A Critical Role of the PINCH-Integrin-linked Kinase Interaction in the Regulation of Cell Shape Change and Migration*

The interaction of cells with extracellular matrix recruits multiple proteins to cell-matrix contact sites (e.g. focal and fibrillar adhesions), which connect the extracellular matrix to the actin cytoskeleton and regulate cell shape change, migration, and other cellular processes. We previously identified PINCH, an adaptor protein comprising primarily five LIM domains, as a binding protein for integrin-linked kinase (ILK). In this study, we show that PINCH co-localizes with ILK in both focal adhesions and fibrillar adhesions. Furthermore, we have investigated the molecular basis underlying the targeting of PINCH to the cell-matrix contact sites and the functional significance of the PINCH-ILK interaction. We have found that the N-terminal LIM1 domain, which mediates the ILK binding, is required for the targeting of PINCH to the cell-matrix contact sites. The C-terminal LIM domains, although not absolutely required, play an important regulatory role in the localization of PINCH to cell-matrix contact sites. Inhibition of the PINCH-ILK interaction, either by overexpression of a PINCH N-terminal fragment containing the ILK-binding LIM1 domain or by overexpression of an ILK N-terminal fragment containing the PINCH-binding ankyrin domain, retarded cell spreading, and reduced cell motility. These results suggest that PINCH, through its interaction with ILK, is crucially involved in the regulation of cell shape change and motility.

Cell-extracellular matrix interactions are critically involved in the embryonic development and many physiological and pathological processes including injury repair, inflammation and metastasis. Upon adhesion to extracellular matrix, cells recruit a highly selective group of membrane and cytoplasmic proteins to the cell-extracellular matrix contact sites, where they connect the extracellular matrix to the actin cytoskeleton and regulate cell shape change, migration, and signal transduction (1–6). Many mammalian adherent cell types grown in culture form morphologically and molecularly distinct cell-matrix adhesion structures, among which the best characterized are focal adhesions (2, 3) and fibrillar adhesions (or extracellular matrix contacts) (5, 7–11). Focal adhesions represent firm substrate attachment sites that typically are arrowhead-shaped and contain clusters of integrins and cytoskeletal and signaling molecules including talin, vescin, focal adhesion kinase, and paxillin. Fibrillar adhesions form between fibrillar matrix such as fibronectin fibrils and actin cytoskeleton. Fibrillar adhesions are more elongated (typical axial ratio, >7) and are rich in integrins, fibronectin, and tensin, but they are deficient in paxillin and several other components of focal adhesions (5, 10, 11). ILK is a common component of both focal adhesions (12–15) and fibrillar adhesions (16). Recent studies have suggested that ILK plays a crucial role in the assembly and functions of the cell-matrix adhesion structures (17–19).

PINCH is a widely expressed and evolutionarily conserved protein comprising primarily five LIM domains. In previous studies, we have shown that PINCH binds to ILK (18, 20) and forms a ternary complex with ILK and CH-ILKBP (21), an ILK C-terminal domain-binding protein, in cells. Mutational studies have shown that the formation of the PINCH-ILK complex is mediated by a direct interaction between the second zinc finger located within the PINCH LIM1 domain and the N-terminal ANK domain of ILK (13, 20). The three-dimensional structure of the PINCH LIM1 domain has recently been solved. It folds into a globular structure consisting of two zinc fingers, each of which comprises two antiparallel β-sheets (22). Consistent with the mutational studies, chemical mapping studies have revealed that many residues in the second zinc finger of the PINCH LIM1 domain undergo large chemical shift changes upon ILK binding (22).

PINCH has been detected in integrin-rich cell-matrix adhesion sites in cells that are spreading on fibronectin (13). The current study is aimed at determining (i) whether PINCH co-localizes with ILK in both focal adhesions and fibrillar adhesions, (ii) the molecular basis underlying the targeting of PINCH to the cell-matrix contact sites, and (iii) the function of the PINCH-ILK interaction in cell spreading and migration.

EXPERIMENTAL PROCEDURES

Cell—Mouse C2C12 cells (from American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum. Rat embryo fibroblasts (REF-52, kindly provided by Dr. James Pipas, the University of Pittsburgh) were cultured in minimal essential medium supplemented with 10% fetal bovine serum. CHO K1 cells were cultured in a-minimal essential medium supplemented with 10% fetal bovine serum.

PINCH Expression Vector Construction and Transfection—DNA fragments encoding the FLAG-tagged full-length or mutant forms (as specified in each experiment) of human PINCH were cloned into the pEGFP-C2 expression vector (CLONTECH). C2C12 cells and CHO K1 cells were transfected with the GFP expression vectors using LipofectAMINE PLUS (Invitrogen) as described (21, 23). CHO K1 cells stably expressing GFP and GFP fusion proteins were selected with 1 mg/ml of G418 (Invitrogen) and cloned as described previously (23, 24).

* This work was supported by National Institutes of Health Grant DK54639 and American Cancer Society Research Project Grant 98-220-01-CSM (to C. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pathology, University of Pittsburgh, 707B Scaife Hall, 3550 Terrace St., Pittsburgh, PA 15261. Tel.: 412-648-2350; Fax: 412-561-4062; E-mail: carywu@imap.pitt.edu.

‡ The abbreviations used are: ILK, integrin-linked kinase; ANK, ankyrin; CH-ILKBP, calponin homology domain-containing ILK-binding protein; CHO, Chinese hamster ovary; GFP, green fluorescent protein.
Recombinant Adenoviral Expression Vector Construction and Infection—The adenoviral expressing vector encoding a FLAG-tagged N-terminal fragment of ILK (residues 1–230) was generated based on a previously described protocol (25). Briefly, the cDNA fragment encoding the FLAG-tagged N-terminal fragment of ILK was cloned into the SalI sites of the pAdTrack-CMV shuttle vector. The shuttle vector plasmid was linearized with PmeI, purified by phenol/chloroform extraction and ethanol precipitation, and mixed with supercoiled pADEFsay-1. The vectors were transferred into Escherichia coli BJS183 by electroporation using a Bio-Rad Gene Pulser electroporator. The bacteria were immediately placed in 1 ml of LB Broth (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl (Fisher) and grown at 37 °C for 1 h. The bacteria were then inoculated onto agar containing LB Broth supplemented with 50 μg/ml of kanamycin. After 16–20 h of growth, colonies were picked and grown in 2 ml of LB Broth containing 50 μg/ml of kanamycin. The clones were screened by digestions with restriction endonucleases PstI and BamHI. The positive supercoiled plasmids were transformed into DH10B cells by electroporation for large scale amplification. The plasmid DNA was digested with HindIII, purified and was used to transfect 293 cells using LipofectAMINE PLUS. The transfected cells were harvested 10 days after transfection. The cells were lysed by three cycles of freezing in a methanol/dry ice bath and rapid thawing at 37 °C, and the lysates containing the recombinant adenovirus were collected. The control adenoviral expression vector encoding a FLAG-tagged N-terminal fragment of ILK (residues 1–249), GFP-FLAG-LIM1 (PINCH residues 63–325), and GFP-FLAG-LIM1-2 (PINCH residues 1–130), the cells expressing the corresponding GFP- and FLAG-tagged PINCH mutants or those expressing GFP as a control were lysed as described above. The lysates (300 μg) were mixed with 50 μl of hybridoma culture supernatant containing monomeric anti-CH-ILKBP antibody (clone 349; Transduction Laboratories). The mouse antibodies were detected with a Rhodamine RedTM-conjugated anti-mouse antibody (Jackson ImmunoResearch Labs, Inc., West Grove, PA). Similar results were obtained with cells plated on fibronectin- or vitronectin-coated surfaces.

Immunoprecipitation—The cells were cultured in complete medium in 60- or 100-mm culture plates. Cell monolayers were rinsed twice with phosphate-buffered saline and directly lysed on the plates with 1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 10 mM Na2P2O7, 2 mM Na3VO4, 100 mM NaF, and protease inhibitors. The protein precipitation with monoclonal anti-CH-ILKBP antibody was previously described (21). Briefly, cell lysates (500 μg) were mixed with 500 μl of hybridoma culture supernatant containing monomeric anti-CH-ILKBP antibody 1D4. The samples were incubated for 3 h, mixed with 40 μl of UltraLink immobilized protein G (Pierce), and then incubated for an additional 1.5 h. The beads were washed four times, and the proteins bound were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with anti-CH-ILKBP antibody 3B5, anti-ILK antibody 65.1, or rabbit polyclonal anti-PINCH antibodies as specified in each experiment. For immunoprecipitation of GFP-FLAG-LIM3s (PINCH residues 1–249), GFP-FLAG-LIM1 (PINCH residues 63–325), and GFP-FLAG-LIM1-2 (PINCH residues 1–130), the cells expressing the corresponding GFP- and FLAG-tagged PINCH mutants or those expressing GFP as a control were lysed as described above. The lysates (300 μg) were mixed with 30 μl of agarose beads conjugated with anti-FLAG antibody M2 (Sigma). The precipitated proteins were released from the beads by boiling in 50 μl of SDS-PAGE sample buffer for 5 min and analyzed by Western blotting (9 μl/bone) with antibodies as specified in each experiment.

Cell Spreading—The cells (as specified in each experiment) were plated on 96-well plates coated with vitronectin (BD Biosciences, Bedford, MS) in Opti-MEM I serum-free medium (Invitrogen). The plates were incubated at 37 °C under a 5% CO2, 95% air atmosphere for different periods of time (as specified in each experiment). The cell morphology were observed under an Olympus IX70 fluorescence microscope equipped with an Hoffman Modulation Contrast system and recorded with a DVC-1310C MagnaFire™ digital camera (Optronics). Unseeded cells were defined as round cells, whereas spread cells were defined as cells with extended processes as described (21, 27, 28). The percentage of cells adopting spread morphology was quantified by analyzing at least 300 cells from three randomly selected fields (>100 cells/field) (21, 27, 28).

Cell Migration—Cell migration was assessed by the ability of the cells to migrate into a cell-free area as previously described (29, 30). Briefly, the cells were plated in complete medium on 24-well plates and grown for 24 h to reach confluence. The monolayers were then wounded by scratching with a plastic pipette tip. After washing, the cells were incubated in complete medium for the indicated times and observed under an Olympus IX70 microscope equipped with an Hoffman Modulation Contrast system. Images of three different segments of the cell-free area were recorded with a DVC-1310C MagnaFire™ digital camera (Optronics), and the distances traveled by the cells at the front in three different segments of the wound were measured.

RESULTS

PINCH Co-localizes with ILK in Both Focal Adhesions and Fibrillar Adhesions—To test whether PINCH co-localizes with ILK in both focal adhesions and fibrillar adhesions, we expressed a GFP-tagged PINCH in mouse C2C12 cells. Cells
Fig. 2. The ILK-binding LIM1 domain, but not LIM5, is required for localization of PINCH to cell-extracellular matrix contact sites. A and B, ILK binding. Lysates of C2C12 cells expressing GFP-FLAG-ΔLIM5 (PINCH residues 1–249), GFP-FLAG-ΔLIM1 (PINCH residues 63–325), or GFP only as a control were mixed with mouse monoclonal anti-FLAG antibody M2. The GFP-FLAG-ΔLIM5 (lane 4), GFP-FLAG-ΔLIM1 (lane 5), and the GFP control (lane 6) immunoprecipitates were analyzed by Western blotting analyses of the ΔLIM1 and ΔLIM5 immunoprecipitates showed that ILK was co-precipitated with ΔLIM5 (Fig. 2B, lane 4) but not with ΔLIM1 (Fig. 2B, lane 5). In control experiments, neither GFP (Fig. 2A, lane 6) nor ILK (Fig. 2B, lane 6) was precipitated with the anti-FLAG antibody from lysates of control cells that express GFP, confirming the specificity of the immunoprecipitation assay. Taken together, these results show that the GFP-FLAG-ΔLIM5, but not GFP-FLAG-ΔLIM1, binds to ILK in cells.

To determine the ability of the PINCH mutants to localize to cell-matrix contact sites, we plated the C2C12 cells expressing GFP-FLAG-ΔLIM1 or GFP-FLAG-ΔLIM5 on fibronectin-coated coverslips and stained them with mouse monoclonal anti-ILK antibody 65.1 (B). Lanes 1–3 were loaded with cell lysates (15 μg/lane) as indicated in the figure. C–F, subcellular localization. C2C12 cells expressing GFP-FLAG-ΔLIM1 (C and D) or GFP-FLAG-ΔLIM5 (E and F) were plated on fibronectin-coated coverslips and stained with mouse monoclonal anti-ILK antibody 65.1 as described in the legend to Fig. 1. GFP-FLAG-ΔLIM1 (C), GFP-FLAG-ΔLIM5 (E), and ILK (D and F) were visualized under a fluorescence microscope equipped with GFP (C and E) and rhodamine (D and F) filters. Bar, 5 μm. MW, molecular mass; I.P., immunoprecipitates.

The ILK-binding LIM1 Domain Is Required for the PINCH Localization to Cell-Matrix Contact Sites—We next analyzed the PINCH domains that are involved in the localization of PINCH to the cell-matrix contact sites. Because PINCH binds to ILK (13, 20), and PINCH and ILK co-localize in cell-matrix contact sites. To test this, we expressed PINCH mutants in which either the N-terminal-most ILK-binding LIM1 domain or the C-terminal-most LIM5 domain is deleted in C2C12 cells. The PINCH mutants were tagged with GFP and a FLAG epitope to facilitate cellular and biochemical analyses. The expression of the GFP- and FLAG-tagged ΔLIM1 (Fig. 2A, lane 2) and ΔLIM5 (Fig. 2A, lane 1) PINCH mutants was confirmed by Western blotting with an anti-GFP antibody. To test whether the PINCH mutants bind to ILK, we immunoprecipitated the GFP- and FLAG-tagged PINCH mutants from the cell lysates with a monoclonal anti-FLAG antibody (Fig. 2A, lanes 4 and 5). Western blotting analyses of the ΔLIM1 and ΔLIM5 immunoprecipitates showed that ILK was co-precipitated with ΔLIM5 (Fig. 2B, lane 4) but not with ΔLIM1 (Fig. 2B, lane 5). In control experiments, neither GFP (Fig. 2A, lane 6) nor ILK (Fig. 2B, lane 6) was precipitated with the anti-FLAG antibody from lysates of control cells that express GFP, confirming the specificity of the immunoprecipitation assay. Taken together, these results show that the GFP-FLAG-ΔLIM5, but not GFP-FLAG-ΔLIM1, binds to ILK in cells.

To determine the ability of the PINCH mutants to localize to cell-matrix contact sites, we plated the C2C12 cells expressing GFP-FLAG-ΔLIM1 or GFP-FLAG-ΔLIM5 on fibronectin-coated coverslips and stained them with mouse monoclonal anti-ILK antibody 65.1. The ILK-binding defective LIM1 deletion mutant (Fig. 2C), unlike the full-length PINCH (Fig. 1), was unable to localize to cell-matrix contact sites where abundant ILK was detected (Fig. 2D), suggesting that the ILK binding is essential for the localization of PINCH to the cell-matrix contact sites. By contrast, the LIM5 deletion mutant was able to co-localize with ILK in cell-matrix contact sites (Fig. 2E and F), indicating that the LIM5 domain, unlike the LIM1 domain, is not required for the localization of PINCH. The level of the LIM5 deletion mutant (Fig. 2E) that was detected in the cell-matrix contact sites, however, appeared lower than that of the full-length PINCH (Fig. 1), suggesting that deletion of LIM5 decreases the efficiency or the stability of the PINCH localization to the cell-matrix contact sites.
To further analyze this, we expressed a PINCH mutant in which LIM3–5 were deleted in C2C12 cells. The expression of the PINCH mutant (LIM1–2) was confirmed by Western blotting with a monoclonal anti-FLAG antibody (Fig. 3A, lane 4). As expected, ILK (Fig. 3B, lane 2) was readily co-immunoprecipitated with GFP-FLAG-LIM1–2 (Fig. 3A, lane 2). In control experiment, no ILK (Fig. 3B, lane 1) was precipitated by the anti-FLAG antibody in the absence of GFP-FLAG-LIM1–2 (Fig. 3A, lane 1), confirming the specificity of the co-immunoprecipitation experiment. Fluorescence microscopic analyses showed that GFP-FLAG-LIM1–2 distributed rather diffusely in cells (Fig. 3, C, E, and G). A closer examination of the cells revealed that in some but not all cells, a very low level of GFP-FLAG-LIM1–2 clusters was present in cell adhesion sites where ILK was clustered (Fig. 3, E and F). Taken together, these results suggest that deletion of the LIM3–5 domains greatly impairs the ability of PINCH to localize to cell-matrix contact sites. Thus, although the C-terminal LIM3–5 domains were not absolutely required for the targeting of PINCH to cell-matrix contact sites, they do play important roles in the regulation of PINCH localization.

Intriguingly, although we have detected ILK clusters in cells overexpressing GFP-FLAG-LIM1–2 (Fig. 3, D and F), they often appeared smaller and less well organized than those in cells that overexpress GFP-FLAG-PINCH (Fig. 1B, GFP-FLAG-LIM1 (Fig. 2D) or GFP alone (data not shown). Abundant paxillin clusters were detected in the GFP-FLAG-LIM1–2 overexpressing cells under the same condition (Fig. 3, G and H). These results suggest that overexpression of LIM1–2, which binds to ILK but fails to localize to the cell-matrix adhesion sites efficiently, negatively influences the localization of ILK to the adhesion sites.

**Overexpression of LIM1–2 Inhibits the PINCH-ILK Interaction**—We next sought to assess the functional significance of the PINCH-ILK interaction. To do this, we generated reagents that allow us to modulate the PINCH-ILK interaction in cells. Because the LIM1–2 fragment binds efficiently to ILK in cells (Fig. 3), we postulated that it could potentially function as a dominant negative inhibitor of the PINCH-ILK interaction. To test this, we transfected CHO cells, which have been widely used in gene transfer experiments because of their high transfection efficiency, with expression vectors encoding either GFP-FLAG-LIM1–2 or GFP alone (as a control). Under the experimental conditions used, the transfection efficiency was ~80% based on the percentages of CHO cells that expressed GFP or GFP-FLAG-LIM1–2. The expression of GFP (Fig. 4A, lane 2) and GFP-FLAG-LIM1–2 (Fig. 4A, lane 3) in the corresponding transfectants was confirmed by Western blotting. To test the
expression of the PINCH-ILK interaction significantly delays the change of cell shape.

Cell migration is a pathologically and physiologically important process that involves dynamic changes of cell shape. The finding that the PINCH-ILK interaction is involved in the cellular control of cell shape change prompted us to test whether it plays a role in cell migration. To do this, we analyzed the migration of cells overexpressing GFP-FLAG-LIM1-2 using a "wound" assay, a well-established in vitro system for measuring cell motility (29, 30). The results showed that the cells overexpressing GFP-FLAG-LIM1-2 migrated much more slowly than the cells overexpressing GFP-FLAG-PINCH, the parental cells, and the GFP control cells (Fig. 5). Thus, consistent with the retardation in cell spreading, overexpression of the LIM1-2 fragment significantly impairs the cell motility.

Overexpression of the PINCH-binding ANK Fragment Inhibits the PINCH-ILK Interaction—Because the N-terminal ANK domain of ILK mediates the binding to PINCH, we hypothesized that overexpressing the ILK N-terminal ANK fragment might also inhibit the PINCH-ILK interaction. To test this, we generated an adenoviral vector encoding a FLAG-tagged ILK N-terminal ANK fragment, which allowed us to express the ILK fragment with high efficiency (typically 80–90%). We infected rat embryo fibroblasts (REF-52) with the recombinant adenovirus encoding the ANK fragment or that encoding an irrelevant protein (β-galactosidase) as a control. The expression of the FLAG-tagged ILK N-terminal ANK fragment in the REF-52 cells that were infected with the adenovirus encoding the ANK fragment (Fig. 7A, lane 3) but not in the uninfected REF-52 (Fig. 7A, lane 1) nor in the control cells that were infected with the β-galactosidase adenovirus (Fig. 7A, lane 2) was confirmed by Western blotting. To determine the effect of overexpression of the ANK fragment on the complex formation between PINCH, ILK, and CH-ILKBP, we immunoprecipitated the PINCH-ILK-CH-ILKBP complex from lysates of the ANK-expressing cells as well as those of the control cells with monoclonal anti-CH-ILKBP antibody 1D4. Similar amount of ILK (Fig. 7C, lanes 3 and 4) was co-precipitated with CH-ILKBP (Fig. 7B, lanes 3 and 4) either in the presence or absence of the ANK fragment, indicating that the interaction between ILK and CH-ILKBP was not altered by the overexpression of the ANK fragment. By contrast, the amount of PINCH associated with ILK was markedly reduced in cells overexpressing the ANK fragment (Fig. 7D, lanes 3 and 4), confirming that overexpression of LIM1-2 significantly inhibits the PINCH-ILK interaction.
expression of the ILK N-terminal ANK fragment inhibits the complex formation between PINCH and ILK.

Effects of the ILK ANK Fragment on Cell Spreading and Migration—To test whether overexpression of the ILK ANK fragment, like that of the PINCH LIM1–2 fragment, also inhibits cell spreading, we plated cells infected with the ANK adenovirus, cells infected with the control adenoviral vector encoding β-galactosidase (lane 2), and REF-52 cells infected with an adenoviral vector encoding a FLAG-tagged ILK N-terminal ANK fragment (residues 1–230) were analyzed by Western blotting with monoclonal anti-FLAG antibody M5. B–D, complex formation. CH-ILKBP was immunoprecipitated from lysates of REF-52 cells expressing the ANK fragment (lane 4) or those of the control cells (lane 3) with monoclonal anti-CH-ILKBP antibody 1D4 as described under “Experimental Procedures.” The immunoprecipitates were analyzed by Western blotting with monoclonal anti-CH-ILKBP antibody 3B5 (B), monoclonal anti-ILK antibody 65.1 (C), and polyclonal anti-PINCH antibodies (D). Lanes 1 and 2 were loaded with cell lysates (13 µg/lane) as indicated. Mr, molecular mass; I.P., immunoprecipitates.

In this study, we have shown that PINCH co-localizes with ILK in both focal adhesions and fibrillar adhesions. Furthermore, we have mapped the domains of PINCH that are involved in the localization of PINCH to the cell-matrix contact sites. Our results indicate that the ILK-binding LIM1 domain is required for the localization of PINCH to the cell-matrix.
contact sites, whereas the C-terminal LIM domains are involved in the modulation of this process. Finally, we have investigated the function of the PINCH-ILK interaction in cell spreading and migration. We have developed two different dominant negative inhibitors of the PINCH-ILK interaction and demonstrated that down-regulation of the PINCH-ILK interaction by overexpression of either inhibitor results in a significant reduction of cell spreading and migration.

The results reported in this paper, together with those of previous studies, shed light on the mechanism by which PINCH and ILK localize to cell-matrix contact sites. The finding that the ILK-binding LIM1 domain is indispensable for the localization of PINCH to cell-matrix adhesion sites (Fig. 2) suggests an essential role of ILK in this process. One explanation for this observation is that ILK simply provides a PINCH-docking site at the cell-matrix adhesion sites. However, two pieces of evidence suggest that the PINCH-ILK interaction is not only crucial for the localization of PINCH but also important for the localization of ILK. First, we have found in previous studies that the PINCH-binding ANK repeat is required for the localization of ILK to cell-matrix adhesion sites (13). Second, we have observed in this study that overexpression of the PINCH LIM1–2 fragment, which reduced but did not completely eliminate the PINCH-ILK interaction, partially inhibited the localization of ILK to cell-matrix contact sites (Fig. 3). Thus, a more likely possibility is that ILK and PINCH form a complex before reaching the cell-matrix adhesion sites and that the formation of such a complex is a prerequisite for the efficient localization of both proteins. The formation of the PINCH-ILK complex could allow multiple, simultaneous interactions mediated by other domains of PINCH and ILK and therefore promote efficient localization of both proteins to the cell-matrix adhesion sites. This model explains why the PINCH-ILK interaction is cru-

FIG. 8. Effect of overexpression of the ILK N-terminal ANK domain on cell spreading and migration. A and B, cell spreading. The spreading of REF-52 cells expressing the ANK fragment, the vector control cells, and the parental REF-52 cells was analyzed as described under “Experimental Procedures.” A shows the morphology of the REF-52 cells expressing the ANK fragment and the vector control cells at 0.5 and 2 h, respectively, after seeding. Bar, 30 μm. The morphology of the parental REF-52 cells was similar to that of the vector control cells (not shown in the figure). The percentage of cells adopting spread morphology 0.5 h after seeding was quantified by analyzing more than 300 cells from three randomly selected fields (B). The data represent the means ± S.D. C and D, cell migration. C, monolayers of the parental REF-52 cells, the β-galactosidase control cells, and the REF-52 cells overexpressing GFP-FLAG-LIM1–2 were wounded at 0 h. The cells were allowed to migrate into the cell-free area for 16 h. Bar, 200 μm. D, distances traveled by the cells at the acellular front. The migration distance was calculated as described under “Experimental Procedures.” The data represent the means ± S.D. of distances traveled by the cells in three different segments of the wound.
cial for the localization of both PINCH and ILK to the cell-matrix adhesion sites. Furthermore, it implies that there exist other PINCH- and/or ILK-mediated interactions that are important for the localization of both proteins. The C-terminal domain of ILK is capable of interacting with several additional components of the cell-matrix adhesion structures including β1 integrins (12), CH-ILKBP (21), paxillin (15), and affixin (31). Deletion of the C-terminal domain abolished the ability of ILK to localize to cell-matrix adhesion sites (13), suggesting that at least one and, more likely, multiple interactions mediated by this domain are required in this process. Mutations in an ILK C-terminal sequence that is 15–33% identical to the paxillin binding sequences in vinculin, focal adhesion kinase, and actopaxin disrupt the localization of ILK to cell-matrix contact sites, suggesting that the interaction with paxillin is involved in this process (15). However, because paxillin is deficient in fibrillar adhesions (5, 10, 11), to which both ILK (16) and PINCH (this report) localize, other ILK C-terminal binding proteins such as β1 integrins, CH-ILKBP/actopaxin/α-parvin, and affixin/β-parvin are likely also involved in this process (12, 21, 32–35). It is worth noting in this regard that CH-ILKBP forms a ternary complex with ILK and PINCH in cells (21), and therefore it is attractive to propose that the formation of the PINCH-ILK-CH-ILKBP complex is a crucial step in the localization of these three proteins to cell-matrix contact sites. The notion that PINCH, ILK, and CH-ILKBP assemble into a complex and thereby localize and function interdependently at the cell-matrix adhesion sites is consistent with recent genetic studies in model organisms such as Caenorhabditis elegans showing that lack of PINCH/UNC-97 (36), ILK/PAT-4 (44), or CH-ILKBP/PAT-6 (45) resulted in an identical defect in the assembly of muscle attachment sites.

In addition to the interactions involving ILK, interactions mediated by the PINCH C-terminal LIM domains likely also contribute to the localization of PINCH, and consequently ILK and CH-ILKBP, to cell-matrix contact sites. Deletion of the LIM3–5 domains greatly impairs the ability of PINCH to localize to cell-matrix contact sites. Deletion of the LIM5 domain also decreased, although to a lesser extent, the efficiency of the localization of PINCH to cell-matrix contact sites. Because the LIM1-containing PINCH N-terminal fragments bind efficiently to ILK (Figs. 2 and 3), the impairment in the localization of the PINCH C-terminal deletion mutants is likely caused by the loss of interactions mediated by the C-terminal LIM domains. It is interesting to note that one of the C-terminal domains, LIM4, interacts with Nck-2 (also known as Nckβ (37) or Grb4 (38)), a Src homology 2 domain- and Src homology 3 domain-containing adaptor protein (39). Because Nck-2 is present in the cell-matrix contact sites in spreading cells, it could represent one of the interactions mediated by the PINCH C-terminal domains that are involved in the localization of PINCH to these sites. The observation that deletion of LIM3–4 further reduces the efficiency of the localization to cell-matrix contact sites (compare Fig. 2E with Figs. 3, C–G) provides evidence supporting, albeit not proving, this possibility. Clearly, future studies are required to test this possibility and to identify other interactions that are involved in this process.

Cell migration is a complex process that is critically involved in embryonic development and many physiological and pathological processes including injury repair, inflammation, and metastasis. Using two different dominant negative inhibitors of the PINCH-ILK interaction, we have demonstrated in this study that PINCH, through its interaction with ILK, plays an important role in the regulation of cell migration. Because cell migration requires coordinated changes of cell shape and inhibition of the PINCH-ILK interaction impairs this process, the defect in cell migration is most likely caused by, at least in part, the disregulation of cell shape change. At the molecular level, this could reflect a need for the PINCH-ILK interaction in physically connecting the transmembrane receptors such as integrins to the actin cytoskeleton. Additionally, PINCH could participate in other events that are critical for cell migration. For example, PINCH, through interactions mediated by Nck-2, could potentially bring proteins that are involved in the actin polymerization into proximity of the cell-matrix adhesion sites and therefore promote cell migration. Nck-2 is known to interact with p21-activated kinase and Wiskott-Aldrich syndrome protein (37, 38), proteins that are directly involved in the regulation of actin polymerization. Although a direct role for Nck-2 in the regulation of actin polymerization remains to be tested, it has recently been shown that Nck-1/Nckα, which is structurally closely related to Nck-2, cooperates with phosphatidylinositol 4,5-bisphosphate to dramatically activate neural-Wiskott-Aldrich syndrome protein/Arp2/3-mediated actin nucleation (40). Additional Nck-2-binding proteins include DOCK180 (41), a Rac-activating protein that is involved in the regulation of membrane ruffling and cell migration (42, 43). Delineation of the functions of PINCH and its associated proteins will be of considerable value to the understanding of the molecular mechanism by which cells regulate shape change, movement, and other processes involving cell-matrix interactions.

Acknowledgments—We thank Drs. Tong-Chuan He and Bert Vogelstein for the pADTrack-CMV and pADEasy-1 vectors and Dr. James Pipas for the REF-52 cells.

REFERENCES

14. Hynes, R. O. (1992) Cell 69, 11–25
15. Jockusch, B. M., Bauback, P., Giehl, K., Kromerker, M., Moschener, J., Rothkegel, M., Rudiger, M., Schluter, K., Stanke, G., and Winkler, J. (1995) Annu. Rev. Cell Dev. Biol. 11, 379–416
16. Burridge, K., and Chrzanowska-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–488
17. Yamada, K. M., and Miyamoto, S. (1995) Curr. Opin. Cell Biol. 7, 681–689
18. Zamar, E., Katz, B. Z., Asta, S., Yamada, K. M., Geiger, B., and Kam, Z. (1999) J. Cell Sci. 112, 1655–1669
19. Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000) J. Biol. Chem. 275, 22607–22610
20. Hynes, R. O., and Destree, A. T. (1978) Cell 15, 875–886
21. Chen, W. T., and Singer, S. J. (1982) J. Cell Biol. 95, 205–222
22. Chen, W. T., Hasegawa, E., Hasegawa, T., Weinstock, C., and Yamada, K. M. (1985) J. Cell Biol. 100, 1103–1114
23. Zamir, E., Katz, M., Posen, V., Erez, N., Yamada, K. M., Katz, B. Z., Lin, S., Lin, D. C., Bershadsky, A., Kam, Z., and Geiger, B. (2000) Nat Cell Biol. 2, 191–196
24. Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C., and Yamada, K. M. (2000) J. Cell Biol. 148, 1075–1090
25. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Fimia, J., Bell, J. C., and D’Carrilho, S. (1996) Nature 379, 91–96
26. Li, F. Zhang, Y., and Wu, C. (1999a) J. Cell Sci. 112, 4589–4599
27. Mulroney, J., Foley, K., Vineberg, S., Barreuther, M., and Grabel, L. (2000) Exp. Cell Res. 258, 332–341
28. Nikolopoulos, S. N., and Turner, C. J. (2001) J. Biol. Chem. 276, 23499–23505
29. Guo, L., Sanders, P. W., Woods, A., and Wu, C. (2001) Am. J. Pathol. 159, 1735–1742
30. D’Carrilho, S., Williams, B., and Hannigan, G. (1999) Trends Cell Biol. 9, 319–323
31. Wu, C. (1999) J. Cell Biol. 144, 4485–4489
32. Zervas, C. G., Gregory, S. L., and Brown, N. H. (2001) J. Cell Biol. 152, 1007–1018
33. Wu, C. (1999b) J. Biol. Chem. 274, 1075–1080
34. Wu, C. (1999c) Mol. Cell. Biol. 19, 2425–2434
35. Wu, C. (1999d) J. Cell Biol. 153, 585–598
36. Wu, C. (1999e) J. Biol. Chem. 274, 4832–4839
37. Wu, C. (1999f) J. Cell Biol. 150, 861–871
38. Wu, C. (1999g) J. Cell Biol. 171, 1391–1397
39. Wu, C. (1999h) Proc. Natl. Acad. Sci. U. S. A. 96, 2509–2514
40. Wu, C., Keivena, V. M., TE, O. T., McDonald, J. A., and Ginsberg, M. H. (1995) Cell 83, 715–724
41. Komorjai, A., Green, L. J., Mervic, M., Yamada, S. S., Yamada, K. M., and

S. M. Goicoechea and C. Wu, unpublished observations.
The PINCH-ILK Interaction Regulates Cell Migration

Humphries, M. J. (1991) J. Biol. Chem. 266, 15075–15079
28. Richardson, A., Malik, R. K., Hildebrand, J. D., and Parsons, J. T. (1997) Mol. Cell. Biol. 17, 6906–6914
29. Giancotti, F. G., and Ruoslahti, E. (1990) Cell 60, 849–859
30. Chen, P., Xie, H., Sekar, M. C., Gupta, K., and Wells, A. (1994) J. Cell Biol. 127, 847–857
31. Yamaji, S., Suzuki, A., Sugiyama, Y., Koide, Y., Yoshida, M., Kanamori, H., Mohri, H., Ohno, S., and Ishigatsubo, Y. (2001) J. Cell Biol. 153, 1251–1264
32. Nikolopoulos, S. N., and Turner, C. E. (2000) J. Cell Biol. 151, 1435–1448
33. Olski, T. M., Noegel, A. A., and Korenbaum, E. (2001) J. Cell Sci. 114, 525–538
34. Wu, C. (2001) J. Cell Sci. 114, 2549–2550
35. Wu, C., and Dedhar, S. (2001) J. Cell Biol. 155, 505–510
36. Hobert, O., Moerman, D. G., Clark, K. A., Beckerle, M. C., and Ruvkun, G. (1999) J. Cell Biol. 144, 45–57
37. Chen, M., She, H., Davis, E. M., Spicer, C. M., Kim, L., Ren, R., Le Beau, M. M., and Li, W. (1998) J. Biol. Chem. 273, 25171–25178
38. Braverman, L. E., and Quilliam, L. A. (1999) J. Biol. Chem. 274, 5542–5549
39. Tu, Y., Li, F., and Wu, C. (1998) Mol. Biol. Cell 9, 3367–3382
40. Rohatgi, R., Nollau, P., Ho, H. Y., Kirschner, M. W., and Mayer, B. J. (2001) J. Biol. Chem. 276, 26448–26452
41. Tu, Y., Kueck, D. F., and Wu, C. (2001) FEBS Lett. 491, 193–199
42. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Genes Dev. 12, 3331–3336
43. Cheresh, D. A., Leng, J., and Klemke, R. L. (1999) J. Cell Biol. 146, 1107–1116
44. MacKinnon, A. C., and Williams, B. D. (2000) Mol. Biol. Cell 11, 515a
45. Lin, X., and Williams, B. S. (2000) Mol. Biol. Cell 11, 515a
