Mechanical forces facilitate actin polymerization at focal adhesions in a zyxin-dependent manner

Hiroaki Hirata[1,2], Hitoshi Tatsumi[3] and Masahiro Sokabe[1,2,3,*]

[1]Cell Mechanosensing Project, ICORP/SORST, Japan Science and Technology Agency, 65 Tsurumai, Showa-ku, Nagoya, Aichi 466-8550, Japan
[2]Department of Molecular Physiology, National Institute for Physiological Sciences, National Institutes of Natural Sciences, 38 Nishigonaka, Myodaiji-cho, Okazaki, Aichi 444-8585, Japan
[3]Department of Physiology, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya, Aichi 466-8550, Japan
*Author for correspondence (e-mail: msokabe@med.nagoya-u.ac.jp)

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Summary

We examined the effects of mechanical forces on actin polymerization at focal adhesions (FAs). Actin polymerization at FAs was assessed by introducing fluorescence-labeled actin molecules into permeabilized fibroblasts cultured on fibronectin. When cell contractility was inhibited by the myosin-II inhibitor blebbistatin, actin polymerization at FAs was diminished, whereas $\alpha_5\beta_1$ integrin remained accumulated at FAs. This suggests that actin polymerization at FAs depends on mechanical forces. To examine the action of mechanical forces more directly, the blebbistatin-treated cells were subjected to a sustained uniaxial stretch, which induced actin polymerization at FAs. These results demonstrate the novel role of mechanical forces in inducing actin polymerization at FAs. To reveal the molecular mechanism underlying the force-induced actin polymerization at FAs, we examined the distribution of zyxin, a postulated actin-regulatory protein. Actin-polymerizing activity was strong at zyxin-rich FAs. Accumulation of zyxin at FAs was diminished by blebbistatin, whereas uniaxial stretching of the cells induced zyxin accumulation. Displacing endogenous zyxin from FAs by expressing the FA-targeting region of zyxin decreased the force-induced actin polymerization at FAs. These results suggest that zyxin is involved in mechanical-force-dependent facilitation of actin polymerization at FAs.

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Key words: Focal adhesion, Mechanical force, Actin polymerization, Zyxin, Integrin

Introduction

Adhesive interactions of a cell with neighboring cells and with extracellular matrices (ECMs) are essential for cellular morphogenesis, migration, proliferation and differentiation. Adherent cells, including fibroblasts, epithelial cells and endothelial cells, develop specialized sites for adhesive interactions with the ECM, called focal adhesions (FAs). Integrins, heterodimeric transmembrane receptors for ECM proteins, are clustered at FAs, where a variety of cytoplasmic proteins, including vinculin, talin and α-actinin, are accumulated. At a FA, integrins are linked to the actin cytoskeleton via a plaque of the cytoplasmic proteins, anchoring the actin cytoskeleton to the ECM (Burridge and Chrzanowska-Wodnicka, 1996; Geiger et al., 2001).

Actomyosin-based contractile forces are transmitted from cells to the ECM at FAs (Harris et al., 1980; Chrzanowska-Wodnicka and Burridge, 1996; Balaban et al., 2001). Stimulation of contractility drives the development of FAs (Chrzanowska-Wodnicka and Burridge, 1996). Actually, inhibition of the contractile forces leads to the disassembly of FAs (Chrzanowska-Wodnicka and Burridge, 1996; Balaban et al., 2001). The size of individual FAs correlates well with the amount of force acting on them (Balaban et al., 2001), and the application of external forces to cells induces an enlargement of FAs (Riveline et al., 2001; Wang et al., 2001; Galbraith et al., 2002; Kaverina et al., 2002). Thus, mechanical forces are crucial for the regulation of FAs.

The linkage between integrin and the actin cytoskeleton at FAs is exposed to mechanical loads. When this linkage is dissected, actin stress fibers are retracted and FAs are disassembled (Pavalko and Burridge, 1991; Rajfur et al., 2002). Therefore, mechanical stability of the linkage is crucial for maintaining the organization of FAs and actin cytoskeleton. The linkage is strengthened in response to mechanical forces acting on integrin clusters (Wang et al., 1993; Choquet et al., 1997; Felsenfeld et al., 1999). Mechanical forces induce an accumulation of filamentous actin (F-actin) at the integrin clusters, and this F-actin is involved in strengthening the integrin–actin-cytoskeleton linkage (Glogauer et al., 1997; Glogauer et al., 1998). Thus, revealing the underlying molecular mechanism in the force-induced accumulation of F-actin might be indispensable for understanding the mechanical regulation of the FA organization. The local accumulation of F-actin could be based, primarily, on the redistribution of pre-existing actin filaments and/or de novo actin polymerization on site. However, the effect of mechanical forces on the redistribution of actin filaments and on actin polymerization at FAs remains largely unknown.

An FA is a site at which monomeric actin is incorporated (Glacy, 1983; Turnacioglu et al., 1998; Fradelizi et al., 2001). Actin regulatory proteins, including the Arp2/3 complex, mammalian Diaphanous (mDia)-related formins and Ena/VASP proteins, have been implicated in the process of actin polymerization at FAs (Beckerle, 1998; Calderwood et al., 2000; Blystone, 2004). Ena/VASP proteins are apparently located at FAs, whereas Arp2/3 and mDia are not, implying that Ena/VASP play a crucial role in the local actin polymerization at FAs.

The FA protein zyxin is involved in the recruitment of Ena/VASP to FAs (Drees et al., 1999; Drees et al., 2000; Nix et al., 2001; Hoffman et al., 2006). Zyxin has several binding partners, including

α-actinin and Ena/VASP, serving as a scaffold at FAs (Beckerle, 1997). Recently, several studies have shown that the dynamics of zyxin at FAs are affected by mechanical forces acting on FAs. Inhibition of actomyosin-based cell contractility induces the dislocation of zyxin from FAs (Rottner et al., 2001). Cyclic stretching and relaxing of cells results in the translocation of zyxin from FAs to nuclei (Cattaruzza et al., 2004) and actin stress fibers (Yoshigi et al., 2005). Lele et al. have demonstrated that the unbinding rate constant of zyxin at FAs increases with decreasing mechanical load on FAs (Lele et al., 2006). These results lead to the hypothesis that mechanical forces acting on FAs facilitate the recruitment of zyxin and Ena/VASP, inducing actin polymerization at FAs. However, it remains unknown whether zyxin is, in fact, involved in the regulation of actin polymerization at FAs.

In the present study, we examine the effect of mechanical forces on actin polymerization at FAs, and demonstrate that mechanical forces facilitate actin polymerization at FAs in a zyxin-dependent manner.

Results

Actin polymerization at zyxin-rich FAs

Human skin fibroblasts grown on fibronectin (FN) developed many FAs, which contained α_5 integrin (Fig. 1A). To assess actin polymerization at FAs, Alexa-Fluor-568-conjugated actin (Alexa568-actin) was applied to the cells in the presence of digitonin. Alexa568-actin was incorporated at FAs located in the peripheral region, with much less being incorporated in the central region of cells (Fig. 1B). The difference in the amount of actin that was incorporated in different regions did not seem to arise from the difference in accessibility of artificially introduced molecules to FAs depending on their location; when the mixture of Alexa568-actin (ca. 42 kDa) and anti-α_5-integrin cytoplasmic-domain antibody (ca. 150 kDa) was applied, the antibody was associated with FAs in both peripheral and central regions, whereas Alexa568-actin was incorporated preferentially at peripheral FAs (supplementary material Fig. S1). Alexa568-actin that was microinjected into living cells was also incorporated preferentially at peripheral FAs (supplementary material Fig. S1). The incorporation of exogenous actin at FAs in permeabilized cells was markedly reduced in the presence of 0.1 μM cytochalasin D, a potent inhibitor of actin polymerization at barbed ends of actin filaments (Fig. 1C-F), indicating that the incorporation is primarily caused by actin polymerization from pre-existing free barbed ends, as shown previously (Chan et al., 1998).

The difference in the level of actin polymerization between peripheral and central FAs might depend on the molecular composition of the FAs. We examined the distribution of the FA protein zyxin, which has a capability to induce actin assembly in an Ena/VASP-dependent manner (Fradelizi et al., 2001). Zyxin was accumulated at peripheral FAs, but less so at central FAs (Fig. 1G,H). VASP was colocalized with zyxin to peripheral FAs (Fig. 1I-K and supplementary material Fig. S2). Sites of zyxin accumulation corresponded to the sites at which Alexa568-actin was incorporated (Fig. 1L-N). These results indicate that actin-polymerizing activity is strong at zyxin-rich FAs.

Accumulation of zyxin is involved in actin polymerization at FAs

We examined whether zyxin is involved in the actin polymerization at peripheral FAs. For this purpose, the green fluorescent protein (GFP)-tagged LIM region of human zyxin (ZYX_{LIM}-GFP) was

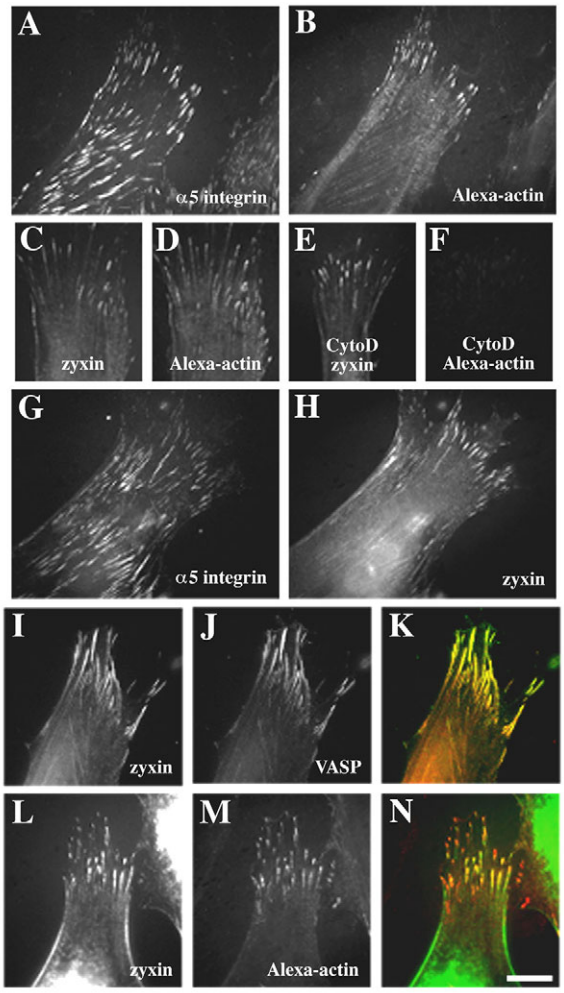

Fig. 1. Actin polymerization and zyxin accumulation at peripheral FAs in cells grown on FN. (A,B) Cells were incubated with Alexa568-actin in 0.003% digitonin for 1 minute. After fixation, the cells were stained for α_5 integrin. (A) α_5 integrin; (B) Alexa568-actin. (C-F) Cells were incubated with Alexa568-actin (D,F) in digitonin supplemented with (E,F) or without (C,D) 0.1 μM cytochalasin D. The cells were fixed and stained for zyxin (C,E). (G,H) A cell that was double stained for α_5 integrin (G) and zyxin (H). (I-K) A cell that was double stained for zyxin (I, red in K) and VASP (J, green in K). (L-N) Cells to which Alexa568-actin was introduced (M, red in N) were stained for zyxin (L, green in N). Scale bar: 20 μm.

expressed, because this region is responsible for recruiting zyxin to FAs and expression of the isolated LIM region causes the displacement of endogenous zyxin from FAs (Nix et al., 2001). When expressed in human skin fibroblasts, ZYX_{LIM}-GFP localized to peripheral FAs (Fig. 2A,G,I). Less endogenous zyxin was accumulated at FAs in cells expressing ZYX_{LIM}-GFP at a higher level (Fig. 2B,E-M). To quantitatively analyze the effect of the expression of ZYX_{LIM}-GFP on the accumulation of endogenous zyxin at FAs, the fluorescence intensity of endogenous zyxin at FAs was averaged and plotted against that of ZYX_{LIM}-GFP for each cell (Fig. 2N; see Materials and Methods). The fluorescence intensity of endogenous zyxin at FAs was negatively correlated with that of ZYX_{LIM}-GFP (Fig. 2N,P). GFP alone neither localized to FAs (Fig. 2C) nor perturbed zyxin accumulation at FAs (Fig. 2D,O,P). VASP was also dislocated from FAs when ZYX_{LIM}-GFP was expressed (Fig. 2P and supplementary material Fig. S3), as previously reported

(Nix et al., 2001). By contrast, the accumulation of α_v integrin at FAs was not affected by the expression of ZYX_{LIM}-GFP (Fig. 2P and supplementary material Fig. S3), indicating that FAs themselves were not disassembled in cells expressing ZYX_{LIM}-GFP. Accumulation of vinculin and palladin, FA proteins that, similar to zyxin, are capable of binding to VASP and α-actinin, was not affected by the expression of ZYX_{LIM}-GFP (Fig. 2P and supplementary material Figs S4 and S5). Expression levels of the endogenous FA proteins zyxin, VASP and α-actinin in cells transfected with ZYX_{LIM}-GFP did not significantly differ from those in cells transfected with GFP or zyxin-GFP (supplementary material Fig. S6).

We assessed actin incorporation at FAs in ZYX_{LIM}-GFP-expressing cells. The incorporation of Alexa568-actin at peripheral FAs was decreased in cells expressing ZYX_{LIM}-GFP (Fig. 3A,B). The average fluorescence intensity of Alexa568-actin at FAs was negatively correlated with that of ZYX_{LIM}-GFP (Fig. 3G,I). Expression of GFP alone did not affect the incorporation of Alexa568-actin at FAs (Fig. 3C,D). A positive correlation between the expression of zyxin-GFP and actin incorporation at peripheral FAs was observed in three out of four cases (Fig. 3E,F,H,I). All these results suggest that zyxin is involved in actin polymerization at FAs.

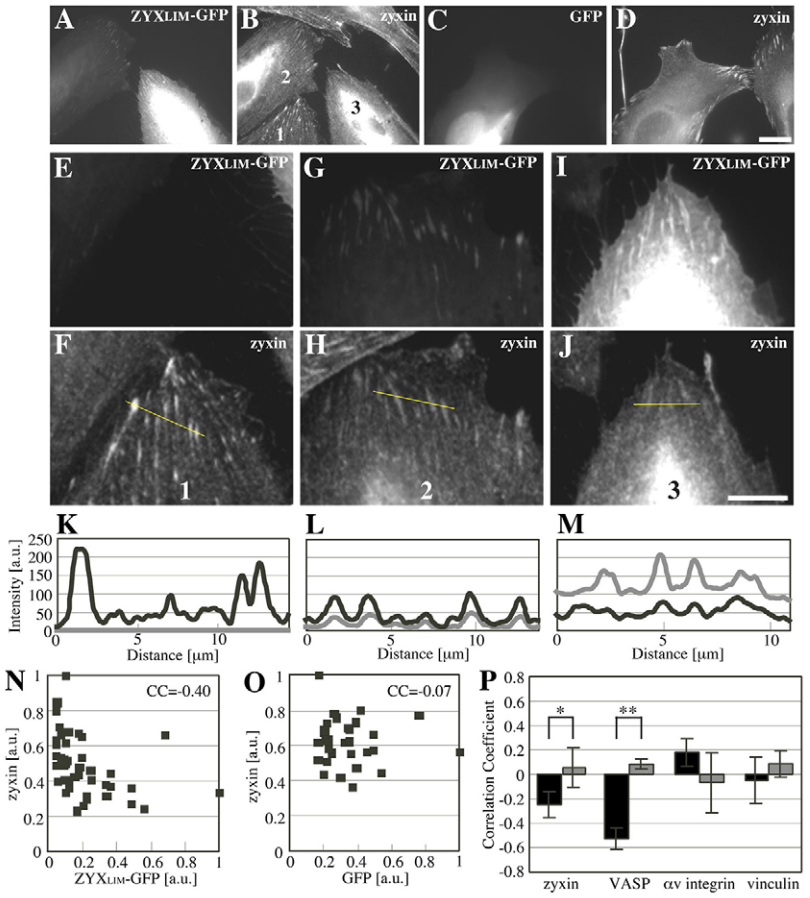

Fig. 2. Displacement of zyxin from FAs by expressing the isolated LIM region (338-572 aa) of human zyxin. (A-D) Cells were grown on FN and transfected with ZYX_{LIM}-GFP (A) or GFP (C). These cells were stained for endogenous zyxin (B and D, respectively). Endogenous zyxin was detected with an antibody against aa 134-150 of human zyxin. (E-J) Magnified images of cells numbered 1 (E,F), 2 (G,H) and 3 (I,J) in B. ZYX_{LIM}-GFP (E,G,I); endogenous zyxin (F,H,J). Scale bars: 20 μm. (K-M) Intensity profiles of the fluorescence images of ZYX_{LIM}-GFP (light-gray lines) and of endogenous zyxin (black lines) along the yellow lines in F,H and J are shown in K,L and M, respectively. (N,O) The averaged fluorescence intensity of endogenous zyxin at FAs was plotted against that of ZYX_{LIM}-GFP (N) or GFP (O) for each cell. Values were normalized with respect to the maximum value on each axis. CC, correlation coefficient. (P) Correlation coefficients of average fluorescence-intensity plots of the FA protein (zyxin, VASP, α_v integrin or vinculin) vs ZYX_{LIM}-GFP (black bars) or GFP (gray bars). Fluorescence images and intensity plots for VASP, α_v integrin and vinculin are shown in supplementary material Figs S3 and S4. Each bar represents the mean ± s.d. of three independent experiments. *P<0.05; **P<0.005 (t-test).

Mechanical forces induce zyxin accumulation at FAs and facilitate local actin polymerization

Both peripheral and central FAs contained the $\alpha_5\beta_1$-integrin heterodimer (supplementary material Fig. S2), talin (Fig. 4A-C) and β_1 integrin in a ligand-binding form (Fig. 4G,H), indicating that both sets of FAs were functional. However, zyxin was accumulated and actin was polymerized preferentially at peripheral FAs (Fig. 1). Two major classes of FAs are found in fibroblasts: the $\alpha_5\beta_1$-integrin-dominated fibrillar adhesion and the $\alpha_v\beta_3$-integrin-dominated focal contact (Singer et al., 1988; Zamir et al., 1999; Katz et al., 2000). Because α_v integrin was preferentially accumulated at peripheral FAs (supplementary material Fig. S2) (Katz et al., 2000) and colocalized with zyxin (supplementary material Fig. S2), zyxin seems to be a constituent of $\alpha_v\beta_3$-integrin-mediated adhesion structures (i.e. focal contacts). Consistently, α-actinin, a constituent of focal contacts (Katz et al., 2000), was also colocalized with zyxin at peripheral FAs (supplementary material Fig. S2). To examine the role of $\alpha_v\beta_3$ integrin in zyxin accumulation and actin polymerization, cells were grown on vitronectin (VN), a ligand for $\alpha_v\beta_3$ integrin. FAs containing α_v integrin were distributed in both peripheral and central regions of cells grown on VN (Fig. 5A,C). However, zyxin was accumulated (Fig. 5B) and actin was incorporated (Fig. 5D) preferentially at peripheral FAs in these cells, indicating that the accumulated α_v integrin alone is not sufficient to induce zyxin accumulation and actin polymerization.

The cytoplasmic molecular composition of FAs is affected not only by integrin species but also by mechanical loads at FAs (Katz et al., 2000). To assess the effect of mechanical forces on zyxin accumulation and actin polymerization at FAs, cells were treated with a myosin-II-specific inhibitor, blebbistatin (Straight et al., 2003), to inhibit actomyosin-based cellular contractile forces. Blebbistatin treatment diminished both zyxin accumulation (Fig. 6A-D) and actin polymerization (Fig. 6E-H) at FAs; however, α_5 integrin was accumulated (Fig. 6C,G) with talin (Fig. 4D-F) and β_1 integrin in a ligand-binding form (Fig. 4I,J). In these cells, a small fraction of α_v-integrin clusters remained, but these clusters did not contain zyxin (Fig. 6I-N), indicating again that accumulation of α_v integrin is not enough to induce zyxin accumulation. Because blebbistatin might affect myosin-II-independent processes (Shu et al., 2005), we also examined the effect of inhibiting Rho kinase, which regulates myosin-II activity (Fukata et al., 2001), with the Rho-kinase-specific inhibitor Y-27632 (Uehata et al., 1997), and obtained similar results (supplementary material Fig. S7). Therefore,

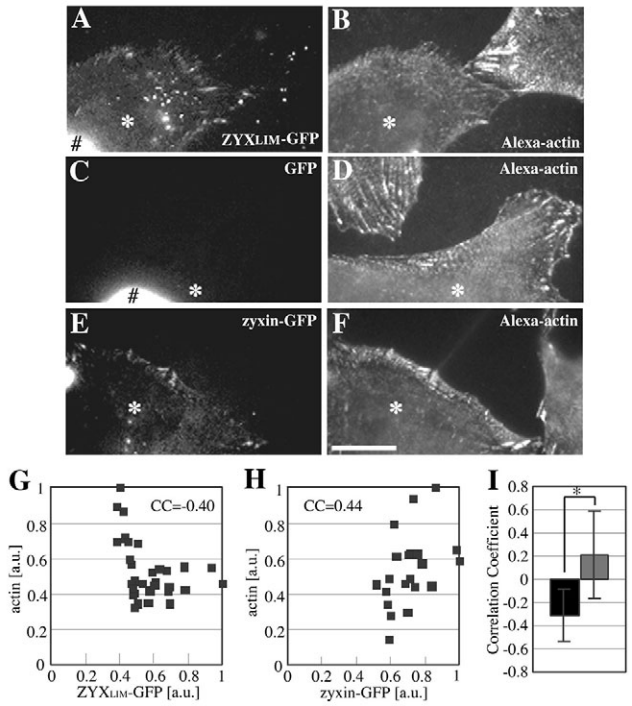

Fig. 3. Actin polymerization at FAs is decreased by displacing zyxin from FAs. Endogenous zyxin was displaced from FAs by expressing the isolated LIM region of human zyxin. (A-F) Alexa568-actin (B,D,F) was introduced into cells transfected with ZYX_{LIM}-GFP (A), GFP (C) or zyxin-GFP (E). Asterisks indicate the cells expressing the exogenous molecules. Large amounts of the exogenous molecules in the cytoplasm were extracted from the cells during the introduction of Alexa568-actin in the presence of digitonin, but ZYX_{LIM}-GFP and GFP in nuclei (# in A,C) were retained. Scale bar: 20 μm. (G,H) The averaged fluorescence intensity of Alexa568-actin at FAs was plotted against that of ZYX_{LIM}-GFP (G) or zyxin-GFP (H) for each cell. Values were normalized with respect to the maximum value on each axis. CC, correlation coefficient. (I) Correlation coefficients of average fluorescence-intensity plots of Alexa568-actin vs ZYX_{LIM}-GFP (black bars) or zyxin-GFP (gray bars). Each bar represents the mean ± s.d. of four independent experiments. *P<0.05 (t-test).

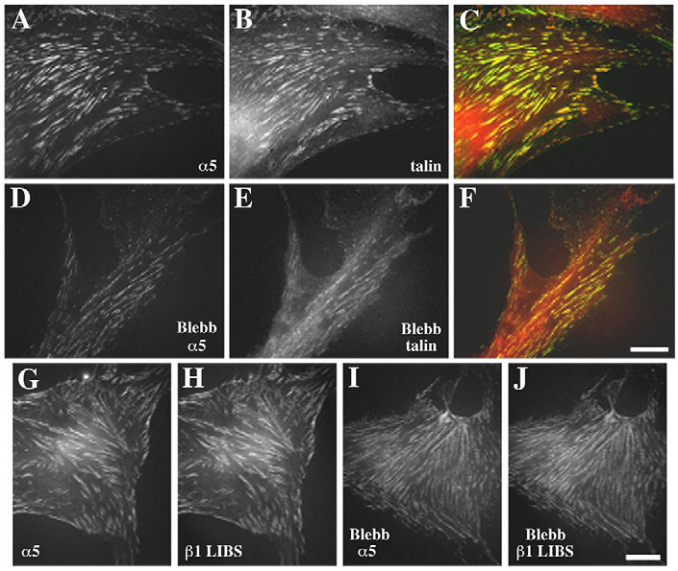

Fig. 4. Talin and β_1 integrin in a ligand-binding form accumulate at both peripheral and central FAs regardless of treatment with blebbistatin. (A-F) Cells grown on FN were treated with (D-F) or without (A-C) 100 μM blebbistatin for 30 minutes, and then fixed and double stained for α_5 integrin (A,D, green in C,F) and talin (B,E, red in C,F). (G-J) Cells treated with (I,J) or without (G,H) 100 μM blebbistatin for 30 minutes were fixed and double stained with anti-α_5-integrin antibody (G,I) and mAb B44, which recognizes the ligand-induced binding site (LIBS) on β_1 integrin (H,J). Scale bars: 20 μm.

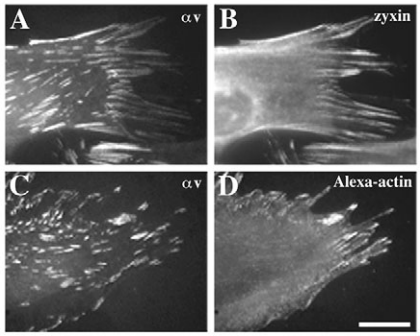

Fig. 5. Accumulation of α_v integrin alone is not sufficient to induce zyxin accumulation and actin polymerization. (A,B) A cell grown on VN was double stained for α_v integrin (A) and zyxin (B). (C,D) Alexa568-actin was introduced into a cell grown on VN (D) and the cell was stained for α_v integrin (C). Scale bar: 20 μm.

zyxin accumulation and actin polymerization at peripheral FAs presumably depend on actomyosin-based mechanical forces.

To examine the action of mechanical forces on zyxin accumulation and actin polymerization more directly, mechanical forces were applied to blebbistatin-treated cells by stretching the elastic silicone substratum to which the cells adhered. Uniaxial stretching of the substratum (50% stretch for 3 minutes) induced both zyxin accumulation (Fig. 7A-D) and actin polymerization (Fig. 8A-D) in blebbistatin-treated cells. Zyxin and polymerized actin were accumulated at peripheral FAs (Fig. 7C,D; Fig. 8C,D), and along F-actin bundles near their ends (Fig. 7G-I; Fig. 8G-I). Central FAs did not yield zyxin accumulation and actin polymerization upon the stretch (red arrows in Fig. 7C and Fig. 8C). When the bundles were oriented at larger angles to the stretch axis, accumulation of zyxin decreased and actin was less polymerized along F-actin bundles (Fig. 7E,F; Fig. 8E,F); quantitative analyses confirmed these angular dependencies (Fig. 7J; Fig. 8J). All these results strongly suggest that mechanical forces are responsible for zyxin accumulation and actin polymerization at FAs.

The role of mechanical forces in zyxin accumulation was also examined without pharmacological treatments. Cells expressing zyxin-GFP were grown on a flexible polyacrylamide substratum coated with FN. When the substratum was locally deformed towards the cell, the fluorescence intensity of zyxin-GFP at FAs decreased (supplementary material Fig. S8 and Movie 1). When the substratum was stretched again, the fluorescence intensity recovered (supplementary material Fig. S8 and Movie 1). These results confirm that mechanical forces regulate zyxin accumulation at FAs.

Zyxin was shown to be involved in actin polymerization at FAs (Fig. 3). We examined the role of zyxin in the stretch-induced actin polymerization observed in blebbistatin-treated cells. When cells expressing ZYX_{LIM}-GFP were treated with blebbistatin, ZYX_{LIM}-GFP was dislocated from peripheral FAs (Fig. 9). When the

substratum was stretched, ZYX_{LIM}-GFP was again accumulated at peripheral FAs in these cells (Fig. 10A), suggesting that the localization of the isolated LIM region to FAs is also force dependent. Stretch-induced accumulation of endogenous zyxin at FAs was decreased by expressing ZYX_{LIM}-GFP (Fig. 10A,B) but not GFP (Fig. 10C,D). Thus, stretch-induced zyxin accumulation was inhibited by ZYX_{LIM}-GFP. Inhibition of zyxin accumulation by expressing ZYX_{LIM}-GFP suppressed stretch-induced actin polymerization at peripheral FAs (Fig. 10E,F). Expression of GFP alone had no effect on stretch-induced actin polymerization (Fig. 10G,H). These results suggest that mechanical force induces the accumulation of zyxin at peripheral FAs, leading to actin polymerization in blebbistatin-treated cells.

Discussion

It has repeatedly been shown that mechanical forces play a crucial role in the molecular assembly of FAs. In the current study, we have demonstrated a novel aspect of mechanical forces: that they facilitate actin polymerization at FAs. Furthermore, we have revealed that the force-dependent accumulation of zyxin at FAs is crucial for this process.

Mechanical-force-induced accumulation of zyxin at FAs

Recently, it has been revealed that mechanical cues affect the distribution of zyxin to FAs. Zyxin dissociated from FAs when the mechanical load on the FAs was reduced by inhibiting the actomyosin interaction, by ablating individual stress fibers with a focused laser or by softening the substratum (Rottner et al., 2001; Lele et al., 2006) (this study). These results suggest that mechanical loads on FAs are required for the recruitment of zyxin to FAs. In the present study, we showed, by stretching the elastic silicone substratum and by deforming locally the polyacrylamide gel substratum, that externally applied mechanical forces induce zyxin accumulation at FAs, thus demonstrating the role of mechanical forces in localizing zyxin to FAs. It has previously been reported that cyclic stretching and relaxing the substrata resulted in the dislocation of zyxin from FAs (Cattaruzza et al., 2004; Yoshigi et al., 2005). Combined with our results, this suggests that sustained mechanical loads are necessary for zyxin accumulation and that a transient decrease in the mechanical force causes dislocation of zyxin from FAs.

We found that zyxin is accumulated preferentially at peripheral FAs. On FN, $\alpha_v\beta_3$ integrin was preferentially accumulated at peripheral FAs, suggesting that $\alpha_v\beta_3$ integrin is involved in recruiting zyxin to FAs. However, accumulation of $\alpha_v\beta_3$ integrin is not sufficient to induce zyxin accumulation, because α_v-integrin clusters in central regions of cells on VN (Fig. 5) or in cells treated with blebbistatin (Fig. 6) were not associated with zyxin. Larger traction forces are presumably exerted at peripheral FAs than at central FAs (Tan et al., 2003). Therefore, the large mechanical loads on peripheral FAs might facilitate the accumulation of zyxin.

Uniaxial stretching of the substratum induced zyxin accumulation and actin polymerization in blebbistatin-treated cells. When F-actin bundles were oriented at larger angles to the stretch axis, zyxin was less accumulated and actin was less polymerized upon the stretch. This suggests that zyxin accumulation is dependent on the amplitude of the stress in the actin-cytoskeleton–FA complexes, because the stress in the actin bundles that are oriented at larger angles to the stretch axis should be smaller. This could be a part of the mechanism in which FAs and the actin cytoskeleton sense and respond to the direction of tension.

The isolated LIM region of zyxin accumulated at FAs in a force-dependent manner, and expression of the LIM region inhibited the force-induced accumulation of endogenous zyxin at FAs. These results indicate that the LIM region of zyxin is crucial for the force-dependent recruitment of zyxin to FAs. The LIM region of zyxin interacts with the FA-associated adapter protein p130Cas (Yi et al., 2002). However, zyxin is distributed to FAs in p130Cas-deficient cells (Yi et al., 2002), indicating that the interaction between zyxin and p130Cas is not necessary for the force-induced recruitment of zyxin to FAs. The zyxin LIM region also binds to cysteine-rich protein (CRP) (Schmeichel and Beckerle, 1994). Because CRP is located at FAs (Sadler et al., 1992), the interaction of zyxin with CRP might contribute to the localization of zyxin. The N-terminal domain of zyxin binds to α-actinin, and this interaction is involved in the recruitment of zyxin to FAs (Drees et al., 1999; Reinhard et al., 1999). However, it is not clear whether the zyxin LIM region affects the interaction between zyxin and α-actinin. CRP and α-actinin could be potential candidates for the molecule responsible for the force-induced recruitment of zyxin to FAs. Future study is needed to resolve this issue.

Little zyxin exists at focal complexes (FXs) – small and short-lived adhesive structures formed just behind the leading edge of a

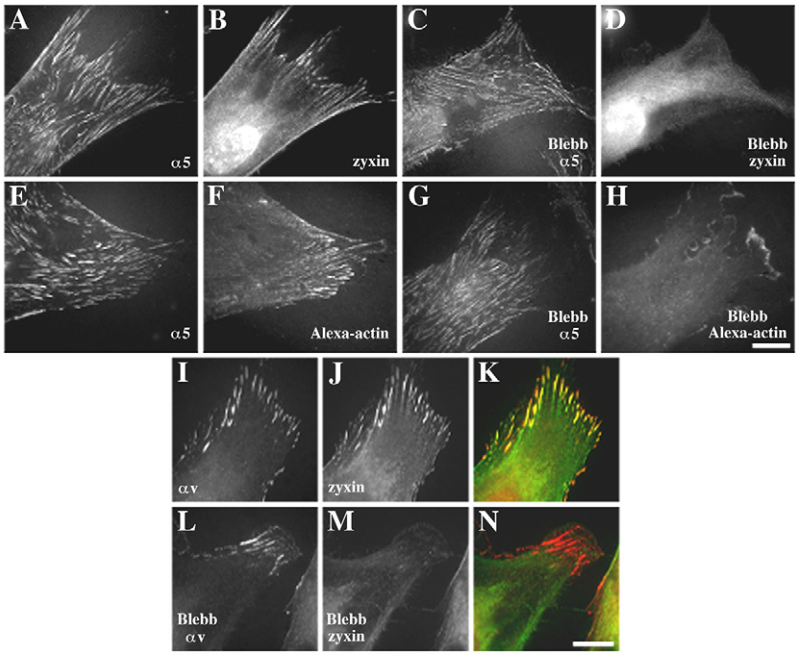

Fig. 6. Inhibition of myosin-II activity diminishes zyxin accumulation and actin polymerization at peripheral FAs. Cells were grown on FN. (A-D) Cells were treated with (C,D) or without (A,B) 100 μM blebbistatin for 30 minutes, and were then double stained for α_5 integrin (A,C) and zyxin (B,D). (E-H) Alexa568-actin (F,H) was introduced into cells treated with (G,H) or without (E,F) 100 μM blebbistatin for 30 minutes. The cells were fixed and stained for α_5 integrin (E,G). (I-N) Cells were treated with (L-N) or without (I-K) 100 μM blebbistatin for 30 minutes, and were then double-stained for α_v integrin (I,L, red in K,N) and zyxin (J,M, green in K,N). Scale bars: 20 μm.

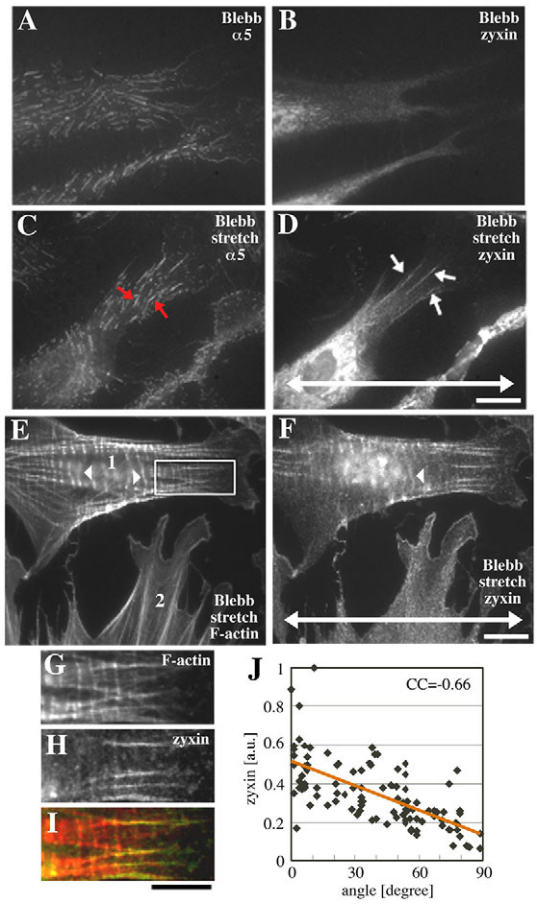

Fig. 7. Stretching of substrata induces zyxin accumulation at peripheral FAs in cells in which myosin II is inhibited. Cells grown on an FN-coated elastic substratum were treated with 100 μM blebbistatin for 30 minutes and then the substratum was uniaxially stretched (50% for 3 minutes) in the presence of blebbistatin. (A-D) Cells with (C,D) or without (A,B) stretching of the substratum were double stained for α5 integrin (A,C) and zyxin (B,D). (D) Arrows indicate accumulated zyxin at peripheral FAs. Double-headed arrow indicates the direction of the stretch axis. (C) Red arrows indicate the central α5-integrin clusters that are not associated with zyxin accumulation. (E-I) Stretched cells were double stained for F-actin (E,G, red in I) and zyxin (F,H, green in I). The boxed area in E is shown at higher magnification in G-I: zyxin was accumulated along F-actin bundles near their ends. (E) Zyxin was accumulated along F-actin bundles that were oriented parallel to the stretch axis (cell 1) but was not found along the bundles perpendicular to the axis (cell 2). Folds perpendicular to the stretch axis (arrowheads in E,F) were generated by relaxing the stretched substrata for observations. Scale bars: 20 μm (A-F); 10 μm (G-I). (J) The fluorescence intensity of zyxin near the end of an F-actin bundle was plotted against the angle of the bundle to the stretch axis. The fluorescence intensities within 6 μm along the F-actin bundle from its tip were measured and normalized with respect to the maximum value. A total of 104 F-actin bundles in 27 cells were plotted. The red line represents the linear fitting. CC, correlation coefficient.

cell (Zaidel-Bar et al., 2003). FXs are formed independently of Rho-kinase-mediated myosin activation (Rottner et al., 1999). When Rho kinase is activated, FXs mature into FAs (Rottner et al., 1999; Totsukawa et al., 2004). This maturation accompanies recruitment of zyxin to the adhesion sites (Zaidel-Bar et al., 2003). These results suggest that the myosin activity mediated by Rho kinase affects zyxin localization to adhesion sites, supporting the notion of the force dependence of zyxin accumulation.

Role of zyxin in actin polymerization at FAs

Zyxin is related in sequence and structure to the bacterial protein ActA (Golsteyn et al., 1997), which is a bacterial factor required for actin polymerization on the bacterial surface within the cytoplasm of the infected host (Domann et al., 1992; Kocks et al., 1992). Therefore, it has been implied that zyxin contributes to the regulation of actin polymerization in mammalian cells. Consistent with this, zyxin mutants with a plasma-membrane- or mitochondria-targeting sequence induced a local actin assembly (Golsteyn et al., 1997; Fradelizi et al., 2001). However, the role of endogenous zyxin in the regulation of actin polymerization remained to be elucidated. In this study, we showed that actin-polymerizing activity is strong at zyxin-rich FAs, and that displacing zyxin from FAs impaired the polymerization, suggesting that accumulation of endogenous zyxin at FAs is crucial for actin polymerization at these sites.

Similar to zyxin, two FA proteins – vinculin and palladin – are capable of binding to Ena/VASP and α-actinin (Critchley 2000; Otey et al., 2005). Displacing zyxin from FAs or zyxin knockout causes dislocation of Ena/VASP from FAs without affecting accumulation of vinculin (Drees et al., 1999; Drees et al., 2000; Nix et al., 2001; Hoffman et al., 2006) (this study) and palladin (this study). These results suggest that vinculin or palladin cannot be an alternative to zyxin in Ena/VASP recruitment and actin incorporation at FAs. The accumulation of VASP at FAs was also dependent on the actomyosin interaction (our unpublished result). Ena/VASP would enhance actin polymerization through its anti-capping effect on the barbed end of F-actin and/or through its interaction with the monomeric actin-binding protein profilin (Krause et al., 2003). Thus, the force-induced accumulation of zyxin would recruit Ena/VASP to FAs and facilitate the local actin polymerization.

FA is an active site for actin polymerization (Glacy, 1983; Turnacioglu et al., 1998; Fradelizi et al., 2001). The actin regulatory protein mDia1 (DIAPH1) is involved in actin polymerization at FAs (Butler et al., 2006; Hotulainen and Lappalainen, 2006). However, some polymerization is still observed at FAs in mDia1-depleted cells, suggesting that mDia1-independent mechanisms also exist (Hotulainen and Lappalainen, 2006). In the present study, we revealed that the accumulation of zyxin enhances actin polymerization at FAs. It is likely that both zyxin-Ena/VASP-dependent and mDia-dependent processes are involved in actin polymerization at FAs. It should be noted that the actin-polymerizing activity at FAs almost disappeared when the cells were treated with blebbistatin or Y-27632. This suggests that, irrespective of the regulatory pathway, actin-polymerizing activity at FAs depends on actomyosin-based mechanical forces. A theoretical study predicted that mDia-induced actin polymerization would be facilitated by mechanical forces (Kozlov and Bershadsky, 2004). However, the exact roles of forces in mDia-dependent actin polymerization at FAs have not been examined experimentally. To date, the zyxin-dependent process presented here is the only experimentally evidenced force-dependent regulatory mechanism of actin polymerization at FAs.

Mechanical forces acting on FAs induce enlargement of FAs (Balaban et al., 2001; Riveline et al., 2001; Wang et al., 2001; Galbraith et al., 2002; Kaverina et al., 2002). However, it is unlikely that force-dependent localization of zyxin to FAs plays a crucial role in the size control of FAs, because the size of accumulations of α_v integrin and vinculin was not apparently changed in cells expressing the isolated LIM region of zyxin (supplementary material Figs S3 and S4), and the morphology of FAs is not altered in zyxin-

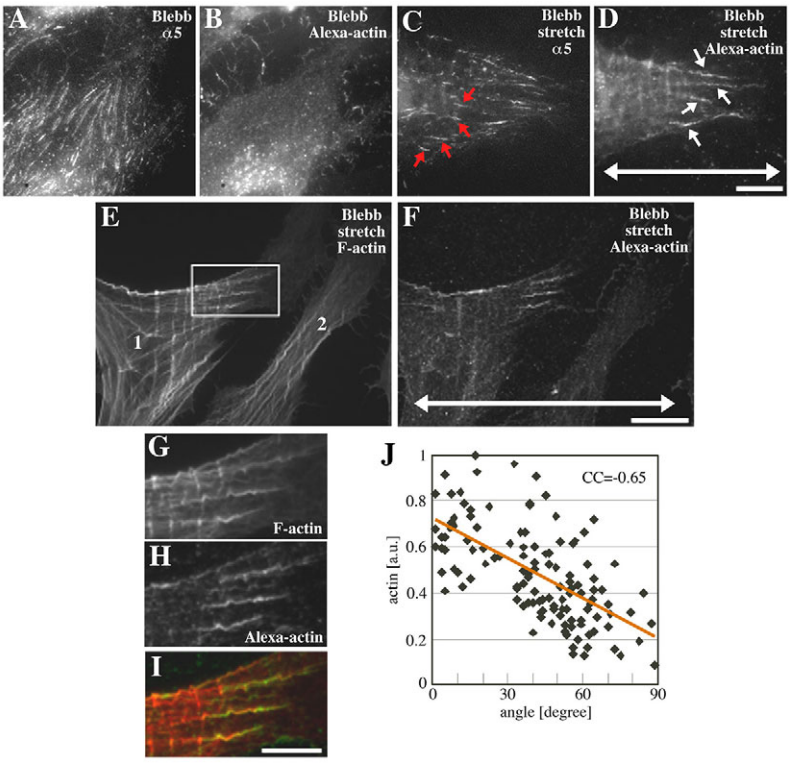

Fig. 8. Stretching of substrata induces actin polymerization at peripheral FAs in cells in which myosin II is inhibited. Cells grown on an FN-coated elastic substratum were treated with 100 μM blebbistatin for 30 minutes and then the substratum was uniaxially stretched, as in Fig. 7. (A-D) Alexa568-actin (B,D) was introduced into cells with (C,D) or without (A,B) stretching of the substratum. The cells were stained for α_5 integrin (A,C). (D) Arrows indicate actin incorporated at peripheral FAs. Double-headed arrow indicates the direction of the stretch axis. (C) Red arrows indicate the central α_5-integrin clusters that are not associated with actin incorporation. (E-I) Alexa568-actin was introduced into cells with stretching of the substratum (F,H, green in I). The cells were stained for F-actin (E,G, red in I). The boxed area in E is shown at higher magnification in G-I: actin was incorporated along F-actin bundles near their ends. (E) Actin was incorporated along F-actin bundles oriented parallel to the stretch axis (cell 1) but was not along the bundles oriented with large angles to the axis (cell 2). Folds perpendicular to the stretch axis were generated as in Fig. 7. Scale bars: 20 μm (A-F); 10 μm (G-I). (J) The fluorescence intensity of Alexa568-actin near the end of an F-actin bundle was plotted against the angle of the bundle to the stretch axis. The fluorescence intensities within 6 μm along the F-actin bundle from its tip were measured and normalized with respect to the maximum value. A total of 123 F-actin bundles in 34 cells were plotted. The red line represents the linear fitting. CC, correlation coefficient.

null fibroblasts (Hoffman et al., 2006). The molecular mechanism regulating the size of FAs in response to forces is still unknown.

Mechanical-force-induced actin polymerization at FAs increases the local amount of F-actin at FAs. Because FAs contain many actin-binding proteins (Geiger et al., 2001), changes in the amount of F-actin at FAs might affect the structure and function of FAs. Consistent with this idea, an FA protein with actin-binding capability, α-actinin, accumulates preferentially at peripheral FAs (Katz et al., 2000), which are the active sites for actin polymerization. α-actinin exhibits correlated motion with F-actin within FAs (Brown et al., 2006; Hu et al., 2007), suggesting its association with F-actin. The accumulation of F-actin at FAs would strengthen the integrin–actin-cytoskeleton linkage (Glogauer et al., 1998). The possible regulation of FAs through F-actin accumulation is based on a balance between actin polymerization at FAs (this study) and retrograde flux of F-actin from FAs (Guo and Wang, 2007; Endlich et al., 2007). We have demonstrated here that mechanical forces induce actin polymerization at FAs and that the polymerization is dependent on the force-induced accumulation of zyxin at FAs. Further studies are needed to reveal the underlying mechanisms of this process, including which molecule is the mechanosensor, how it senses mechanical forces, how the sensed forces are used for zyxin accumulation, and how zyxin acts on other molecules and facilitates actin polymerization at FAs.

Materials and Methods

Cell culture

Human foreskin fibroblasts (Hs-68 cells), were cultured in Dulbecco's modified Eagle's medium (Sigma Chemical, St Louis, MO) supplemented with 10% fetal bovine serum (Nipro, Osaka, Japan) at 37°C in 5% CO_2. For experiments, cells were grown for 15 hours on glass coverslips, elastic silicone (polydimethylsiloxane elastomer) chambers (Strex, Osaka, Japan) or polyacrylamide-gel substrata. Glass coverslips and silicone chambers were pre-coated with either 100 μg/ml FN (Sigma Chemical) or VN (Life Laboratory, Yamagata, Japan).

Antibodies

Mouse anti-talin, -vinculin and -β-actin monoclonal antibodies (mAbs), and the rabbit anti-zyxin polyclonal antibody were purchased from Sigma Chemical. The mouse anti-zyxin mAb (clone 2C10-4A7) was from Abnova (Taipei, Taiwan). The rabbit anti-α_5-integrin antibody was from Biogenesis (Poole, UK). The mouse anti-α_v-integrin mAb was from Calbiochem (San Diego, CA). The B44 mAb, which recognizes the ligand-induced binding site on β_1 integrin (Ni et al., 1998), and the mouse anti-$\alpha_5\beta_1$-integrin mAb were from Chemicon (Temecula, CA). The mouse anti-α-actinin mAb was from Upstate (Lake Placid, NY). The rabbit anti-VASP antibody was from Immunoglobe (Himmelstadt, Germany). The rabbit anti-palladin antibody was from Proteintech Group (Chicago, IL). The rabbit anti-GFP antibody was from Medical and Biological Laboratories (Nagoya, Japan). Alexa-Fluor-488-conjugated chicken anti-rabbit IgG, Alexa-Fluor-546-conjugated goat anti-mouse IgG, Alexa-Fluor-594-conjugated chicken anti-rabbit IgG and Alexa-Fluor-488-conjugated goat anti-mouse IgG antibodies, and Alexa488-phalloidin were from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-conjugated anti-mouse and -rabbit IgG antibodies were from GE Healthcare (Little Chalfont, UK).

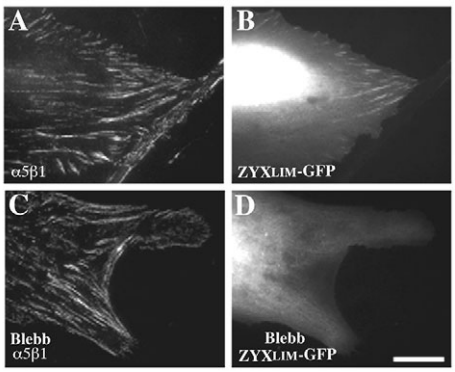

Fig. 9. Inhibition of myosin-II activity diminishes the accumulation of ZYX_{LIM}-GFP at peripheral FAs. Cells expressing ZYX_{LIM}-GFP (B,D) were treated with (C,D) or without (A,B) 100 μM blebbistatin for 30 minutes and were then stained for $\alpha_5\beta_1$ integrin (A,C). Scale bar: 20 μm.

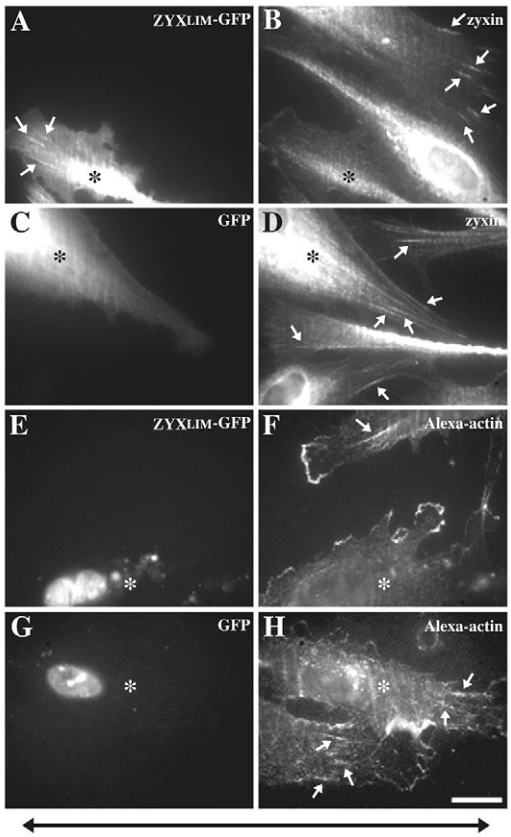

Fig. 10. Expressing ZYX_{LIM}-GFP suppresses stretch-induced zyxin accumulation and actin polymerization at FAs. Cells transfected with either ZYX_{LIM}-GFP or GFP were grown on FN-coated elastic substrata and were treated with 100 μM blebbistatin for 30 minutes. The substrata were then uniaxially stretched as in Fig. 7. (A-D) Cells transfected with ZYX_{LIM}-GFP (A) or GFP (C) were stained for zyxin (B,D) after stretching of the substrata. Arrows in A indicate accumulated ZYX_{LIM}-GFP, and arrows in B and D indicate accumulated zyxin. Asterisks indicate the cells expressing the exogenous molecules. (E-H) Alexa568-actin (F,H) was introduced into cells transfected with ZYX_{LIM}-GFP (E) or GFP (G) after the stretching of substrata. Arrows in F and H indicate actin incorporated at peripheral FAs. Stretch-induced accumulation of ZYX_{LIM}-GFP at FAs could not be observed after introducing exogenous actin molecules, probably because the extent of the stretch-induced accumulation is low and the accumulated ZYX_{LIM}-GFP would be extracted from the cells during the introduction of actin molecules in the presence of digitonin. The double-headed arrow (bottom) indicates the direction of the stretch axis. Folds perpendicular to the stretch axis were generated as in Fig. 7. Scale bar: 20 μm.

Plasmid constructs and transfection

The GFP-tagged human zyxin construct was kindly provided by Jürgen Wehland and Klemens Rottner (German Research Centre for Biotechnology, Germany). Using this construct as a template, a fragment of zyxin encoding the LIM region (amino acids 338-572) was amplified by polymerase chain reaction with primers 5′-ATCAAGAATTCACCATGGAGAACCAAAACCAGGTGCGCTCCCC-3′ and 5′-TATCTGGATCCTTGGTCTGGGCTCTAGCAGTGTGGCAC-3′. The zyxin fragment was subcloned into *Eco*RI-*Bam*HI restriction sites of the pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA). The construct was verified by DNA sequencing. The GFP-tagged α-actinin construct was kindly provided by Carol A. Otey (University of North Carolina at Chapel Hill, NC).

Hs-68 cells on glass coverslips, silicone chambers or polyacrylamide substrata were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Cells were analyzed 40-48 hours after transfection.

Immunofluorescence

Cells were fixed and permeabilized for 30 minutes with 4% formaldehyde and 0.2% Triton X-100 in cytoskeletal stabilizing (CS) buffer (137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 5.5 mM glucose, 2 mM EGTA and 5 mM PIPES, pH 6.1) (Conrad et al., 1989). This was followed by blocking with 1% skimmed milk (Becton, Dickinson and Company, Franklin Lakes, NJ) in CS buffer for 30 minutes. The cells were then incubated with primary antibodies for 40 minutes, washed and further incubated with secondary antibodies for 40 minutes. Antibodies were diluted to 1:100 in CS buffer containing 1% skimmed milk.

The immunofluorescence intensity of zyxin was higher at peripheral FAs than central FAs (see Results); the heterogeneity of fluorescence intensity was not due to an artifact of immunostaining, because zyxin-GFP also accumulated preferentially at peripheral FAs (supplementary material Fig. S1).

Actin-polymerization assay

The stock solution of 100 μM Alexa568-actin (Molecular Probes) in 1 mM HEPES, 0.2 mM $MgCl_2$ and 0.2 mM ATP (pH 7.5) was filtered (pore 0.22 μm; Millipore, Bedford, MA) before use. The stock solution was diluted to 0.4 μM Alexa568-actin with permeabilization buffer (CS buffer supplemented with 0.003% digitonin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin and 0.1 μM phenylarsine oxide), and cells were incubated with the diluted actin solution for 1 minute at room temperature. Digitonin-treated plasma membrane is permeable to molecules of 200 kDa (Schulz, 1990) and to streptavidin-conjugated quantum dots (ca. 10 nm in diameter) (Hirata et al., 2004; Hirata et al., 2007), which are much larger than actin molecules (42 kDa and ca. 6 nm in diameter) (Bremer et al., 1991). In some cases, cells were pre-treated with 100 μM blebbistatin (Toronto Research Chemicals, North York, Canada) or 40 μM Y-27632 (Calbiochem). The majority of actin stress fibers were disassembled by treatment with 100 μM blebbistatin for 30 minutes or with 40 μM Y-27632 for 60 minutes, but some residual bundles of F-actin were still observed. Cytochalasin D (Sigma Chemical) was added to the permeabilization buffer when indicated (Fig. 1). After the incubation, cells were fixed with 4% formaldehyde and 0.2% Triton X-100 in CS buffer.

Microinjection

Micropipettes were made from glass capillaries with a diameter of 1 mm (Clark Electromedical Instruments, Pangbourne, UK) by pulling with a Flaming/Brown micropipette puller (P-87, Sutter Instrument, Novato, CA). Alexa568-actin (25 μM) in 1 mM HEPES, 0.2 mM $MgCl_2$ and 0.2 mM ATP (pH 7.5) was filtered (pore 0.22 μm), loaded into the micropipettes, and injected into cells using a microinjector (IM-16, Narishige, Tokyo) and a micromanipulator (MC35A, Narishige). At 1 minute after injection, cells were fixed and stained for α_5 integrin.

Stretching-cell assay

Cells grown on an elastic silicone chamber were treated with 100 μM blebbistatin for 30 minutes and then the chamber was uniaxially stretched to 150% of its original length (50% stretch) for 3 minutes in the presence of blebbistatin. The stretched cells were used for the actin-polymerization assay and/or immunofluorescence staining.

Polyacrylamide substratum

Polyacrylamide-gel substrata coated with FN were prepared as described previously (Dembo and Wang, 1999); concentrations of acrylamide and bis-acrylamide were 5% and 0.1%, respectively. The polyacrylamide substratum was deformed with a glass microneedle. The microneedles were prepared from glass capillaries with a diameter of 1 mm (G-1, Narishige, Tokyo) using a Flaming/Brown micropipette puller (P-97, Sutter Instrument, Novato, CA). The tip of the microneedle was removed before use to increase bending rigidity. The microneedle was inserted into the polyacrylamide substratum and displaced with a micromanipulator (MC-35A, Narishige). Experiments were carried out in the standard external solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4).

Fluorescence microscopy and image analysis

The cells were observed with an epifluorescence microscope (IX70, Olympus, Tokyo) equipped with an oil-immersion objective (NA 1.40, 100×; PlanApo, Olympus) and a charge-coupled device camera (Micromax, Princeton Instruments, Trenton, NJ). Acquired images were analyzed off line with the public domain Object-Image program (version 2.08).The average fluorescence intensity of a particular FA protein or incorporated fluorescent actin at FAs in a cell was calculated as follows: the five FAs with the highest immunofluorescent intensity (in immunofluorescence experiments) or EGFP intensity (in actin-polymerization experiments) were chosen in each cell for analyses. The mean fluorescence intensity of the FA protein or incorporated actin at the five FAs was calculated in each cell. The correlation analysis of fluorescence intensities of two different proteins at FAs was carried out by plotting the mean value of one protein against the mean value of the other. When the seven brightest FAs were chosen in each cell and used for the analyses, we obtained essentially the same results (data not

shown). We could not choose the ten brightest FAs for analyses, because ten typical FAs were not always found in single cells.

Immunoblot

Cells were lysed with 2× lithium dodecyl sulfate sample buffer (Invitrogen) containing 2.5% β-mercaptoethanol. Equal amounts of lysate were resolved by SDS-PAGE (3-8% Tris-acetate gel; Invitrogen), transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA) and probed with antibodies. Immunoreactive bands were detected with HRP-conjugated anti-mouse or -rabbit IgG antibody and visualized by metal-enhanced DAB staining (Sigma Chemical). Antibodies were diluted 1:1000 in PBS containing 1% skimmed milk.

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