

A Novel Integrin-linked Kinase-binding Protein, Affixin, Is Involved in the Early Stage of Cell-Substrate Interaction

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Abstract. Focal adhesions (FAs) are essential structures for cell adhesion, migration, and morphogenesis. Integrin-linked kinase (ILK), which is capable of interacting with the cytoplasmic domain of $\beta 1$ integrin, seems to be a key component of FAs, but its exact role in cell-substrate interaction remains to be clarified. Here, we identified a novel ILK-binding protein, affixin, that consists of two tandem calponin homology domains. In CHO cells, affixin and ILK colocalize at FAs and at the tip of the leading edge, whereas in skeletal muscle cells they colocalize at the sarcolemma where cells attach to the basal lamina, showing a striped pattern corresponding to cytoplasmic Z-band striation. When CHO cells are replated on fibronectin, affixin

and ILK but not FA kinase and vinculin concentrate at the cell surface in blebs during the early stages of cell spreading, which will grow into membrane ruffles on lamellipodia. Overexpression of the COOH-terminal region of affixin, which is phosphorylated by ILK *in vitro*, blocks cell spreading at the initial stage, presumably by interfering with the formation of FAs and stress fibers. The coexpression of ILK enhances this effect. These results provide evidence suggesting that affixin is involved in integrin-ILK signaling required for the establishment of cell-substrate adhesion.

Key words: affixin • cell spreading • focal adhesion • integrin-linked kinase • integrin

Introduction

Embryogenesis, metamorphosis, and wound healing depend critically on the ability of cells to form specific contacts with the extracellular matrix (ECM)¹ (Jockusch et al., 1995). In addition to guaranteeing the mechanical adhesion of cells, these cell-ECM interactions also trigger signal transduction pathways that lead to cell proliferation, differentiation, apoptosis, migration, and specific gene expressions (Hughes and Pfaff, 1998). The contact sites are defined by morphologically discrete structures that mediate between the intra- and extracellular regions. Focal adhesions (FAs) represent specialized structures observed at sites of tight juxtaposition between the cell surface and ECM in many types of cultured cells. Although typical FAs are only rarely found in organisms, they have been proven to be an attractive model for the analysis of the architecture

and regulation of cell-ECM contact in general. At these sites, actin filaments are bound to transmembrane glycoproteins, integrins, through a complex of structural “plaque” proteins that include vinculin, talin, and α -actinin. In addition to these components, signal transduction molecules such as FA kinase (FAK) and Src are also concentrated at FAs (Miyamoto et al., 1995). Indeed, in extremely integrated cell functions such as cell migration the integrin signals are activated at FAs by ECM stimulation and transmitted to intracellular components, producing a dynamic regulation of cytoskeletal organization. Importantly, although sufficiently rigid to provide strong adhesion, FAs are highly dynamic and can be reversibly assembled and disassembled in response to both internal and external signals. For example, the process of cell migration of cultured fibroblasts consists of (a) extending membrane protrusions called lamellipodia or filopodia to form initial cell-substrate attachments, (b) *de novo* formation of initial FAs at the tip of these membrane protrusions, (c) development of the mature forms of the FA complex and actin stress fibers (SFs) through the organization of several cytoskeletal proteins, and (d) disruption of FA complexes at the rear of the cell and retraction of the tail (Lauffenburger and Horwitz,

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¹Abbreviations used in this paper: CH, calponin homology; ECM, extracellular matrix; FA, focal adhesion; FAK, FA kinase; GST, glutathione S-transferase; ILK, integrin-linked kinase; MBP, myelin basic protein; SF, stress fiber.

1996). However, how matrix molecules, integrins, cytoskeletal molecules, and signaling molecules collaborate to promote such integrated events remains to be determined.

Integrins, the major transmembrane glycoproteins in FAs, consist of two different subunits (α and β chains) that form a heterodimer (Hynes, 1992). Although their extracellular domains together form a ligand-linking site, their cytoplasmic domains are considered to provide attachment sites for cytoplasmic structural and signaling molecules. So far, to understand the molecular basis of integrin signaling, many molecules that can bind directly to the cytoplasmic tails of integrins have been successfully identified. One of these is integrin-linked kinase (ILK), which is a ubiquitously expressed serine–threonine protein kinase capable of interacting with the integrin β cytoplasmic domain (Hannigan et al., 1996). Although the first report on ILK demonstrated that its activity is regulated by cell–ECM interaction and that it plays a role in cell adhesion, the multifunctional aspects of this kinase being involved in signal transduction pathways including insulin and Wnt signaling were demonstrated in subsequent works (Hannigan et al., 1996; Delcommenne et al., 1998; Novak et al., 1998). On the other hand, recent studies have suggested the possibility that ILK is involved in the process of FA formation: the overexpression of ILK promotes the colocalization of $\alpha 5 \beta 1$ integrin and fibronectin with vinculin (Wu et al., 1998) and ILK colocalizes with $\alpha 5 \beta 1$ integrin and FAK in FAs (Li et al., 1999). However, the underlying molecular mechanism by which ILK is involved in the regulation of FA formation is still unclear.

In this study, we identified a novel calponin homology (CH) domain–containing protein, affixin, which interacts specifically with the kinase domain of ILK. Immunocytochemical analyses demonstrate that affixin colocalizes with ILK at FAs and at the tip of the leading edge. Furthermore, in cells replated on fibronectin-coated coverslips affixin shows codistribution with ILK in bleb-like initial membrane protrusions at a very early stage of cell spreading. The introduction of the COOH-terminal half of affixin, which binds ILK and is phosphorylated by ILK *in vitro*, inhibits the development of FAs and SFs and blocks the cell spreading process at a very early stage. Interestingly, when overexpressed in well-spread CHO cells, the COOH-terminal half of affixin also disrupts preformed FAs and SFs, but sufficient activity is observed only when ILK is coexpressed. These results suggest that affixin may be one of the downstream targets of ILK, which works at a very early stage of cell–substrate adhesion to allow the formation of FAs and SFs.

Materials and Methods

Yeast Two-Hybrid Library Screening

cDNAs corresponding to full-length human ILK were amplified from a human bone marrow cDNA library (CLONTECH Laboratories, Inc.) by PCR. These fragments were subcloned into pAS2-1, and the resultant ILK–pAS2-1 was used to screen human bone marrow and fetal liver cDNA libraries (CLONTECH Laboratories, Inc.) in Y190(a) yeast strain. Cotransformants of the bait and library plasmids were grown for 3–7 d at 30°C on minimum essential plates lacking histidine, tryptophan, leucine, and uracil and containing 35 mM 3-aminotriazole (Sigma-Aldrich). Positive colonies were further screened for β -galactosidase activity according

to the manufacturer's instructions (CLONTECH Laboratories, Inc.). Y187(a) yeast strain was also used only for β -galactosidase assay to verify the two-hybrid interactions. Approximately 1×10^6 and 4×10^6 clones were screened, respectively, and seven independent clones positive for growth on plates lacking histidine and β -galactosidase activity were identified. Sequencing analysis revealed that five of these clones contained cDNA inserts encoding a 570-bp overlapping sequence. Subsequent back-screening against phage cDNA libraries from NEC and Jurkat cell lines to obtain the corresponding full cDNA resulted in the identification of several affixin cDNAs with the same ORF but containing different 5' or 3' noncoding regions.

Northern Blot Analysis

To analyze the tissue distribution of mRNA expression, multiple tissue Northern blot membrane (CLONTECH Laboratories, Inc.) was probed with a ^{32}P -labeled human affixin cDNA probe, corresponding to amino acid residues 206–481 of the I-affixin prepared using a random-primed DNA labeling kit (Amersham Pharmacia Biotech). The hybridization was performed according to the manufacturer's instructions (CLONTECH Laboratories, Inc.), and an x-ray film was exposed at -80°C for 10 d with an intensifying screen.

Affixin and ILK Mutations

Affixin and ILK deletion mutants were generated by PCR using appropriate primers. Point mutations in ILK mutants (E359K, K220M, K220A) were introduced using a QuickChange site-directed mutagenesis kit (Stratagene). The fidelities of the amplified sequences were all verified by DNA sequencing.

Cell Culture

CHO-K1 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in F-12 medium containing 10% FCS (Cell Culture Technologies, Inc.), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. COS-7 cells were cultured under the same conditions as those for CHO-K1, except for the use of DME instead of F-12 medium. cDNA transfection was performed by either electroporation for the immunoprecipitation assay or lipofection using a Fugene6 transfection reagent (Roche) for immunofluorescence analysis. When performing the replating assay, CHO-K1 cells were transfected with the appropriate expression plasmids, harvested 48 h later by incubating in 0.05% trypsin in PBS containing 0.02% (wt/wt) EDTA, washed two times with PBS, and replated on fibronectin-coated coverslips.

Antibodies

The antibodies used in this study were anti-Flag and antivinculin monoclonal antibodies (Sigma-Aldrich), anti-FAK monoclonal antibodies (Transduction Laboratories), anti-ILK monoclonal antibody (Upstate Biotechnology), anti- α -actinin monoclonal antibody (provided by Yukiko Hayashi, National Institute of Neuroscience, NCNP, Japan), anti-T7 monoclonal antibody (Novagen), and FITC-phalloidin and rhodamine-phalloidin (Molecular Probes). Antiaffixin antibodies were generated in rabbits using glutathione *S*-transferase (GST)–ss-affixin as an antigen and affinity purified with the antigen before use.

SDS-PAGE and Immunoblot Analysis

For analysis of affixin expression in various rat tissues, each organ was excised from 10-wk-old Sprague Dawley rats deeply anesthetized with diethyl ether, washed with ice-cold PBS, and frozen immediately in liquid nitrogen. The tissue blocks were crushed using a Cryo-Press (Diatron) precooled in liquid nitrogen, and the resultant powder was suspended in 10 vol (vol/wt) of SDS sample buffer, homogenized with a Polytron homogenizer (Kinematica), and sonicated. 15- μg aliquots of these samples were loaded in each lane. Electrophoresis was carried out by one-dimensional SDS-PAGE (10 or 12% polyacrylamide). The separated proteins were transferred onto PVDF membranes, which were subsequently blocked with 5% skimmed milk. The membranes were treated with appropriate antibodies, and antibody reactions were visualized by a chemiluminescence ECL system (Amersham Pharmacia Biotech).

Immunoprecipitation Assay

Cells cultured in 10-cm dishes were suspended in 200 μ l lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10 μ g/ml leupeptin, 1 mM PMSF, 1% Triton X-100, 0.1% deoxycholate, and 0.1% SDS. After a 30-min incubation on ice, the lysates were clarified by centrifugation at 14,000 rpm for 30 min. 15 μ l of protein G-Sepharose (Amersham Pharmacia Biotech) conjugated with 2 μ g of affinity-purified anti-affixin, anti-Flag antibodies, or control normal rabbit IgG were incubated with the cell lysates for 1 h at 4°C. After washing with lysis buffer, the immunocomplex was solubilized by adding SDS sample buffer to the resin.

Immunofluorescence Microscopy

CHO-K1 cells or those transfected with expression plasmids were cultured on fibronectin-coated coverslips for 48 h and, after washing with PBS, fixed with 1 or 2% formaldehyde in PBS for 15 min and then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. In some experiments, cells were fixed with 100% methanol. The cells were blocked with PBS containing 10% calf serum for 1 h at room temperature and then treated with appropriate primary antibodies for 45 min at 37°C in a moist chamber. After washing with PBS containing 0.05% Tween 20, the cells were incubated with secondary antibodies (Cy3-conjugated goat anti-rabbit [Amersham Pharmacia Biotech] and Alexa488-conjugated goat anti-mouse antibodies [Molecular Probes]) at 37°C for 45 min. After washing, samples were observed under a fluorescence microscope (BX50; Olympus) equipped with a cooled CCD camera (Photometrics). Human skeletal muscle sample was obtained by biopsy for diagnostic purpose with informed consent. Thin sections of 6- μ m thickness were fixed for 10 min with 100% acetone at -20°C, blocked for 15 min at 37°C with 2% BSA and 0.5% goat serum in PBS, and then processed for immunofluorescence analysis. Confocal microscopic analysis was performed using a Bio-Rad Laboratories Radiance 2000 scan head mounted on a Nikon Eclipse E600 microscope.

Purification of Recombinant Affixin and Its Mutant from *Escherichia Coli*

GST-ss-affixin and GST-RP2 fusion proteins were induced in *E. coli* with isopropyl β -D-thio-galactopyranoside (Amersham Pharmacia Biotech), and the proteins were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The GST linker sites of these fusion proteins were digested with PreScissionTM protease (Amersham Pharmacia Biotech) according to the manufacturer's protocol, and the excised recombinant proteins eluted from the resin were dialyzed against the appropriate buffers before use.

In Vitro Kinase Assay

COS-7 cells transfected with expression vectors encoding Flag-tagged ILK or its mutants were lysed in 20 mM Hepes, pH 7.0, 150 mM NaCl, 1 mM EDTA, 10 μ g/ml leupeptin, 1 mM PMSF, 1% Triton X-100, and 0.1% deoxycholate. Immunoprecipitates by anti-Flag antibody were extensively washed with lysis buffer and then kinase reaction buffer (50 mM Hepes, pH 7.0, 10 mM MnCl₂, 10 mM MgCl₂, 2 mM NaF, 1 mM Na₃VO₄) and subjected to protein kinase assays in 20 μ l kinase reaction buffer containing 10 μ Ci [γ -³²P]ATP and an appropriate substrate (myelin basic protein [MBP] or recombinant affixin). After incubation for 60 min at 30°C, the reaction mixture was resolved by 10% SDS-PAGE, and bands were visualized by a Bio-imaging analyzer system (BAS2000; Fuji).

Results

Cloning of a Novel ILK-binding Protein, Affixin

Two-hybrid screening against human bone marrow and fetal liver cDNA libraries using full-length human ILK as bait resulted in the identification of a cDNA sequence encoding a novel protein of 364 amino acids (predicted molecular mass is 41.71 kD) as the longest ORF (Fig. 1; described in Materials and Methods). Another candidate for initiation codon with the preceding Kozak consensus sequence (Fig. 1, arrowhead) predicts a shorter product of

350 amino acids (40.13 kD). We named these products l- and s-affixin, respectively, from the Latin word "affixa," meaning fixtures, because they are involved in cell-substrate adhesion (see below). A similarity search revealed that there are affixin homologues in *Caenorhabditis elegans* and *Drosophila melanogaster* (Fig. 1; see below). During this analysis, a cDNA clone, CGI-56, containing a sequence identical to affixin except for the 5' sequence, was identified as one of the human genes conserved in *C. elegans* (Lai et al., 2000). The remaining two positive clones obtained in the two-hybrid screening also encoded novel proteins containing sequences homologous to the ILK-binding region (see below) of affixin, suggesting that affixin comprises a novel protein family (data not shown).

Affixin Is a Novel Member of the CH Domain-containing Protein Family

Close inspection of the sequence of affixin revealed an internal sequence repeat that shows a significant sequence similarity to the actin-binding regions of several actin cross-linking proteins, such as β -spectrin, α -actinin, and dystrophin (Fig. 1 and Fig. 2 A). In these actin cross-linking proteins, the sequences are composed of 240 amino acid residues containing a tandem repeat of two CH domains, followed by an extended rod domain with a coiled coil structure (spectrin repeats; Fig. 2 B). However, the affixin sequence exhibits several unique features. First, as shown in Fig. 2 B, most of the affixin molecule corresponds to only two repeated CH domains with short flanking NH₂- and COOH-terminal sequences and lacks the rod domain. Second, the two tandem CH domains in actin cross-linking proteins, CH1 (NH₂-terminal) and CH2 (COOH-terminal), show weaker homology to each other in contrast to their intermolecular homology. On the other hand, both CH domains of affixin show higher homology to the CH1 domains of actin cross-linking proteins (Fig. 2, A and B). Third, the homology between actin cross-linking proteins and affixin is restricted to the NH₂-terminal half of the CH domains. The sequence of the COOH-terminal half of affixin is highly diverged, although the COOH-terminal hydrophobic moieties that have been suggested to be important for the actin binding of these actin cross-linking proteins are partially conserved (Fig. 2 A, underline; Carugo et al., 1997). Additionally, the COOH-terminal regions in the affixin CH domains show divergence from the consensus residues that are essentially conserved in CH domain-containing proteins such as calponin (Fig. 2 A, asterisks). Interestingly, recent progress in genome projects has revealed that affixin has counterparts in other species. *C. elegans* T21D12.4 shows 64% overall similarity and 45% identity, whereas *D. melanogaster* AAF49016 shows 71% similarity and 58% identity (Fig. 1 and Fig. 2 B). Taken together, we conclude that affixin is a unique member of the CH domain-containing proteins, which is well conserved evolutionary from worms to humans.

Affixin Is Highly and Ubiquitously Expressed in Muscles and Various Tissues

Tissue distribution of affixin was examined by Northern and Western blot analysis. Predicted affixin transcripts (1.4 kb) were detected in heart, skeletal muscle, spleen,

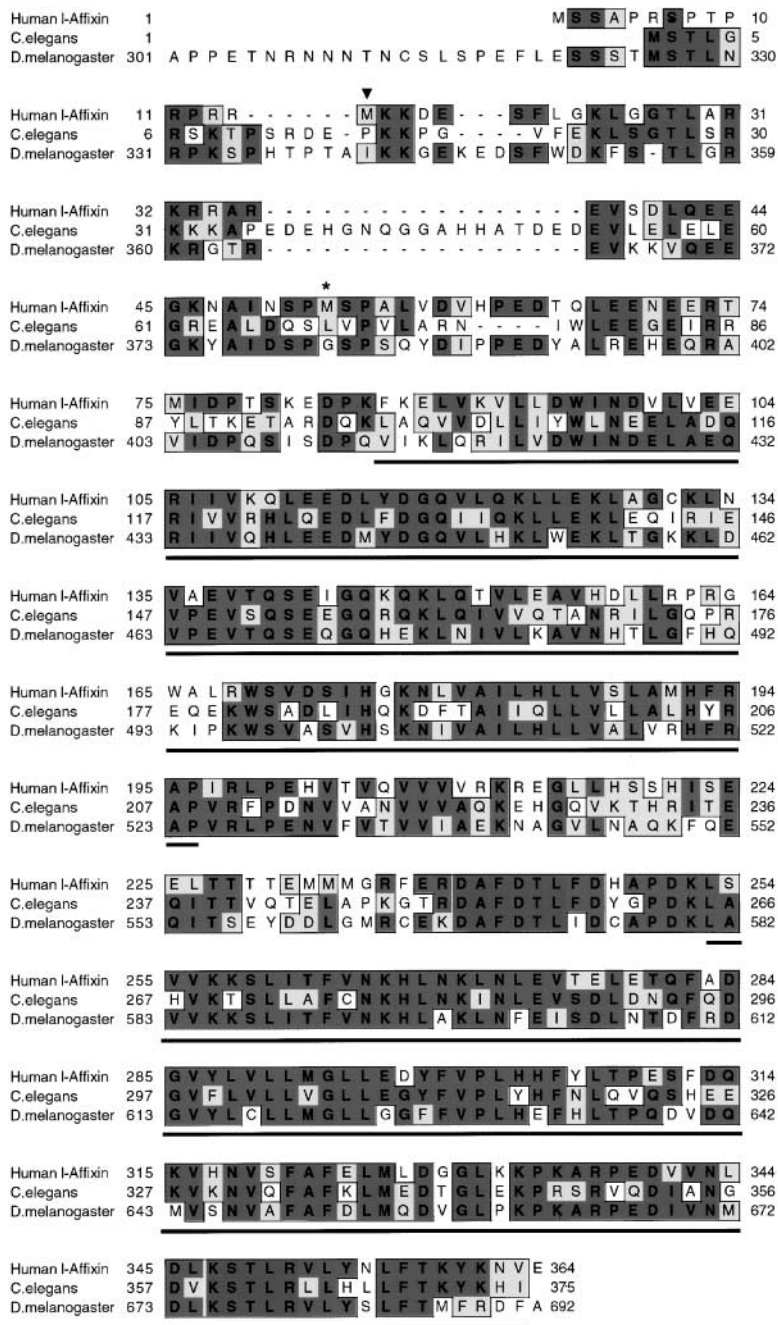


Figure 1. Predicted amino acid sequence of human affixin. Predicted amino acid sequence of human affixin (sequence data available from GenBank/EMBL/DBJ under accession no. AB048276) is aligned with the predicted affixin homologue in *C. elegans* (accession no. T21D12.4) and *D. melanogaster* (accession no. AAF49016) based on analysis using clustal W. Residues similar and identical to those of affixin are lightly and heavily shaded, respectively. The second and third alternative initiation methionines are indicated by an arrowhead and an asterisk, respectively. Two CH domains are underlined.

kidney, liver, small intestine, placenta, lung, and peripheral blood leukocyte, with the highest expression in skeletal muscle and heart (Fig. 3 A). On the other hand, four additional larger transcripts (2.8, 3.5, 5.0, and 6.5 kb) were also observed in several human tissues, especially in skeletal muscle. Affinity-purified polyclonal antibodies raised against the full-length amino acid sequence of ss-affixin (see below) specifically detected two bands (47.4 and 50.5 kD, respectively) in CHO and NIH3T3 cells, each of which shows the same migration rate as l- and s-affixin overexpressed in COS-7 cells (Fig. 3 B, arrows). It should be noted that the expression vector encoding l-affixin also induced the expression of a protein with lower molecular weight corresponding to s-affixin. This may indicate that

the identified two initiation codons (Fig. 1) are simultaneously active in this construct, although the possibility of specific proteolysis of l-affixin cannot be formally excluded. Consistent with Northern blot analysis, affixin is detected ubiquitously, with higher expression in heart and skeletal muscle (Fig. 3 C). In the spleen, the antibody also detected a smaller band strongly showing the same migration rate as overexpressed ss-affixin, which is considered to be expressed using a more downstream initiation codon with the preceding Kozak consensus sequence (Fig. 1, asterisk; data not shown). In fact, we have obtained an affixin cDNA species from the T cell lymphoma-derived Jurkat cell line library with an alternatively spliced 5' sequence in which a stop codon appears in front of the

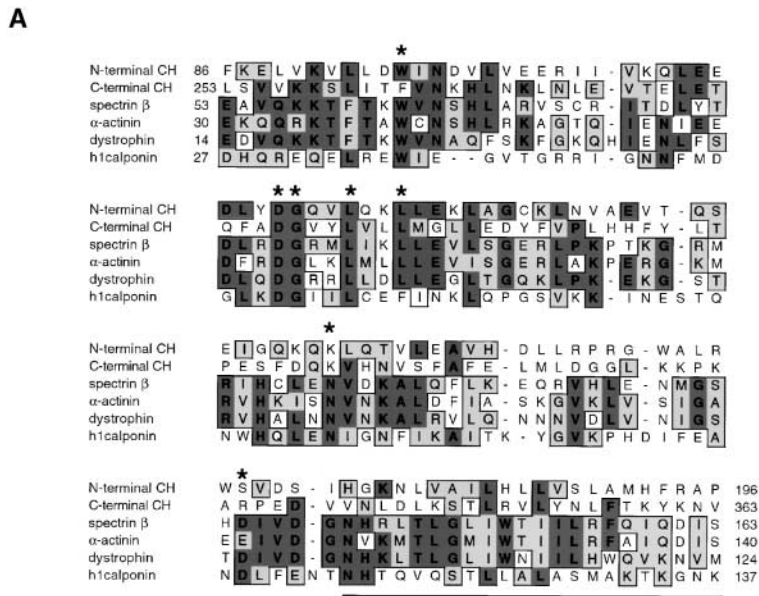
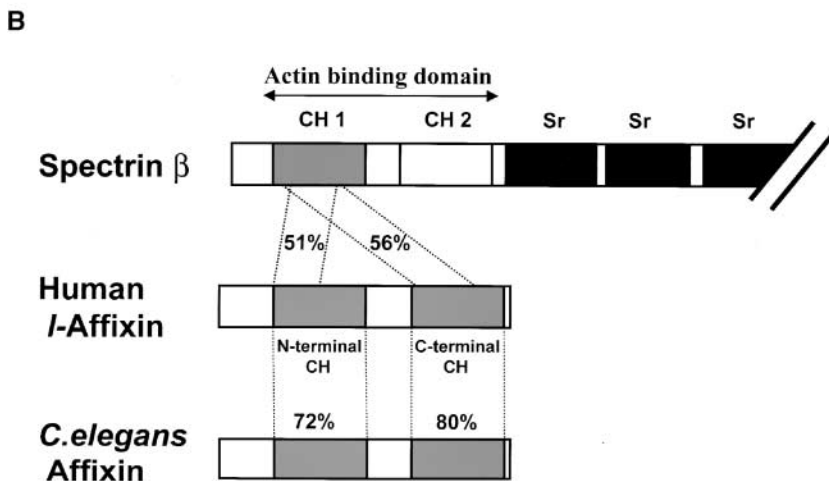


Figure 2. Affixin is a novel CH domain-containing protein. (A) Alignment of the two CH domains of human affixin (NH₂-terminal CH and COOH-terminal CH) with the first CH domains in the actin-binding region of three human actin cross-linking proteins (spectrin β , sequence data available from GenBank/EMBL/DBJ under accession no. M96803; α -actinin, accession no. P12814; dystrophin, accession no. P11532) and the single CH domain of human calponin H1 (accession no. SWP51911). Residues similar and identical to those of affixin are lightly and heavily shaded, respectively. Asterisks at the top indicate the consensus residues that are essentially conserved among CH domain-containing proteins (Carugo et al., 1997). The underlined sequence indicates the COOH-terminal hydrophobic residues conserved in the first CH domains of the actin-linking proteins. (B) Schematic diagram of the overall structure of the predicted affixin protein. Human affixin comprises two tandem CH domains, both of which show the closest similarity to the first CH domain in the actin-binding site of human β -spectrin. In contrast to spectrin, affixin is unique in that it lacks the triple-helical repeats (Sr, spectrin repeats). A *C. elegans* homologue of affixin (accession no. T21D12.4) is also shown at the bottom.



third initiation codon mentioned above. Taken together, these results suggest that ss-affixin, another affixin isoform, is highly expressed and functions in spleen.

Analysis of the Interaction between ILK and Affixin

Fig. 4 A demonstrates that affixin is coprecipitated with ILK by anti-affixin antibody from CHO cell extract, indicating the endogenous interaction of these proteins in vivo. The following experimental results shown in Fig. 4 further reveal the binding sites on each molecule in detail. Since the cDNA clones obtained by the first two-hybrid screening contained overlapping sequences corresponding to 242–364 amino acids of affixin, the ILK-binding site on affixin was considered to be around the second CH domain. The subsequent two-hybrid analysis using a series of affixin deletion mutants revealed that the amino acid sequence 258–364, corresponding to the second CH domain, is sufficient for ILK binding, whereas mutants (I-AFX 292–364) lacking the NH₂-terminal half of the second CH domain lose ILK-binding activity (Fig. 4 B). This interac-

tion was further confirmed by coimmunoprecipitation assays performed in COS-7 cells: full-length and an NH₂-terminal-truncated mutant of I-affixin, RP2, were coimmunoprecipitated with Flag-tagged full-length ILK by anti-Flag antibody, whereas a COOH-terminal truncated mutant, RP1, was not (Fig. 4 C). These results indicate that the second but not the first CH domain is required and sufficient for the interaction with ILK.

Next, we determined the affixin-binding site on ILK using a similar approach. Two-hybrid assays revealed that the COOH-terminal kinase domain but not the preceding ankyrin repeats or pleckstrin homology domain is necessary and sufficient for the interaction with affixin (data not shown). Coimmunoprecipitation assays in COS-7 cells, the results of which are shown in Fig. 4 D, provide further insight into the requirement for the interaction. We constructed a Flag-tagged kinase-deficient mutant of ILK (K220M) by substituting the conserved lysine residue in the ATP-binding site of the kinase domain with methionine and found that the mutant coimmunoprecipitates with T7-tagged ss-affixin. Thus, we concluded that the ki-

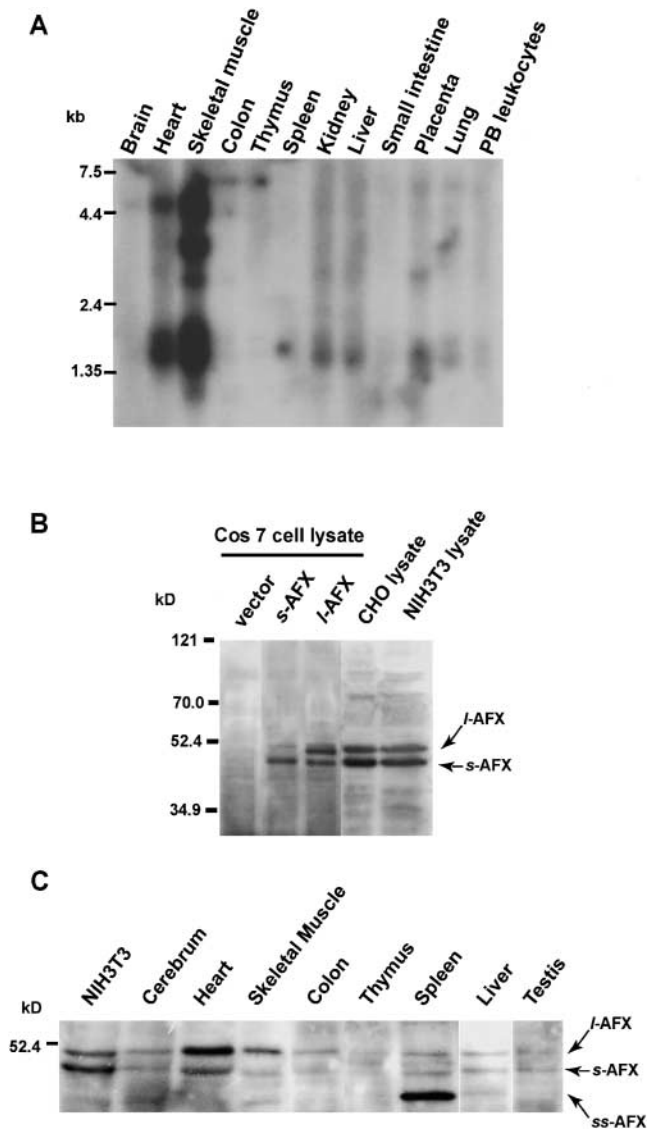


Figure 3. Expression of affixin mRNA and protein in various tissues and cell lines. (A) Human multiple Northern blots (CLONTECH Laboratories, Inc.) on which 2 μ g of polyA RNAs isolated from the indicated tissues were probed with an affixin cDNA fragment (206–481 bp). (B) Western blot analysis of total lysates of CHO-K1 (lane 4) and NIH3T3 (lane 5) cells probed with the affixin antibody. In lanes 1–3, total lysates of COS-7 cells transfected with pMe18s vector alone (lane 1), s-affixin (lane 2), and I-affixin (lane 3) are loaded. Note that s-affixin is also detected in lysate of cell transfected with an expression vector encoding I-affixin. (C) 15 μ g of various tissue lysates from adult rat was loaded and analyzed with the affixin antibody as indicated. Note that a strong band with a lower molecular weight was specifically detected in spleen. The migration rate of this band is the same as that of ss-affixin that is expressed using the third initiation methionine (data not shown; see Fig. 1). AFX, affixin.

nase activity of ILK is not necessary for this interaction. However, another ILK mutant, E359K, which has been suggested to be kinase deficient (Novak et al., 1998; see below), showed greatly reduced interaction with affixin. This indicates that the glutamic acid residue located in the

activation loop of the kinase domain of ILK is critical for the interaction.

Affixin Is a Direct Substrate for ILK In Vitro

Since the kinase domain of ILK is sufficient to associate with affixin, we next tested whether affixin can be phosphorylated by ILK in vitro. In Fig. 5 A, Flag-tagged wild-type or kinase-deficient ILK was overexpressed in COS-7 cells, and the kinase activity in the anti-Flag antibody immunoprecipitates was assayed using MBP as a substrate. The immunoprecipitates from cells expressing wild-type ILK showed enhanced kinase activity, whereas those from ILK (K220A)- and ILK(K220M)-expressing cells showed the background level of the activity. Surprisingly, the immunoprecipitate of ILK(E359K) reproducibly showed kinase activity comparable to wild-type ILK, suggesting that this mutant is not kinase deficient, although we do not know the reason for the discrepancy with the previous data (Novak et al., 1998). When we used recombinant affixin purified from *E. coli* as a substrate, the wild-type ILK immunoprecipitates but not the K220M immunoprecipitates phosphorylated affixin to the same extent as MBP, suggesting that affixin is a good substrate for ILK in vitro (Fig. 5 B). Consistent with the results on the narrowed binding site of the ILK–affixin interaction, the deletion mutant of affixin, RP2, was also phosphorylated by the ILK immunoprecipitates to a similar extent (Fig. 5 B). We also examined the possibility that affixin affects the kinase activity of ILK, since as shown in Fig. 4 D the activation loop of the kinase domain of ILK is involved in the interaction with affixin. However, the addition of recombinant affixin to the ILK immunoprecipitates did not affect 32 P incorporation into MBP (data not shown), suggesting that affixin can be a substrate but not a regulator of ILK kinase activity.

Affixin Colocalizes with ILK at the Focal Contacts of CHO Cells and the Sarcolemma of Skeletal Muscle Cells

To investigate the physiological significance of the ILK–affixin interaction, we next examined the cellular localization of affixin and ILK in well-spread CHO cells cultured on fibronectin-coated coverslips. As shown in Fig. 6, affixin localizes at FAs identified by staining with antivinculin and antipaxillin antibody (Fig. 6, A and B; data not shown). Furthermore, affixin staining is also detected at the tip of the leading edge, which is negative for vinculin staining (Fig. 6, A and B, arrowheads). Double staining of affixin and ILK revealed that these proteins are colocalized at both FAs and at the tip of the leading edge. The colocalization with ILK was also observed for overexpressed RP2 but not for RP1 (data not shown), consistent with the molecular interaction between ILK and affixin detected in the yeast two-hybrid and immunoprecipitation assays described above (Fig. 4, B and C). As can be seen in Fig. 6 A, paraformaldehyde fixation but not methanol fixation (Fig. 6 C) of cells also revealed filamentous staining of affixin outside dot-like FA structures, which seems to correspond to actin SFs. Immunostaining of overexpressed T7-tagged ss-affixin with anti-T7 antibody finally confirmed this observation: affixin localizes at FAs (Fig. 6, E and F, arrowheads), at the leading edge (data not shown)

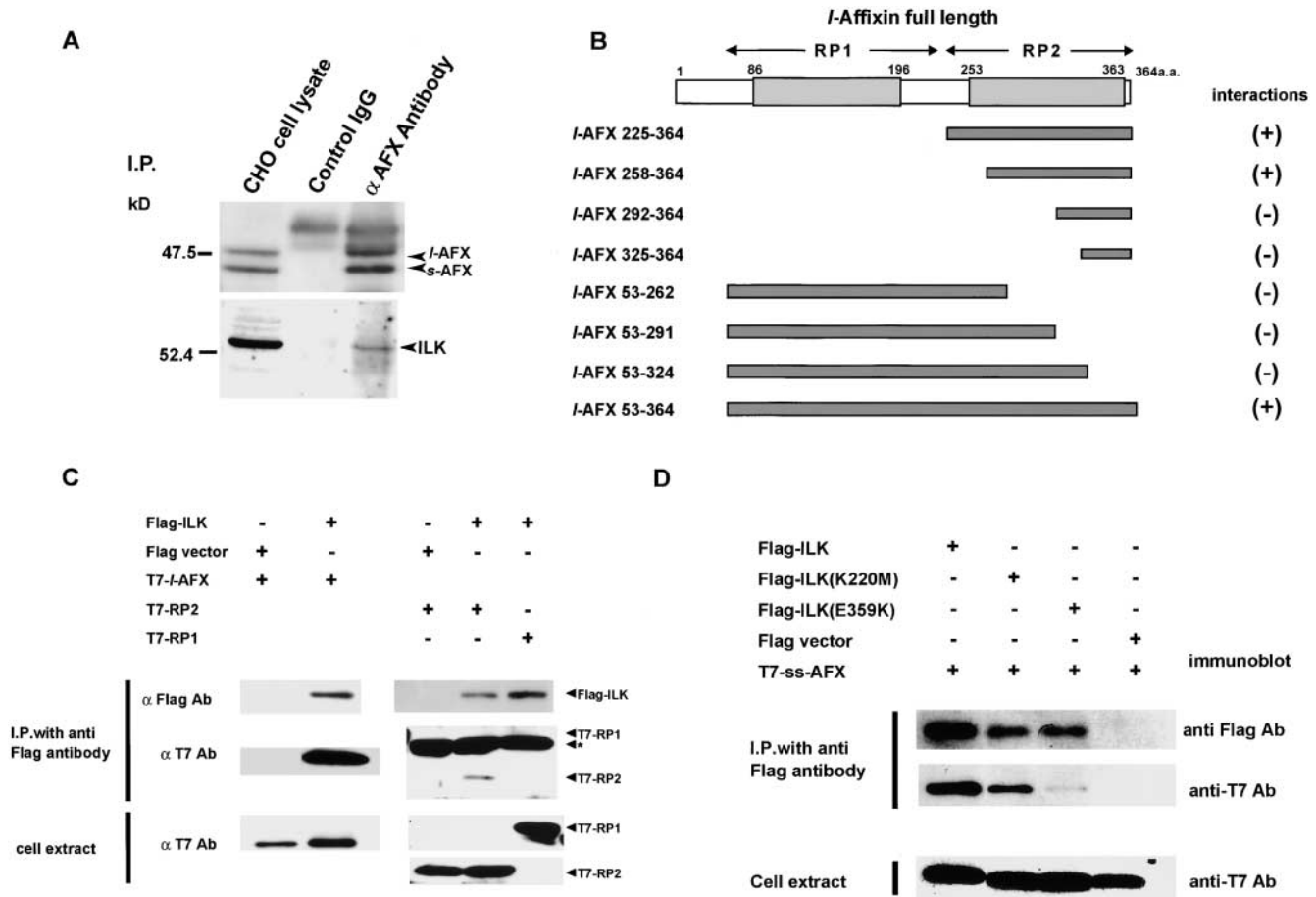


Figure 4. Detailed analysis of the affixin–ILK interaction. (A) Coimmunoprecipitation of ILK with endogenous affixin. Subconfluent CHO cells were lysed in immunoprecipitation buffer. Antiaffixin and control IgG immunoprecipitates were subjected to SDS-PAGE followed by Western blotting with antiaffixin (top) and ILK (bottom) antibodies. (B) Mapping of the ILK-binding region on affixin molecule by yeast two-hybrid binding assay. cDNA fragments encoding human I-affixin deletion mutants and full-length ILK were subcloned into pAS2-1 and pGAD424 vectors, respectively. These vectors were cotransformed into yeast Y187(a), and 5 d later the interactions were examined by β -galactosidase filter assay. (C) COOH-terminal half of affixin is coimmunoprecipitated with ILK from COS-7 cells. Expression vectors encoding the T7-tagged ss-affixin (left) or its NH₂-terminal (RP1) or COOH-terminal (RP2) half (right) were cotransfected into COS-7 cells with pME18s-Flag expression vectors with or without a full-length ILK cDNA insert. The immunoprecipitation assay was performed using anti-Flag antibody, and immunocomplexes were subjected to immunoblot analysis with anti-Flag and anti-T7 antibody. The asterisk indicates a band corresponding to the IgG light chain. (D) The binding of T7-tagged ss-affixin with Flag-tagged ILK point mutants was examined by immunoprecipitation assays in COS-7 cells. Immunoprecipitation was performed with anti-Flag monoclonal antibody, whereas Western blot analysis of the immunocomplex was carried out using anti-T7 and anti-Flag antibody. ILK(E359K) and ILK(K220M) represent point mutants in which glutamate 359 in the activation loop or lysine 220 in the ATP-binding region of the ILK kinase domain is replaced with lysine or methionine, respectively. Note that K220M but not E359 coimmunoprecipitates with affixin.

and along actin SFs linking separate FAs (Fig. 6, E–H). Since affixin is highly expressed in muscle cells, we next examined the localization of affixin and ILK in skeletal muscle cells. Consistent with the localization of the muscle-specific integrin β 1 isoform, β 1D, reported to date (Vachon et al., 1997), immunohistochemical analysis of human skeletal muscle cells revealed that affixin and ILK colocalize at the sarcolemma, although affixin but not ILK staining is also detected in cytoplasm (Fig. 7, A–F). Close inspection of the sarcolemma staining of these proteins using confocal microscopy revealed their striated distributions on the membrane, to which Z-bands identified by α -actinin-staining anchor (Fig. 7, G–I, arrowheads). These results indicate that affixin and ILK are concentrated to the region on

the sarcolemma where Z-bands attach, which are the corresponding structures of FAs in cultured cells.

Affixin and ILK May Be Involved in the Early Phase of FA Formation

Because the leading edge is a site where the formation and growth of de novo cell–substrate adhesions actively occur, the above results on the distribution of affixin and ILK at the tip of the leading edge suggest the possibility that their interaction plays a role in the initial phase of FA formation. This notion was supported by analyzing their localization in reseeded CHO cells that are actively spreading. When CHO cells are harvested in trypsin/EDTA solution

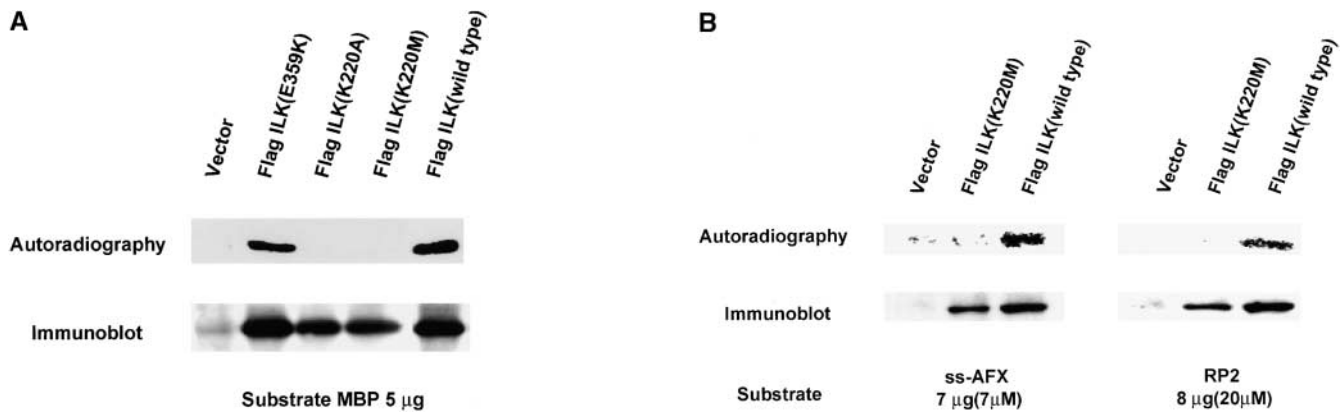


Figure 5. ILK phosphorylates affixin in vitro. (A) Kinase activity of wild-type ILK and its point mutants overexpressed in COS-7 cells. Flag-tagged ILK or its mutants were overexpressed and immunoprecipitated from COS-7 cell lysates with monoclonal anti-Flag antibody. Kinase activities of the resultant immunocomplexes were examined using MBP as a substrate. Top, autoradiography showing ^{32}P incorporation into MBP; bottom, Western blot analysis of the precipitated Flag-tagged ILK or its mutants using polyclonal anti-Flag antibody. (B) Full-length and COOH-terminal half of ss-affixin are phosphorylated by ILK in vitro. Recombinant full-length and COOH-terminal half of ss-affixin (RP2) were used as substrates to estimate the kinase activity of the ILK immunocomplexes prepared as described in A.

and reseeded on fibronectin-coated coverslips, their cell-substrate adhesions are gradually restored, and their shape changes from round to spread within 4 h (Bauer et al., 1993). During the early stages of this spreading process (1 h after replating) when the cells attach to the substrate with a limited central area of round cell body, most cells transiently develop many spherical out-pouchings of the plasma membrane, blebs, which are ultimately replaced by flat ruffles or small lamellipodia. As shown in Fig. 8, A–C, high concentrations of affixin and ILK are observed in these blebs during the early stages of cell spreading when the cells still show irregular dot-like staining of vinculin in their inner area (Fig. 8 D). Overexpressed affixin and ILK also show similar distribution in blebs (Fig. 8, G and H). On the other hand, FAK was not observed in these peripheral membrane protrusions even when blebs have developed into more flattened lamellipodia-like structures (Fig. 8, E and F). Actin filament identified by rhodamine-phalloidin staining was observed only in restricted numbers of blebs (data not shown), but at later stages when cells have started to spread, short actin bundles started to appear in the peripheral lamellae from the dot-like structures at which affixin localizes (Fig. 8, I and J). These results indicate that affixin and ILK are recruited into nascent cell-substrate adhesion structures at a very early stage of cell spreading faster than FAK and vinculin from which mature FAs and SFs develop.

Next, to obtain further insight into the roles of affixin and ILK in the initial phase of cell-substrate adhesion, the effects of the overexpression of ss-affixin and its mutants, RP1 and RP2, were investigated in this replating assay of CHO cells (Fig. 9 A). 1 h after reseeding onto fibronectin-coated coverslips, >70% of the cells overexpressing each affixin construct still displayed a round morphology, although cells expressing RP1 tended to show flatter shape (Fig. 9, B and C, and Fig. 1 H). On the other hand, 4 h after cell replating a drastic deleterious effect of RP2 overexpression on cell spreading was observed: in contrast to cells overexpressing ss-affixin or RP1, many of which ex-

hibited a well-spread flat shape (round cells are <40%), >70% of RP2-expressing cells still remained in circular or oval shape with poor membrane extensions (Fig. 9, B and C, and Fig. 4 H). Close inspection of the RP2-overexpressing cells revealed that they were arrested and unable to proceed from the early stage of cell spreading with peripheral blebs (Fig. 9 D). In these cells, vinculin weakly localized at inner FA-like structures, whereas filamentous actin formed a weak cortical ring with a high concentration within limited numbers of blebs (Fig. 9 D). On the other hand, cells overexpressing RP1 again looked more actively spread compared with cells expressing wild-type affixin (ss-affixin), suggesting that RP1 and RP2 have opposite effects on the establishment of cell-substrate adhesion (Fig. 9 B and Fig. 4 H).

Interestingly, if the overexpression of the affixin mutants was induced in cells that have already established well-developed cell-substrate adhesions, the effect of RP2 overexpression would be strongly restricted (Fig. 10). Although the overexpression of neither ss-affixin nor ILK affects cell morphology (data not shown), limited numbers (10%) of cells expressing RP2 but not RP1 (<3%) show an aberrant spherical morphology instead of the typical well-spread shape (Fig. 10 B, arrowheads). In these cells, FAs and SFs are disrupted, exhibiting similar vinculin and F-actin staining as observed in reseeded cells (data not shown; Fig. 9 D). Again, cells expressing RP1 tended to show an enhanced well-spread shape compared with control CHO cells. Interestingly, the coexpression of ILK with RP2 but not kinase-deficient ILK(K220M) or ILK(E359K), that is kinase active but incompetent to bind affixin (Figs. 4 and 5), increased the population of aberrant round-shaped cells ($\leq 50\%$; Fig. 10, E and F). This effect of ILK was not observed in cells expressing RP1 (Fig. 10 C). Therefore, these results indicate that the second CH domain of affixin requires the interaction with active ILK to exert the deleterious effects on cell-substrate adhesion, whereas it does not in cells that are actively spreading (Fig. 9 B compared with Fig. 10, B and D).

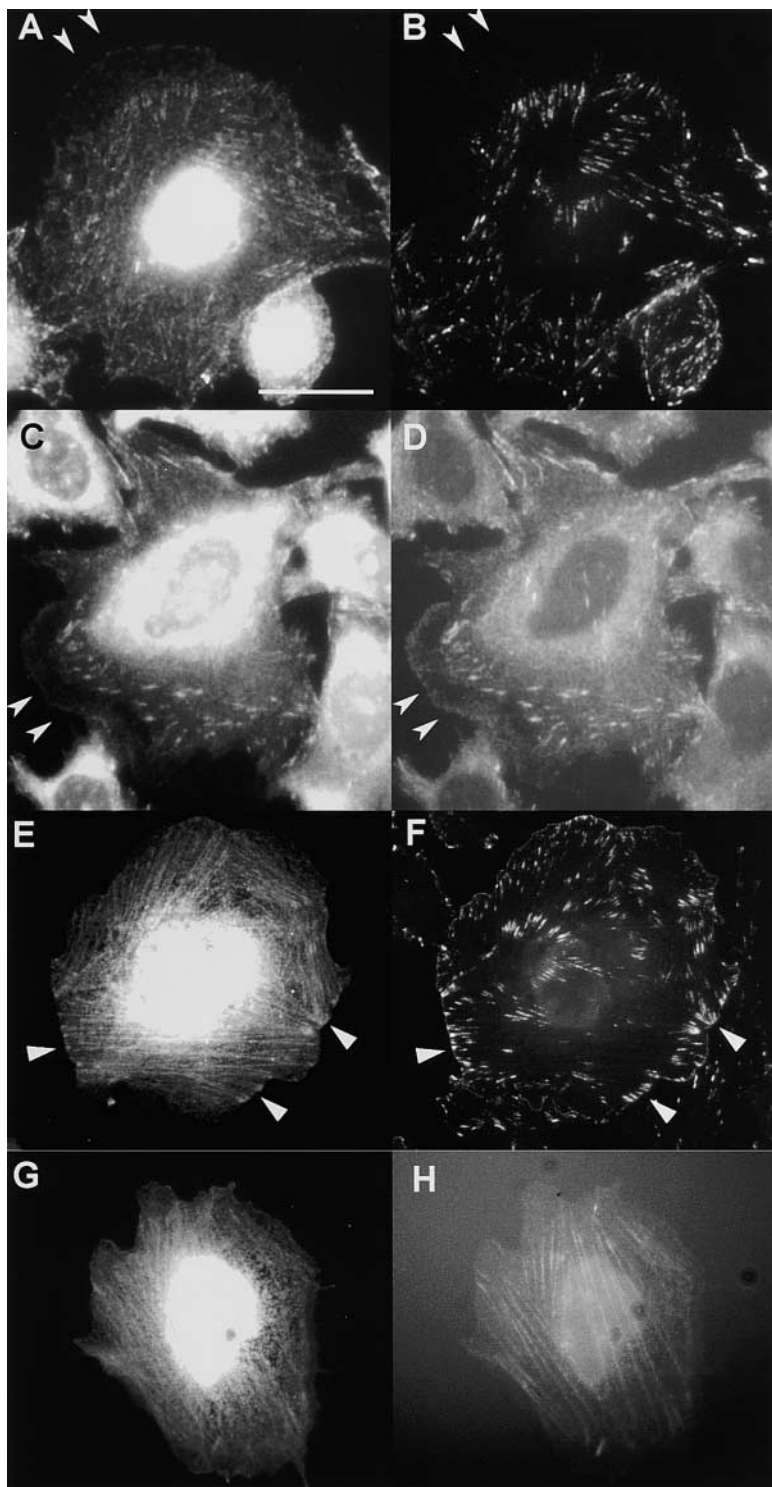


Figure 6. Colocalization of affixin with ILK at FAs and the tip of the leading edge in CHO cells. Immunofluorescence staining with anti-affixin antibody of CHO cells (A and C) or with anti-T7 antibody of CHO cells transfected with T7-tagged affixin (E and G). Cells were stained simultaneously with anti-vinculin (B and F), anti-ILK (D) antibodies, or rhodamine-phalloidin (H). In E–H, CHO cells are reseeded on fibronectin-coated coverslips and fixed after 4-h spreading. Note that affixin and ILK are colocalized at FAs visualized by vinculin staining (A, B, and E and F, arrowheads) and at the tip of the leading edge (A–D, arrowheads). In C and D, fixation was performed with 100% cold methanol, whereas in other cases 1 (A and B) or 2% (E–H) paraformaldehyde in PBS was used. Intense signals from nuclei or perinuclear region represent nonspecific staining of the anti-affixin antibody observed depending on the fixation conditions. Bar, 25 μ m.

Discussion

Affixin, a Novel Component of FAs, Is Recruited with ILK into the Nascent Structure Formed during the Very Early Stages of Cell Spreading

Here, we report a novel CH domain-containing protein named affixin as a binding protein for ILK, a serine-threonine protein kinase that interacts with the cytoplasmic re-

gion of β 1 integrin. Affixin and ILK associate *in vivo* and colocalize at FAs in well-spread cultured cells, suggesting that affixin is a novel component of FAs that may be involved in integrin–ILK signaling at this specific structure. Immunofluorescence analysis of cells in the spreading process after replating on fibronectin indicates that endogenous and exogenously expressed affixin and ILK accumulate in cell surface blebs observed during the very early phase of the spreading process. These blebs have been shown to be spherical out-pouchings of the plasma mem-

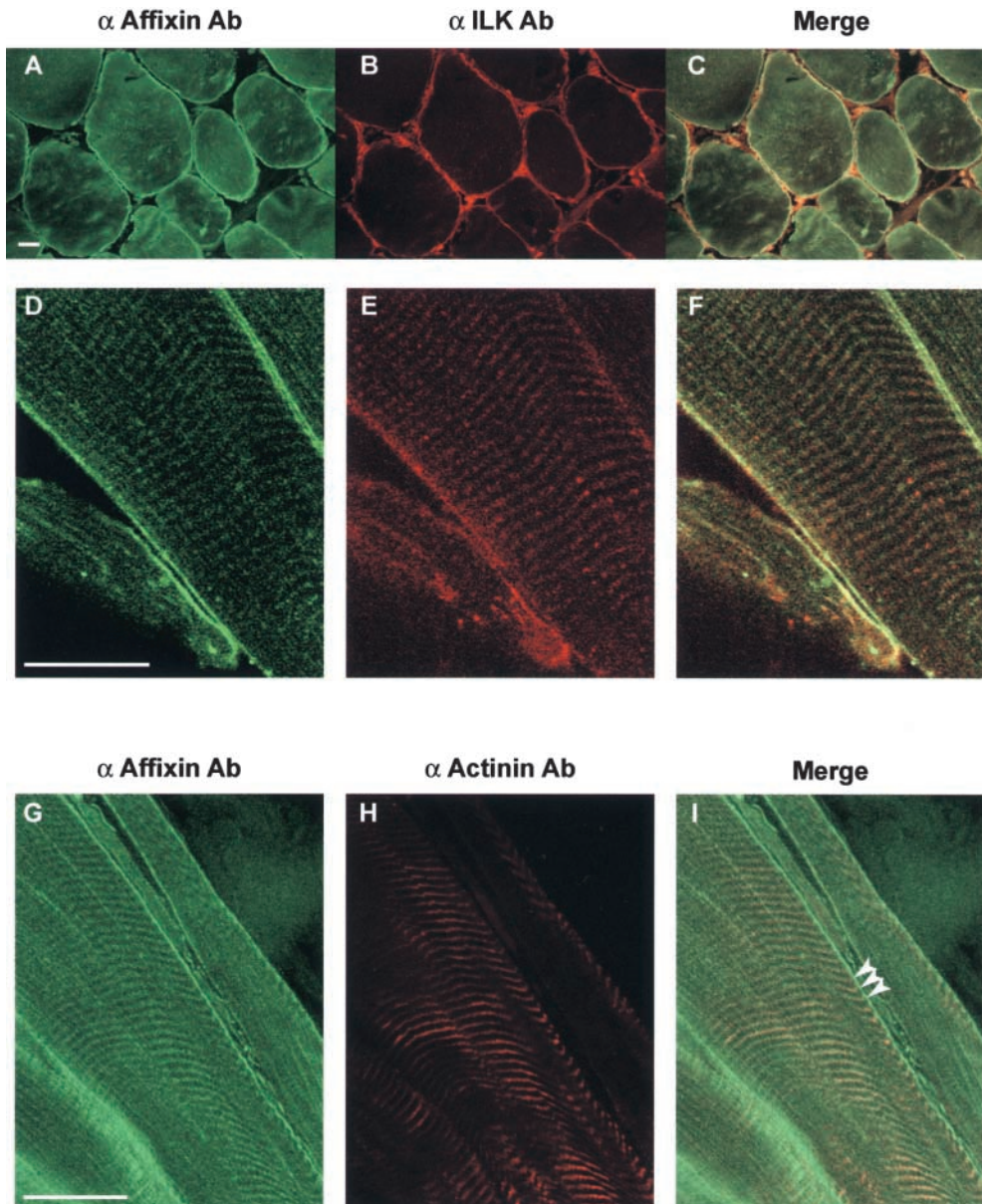


Figure 7. Affixin and ILK localize at the sarcolemma of skeletal muscle cells. Immunofluorescence analysis of cross (A) or longitudinal (B and G) sections of human skeletal muscle with anti-affixin antibody. Cells were stained simultaneously with anti-ILK antibody (B and E) and anti- α -actinin antibody (H). Merged views are also demonstrated (C, F, and I). Observations were performed using confocal microscopy, and in D–I optical sections (0.4 μ m) that tangentially cut off the sarcolemma were selected to demonstrate the striped pattern of affixin and ILK staining on the sarcolemma. In this section, staining of Z-bands with anti- α -actinin almost disappears, but in some regions the completely matched correlation between affixin staining and Z-band is clearly demonstrated. Note that affixin concentrated in the sites of the sarcolemma where Z-bands anchor (I, arrowheads). Bars, 25 μ m.

brane that are commonly observed at the periphery of eukaryotic cells as they spread on a substrate or at the leading edge of moving cells (Bereiter-Hahn et al., 1990). They are considered to be transient structures that are driven by hydrodynamic forces and finally result in other types of protrusions such as membrane ruffles on lamellipodia (Cunningham, 1995). They also correspond to the membrane ruffles where endogenous β -integrin and another ILK-binding protein, PINCH, have been shown to concentrate during cell spreading (Tu et al., 1999). These results suggest that integrin–ILK signaling plays a role in the early phase of cell spreading, and affixin and PINCH also participate in this process. Importantly, vinculin and FAK do not accumulate in these regions at this stage (Fig. 8, D and F), suggesting that ILK and affixin are recruited into these nascent substrate adhesion sites in advance of these FA components. We further demonstrate that during the later stage of cell spreading, affixin shows punctuate staining at lamelli-

podia from which short actin bundles emanate (Fig. 8, I and J) and finally colocalizes with vinculin and FAK at FAs (Fig. 6, A and E; data not shown). In well-spread cells, ILK and affixin are also detected at the tip of the leading edge of lamellipodia where formation and growth of de novo cell–substrate adhesions actively occur. These results strongly indicate that affixin is involved in integrin–ILK signaling required for the nascent cell–substrate adhesion structures, which will develop into mature FAs.

FAs form and disappear continuously during cell locomotion and the cell spreading process, and the underlying molecular basis of this dynamic nature of FAs has been extensively investigated. FAK, one of the major protein kinases accumulating at FAs, was thought to function in the initial step of FA formation, partially because many FA components are tyrosine phosphorylated during the FA formation. However, recent results revealed that FAs can be formed even in the absence or inhibition of FAK activ-

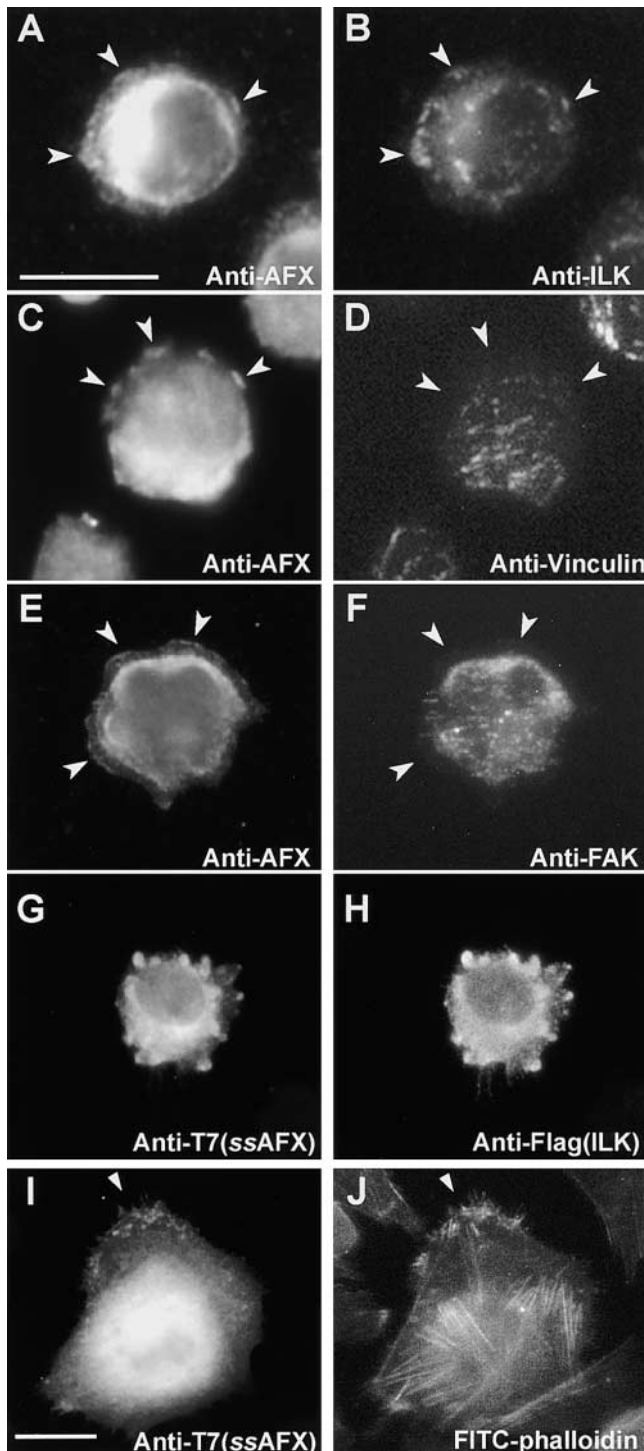


Figure 8. Distribution of affixin during the early stages of the cell spreading process. CHO cells (A, C, and E) or those cotransfected with T7-tagged affixin and Flag-tagged ILK (G and I) were replated on fibronectin-coated coverslips and 1 h later fixed and stained with antiaffixin or anti-T7 antibodies as indicated. Cells were stained simultaneously with anti-ILK (B), antivinculin (D), anti-FAK (F), anti-Flag (H) antibodies, or FITC-phalloidin (J). Note that affixin is concentrated in peripheral blebs (A–F, arrowheads), whereas in well-spread cells dot-like staining is observed from which F-actin bundles emanate (I and J). Fixation is 100% methanol (A–F), 2% paraformaldehyde (G–J). Bars, 25 μ m.

ity, implying the presence of more essential signaling molecules regulating the formation of the FA complex (Ilic et al., 1995). The recent finding of ILK, which is activated within 30–45 min after plating on fibronectin (Delcommenne et al., 1998) and is localized to FAs in CHO cells (Li et al., 1999), suggests the new possibility that this serine–threonine kinase is involved in FA formation. In this context, the present results are important in not only supporting these notions about the physiological function of ILK signaling but also suggesting the involvement of affixin in this ILK function.

Affixin Is a Possible ILK Substrate That Transmits Integrin–ILK Signaling for the Initial FA Formation

Yeast two-hybrid assays and immunoprecipitation assays in COS-7 cells revealed that the kinase domain of ILK interacts specifically with the second CH domain of affixin. The glutamic acid residue located in the activation loop of the ILK kinase domain is further demonstrated to be critical for the interaction. Consistently, in vitro kinase assay showed that the second CH domain can be a substrate for ILK, suggesting the possibility that affixin is a novel in vivo substrate for ILK, which links integrin–ILK signaling to the initial FA formation. This was further supported by showing the deleterious effects of an affixin deletion mutant, RP2, corresponding to the second CH domain, on FA formation: when CHO cells overexpressing RP2 were reseeded on fibronectin, >75% of the cells retained their round form and could not develop FAs and SFs. Closer inspection further suggested that the cells were completely blocked at the initial phase of the spreading process with many surface blebs. Interestingly, this effect of RP2 on FA formation was not significantly observed if it was expressed in cells which have already established FAs. Only a limited number (<10%) of cells expressing RP2 alone showed a round shape, and the number was dramatically increased to levels comparable to those of spreading cells when wild-type ILK was cotransfected. Because this enhancement was not observed by cotransfection of kinase-deficient ILK K220M or an affixin-binding-deficient ILK mutant, ILK(E359K), these results indicate that the effect of RP2 is dependent on the interaction with active ILK, probably on the phosphorylation by ILK. The apparent discrepancy of the effects of RP2 on well-spread cells and on actively spreading cells can be reasonably explained by assuming that integrin–ILK signaling evoked by initial cell–substrate interaction (Delcommenne et al., 1998) mimics the effect of ILK overexpressing in actively spreading cells. Although further studies will be needed, the present result about the positive effect of RP1, the first CH domain, on cell spreading may suggest that this domain is also involved in the interaction with the downstream target for FA formation in a different way from RP2.

Amino acid sequence analysis of affixin revealed it to represent a novel member of the CH domain-containing protein family that is conserved from worms to mammals. Considering that most CH domain-containing proteins have been shown to be related to the actin cytoskeleton (Stradal et al., 1998), the putative molecular target of affixin may be the actin cytoskeleton. This notion is further supported by the fact that both CH domains in affixin ex-

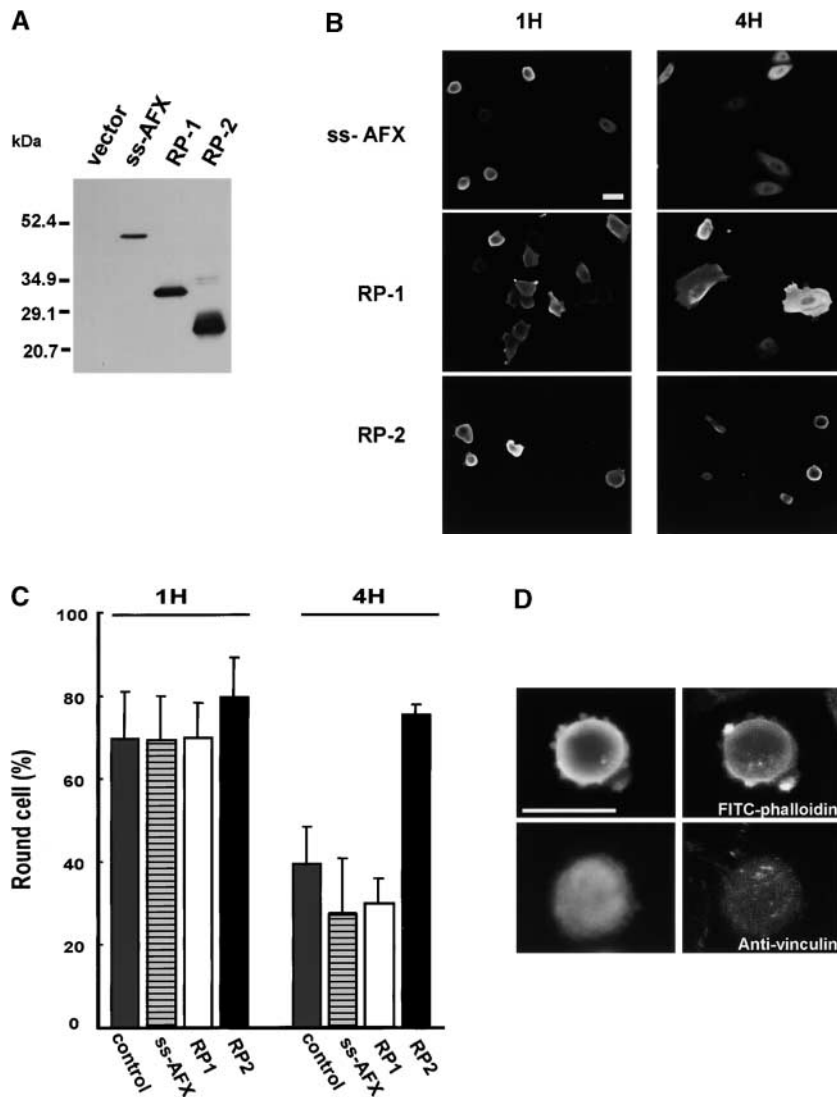


Figure 9. Overexpression of the affixin deletion mutant, RP2, inhibits the progression of the cell-spreading process. (A) Western blot analysis showing relative expression levels of each construct in CHO-K1 cells. The total lysates of CHO-K1 transfected with indicated expression vectors were prepared 48 h after transfection and analyzed using anti-T7 antibody. (B) CHO cells transfected with T7-tagged ss-affixin or its deletion mutants (RP1 or RP2) were replated on fibronectin-coated coverslips 48 h after transfection. 1 or 4 h later, the cells were fixed and stained with anti-T7 polyclonal antibody. (C) The numbers of transfected cells with round morphology were counted at 1 and 4 h after replating. As control, CHO cells treated in a manner similar to the transfected cells were stained with rhodamine-phalloidin and counted. Note that cells transfected with RP2 but not ss-affixin or RP1 are blocked in the round morphology even at 4 h after reseeding. The values given represent mean values (\pm SD) of three independent experiments. (D) Cells overexpressing T7-tagged RP2 were doubly stained with anti-T7 polyclonal antibody (left) and FITC-phalloidin or antivinculin monoclonal antibody as indicated. Bars, 25 μ m.

hibit the closest homology to those of the actin-binding regions of actin cross-linking proteins such as β -spectrin and α -actinin. Hence, it is quite possible that affixin also binds directly to F-actin. In fact, we observed that although weakly, endogenous affixin localizes to SFs in cultured cells (Fig. 6, A, E, and G). However, despite extensive efforts, we have not succeeded in detecting the direct interactions of affixin or RP2 with F-actin and cannot yet discuss the molecular mechanism by which affixin plays critical roles in FA development in detail. It may be due to the unique divergence of affixin from the sequence of actin-linking proteins. Of course, it is possible that the phosphorylation of affixin by ILK is required for its interaction with F-actin. We are now conducting further experiments to address these issues as one of the major extensions of the present study.

Affixin and ILK in Muscle Cells

Northern and Western blot analyses showed that affixin is highly expressed in skeletal muscle and heart, suggesting its role in cell-substrate interaction in muscle cells. Interestingly, the mutation/depletion of the *C. elegans* homologue

of β -integrin (*pat-3*) or ILK-binding protein, PINCH (*unc-97*), from the embryo was reported to result in a similar phenotype called "pat" showing paralysis and elongation arrest at the twofold stage due to defects in the integrity of myofibril structures in the body wall muscle (Williams and Waterston, 1994; Gettner et al., 1995; Dedhar et al., 1999; Hobert et al., 1999). Since the body wall muscles of these mutants show disorganized dense bodies (structural analogues of FAs in cultured cells) to which β -integrin/PAT-3, vinculin/DEB-1, and PINCH/UNC-97 are localized, it has been suggested that defects in the development of dense bodies are the primary cause of the phenotype (Gettner et al., 1995; Hobert et al., 1999). Interestingly, we recently observed that the *C. elegans* embryo from which the expression of ILK or affixin homologues (CAB77052 or T21D12.4, respectively) was deleted by the RNA interference method shows pat phenotype characterized by an arrest during its development at twofold stage and paralysis (Sugiyama, Y., unpublished results). Although we have not confirmed that these are primarily caused by defects in muscle attachments, our present results showing ILK and affixin play important roles in FA development, and it

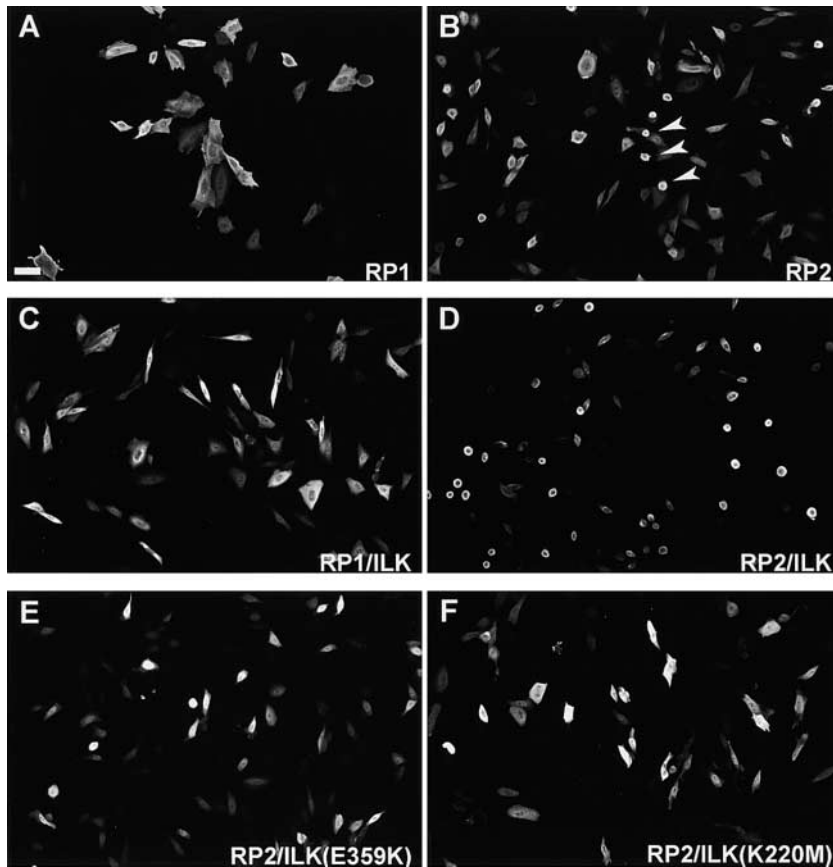


Figure 10. Overexpression of the COOH-terminal half of affixin in well-spread cells induces ILK-dependent disruption of cell-substrate interaction. T7-tagged affixin mutants (RP1 or RP2) were overexpressed alone (A and B), with Flag-tagged ILK (C and D), or with ILK point mutants (E and F) in CHO cells as indicated. 48 h after transfection, the cells were stained with anti-T7 polyclonal antibody. Note that overexpression of RP2 but not RP1 induces cell rounding, and this effect of RP2 is greatly enhanced by the coexpression of ILK but not ILK(E359) or ILK(K220M). Bar, 25 μ m.

might be reasonable to speculate that these embryo also have defects in dense body formation. Consistently, immunohistochemical analysis of human skeletal muscle demonstrated that affixin and ILK colocalize to the sarcolemma showing a striated pattern matching cytoplasmic Z-band striation, suggesting that these proteins accumulate at sites where the Z-band attaches to the sarcolemma, which correspond to dense bodies in *C. elegans* body wall muscle cells. Taken together, these results imply the possibility that affixin and ILK are also important for muscle development and function.

During the revision of this manuscript, a novel CH domain protein similar to affixin, actopaxin, was reported as a paxillin-binding protein. One of the clones we obtained in the two-hybrid screening using ILK as bait, FL29, was identical to actopaxin, indicating that this protein also binds ILK. Affixin and actopaxin are products of distinct genes with different expression patterns but share common features such as localization to focal contact.

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