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

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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 α5β1 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 results suggest that zyxin is involved in mechanical-force-dependent facilitation of actin polymerization at FAs.

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 (Burrage 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 α5 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-α5-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\textsubscript{LIM}-GFP) was 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\textsubscript{LIM}-GFP localized to peripheral FAs (Fig. 2A,G,I). Less endogenous zyxin was accumulated at FAs in cells expressing ZYX\textsubscript{LIM}-GFP at a higher level (Fig. 2B,E-M). To quantitatively analyze the effect of the expression of ZYX\textsubscript{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\textsubscript{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\textsubscript{LIM}-GFP (Fig. 2P,N,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\textsubscript{LIM}-GFP was expressed (Fig. 2P and supplementary material Fig. S3), as previously reported.

![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 α5 integrin. (A) α5 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,H) and zyxin (G,H) A cell that was double stained for α5 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.](image-url)
(Nix et al., 2001). By contrast, the accumulation of α5 integrin in FAs was not affected by the expression of ZYXLIM-GFP (Fig. 2P and supplementary material Fig. S3), indicating that FAs themselves were not disassembled in cells expressing ZYXLIM-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 ZYXLIM-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 ZYXLIM-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 ZYXLIM-GFP-expressing cells. The incorporation of Alexa568-actin at peripheral FAs was decreased in cells expressing ZYXLIM-GFP (Fig. 3A,B). The average fluorescence intensity of Alexa568-actin at FAs was negatively correlated with that of ZYXLIM-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.

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

Both peripheral and central FAs contained the α5β1-integrin heterodimer (supplementary material Fig. S2), talin (Fig. 4A-C) and β1 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 α5β1-integrin-dominated fibrillar adhesion and the αvβ3-integrin-dominated focal contact (Singer et al., 1988; Zamir et al., 1999; Katz et al., 2000). Because αv 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 αvβ3-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 α5β1 integrin in zyxin accumulation and actin polymerization, cells were grown on vitronectin (VN), a ligand for αvβ3 integrin. FAs containing αv 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 αv 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, α5 integrin was accumulated (Fig. 6C,G) with talin (Fig. 4D-F) and β1 integrin in a ligand-binding form (Fig. 4L,J). In these cells, a small fraction of αv integrin clusters remained, but these clusters did not contain zyxin (Fig. 6I-N), indicating again that accumulation of αv 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,
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 ZYX1LM-GFP were treated with blebbistatin, ZYX1LM-GFP was dislocated from peripheral FAs (Fig. 9). When the
substrum 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, α_5β_3 integrin was preferentially accumulated at peripheral FAs, suggesting that α_5β_3 integrin is involved in recruiting zyxin to FAs. However, accumulation of α_5β_3 integrin is not sufficient to induce zyxin accumulation, because α_5-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 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
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 (Crichtley 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; Turmacioglu et al., 1998; Fradelizi et al., 2001). The actin regulatory protein mDia1 (DIAP1) 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 α5 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-
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% CO2. For experiments, cells were grown for 15 hours on glass coverslips, elastic silicone (polymethylsiloxane 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-α5-integrin antibody was from Biogenesis (Poole, UK). The mouse anti-α5-integrin mAb was from Calbiochem (San Diego, CA). The B44 mAb, which recognizes the ligand-induced binding site on β1 integrin (Ni et al., 1998), and the mouse anti-αβ1-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 Immunoglobulin (Himmelsstadt, Germany). The rabbit anti-palladin antibody was from Proteintech Group (Chicago, IL). The rabbit anti-GFP antibody was from Immunoglobulin (Himmelstadt, Germany). The rabbit anti-ZYXLIM-GFP antibody was from GE Healthcare (Little Chalfont, UK).

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 α5 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 α5-integrin clusters that are not associated with actin incorporation. (E-I) Alexa568-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.
Cells transfected with ZYXLIM-GFP (A) or GFP (C) were stained for zyxin (B,D) after stretching of the substrata. Arrows in A indicate accumulated ZYXLIM-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 containing 1% skimmed milk. 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 stained for 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 MgCl₂ 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) (Bremner 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 major 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**

Microinjections 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 MgCl₂ 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 (MC-35A, Narishige). At 1 minute after injection, cells were fixed and stained for α5 integrin.

**Streching-cell assay**

Cells growing 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 CaCl₂, 0.8 mM MgCl₂, 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, 100X; 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 immunofluorescence intensity (in immunofluorescence experiments) or EGF 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. Immune-reactive 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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