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Affixin interacts with α-actinin and mediates integrin signaling for reorganization of F-actin induced by initial cell–substrate interaction

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The linking of integrin to cytoskeleton is a critical event for an effective cell migration. Previously, we have reported that a novel integrin-linked kinase (ILK)–binding protein, affixin, is closely involved in the linkage between integrin and cytoskeleton in combination with ILK. In the present work, we demonstrated that the second calponin homology domain of affixin directly interacts with α-actinin in an ILK kinase activity–dependent manner, suggesting that integrin–ILK signaling evoked by substrate adhesion induces affixin–α-actinin interaction. The overexpression of a peptide corresponding to the α-actinin–binding site of affixin as well as the knockdown of endogenous affixin by small interference RNA resulted in the blockade of cell spreading. Time-lapse observation revealed that in both experiments cells were round with small peripheral blebs and failed to develop lamellipodia, suggesting that the ILK–affixin complex serves as an integrin-anchoring site for α-actinin and thereby mediates integrin signaling to α-actinin, which has been shown to play a critical role in actin polymerization at focal adhesions.

Introduction

Cell motility is a basic element for many cellular activities such as leukocyte migration, wound healing, and development (Clark and Brugge, 1995). In general, cell motility is divided into several processes, namely the extension of the cell process at the cell front, formation of the ECM linkage, force generation, and release from adhesion at the rear end (Sheetz et al., 1998). In particular, the former two processes at the cell front play a critical role in cell spreading. Membrane protrusions that adhere to the substratum during the extension process can develop into a dominant lamellipodium and generate forward cell movement (Sheetz et al., 1998). On the other hand, when the membrane protrusions fail to adhere to the substratum, the extension will fold back upon itself. Thus, it is important for cells to establish stable adhesion between protrusions and ECM for effective migration (Condeelis, 1998). This substrate adhesion is largely due to the integrin family among the ECM receptor (Lauffenburger and Horwitz, 1996). Several reports that liganded integrins increase their avidity by their cytoskeleton binding indicate that integrins participate as an important interface between ECM and cytoskeleton in the adhesion process (Choquet et al., 1997; Nishizaka et al., 2000). The integrins bind to a number of cytoskeletal proteins through their cytoplasmic domain, including talin, α-actinin, and filamin, all of which bind F-actin (Liu et al., 2000). These integrin-binding proteins bind to another cytoskeletal or scaffold protein, and these hierarchical multiple protein complexes are considered to mediate the attachment of integrins to actin filaments (Miyamoto et al., 1995). However, the complex mechanisms underlying integrin–cytoskeletal linkage regulation remain to be elucidated.

Integrin-linked kinase (ILK) is a ubiquitously expressed serine/threonine protein kinase capable of interacting with the cytoplasmic domains of integrin β1 and β3 (Hannigan et al., 1996). Several reports have demonstrated that ILK is involved in the integrin-dependent cell adhesion, spreading, and cell shape change in cultured cells (Hannigan et al., 1996; Huang

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Abbreviations used in this paper: ABD, actin-binding domain; CH, calponin homology; DIC, differential interference contrast; FA, focal adhesion; FN, fibronectin; ILK, integrin-linked kinase; siRNA, small interference RNA; SF, stress fiber.

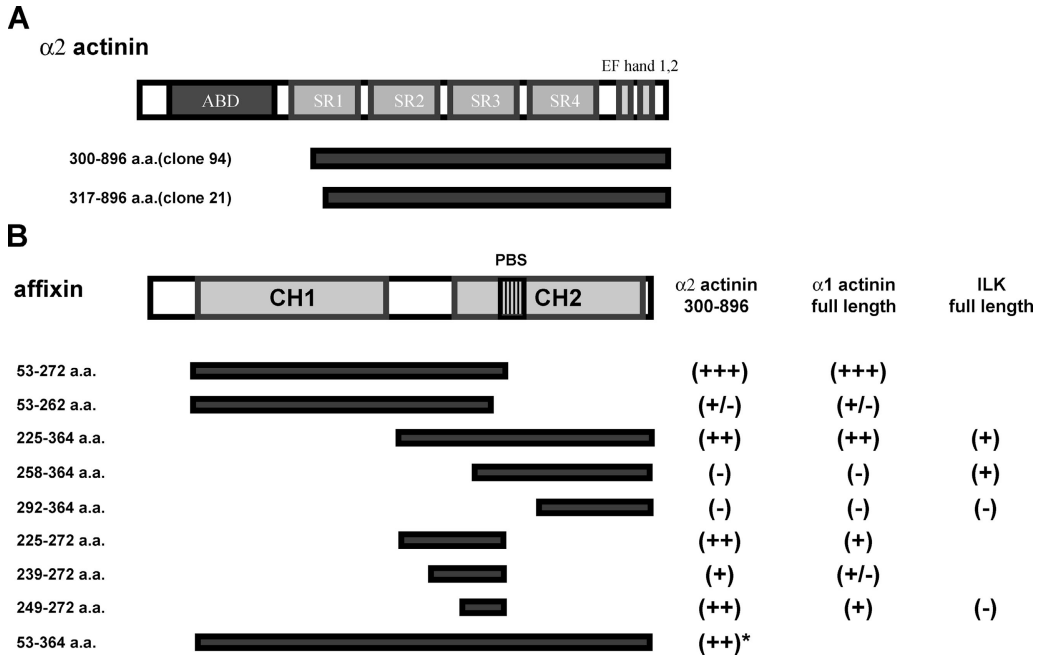

Figure 1. **Mapping of α-actinin–binding region on affixin molecule by yeast two-hybrid assay.** (A) Isolated positive clones by yeast two-hybrid screening. (B) Specific binding site of α-actinin on affixin molecule. cDNA fragments encoding human *l*-affixin deletion mutants and full-length α1-actinin were subcloned into pAS2-1 and pGAD10 vectors, respectively. These vectors, obtained clone 94, and pGAD424 full-length ILK were cotransformed into yeast Y187(a) in the indicated combinations, and 5 d later the interactions were examined by β-galactosidase filter assay.

and Wu, 1999; Zhang et al., 2002b). Recently, we have shown that ILK binds to a novel focal adhesion (FA) protein, named affixin (β-parvin), that consists of two tandem calponin homology (CH) domains and belongs to a novel family of FA proteins, together with other homologous proteins such as actopaxin/CH-ILKBP/α-parvin (α-parvin) and γ-parvin (Olski et al., 2001; Tu et al., 2001; Yamaji et al., 2001; Nikolopoulos and Turner, 2002). In CHO-K1 cells replated on fibronectin (FN), affixin and ILK are concentrated on the cell surface in blebs and then recruited into nascent substrate adhesion sites in advance of other FA components. In well-spread cells, affixin is then distributed at FA and leading edge with ILK as well as along stress fibers (SFs; Yamaji et al., 2001). Consistent with these subcellular localizations, affixin, as well as α-parvin, were suggested to mediate integrin–ILK signaling for actin organization and thus have roles in cell spreading and adhesion. For example, the overexpression of the COOH-terminal region of affixin, which is phosphorylated by ILK in vitro, blocks cell spreading at the initial stage. The coexpression of ILK enhances this effect. Thus, we suggested that affixin is involved in integrin–ILK signaling required for the development of nascent cell–substrate adhesion structures to mature FAs (Yamaji et al., 2001). In platelets, we noted that ILK stably forms a complex with affixin, and thrombin stimulation induces their association with integrin β3, which is followed by ILK activation and their subsequent incorporation into the Triton X-100–insoluble membrane–cytoskeletal fraction (Yamaji et al., 2002). On the basis of these results, we suggested that ILK and affixin play critical roles in cell spreading, particularly for the initial formation of FA and integrin–cytoskeletal linkage. Interestingly, α-parvin was reported to form a complex with not only ILK, but also with PINCH and paxillin, and the analysis of the binding-defective point mutant revealed that a correct complex formation is essential for their FA localization (Nikolopoulos and Turner, 2002; Zhang et al., 2002a). Although these results indicated that affixin and its family are closely correlated with the initial formation of FA via integrin–ILK signaling, the downstream target and the precise roles of affixin in the initial maturation process of FA are unknown.

In the present work, we demonstrated that affixin interacts with α-actinin through its second CH domain. Their association depends on kinase activity of ILK in vitro and the FN-induced integrin stimulation in vivo. The overexpression of the specific α-actinin–binding site of affixin fused with GFP (GFP-affixin$^{249–272}$) resulted in the unique inhibition of affixin–α-actinin interaction and the perturbation of Mena localization, which in turn resulted in the blockade of cell spreading. Furthermore, we designed small interference RNA (siRNA) that specifically down-regulated affixin expression, and noted that affixin knockdown cells formed multiple blebs, which was followed by the inhibition of FA formation and cell spreading as observed in GFP-affixin$^{249–272}$–overexpressing cells. These results suggest that affixin plays a critical role in integrin–cytoskeletal linkage during maturation of nascent FA and SF extensions.

Results

Affixin interacts with α-actinin via COOH-terminal CH domain in the yeast two-hybrid assay

To identify the downstream target of affixin that induces FA formation, we screened a human skeletal muscle cDNA library using the yeast two-hybrid system as described previously (Yamaji et al., 2001). As a bait, we used affixin 53–272 fused to the Gal4 DNA-binding domain because full-length

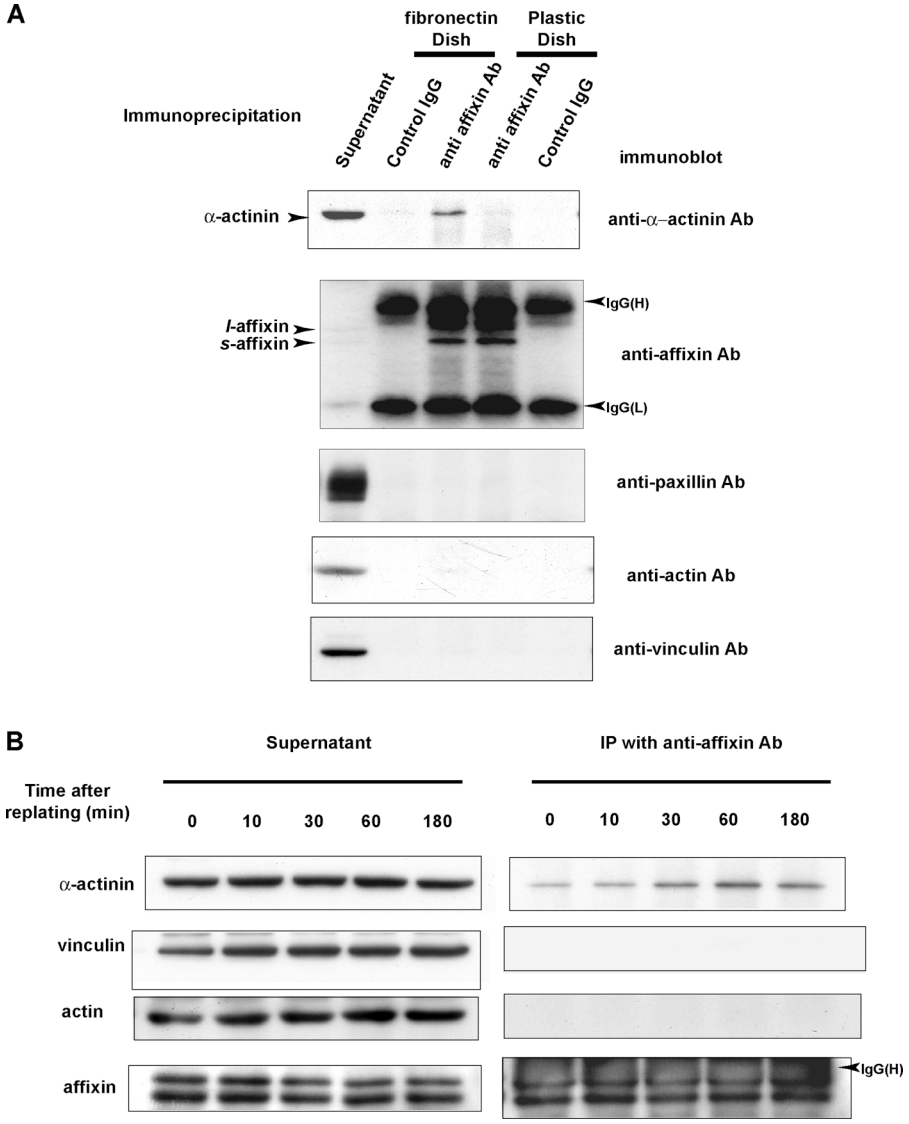

Figure 2. **α-Actinin interacts with affixin in an integrin stimulation–dependent manner.** (A) CHO-K1 cells were lysed in a lysis buffer 3 h after replating on FN-coated dishes or nonadhesive plastic dishes. The anti-affixin antibody and control IgG immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with anti-α-actinin, -affixin, -paxillin, -actin, and -vinculin antibodies. Note that lysate from FN-coated dishes (but not nonadhesive plastic dishes) exhibited coimmunoprecipitation of α-actinin with endogenous affixin. On the other hand, the negative interaction between affixin and actin (or paxillin and vinculin) demonstrates that the association is specific and is not the result of contamination of large cytoskeletal pellets. (B) HT1080 cells were trypsinized, washed three times in PBS, and replated on FN-coated dishes. After the cells were collected by lysis buffer at times indicated, the lysates were subjected to immunoprecipitation assay by anti-affixin antibody. Note that the coimmunoprecipitation of endogenous affixin and α-actinin increases gradually after replating with a peak at 60 min.

affixin causes weak self-transactivation in the system. Two positive independent clones (clones 21 and 94) were isolated, both of which corresponded to α2-actinin cDNA (GenBank/EMBL/DDBJ accession no. NM001103) fragments lacking the NH_2-terminal actin-binding domain (ABD) and a part of spectrin repeat 1 (Fig. 1 A). The specificity of this interaction was further confirmed by β-galactosidase assay in the other yeast strain, Y187(a) (unpublished data). Using clone 94 (α2-actinin 300–896), we searched the specific binding site of α-actinin on the affixin molecule by the yeast two-hybrid assay. As a result, 23 amino acids (aa 249–272) within the CH2 domain of affixin were revealed to be necessary and sufficient for their binding (Fig. 1 A). Notably, another affixin-binding protein, ILK, which has been shown to interact with the CH2 domain, did not bind to this α2-actinin–binding region (aa 249–272), and the narrowed ILK-binding site of affixin (aa 258–364) did not exhibit interaction with α2-actinin. On the other hand, the deletion of aa 258–291 of the narrowed ILK-binding site disrupted ILK binding (see mutant aa 292–364), suggesting that these amino acids, some of which are included in the α2-actinin–binding region, are indispensable for ILK binding. Together, these results suggest that the binding sites for α2-actinin and ILK are differentiated on the affixin molecule, even if they are not completely independent.

To date, many types of α-actinin paralogues have been reported (de Arruda et al., 1990; Witke et al., 1993). Because α2-actinin identified in Fig. 1 A is a muscle-specific type variant, we tested whether affixin also binds to the nonmuscle-type α-actinin, α1-actinin. For this purpose, we used a full-length construct of chicken α1-actinin showing 97% identity with human α1-actinin (GenBank/EMBL/DDBJ accession no. P05094). As shown in Fig. 1 B, affixin$^{249–272}$ was revealed to be necessary and sufficient for interaction with full-length α1-actinin.

Endogenous affixin binds to α-actinin in a substrate adhesion–dependent manner

Next, we examined whether affixin endogenously interacts with α-actinin in mammalian cells. Recently, we have reported that in resting platelets, affixin is recruited into the integrin complex together with ILK in response to thrombin stimulation. The tripartite complex then gradually translo-

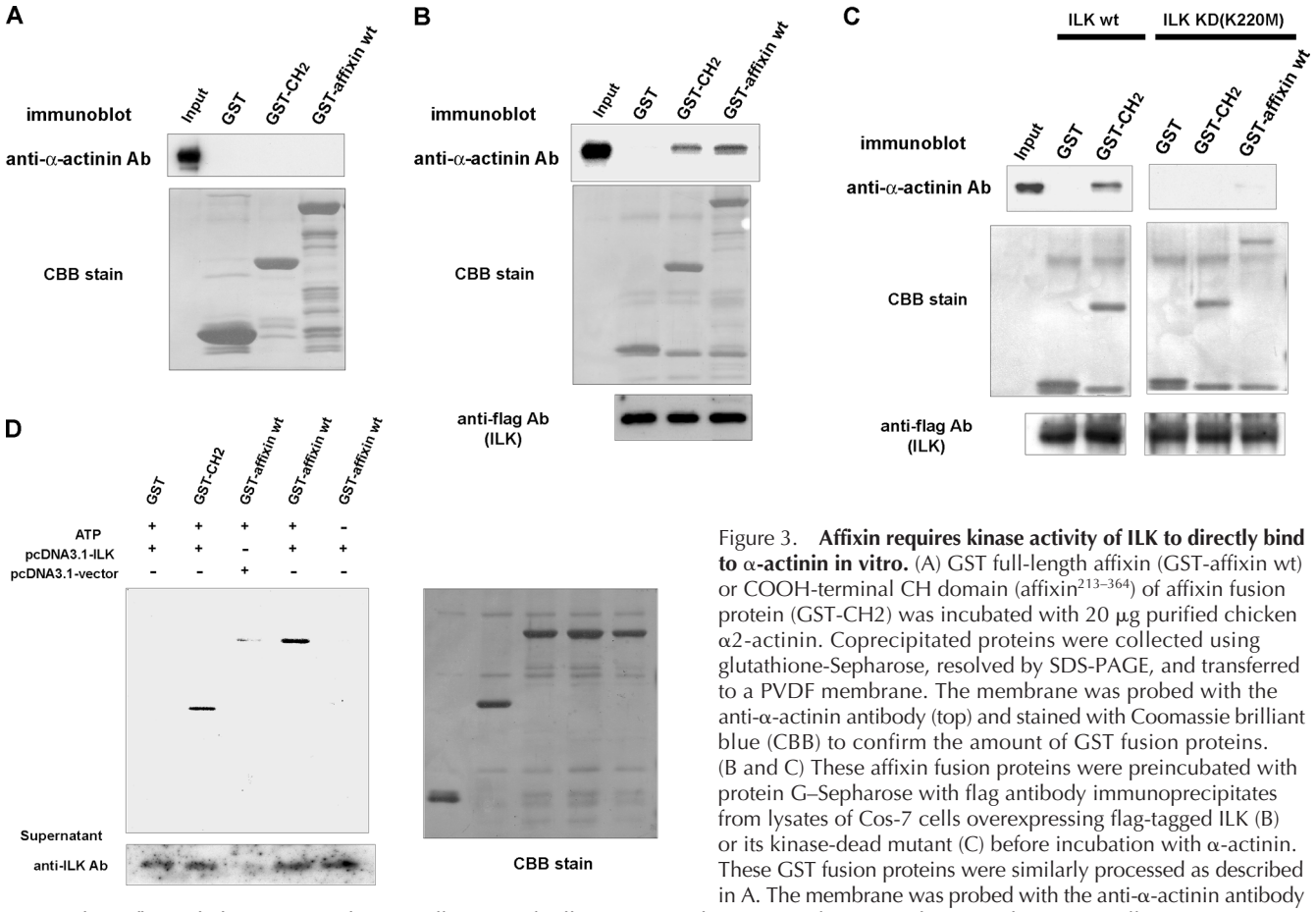

Figure 3. **Affixin requires kinase activity of ILK to directly bind to α-actinin in vitro.** (A) GST full-length affixin (GST-affixin wt) or COOH-terminal CH domain (affixin$^{213–364}$) of affixin fusion protein (GST-CH2) was incubated with 20 μg purified chicken α2-actinin. Coprecipitated proteins were collected using glutathione-Sepharose, resolved by SDS-PAGE, and transferred to a PVDF membrane. The membrane was probed with the anti-α-actinin antibody (top) and stained with Coomassie brilliant blue (CBB) to confirm the amount of GST fusion proteins. (B and C) These affixin fusion proteins were preincubated with protein G–Sepharose with flag antibody immunoprecipitates from lysates of Cos-7 cells overexpressing flag-tagged ILK (B) or its kinase-dead mutant (C) before incubation with α-actinin. These GST fusion proteins were similarly processed as described in A. The membrane was probed with the anti-α-actinin antibody (top) and anti-flag pAb (bottom). (D) The GST-affixin specifically interacts with α-actinin after preincubation with ILK. GST-affixin wt, GST-CH2, or GST alone was separated by SDS-PAGE after preincubation with in vitro–translated ILK in the presence or absence of 10 μM ATP in phosphorylation buffer, and was transferred to membrane. Blot overlay assay was done using 10 μg/ml purified α-actinin after blocking.

cates to the Triton X-100–insoluble fraction, biochemically corresponding to the actin-based membrane–cytoskeletal fraction (Yamaji et al., 2002). On the basis of these observations, we speculated that the interaction of affixin with α-actinin may be dependent on integrin stimulation. In Fig. 2 A, CHO-K1 cells were cultured on FN-coated dishes or remained in suspension on nonadhesive plastic dishes for 3 h, and the cell lysates were subjected to immunoprecipitation analysis using the anti-affixin antibody. As shown in Fig. 2 A, a moderate amount of α-actinin was coimmunoprecipitated with affixin from the cells cultured on FN dishes, but not from those cultured on plastic dishes. In contrast, paxillin, which was reported to interact with α-parvin, as well as actin and vinculin, failed to coimmunoprecipitate with affixin under either condition. These results indicate that affixin interacts with α-actinin, but not with paxillin, in an adhesion-dependent manner in mammalian fibroblasts.

To further analyze the adhesion dependence of the affixin–α-actinin interaction, we observed the time course of their coimmunoprecipitation during cell spreading on FN-coated dishes after replating. As shown in Fig. 2 B, the amount of affixin–α-actinin complex gradually increased with a peak at 60 min, when cell spreading was most active (unpublished data). These results suggest that affixin–α-actinin interaction is dependent on the initial cell–substrate interaction.

Affixin binds to α-actinin in vitro in an ILK kinase activity–dependent manner

To confirm the direct interaction between affixin and α-actinin, we performed an in vitro pull-down assay using bacterially purified GST-affixin, or GST-CH2 (affixin$^{213–364}$; previously called RP2). For this purpose, the GST fusion proteins were incubated with purified α-actinin, and the bound proteins were subjected to immunoblotting with the anti-α-actinin antibody. As shown in Fig. 3 A, the results reproducibly did not exhibit any interaction between purified affixin and α-actinin. However, the result that the endogenous interaction between affixin and α-actinin was only observed when cells were plated on FN-coated dishes (Fig. 2 A) made us hypothesize further that some post-translational modification may be required for the interaction. In this respect, it should be noted that ILK has been shown to be acutely activated in response to integrin signaling (Delcommenne et al., 1998), and that the CH2 domain of affixin can be effectively phosphorylated by ILK in vitro (Yamaji et al., 2001). Together with the present results that the α-actinin–binding site of affixin is very close to the ILK-binding site, it is plausible that the affixin–α-actinin interaction is induced by the phosphorylation of the CH2 domain of affixin by ILK. The results in Fig. 3 (B and C) strongly support this notion: when preincubated with immunoprecipitated ILK

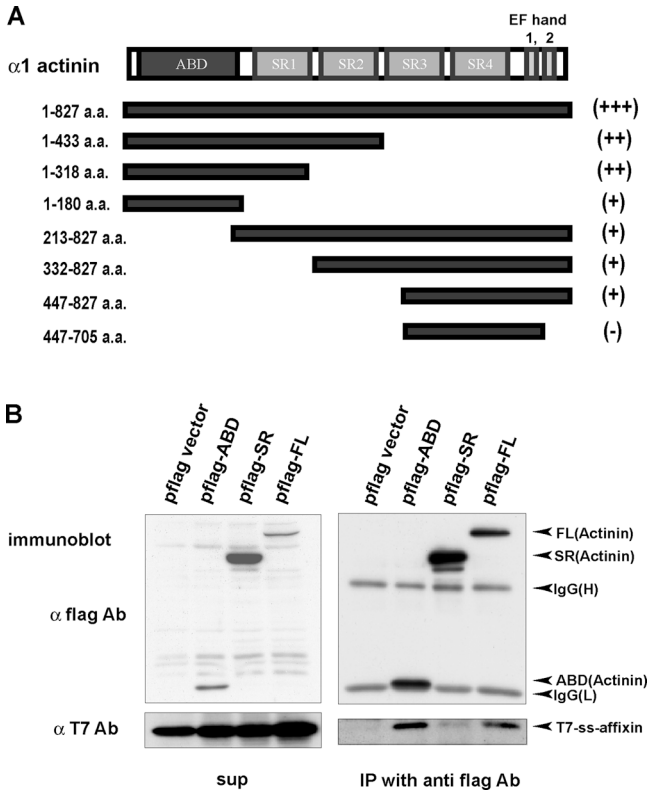

Figure 4. **α-Actinin binds to affixin through its ABD.** (A) Interaction between affixin and α-actinin in the two-hybrid system. cDNA fragments encoding chicken α1-actinin deletion mutants were subcloned into pGAD424 vectors. These vectors were cotransformed with pAS2-1 affixin$^{53–272}$ into yeast Y187(a), and the interaction was investigated by β-galactosidase filter assay. (B) The ABD of α-actinin was coimmunoprecipitated with affixin. The expression vector encoding the flag-tagged α-actinin or its ABD or ABD-truncated α-actinin mutant (SR) was cotransfected into Cos-7 cells with pSRD-T7-affixin. Immunoprecipitation assay was performed using the anti-flag antibody, and immunocomplexes were subjected to immunoblot analysis with anti-flag monoclonal and anti-T7 antibodies.

under a condition in which GST-affixin was effectively phosphorylated by ILK (Yamaji et al., 2001), GST-affixin as well as GST-CH2, but not GST alone, became competent to interact with α-actinin. Importantly, similar effects were not observed when a kinase-dead mutant of ILK, ILK(K220M), was used for preincubation instead of ILK (Fig. 3 C; Yamaji et al., 2001). Considering that ILK (K220M) can associate with affixin (Yamaji et al., 2001), these results indicated that the interaction of affixin with α-actinin is dependent on ILK kinase activity, but not on its ability to associate with ILK. To further confirm direct interaction between affixin and α-actinin, we purified GST-affixin by SDS-PAGE after preincubation with in vitro–translated ILK in the presence or absence of ATP, and examined its binding to purified α-actinin by blot overlay assay. Fig. 3 D shows again that GST-affixin specifically interacts with α-actinin, only when it was preincubated with ILK in the presence of 10 μM ATP in phosphorylation buffer. These results indicate that affixin directly associates with α-actinin only when its CH2 domain is phosphorylated by ILK.

Both the NH_2- and COOH-terminal regions of α-actinin bind to affixin

α-Actinin is composed of three domains, an NH_2-terminal ABD, spectrin-like repeats (SP), and a COOH-terminal region containing Ca^{2+}-binding motifs (EF hands; Puius et al., 1998). To identify the binding region of α-actinin for affixin, we constructed various deletion mutants of α1-actinin and tested their interaction with affixin$^{53–272}$ by yeast two-hybrid assay. Fig. 4 A shows that the most COOH-terminal region of α1-actinin outside of the second half of EF hand motifs is essential for the interaction. However, the most surprising result was that the NH_2-terminal region from ABD to SP-1, which was almost lacking in the initially cloned α-actinin fragment (clones 22 and 94), demonstrated strong interaction with affixin. The results were further confirmed by coimmunoprecipitation assays performed using Cos-7 cells: flag-tagged full-length and ABD of α-actinin were coimmunoprecipitated with T7-tagged full-length affixin by the anti-T7 antibody, whereas a truncated mutant (SR) lacking ABD showed only a very weak coimmunoprecipitation activity (Fig. 4 B). These results suggest that the two distinct regions of α-actinin, which correspond to the ABD and the COOH-terminal region, are involved in the interaction with affixin. Considering that α-actinin forms an anti-parallel dimer (Puius et al., 1998), the present results may suggest that affixin recognizes the terminal structure of the α-actinin dimer in which the NH_2-terminal and COOH-terminal regions are located in close vicinity. We also observed that flag-α-actinin overexpressed in CHO-K1 cells was coimmunoprecipitated with endogenous affixin by anti-affixin antibody (unpublished data).

α-Actinin colocalizes with affixin in FA, leading edge, and along fine SFs

Previously, we had demonstrated that affixin and ILK are colocalized with α-actinin at the striated structures distributed on the sarcolemma of human skeletal muscles, to which Z-bands anchor. On the other hand, both affixin and α-actinin have been shown to localize at FA, the tip of the leading edge, and SFs in fibroblasts (Lazarides and Burridge, 1975; Yamaji et al., 2001). To confirm their colocalization in a single fibroblast, we doubly immunostained for overexpressed (Fig. 5, A–C) as well as endogenous (Fig. 5, D–I) α-actinin and affixin in CHO-K1 cells. In cells with prominent SFs and FAs, their overall distribution rather differed (Fig. 5, A–F). However, a close inspection revealed that affixin and α-actinin exhibited partial colocalization in particular structures, such as the tip of leading edge (Fig. 5, A–C; arrows) and the junction between SFs and FAs (Fig. 5, A–F, arrowheads). They also showed colocalization in fine SFs, but not in the well-developed thick SFs (Fig. 5, A–C; unpublished data). Importantly, their colocalization was clearly observed at the tip of leading edge in a population of cells with well-developed lamellipodia and completely lacked FAs (Fig. 5, G–I; arrows). These results are well consistent with the biochemical coimmunoprecipitation assay shown in Fig. 2, and suggest that affixin and α-actinin interact in the nascent cell–substrate complex in the leading edge. Affixin may remain to interact with α-actinin in mature FAs and nascent

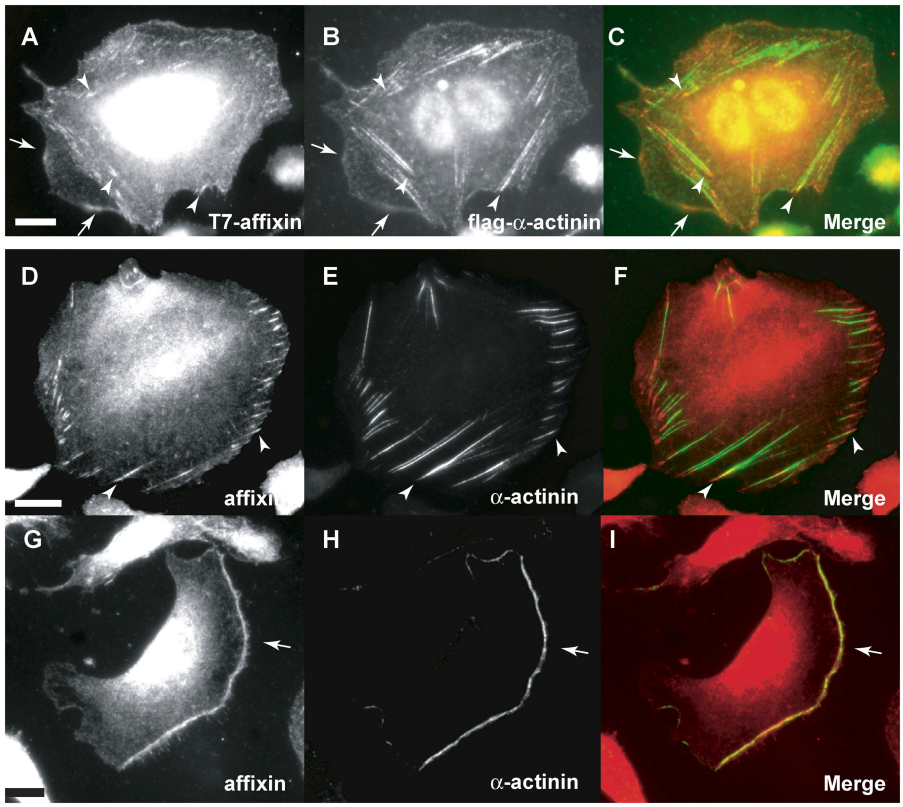

Figure 5. **α-Actinin colocalizes with affixin at leading edge, FA, and along SFs.** Double-immunofluorescence staining with anti-T7 and anti-flag mAbs of CHO-K1 cells transfected with T7-tagged affixin and flag-tagged α1-actinin (A–C) or with anti-affixin and anti-α-actinin antibodies of CHO-K1 cells (D–I). Note that both affixin and α-actinin are co-localized at FAs (A–F, arrowheads), at the tip of the leading edge (A–C, G–I; arrows). In A–C, fixation was performed using 2% PFA in PBS, whereas in D–I, 0.5% PFA in PBS was used. Bars, 10 μm.

fine SFs, but may be excluded from mature SFs in which α-actinin is considered to cross-bridge actin filaments.

Affixin$^{249–272}$ specifically disrupts affixin–α-actinin interaction and thus inhibits spreading of mammalian cells

To examine the physiological importance of affixin–α-actinin interaction within cells, we next overexpressed GFP-tagged affixin$^{249–272}$ in CHO-K1 cells, which contain the minimum α-actinin–binding site of affixin in yeast two-hybrid assay (Fig. 1), and actually binds to α-actinin in CHO-K1 cells (Fig. 6 A). Immunoprecipitation analysis using the anti-affixin antibody revealed that the coimmunoprecipitation of α-actinin, but not ILK with affixin, was significantly reduced in GFP-affixin$^{249–272}$–overexpressing cells (Fig. 6 B). When quantified, the signal intensity of coprecipitated α-actinin in GFP-affixin$^{249–272}$–overexpressing cells is >10-fold lower than those in control GFP–overexpressing cells reproducibly. These results indicate that GFP-affixin$^{249–272}$ can be used as a dominant-negative mutant to specifically disrupt affixin–α-actinin interaction in vivo.

Fig. 7 (A and B) shows that, 24 h after transfection, ~40% of the cells overexpressing GFP-affixin$^{249–272}$ displayed a round morphology, compared with the control cells expressing GFP alone (round cells <10%). The effect was further enhanced when cells were reseeded: when cells were harvested in trypsin/EDTA solution 24 h after transfection and reseeded on FN-coated coverslips, >90% cells overexpressing GFP-affixin$^{249–272}$ exhibited a round morphology 24 h after reseeding (see Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1). In these round cells, α-actinin was observed as dotlike structures at the cell periphery and F-actin was concentrated in a limited number of peripheral blebs (Fig. 7 C, a–d). Double staining with anti-affixin and anti-Mena antibodies revealed that their colocalization at the FAs in normal cells (unpublished data) was also obstructed by the expression of GST-affixin$^{249–272}$ (Fig. 7 C, e and f).

It has been shown that α-parvin binds to paxillin through its paxillin-binding sequence embedded in the NH_2-terminal region of the CH2 domain. Because affixin also has conserved sequence of paxillin-binding sequence, which is partially overlapped by the affixin$^{249–272}$ (Fig. 1), the observed effects of affixin$^{249–272}$ may be due to the interference of endogenous interaction between α-parvin and paxillin. To exclude this possibility, we constructed affixin$^{249–262}$, an affixin fragment completely devoid of paxillin-binding sequence, with the GFP tag, and confirmed that this shorter fragment of affixin also exhibited similar deleterious effects on cell spreading to the affixin$^{249–272}$ (unpublished data).

To obtain further insight into the roles of affixin–α-actinin interaction in cell spreading, cells overexpressing GFP-affixin$^{249–272}$ were observed by time-lapse video microscopy (Fig. 7 A; Videos 1–4, available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1). 24 h after transfection, cells overexpressing GFP-affixin$^{249–272}$ with a round shape were noted to actively protrude many blebs peripherally. However, these blebs did not further develop into lamellipodia as observed in normal cells, but were retracted into the cell body within 30 min (Fig. 7 A, d; indicated by arrows). It should be noted that these round cells still attached on the slides and actively moved in a very limited area, indicating that this round morphology did not reflect the process of apoptosis. These effects of GFP-affixin$^{249–272}$ suggest that af-

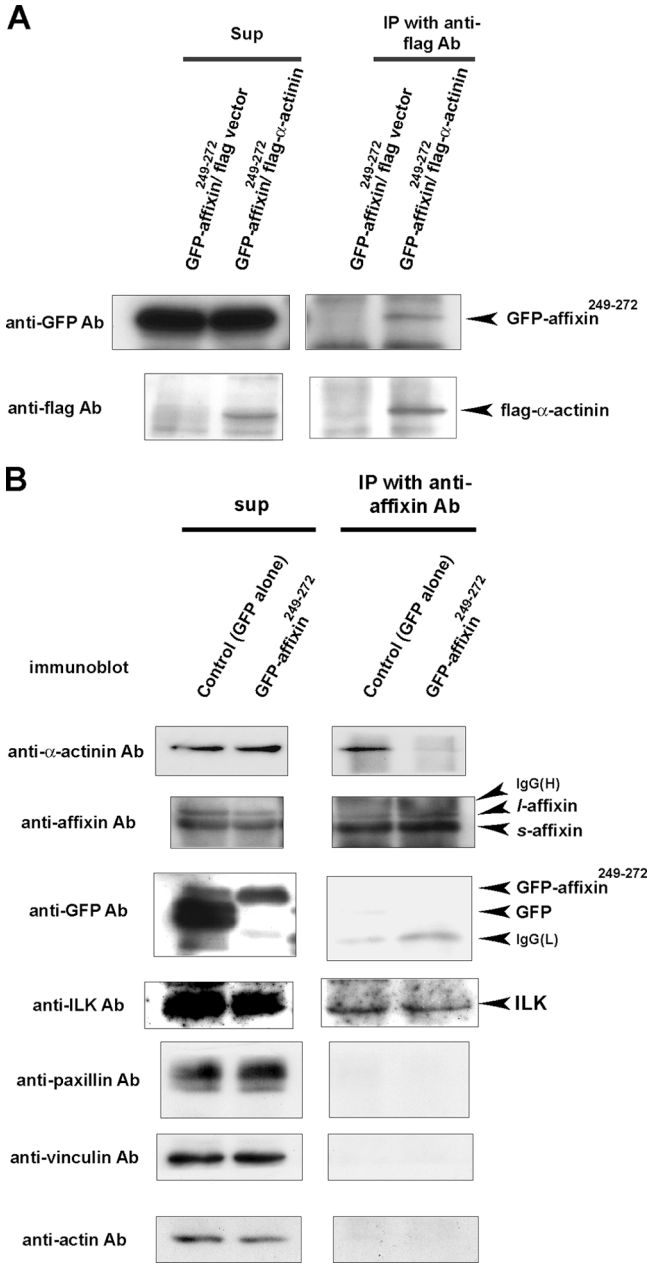

Figure 6. **Affixin$^{249–272}$ specifically inhibits α-actinin–affixin interaction.** (A) The minimum α-actinin–binding site of affixin was coimmunoprecipitated with α-actinin. The expression vector encoding the flag-tagged α-actinin was cotransfected into CHO-K1 cells with pEGFP-affixin$^{249–272}$. Immunoprecipitation assay was performed using the anti-flag antibody, and immunocomplexes were subjected to immunoblot analysis with monoclonal anti-flag and anti-GFP antibodies. (B) Endogenous affixin was immunoprecipitated from lysates of CHO-K1 cells harvested from fully spreading cells transfected with GFP-affixin$^{249–272}$ or GFP vector alone and cultured for 24 h. Affixin immunoprecipitates were analyzed by Western blotting with anti-α-actinin, affixin, GFP, ILK, paxillin, vinculin, and actin antibodies as indicated. Note that the coimmunoprecipitation of α-actinin, but not ILK, was abolished in GFP-affixin$^{249–272}$–expressing cells.

fixin–α-actinin interaction does not contribute to the initial formation of the cell protrusions in itself, but rather to their stabilization and further development into lamellipodia, which establish firm and broad interaction with the substrate.

Loss of affixin expression results in the blockade of FA formation, lamellipodial development, and cell spreading

To further confirm the essential role of affixin in cell spreading, we next used the siRNA to monitor the effects of knockdown of affixin expression. We synthesized and introduced three affixin siRNAs or an irrelevant control RNA into human fibroblasts (HT1080 and IMR-90) or HeLa cells. As shown in Fig. 8, the two affixin-targeted siRNAs #1 and #3 specifically suppressed the expression of affixin in HT1080, IMR-90 (Fig. 8 A), and HeLa cells (unpublished data), whereas the expression of other affixin-interacted or cytoskeletal proteins such as ILK, α-actinin, vinculin, and actin were not affected. The differential interference contrast (DIC) images of these affixin-deficient cells transfected with affixin siRNA #3 revealed a marked increase in the number of multiple blebbing cells compared with control cells (Figs. 8, B and C). Time-lapse observations by DIC microscopy revealed that these blebs were repeatedly protruding from and retracting into the cell bodies, but could not develop into mature lamellipodia, which was quite similar to those of CHO-K1 cells overexpressing GFP-affixin$^{249–272}$ (Fig. 8 C; Videos 5 and 6, available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1). We also confirmed by TUNEL staining that affixin RNA interference does not induce apoptosis within 48 h after siRNA transfection (unpublished data). In the immunofluorescence microscopy, well-developed thick SFs and FAs were not observed in these affixin-deficient cells, but actin condensation in the cell periphery (Fig. 9 E) and diffuse staining of α-actinin (Fig. 9 K) and vinculin (Fig. 9 Q) in cytosol were detected instead. These results indicate that affixin plays a critical role in the process of FA formation, lamellipodial development, and cell spreading.

Discussion

Affixin mediates anchoring of α-actinin to FAs in an adhesion-dependent manner

α-Actinin is an actin cross-linking protein abundant at FAs (Maruyama and Ebashi, 1965; Lazarides and Burridge, 1975; Podlubnaya et al., 1975), and has been suggested to play important roles in nascent FA assembly and SF extensions from the integrin-based cell–substrate adhesion complex. For example, recent analyses of the GFP-α-actinin dynamics revealed that once the interaction between the tips of protrusions and ECM stabilizes, α-actinin begins to localize in small foci at the leading edge, which then grow in size and extend small fiberlike structures toward the cell body (Edlund et al., 2001; Laukaitis et al., 2001). On the other hand, α-actinin was demonstrated to be critical for the correct positioning of zyxin at FAs, which is suggested to induce actin polymerization at FAs independent of the Arp2/3 complex by forming a complex with a mammalian member of the Ena/VASP family, Mena (Drees et al., 1999; Reinhard et al., 1999; Fradelizi et al., 2001). Despite these lines of evidence on the role of α-actinin in FA development, the molecular mechanism underlying α-actinin recruitment to nascent adhesion sites is less understood. One of the possible mechanisms underlying α-actinin targeting suggested to date is its direct interaction with integrins because α-actinin was re-

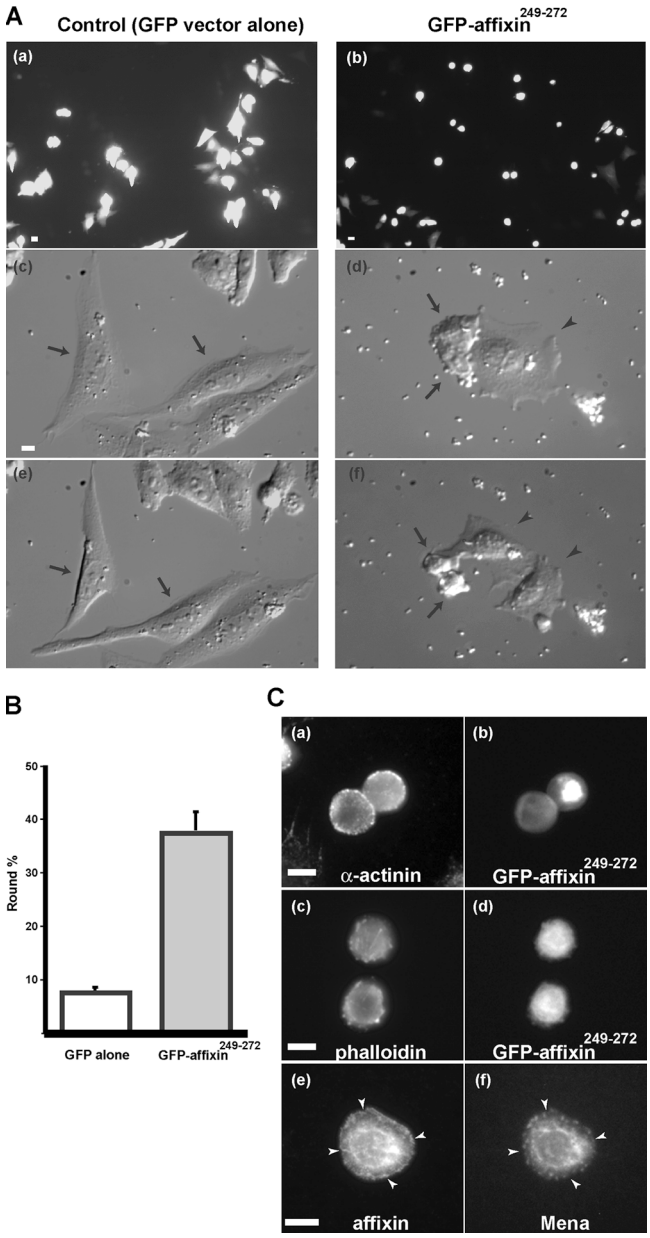

Figure 7. **Affixin$^{249–272}$–expressing cells showed blockade of cell spreading and abnormal distribution of F-actin, α-actinin, and Mena.** (A) CHO-K1 cells transfected with GFP-affixin$^{249–272}$ or GFP vector were cultured on FN-coated plates and video microscopy data were collected from 24 to 48 h after transfection at 37°C in a humidified atmosphere of 5% CO_2. Fluorescence microscopic data obtained 38 h after transfection to only visualize GFP-expressing cells under low magnification (a and b; Videos 1 and 2) are shown, and DIC images obtained at 24 h (c and d) or 30 h (e and f) demonstrate cell morphology under enhanced magnification. In c–f, arrows indicate GFP-expressing cells that were confirmed by fluorescence microscopy (not depicted) and arrowheads indicate GFP-negative cells that divided during time-lapse observation (see Videos 3 and 4). Note that cells transfected with GFP-affixin$^{249–272}$ were arrested at the early stage of cell spreading with peripheral blebs. Bars: 20 μm (A, in a and b), 10 μm (C). An animated time-lapse version of this figure is available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1. (B) The percentage of round cells among GFP-positive cells were estimated 24 h after transfection. The values provided represent mean values (±SD) of three independent experiments. (C) GFP-affixin$^{249–272}$ was overexpressed in CHO-K1 cells, and 24 h later the cells were fixed with 100% cold methanol (a, b, e, and f) or 2% PFA (c and d) and stained with the anti-α-actinin antibody (a) or rhodamine phalloidin (c). In e and f, GFP-expressing cells were doubly stained with anti-affixin (e, Cy3) and Mena (f, Cy5). Note that in GFP-affixin$^{249–272}$–overexpressing cells, α-actinin demonstrated punctate staining at cell periphery, whereas F-actin formed a weak peripheral staining with a high concentration in a limited number of blebs. Colocalization of affixin and Mena was not observed.

vealed to bind directly to the cytoplasmic domains of the β1 subunit in vitro (Otey et al., 1990; Pavalko and Burridge, 1991). Greenwood et al. (2000) further demonstrated that the binding of PtdIns (3,4,5)-P3 to α-actinin disrupts its interaction with the integrin β1 and β3 subunits in PDGF-treated cells, providing a modification mechanism underlying the interaction of α-actinin at FAs.

In this work, we demonstrated that an ILK-binding protein, affixin, directly interacts with α-actinin through its second CH domain; thereby providing a novel molecular basis of α-actinin targeting to FAs. Previously, we demonstrated that during cell spreading observed after replating, ILK and affixin are both recruited into cell surface blebs formed at a very early stage before FAK and vinculin. Later, affixin localizes as small dots in the lamellipodia from which short actin bundles emanate. These features of affixin localization during the initial establishment of integrin-based adhesion are well consistent with the dynamics of α-actinin stated in the previous paragraph, suggesting a possibility that both proteins cooperate to transmit initial integrin signal to F-actin organization. In the present work, we confirmed that affixin and α-actinin are mainly colocalized at the tip of lamellipodia, where nascent cell–substrate interactions intensively occur. Furthermore, we also revealed biochemically that in vivo interaction between endogenous affixin and α-actinin was dependent on integrin-mediated substrate adhesion, and peaked at an initial phase of cell spreading. The functional importance of the affixin–α-actinin interaction in the initial integrin signaling was then demonstrated by the observation that introduction of the minimum α-actinin–binding sequence of affixin, affixin$^{249–272}$, which disrupts endogenous interaction of affixin with α-actinin, severely interfered with cell spreading. The resultant round cells lacked FAs, well-developed lamellipodia, and SFs, but manifested peripheral small blebs in which F-actin often aggregates. Observation by time-lapse video microscopy revealed that these cells showed jerky movement and actively protruding small blebs, but failed to spread by developing lamellipodia from these blebs. These results are essentially reproduced in siRNA experiments in which affixin expression was specifically knocked down. Together, these data strongly support the notion that the affixin–α-actinin interaction plays an essential role in the recruitment of α-actinin to nascent FAs, where α-actinin is considered to trigger robust actin polymerization by interacting with a zyxin–Mena complex and thus induces the stabilization of the nascent substrate adhesion, lamellipodial development, and formation of SFs (Drees et al., 1999; Fradelizi et al., 2001).

Kinase activity of ILK is essential for affixin–α-actinin interaction

ILK has been demonstrated to be activated by integrin signaling evoked by the interaction with the substrate

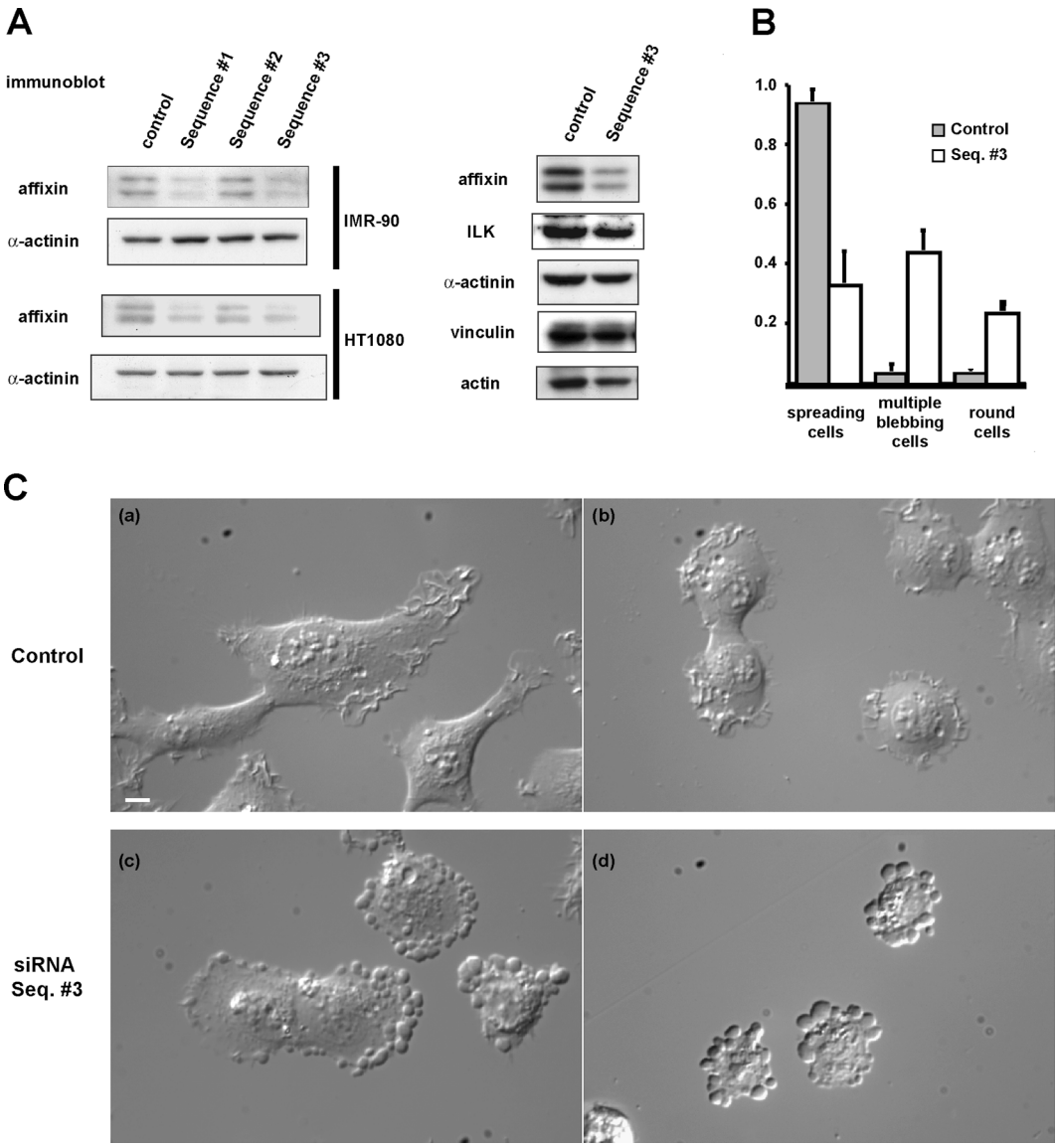

Figure 8. **Effects of affixin knockdown by siRNA on cell morphology.** (A) IMR-90 (left) and HT1080 (both panels) human fibroblasts were transfected with the indicated siRNA duplex. The specificity of each siRNA was assessed by immunoblot analysis 48 h after transfection. Note that #1 and #3 siRNAs specifically suppressed affixin expression without significant effects on the expression of α-actinin (both panels), ILK, vinculin, or actin (right). (B) The percentage of cells with multiple blebs or round morphology was estimated 48 h after transfection. The values provided represent mean values (±SD) of three independent experiments. (C) DIC images of cells transfected with control RNA (a and b) or affixin #3 siRNA (c and d). Note that #3 siRNA-transfected cells were surrounded by various sized spherical out-pouchings called blebs. Although relatively well-spread cells with multiple blebs were observed (c), they moved slowly and gradually reduced in size, and finally resulted in a round morphology (d). Bar, 10 μm. An animated time-lapse version of this process is shown in Videos 5 and 6, available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1.

(Delcommenne et al., 1998). Our analysis of thrombin-activated platelets revealed that its kinase activity is acutely enhanced within 90 s after the stimulation, which is followed by the incorporation of the integrin–ILK–affixin complex into 1% Triton X-100–insoluble, membrane-skeletal structures (Yamaji et al., 2002). Because the CH2 domain of affixin is efficiently phosphorylated by ILK in vitro, and because the binding regions of ILK and α-actinin on affixin are located close to each other (Yamaji et al., 2001), it is plausible that the interaction between α-actinin and affixin is triggered by phosphorylation of affixin by ILK in response to substrate adhesion. Indeed, we demonstrated here that the direct interaction between the CH2 domain of affixin and α-actinin was only observed when the CH2 domain was preincubated with ILK, but not with the kinase-dead mutant of ILK, in vitro. Although further analyses including the determination of ILK phosphorylation sites on affixin are required to completely verify our hypothesis, the present results are well consistent with the concept that ILK activation by integrin signaling and the subsequent phosphorylation of affixin is critical to the initial establishment of cell–substrate adhesion. It should be also noted that the time course of the increase in affixin–α-actinin interaction after cell replating (Fig. 2 B) well cor-

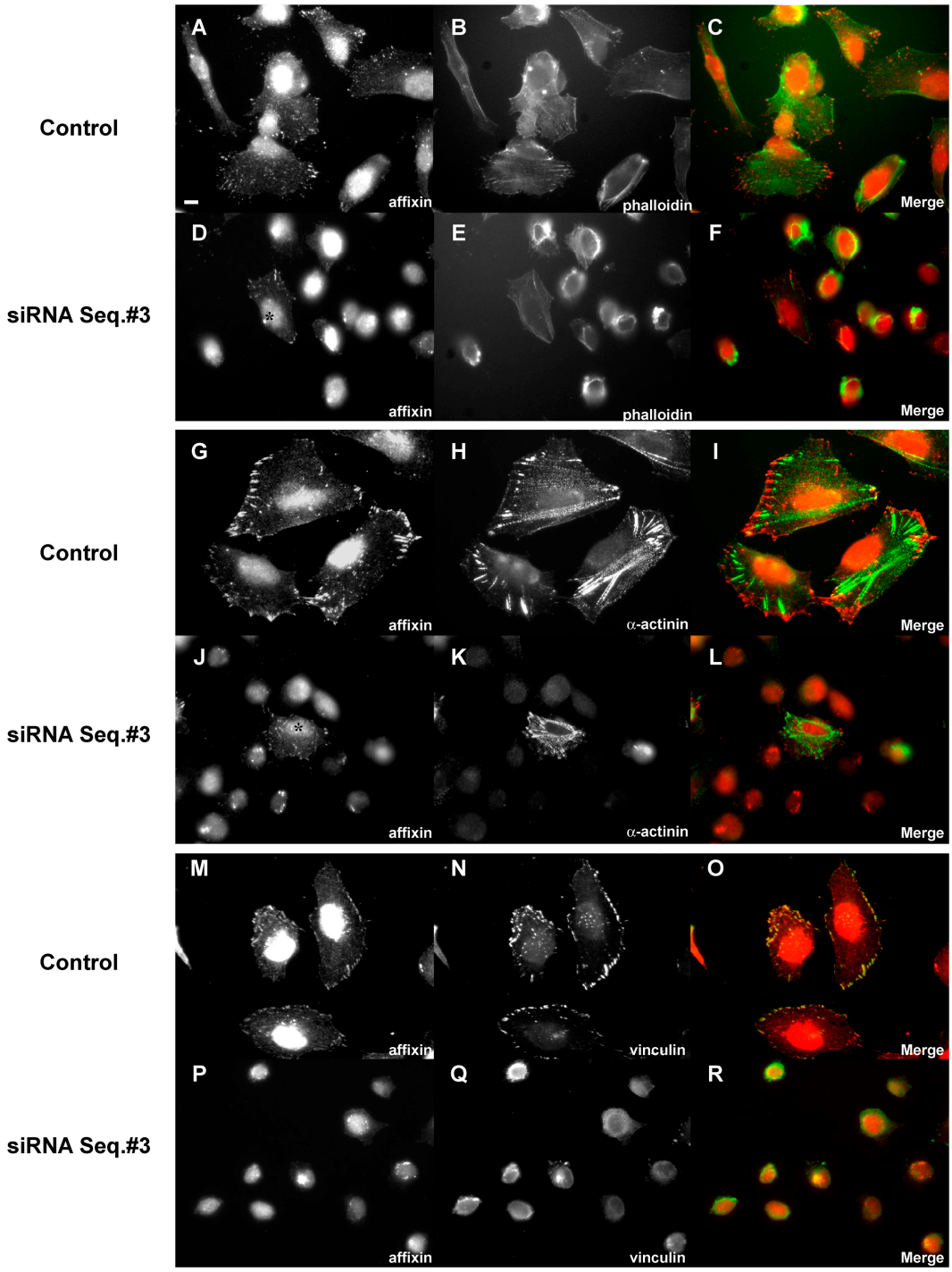

Figure 9. **Knockdown of affixin expression impaired FA and SF formation.** HT1080 cells were transfected with control RNA or affixin-targeted siRNA #3 duplex as indicated. 48 h after transfection, the cells were fixed with 0.5% PFA, permeabilized with 0.2% Triton X-100, and double stained with affixin antibody (A, D, G, J, M, and P) and FITC-phalloidin (B and E), or anti-α-actinin antibody (H and K) or anti-vinculin antibody (N and Q). Merged views of each staining are also presented in C, F, I, L, O, and R. Asterisks indicate cells that were considered to evade affixin siRNA transfection. Nuclear stainings in the affixin stain (A, D, G, J, M, and P) are nonspecific signals raised by the secondary antibody under this fixation method. Note that the expression of affixin was markedly reduced in #3 siRNA-transfected cells. Bar, 10 μm.

relates with that of ILK activation induced by cell–substrate adhesion (Delcommenne et al., 1998).

Previously, we demonstrated that the overexpression of affixin$^{258–364}$ corresponding to the entire CH2 domain in well-spread CHO-K1 cells does not severely interfere with cell adhesion unless ILK, but not the kinase-defective point mutant of ILK (K220M), is coexpressed or the cells are subjected to replating. On the other hand, we observed here that the overexpression of the smaller affixin fragment, affixin$^{249–272}$, corresponding to the first portion of the CH2 domain and lacking ILK-binding activity, exerts significant deleterious effects on cell–substrate adhesion even without

ILK coexpression or replating. This may indicate that phosphorylation of the CH2 domain by ILK induces a conformational change of the CH2 domain of affixin, which enables affixin to interact with α-actinin to evoke the subsequent maturation of the FA complex.

Functional divergence between affixin and actopaxin/CH-ILKBP/α-parvin

In the course of the present work, we also noted that affixin does not interact with paxillin. This is in sharp contrast with α-parvin, which has been demonstrated to interact with paxillin through the paxillin-binding sequence domain located in the first half of the CH2 domain. Considering that α-parvin does not interact with α-actinin (Nikolopoulos and Turner, 2000, 2002), these results provide very interesting functional divergence between the two closely related members of the parvin family, both of which bind to ILK. It was reported that α-parvin directly interacts with F-actin, but affixin does not (Yamaji et al., 2001). This functional divergence may explain the difference in the effect of the CH2 domain overexpression on cell spreading: in contrast with affixin, the morphological effect of the overexpression of the α-parvin CH2 domain was revealed to be rather weak (Nikolopoulos and Turner, 2000; Tu et al., 2001). Furthermore, the α-parvin mutant, F271D, which exhibits impairment in binding to paxillin, has not been reported to cause any morphological changes without the inhibition of the correct FA localization of this mutant (Tu et al., 2001; Nikolopoulos and Turner, 2002). Finally, α-parvin siRNA have revealed no obvious effect on their morphology and FA formation (Fukuda et al., 2003). These results suggest that affixin has some distinct roles from α-parvin in cell spreading despite their close similarity in their amino acid sequence.

Role of affixin during early FA formation

Recently, Rosenberger et al. (2003) have reported that affixin interacts with αPIX, a PAK-interacting protein that has GEF activity for Rac1 and possibly for Cdc42. In a previous report, we demonstrated that, in contrast to the CH2 domain, overexpression of the CH1 region of affixin promotes cell spreading of CHO-K1 cells, and speculated that this region may be the site that interacts with the downstream target of ILK–affixin signaling (Yamaji et al., 2001). Consistent with this prediction, we confirmed that the CH1 domain is the site that interacts with αPIX, and the overexpression of CH1 enhances Rac and Cdc42 activities via αPIX (Mishima et al., 2004). These results suggest that affixin also participates in the activation of Rac and Cdc42 by associating with αPIX through its CH1 domain. This activity of affixin should result in enhanced actin polymerization through the activation of various downstream effectors of Rac1/Cdc42, including Mena/VASP and WASP-Arp2/3. In addition, PIX was suggested to be responsible for the recruitment of PAK1 to integrin-based focal contacts, which is activated by Rac1/Cdc42 (Manser et al., 1998) and causes filopodia formation and membrane ruffles via the LIM kinase–ADF/cofilin pathway (Edwards et al., 1999; Zebda et al., 2000). Therefore, these results suggest that affixin is not only a downstream mediator of integrin–ILK signaling, but is also a scaffold protein on which all these key players of actin polymerization converge in concert with α-actinin and PIX (Fig. 10). This protein complex formed around the initial integrin-based substrate adhesion site may synergistically evoke acute actin polymerization, which results in the rapid stabilization of the nascent cell–substrate interaction, lamellipodia development, and SF formation. Dramatic effects of affixin knockdown on cell spreading are consistent with this hypothesis on the central role of ILK–affixin signaling in initial integrin signaling. Then, the fact that the introduction of the minimum α-actinin–binding site caused essentially similar effects on affixin siRNA suggests that the interaction between affixin and α-actinin is one of the critical components of this nascent integrin signaling mediated by the ILK–affixin system.

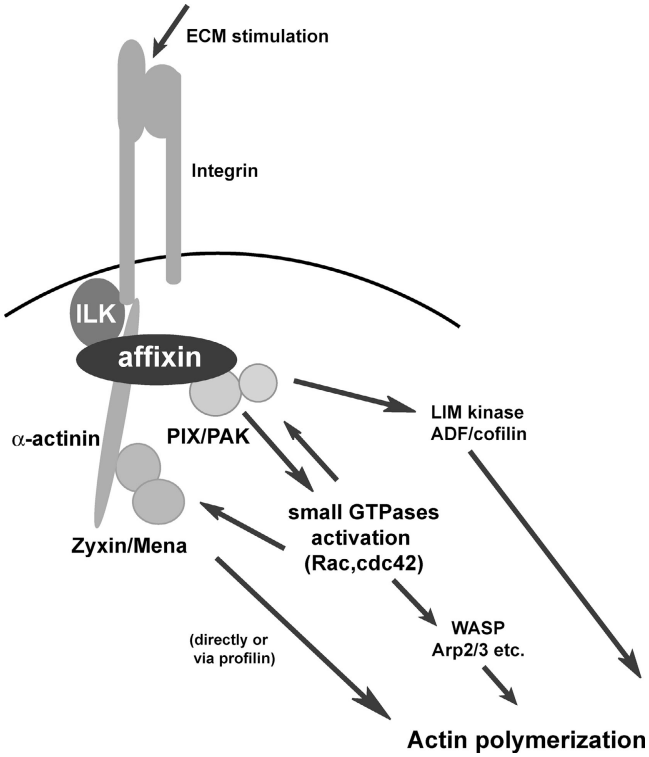

Figure 10. **Schematic illustration of the role of affixin.** The integrin–ECM interaction acutely activates ILK, which is considered to induce phosphorylation of the COOH-terminal CH domain of affixin. It triggers the interaction between affixin and α-actinin, which promotes the recruitment of α-actinin into these nascent FAs. Zyxin and its binding partner, Mena, are recruited into FAs by their interaction with α-actinin. On the other hand, the NH_2-terminal CH domain of affixin is considered to transmit integrin–ILK signals to activate Cdc42/Rac1 through interaction with αPIX, which results in the recruitment of PAK to FAs. Affixin-mediated formation of the signaling complex at nascent FAs should cooperatively promote actin polymerization and lead to cell spreading.

Materials and methods

Reagents

Anti-ILK and anti-α-actinin mAbs were obtained from Upstate Biotechnology; anti-paxillin and anti-Mena mAbs were from Transduction Laboratories; anti-vinculin, anti-actin, and anti-flag mAbs were from Sigma-Aldrich; anti-flag pAb was from Zymed Laboratories; and anti-α-actinin and anti-T7

pAbs were from Santa Cruz Biotechnology, Inc. Anti-affixin antibodies were generated as described previously (Yamaji et al., 2001). The chicken α1-actinin cDNA was provided by Dr. Michihiro Imamura (National Institute of Neuroscience, Tokyo, Japan).

Cell culture

CHO-K1, Cos-7, IMR-90, and HT1080 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Ham's F12 medium (CHO-K1), or DME (Cos-7 and IMR-90) or MEM (HT1080), containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. cDNA transfection was performed by either electroporation for the immunoprecipitation assay or lipofection using a FuGENE™ 6 transfection reagent (Roche) for immunofluorescence analysis.

Immunoprecipitation assay

Cells cultured in 10-cm dishes were suspended in 200 μl lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM PMSF, 2 mM sodium fluoride, and 1.5% Triton X-100. In experiments to analyze cell spreading on FN, cells were trypsinized, washed three times in PBS, plated onto FN-coated 10-cm dishes, and collected at appropriate times using another lysis buffer containing 20 mM Hepes, pH 7.5, 50 mM NaCl, 10 μg/ml leupeptin, 1 mM PMSF, 2 mM sodium fluoride, 100 μM $CaCl_2$, and 0.75% Triton X-100. After a 30-min incubation on ice, the lysates were clarified by centrifugation at 15,000 rpm for 30 min (clearance of cell extracts with high speed centrifugation was always performed to exclude large cytoskeletal structures), and then incubated with 10 μl protein G–Sepharose (Amersham Biosciences) conjugated with 2 μg anti-affixin pAb, anti-flag mAb, or an equal amount of control normal IgG, for 1 h at 4°C. After washing with each lysis buffer, the immunocomplex was solubilized by adding SDS sample buffer to the resin and subjected to standard Western blot analysis.

Pull-down and blot overlay assay

Immunoprecipitates obtained using protein G–Sepharose conjugated with anti-flag mAb from Cos-7 lysates overexpressing flag-ILK or flag-ILK-KD (K220M) were mixed with purified recombinant GST-affixin, CH2, or GST control (15 μg) in a phosphorylation buffer (50 mM Hepes, pH 7.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$, and 2 mM sodium fluoride) with 10 μM ATP as described previously (Yamaji et al., 2001). After incubation for 60 min at 30°C, glutathione-Sepharose 4B was added to the mixture and was incubated for 30 min at 4°C. After washing with PBS containing 0.05% Tween 20, resins (containing both protein G–Sepharose and glutathione-Sepharose 4B) were resuspended in binding buffer (50 mM Hepes, pH 7.0, 20 mM NaCl, and 0.5% Triton X-100) containing 20 μg/ml purified chicken α-actinin (Sigma-Aldrich) and were incubated for 3 h at 4°C. After incubation, resins were washed again with binding buffer three times, and were resolved by 10% SDS-PAGE, blotted on PVDF membranes, and treated with the anti-α-actinin monoclonal or anti-ILK polyclonal antibody.

Blot overlay was performed as follows: after preincubation with in vitro–translated ILK (Promega) in the phosphorylation buffer, GST-affixin was purified by SDS-PAGE and transferred to a PVDF membrane. After blocking, the membrane was overlaid with 10 μg/ml purified α-actinin (Sigma-Aldrich) in overlay buffer (20 mM Hepes, pH 7.5, 140 mM NaCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 2 mM sodium fluoride, 0.1% BSA, 0.3% NP-40, and 4 mM DTT) for 3 h at RT. Bound α-actinin was revealed by standard Western blot analysis using anti-α-actinin antibody.

Immunofluorescence microscopy

CHO-K1 cells or those transfected with expression plasmids were cultured on FN-coated coverslips for 24 h, and after washing with PBS, were fixed with 2% formaldehyde in PBS for 15 min and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT. In anti-affixin staining experiments, cells were fixed with 100% methanol. After blocking, the cells were treated with appropriate primary antibodies for 45 min at 37°C, washed with PBS containing 0.05% Tween 20, and incubated with secondary antibodies (Cy3-conjugated goat anti–rabbit [Amersham Biosciences] and Alexa 488®–conjugated [Molecular Probes, Inc.] or Cy5-conjugated [Amersham Biosciences] goat anti–mouse Ig antibodies) at 37°C for 45 min.

siRNA

21-base sequences of the human affixin gene, targeting #1; 5′-AAGCUGAAUGUGGCUGAGGUG-3′, #2; 5′-AAGCAGUACAUGACCUGCUGC-3′, and #3; 5′-AAGCUGAAUUUGGAGGUGACG-3′ (sense sequences), were designed on the basis of a method described previously (Elbashir et al., 2002) and blasted to assess specificity. The target siRNA duplexes and a control nonsilencing siRNA (16-base overlap with that of *Thermotoga maritimia*) were synthesized and purchased from QIAGEN. HT1080, IMR-90, and HeLa cells were transfected with each siRNA duplex using TransMessenger™ transfection reagent (QIAGEN). For immunofluorescence microscopy, HT1080 cells were cultured on FN-coated coverslips for 48 h, fixed with 0.5% PFA in PBS for 15 min, and then similarly treated with other immunostains.

Online supplemental material

CHO-K1 cells cultured on FN-coated plates were transfected with either the GFP vector or GFP-affixin$^{249–272}$. For the replating experiment, cells were trypsinized 24 h after transfection, washed three times in PBS, and plated onto FN-coated plates. HT1080 cells transfected with either control or affixin-targeted siRNA duplex were cultured on glass-bottom dishes for 36 h before time-lapse observations. Immunofluorescence and DIC images were collected using and inverted microscope (model DM IRB; Leica) with the 10× objective every 30 min or the 40× every 10 min, as indicated. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200308141/DC1.

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