their turnover rate by determining the time period between formation and dissolution of the α-actinin spots.

RESULTS

Dependence of Rho activity on FAK expression

To test whether FAK might participate in regulating Rho activity, polyclonal FAK+/+ or FAK–/– primary mouse embryo fibroblasts were plated in dishes coated with FN, lysed at various times and Rho activity assayed. Following attachment to fibronectin, FAK+/+ control fibroblasts showed a transient decrease in Rho activity at the early stage of

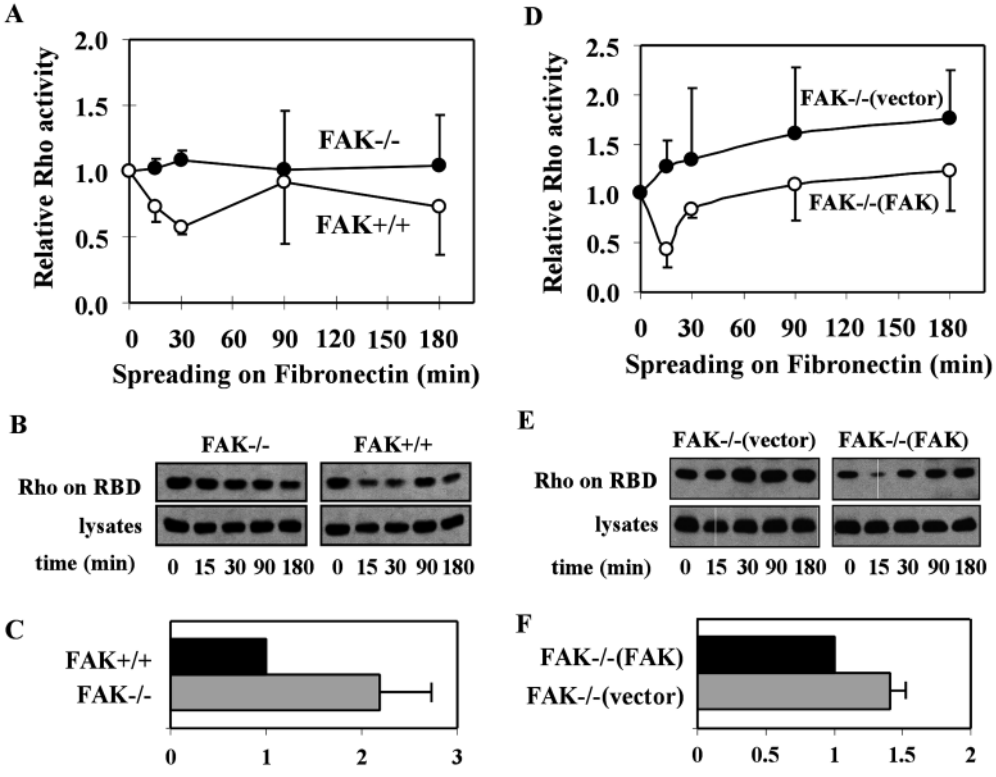

Fig. 1. Rho activity in FAK–/– and FAK+/+ cells spreading on fibronectin. (A) Cells were serum-starved for 16 h, suspended in serum-free medium for 60 minutes, and replated on dishes coated with fibronectin (25 μg/ml) for the indicated times. Cells were extracted and Rho assayed as described in Materials and Methods. Data are means ± standard deviations from three experiments. The decrease in Rho activity in FAK+/+ cells was statistically significant (P<0.05 at 15 minutes and <0.005 at 30 minutes). (B) Rho blots from one of the experiments described in A. (C) Comparison of Rho activity in FAK+/+ and FAK–/– suspended cells (time 0 in a) in three experiments (P=0.03). (D) FAK–/– cells stably expressing wild-type FAK or control cells with empty vector were designated as FAK–/–(FAK) and FAK–/–(vector), respectively. Cells were plated on FN as in A and Rho assayed. Data are means ± standard deviations from three experiments. The decrease in Rho activity in FAK+/+ cells at 15 minutes was statistically significant (P<0.025) (E) Rho blots from one of the experiments described in D. (F) Rho activity in FAK–/–(vector) and reconstituted FAK–/–(FAK) cells in suspension (time 0 in d) in three experiments (P=0.01).

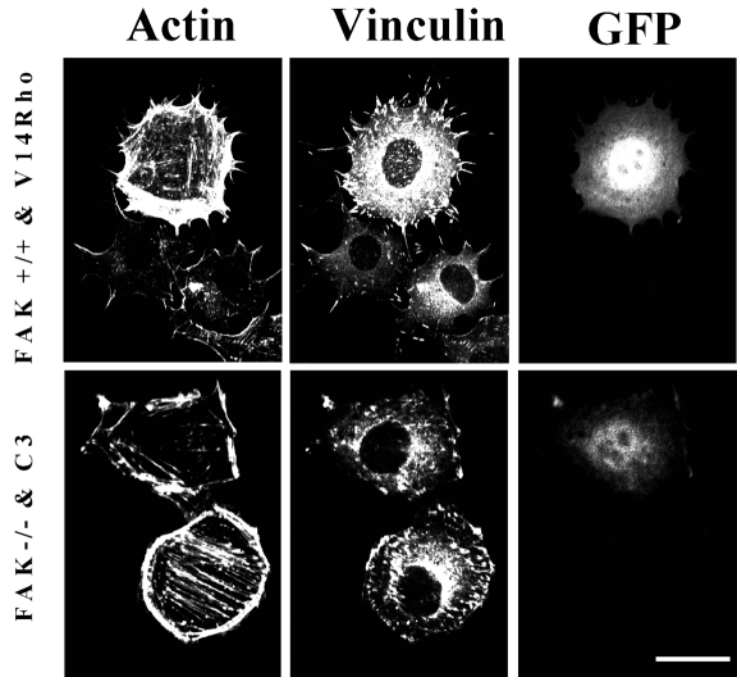

Fig. 2. Cytoskeletal organization. FAK+/+ or FAK–/– cells were transfected with 0.4 μg (per 6-cm dish cells) pcDNA3-HA-V14-Rho or pEF-myc-C3, respectively. pEGFP vector was co-transfected for the identification of the transfected cells. Fluorescence images of actin filaments, focal adhesions and GFP in the same cells are shown. Separate staining showed that virtually all the GFP positive cells were also positive for anti-myc or anti-HA staining (data not shown).

spreading (Fig. 1A and B), similar to Swiss 3T3 cells (Ren et al., 1999). By contrast, this inhibition was virtually absent from the FAK–/– fibroblasts (Fig. 1A and B).

To determine whether this difference was directly attributable to the presence or absence of FAK, a cell line prepared from the FAK–/– cells stably transfected with wild-type FAK was examined. These cells contain levels of FAK that are slightly lower than the endogenous (Sieg et al., 1999). Re-expression of FAK restored the adhesion-induced inhibition of Rho (Fig. 1D and E), as well as their fibroblastic morphology (Sieg et al., 1999; data not shown). These cells spread more rapidly than the early passage MEFs, which most likely accounts for the faster time course. These results demonstrate that FAK is required for the adhesion-dependent inhibition of Rho. We also noted that Rho activity was somewhat higher in FAK–/– cells even in suspension (Fig. 1C and F). Whether this result is due to effects of FAK on upstream Rho regulators even in the absence of cell adhesion or to changes in gene expression due to the presence of FAK is currently unknown. Additional experiments revealed that these primary fibroblasts did not decrease Rho activity or stress fibers upon extended serum starvation, nor did they show significant increases upon stimulation with serum or lysophosphatidic acid (data not shown). Thus, cell adhesion may be the primary regulator of Rho activity in this system.

Cytoskeletal organization

Consistent with these data, vinculin-containing focal adhesions were highly abundant in FAK–/– cells during cell spreading on fibronectin, whereas fewer focal adhesions were formed in FAK+/+ cells. In addition, FAK–/– cells had focal adhesions over the entire basal surface and generally adopted a nonpolarized, circular shape, unlike FAK+/+ cells that had adhesions primarily at the cell edges and a more elongated, polygonal morphology (Ilic et al., 1995; Fig. 2). To test whether changes in Rho activity could account for these differences, low doses of DNA encoding C3 exoenzyme were transfected into FAK–/– cells. Cells were co-transfected with a vector coding for green fluorescent protein (GFP) to identify expressing cells, then replated on FN-coated coverslips. In FAK–/– cells transfected with C3, high expressors showed the poorly spread, dendritic morphology typical of cells treated with high doses of C3 (not shown), however, low expressors spread well, and developed a more polygonal shape with fewer focal adhesions than untransfected cells (Fig. 2). Indeed, their appearance was similar to FAK+/+ cells. Cells transfected with GFP only were undistinguishable from untransfected cells (not shown).

As a further test, FAK+/+ cells were transfected with V14 Rho together with GFP. The V14Rho-expressing FAK+/+ cells showed significantly more focal adhesions and were more circular than untransfected cells (Fig. 2). Notably, the focal adhesions were distributed over the entire basal surface. Thus, expression of V14Rho shifts the phenotype of FAK+/+ cells toward that of FAK–/– cells. These results demonstrate that directly altering Rho activity recapitulates to some extent the effects of FAK expression on focal adhesions and cell morphology. The data are therefore consistent with the hypothesis that FAK inhibits Rho.

Focal adhesion dynamics

To gain additional insights into cytoskeletal dynamics, cells were transfected with a cDNA encoding GFP fused to the N terminus of full length α-actinin. This fusion protein localized strongly to focal adhesions and also labeled actin filaments in stress fibers and membrane ruffles, thereby allowing visualization of all of these structures in living cells. Cells were replated on fibronectin-coated coverslips and time lapse fluorescent images recorded. At early times after replating while the cells were rapidly spreading, GFP-α-actinin labeled small focal adhesions/complexes at the actively spreading edges of the cells. Typical time lapse sequences are shown in

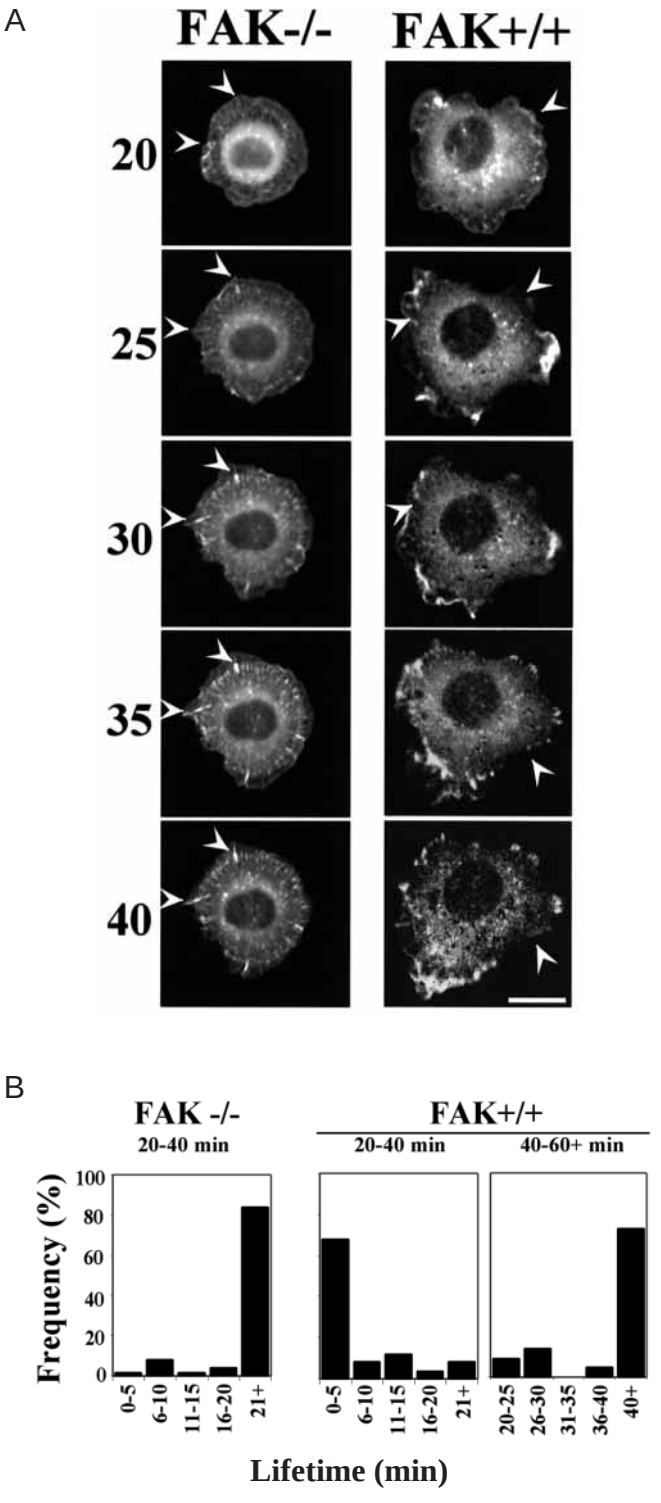

Fig. 3. Focal adhesion dynamics in FAK–/– and FAK+/+ cells. Cells transiently transfected with pGFP-α-actinin were replated on FN-coated glass coverslips as described in Fig. 1. (A) Typical time courses of GFP-α-actinin are shown for FAK+/+ cells (right panel) and FAK–/– cells (left panel). Arrowheads indicate individual focal adhesions that persist for FAK–/– cells or that rapidly disappear for FAK+/+ cells. (B) Quantification of focal adhesion turnover rate was carried out from time lapse analysis of images similar to A. Data are summarized in bar graphs showing percentage of focal adhesions as a function of lifetime.

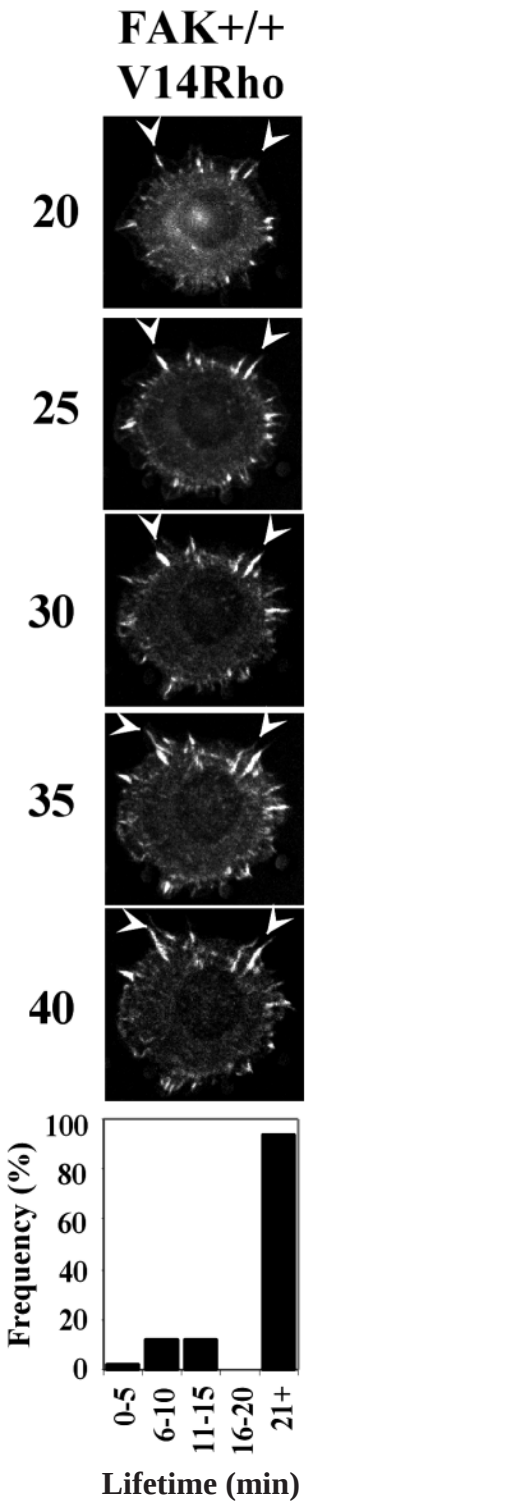

Fig. 4. Effect of V14-Rho on Cytoskeletal dynamics in FAK+/+ cells. Cells transiently transfected with V14-Rho and GFP-a-actinin were monitored to assess focal adhesion dynamics. Time lapse images are shown in the upper panels. The V14-Rho transfected cells were fixed after the experiments and confirmed by immunofluorescence staining with an anti-HA antibody (data not shown). Bottom panel shows the quantification of focal adhesion turnover obtained as described in Fig. 3.

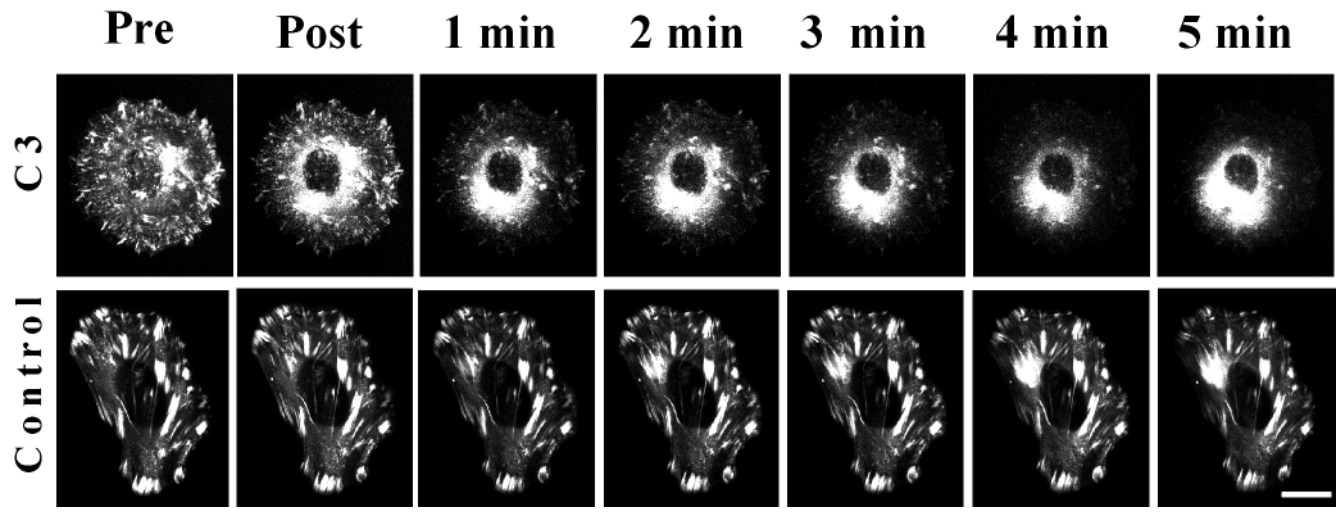

Fig. 5. Effect of C3 on focal adhesions in FAK–/– cells. FAK–/– cells expressing GFP-α-actinin were micro-injected with 1 mg/ml C3 (upper panels) or buffer only (lower panels) and the focal adhesions monitored as before.

Fig. 3. In FAK+/+ cells, adhesions were rapidly assembled and disassembled, rarely lasting for more than 5 minutes. Quantitation of results in the interval between 20 and 40 minutes showed that in the FAK+/+ cells most of the adhesions lasted less than 2 minutes (Fig. 3B). At later times, after spreading was complete (>40 minutes), stability of focal adhesions in these cells increased, though turnover was still detectable (Fig. 3B).

In FAK–/– cells, focal adhesions/complexes also formed rapidly after plating, but were substantially more stable, with the majority lasting more than 20 minutes (Fig. 3A and B). Interestingly, these complexes initially appeared primarily at the spreading edges of the cells under lamellipodia. Once assembled, they often grew larger or shifted location but generally persisted for the length of the experiment. As spreading of FAK–/– cells proceeded and cellular area increased, newer adhesions formed at the edge but the older adhesions remained. The greater stability of focal adhesions in FAK–/– cells therefore resulted in focal adhesions being distributed over the entire basal surface (Fig. 3A). These results show that Rho activity correlated inversely with focal adhesion turnover, with high turnover in cells that had low Rho activity and vice versa. The data also show that the unusual central focal adhesions in FAK–/– cells are a consequence of the reduced turnover.

Effects of activating or inhibiting Rho

To test whether the differences in focal adhesion turnover might be attributed to differences in Rho activity, FAK+/+ cells were co-transfected with GFP-α-actinin plus V14 Rho. The cells were then replated on FN-coated coverslips and focal adhesion lifetime assayed by time lapse imaging. Cells were again examined during the period of rapid spreading. In FAK+/+ cells co-transfected V14 Rho, focal adhesion turnover was dramatically reduced, reaching a level similar to the FAK–/– cells (Fig. 4). These results show that elevating Rho activity increases focal adhesion lifetime.

To test the rate of focal adhesion disassembly when Rho activity is decreased, FAK–/– cells transfected with the vector for GFP-a-actinin were plated on FN. After cells were fully spread (times >40 minutes), they were microinjected with C3 protein to inhibit Rho. Focal adhesions were then monitored as before. Cells injected with buffer alone sometimes retracted slightly immediately after injection, but retained focal adhesions (Fig. 5). By contrast, C3 caused their disappearance within minutes. As C3 inactivates Rho via an enzymatic reaction that cannot occur instantly after injection (Aktories, 1997), the rate of focal adhesion disruption in this experiment likely underestimates the true rate of turnover after loss of Rho activity. These results support the hypothesis that changes in Rho activity can account for the difference in focal adhesion turnover between FAK+/+ and FAK–/– cells.

DISCUSSION

These results demonstrate three key points. First, FAK is required for the transient decrease in Rho activity seen at early times during cell spreading when cells are rapidly extending lamellipodia and increasing their contact area with the substratum. Second, rapid focal adhesion turnover correlates with low Rho activity. Focal adhesions are unstable in rapidly spreading FAK+/+ cells but their stability increases at later times when Rho activity is higher. In FAK–/– cells where Rho activity is constitutively high, focal adhesions are stable at all times after plating. Third, changes in Rho activity appear to be able to account for the difference in focal adhesion turnover rates. Expressing V14Rho in FAK+/+ cells increases focal adhesion stability to the level seen in FAK–/– cells and induces a more circular shape with abundant focal contacts that cover much of the ventral surface, similar to FAK–/– cells. Conversely, inactivating Rho with high doses of C3 triggers rapid loss of focal adhesions or, at low levels, induces a more polarized morphology similar to FAK+/+ cells. These data support the hypothesis that Rho mediates some of the effects of FAK on the cytoskeleton.

Total FAK phosphorylation is increasing during the period of low Rho activity though does not reach its maximum until later when spreading is complete and focal adhesions have

formed (Burridge et al., 1992; Guan et al., 1991; Schlaepfer et al., 1994). Thus, the effect does not precisely correlate with total FAK phosphorylation, though there might be individual sites on FAK whose phosphorylation follows a similar pattern. In this regard, it is intriguing that FAK has been shown to interact with a RhoGAP named GRAF (GAP for Rho Associated with FAK), isolated from an embryonic chicken library (Hildebrand et al., 1996). This interaction was due to binding of the GRAF SH3 domain to a proline rich sequence in the C-terminus of FAK, and did not require FAK phosphorylation. Although GRAF has a restricted tissue distribution in the chicken, related proteins in mammalian systems have not been characterized. Thus, it seems plausible that a RhoGAP similar to GRAF could be responsible for the effects we observe.

Previous work has demonstrated that Rho activation induces focal adhesion formation and FAK activation (Kumagai et al., 1993; Barry et al., 1997). Our results show that once activated or localized, FAK induces Rho down-regulation. Rho, in turn, controls focal adhesion stability. The data therefore reveal the existence of a regulatory circuit that should result in homeostasis, preventing excessive formation of adhesions. Loss of this regulatory loop is likely to explain the defect in motility of FAK–/– cells (Ilic et al., 1995). Abnormal migration probably underlies defective gastrulation in FAK–/– mice, accounting for the embryonic lethality. In summary, our study shows that FAK is required for Rho inhibition to promote focal adhesion turnover and cell migration.

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