Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading and migration

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Summary

Integrin-linked kinase (ILK) is a multi-domain protein that plays important roles at cell-extracellular matrix (ECM) adhesion sites. We describe here a new LIM-domain containing protein (termed as PINCH-2) that forms a complex with ILK. PINCH-2 is co-expressed with PINCH-1 (previously known as PINCH), another member of the PINCH protein family, in a variety of human cells. Immunofluorescent staining of cells with PINCH-2 specific antibodies show that PINCH-2 localizes to both cell-ECM contact sites and the nucleus. Deletion of the first LIM (LIM1) domain of PINCH-2 abolished the ability of PINCH-2 to form a complex with ILK. The ILK-binding defective LIM1-deletion mutant, unlike the wild type PINCH-2 or the ILK-binding competent LIM5-deletion mutant, was incapable of localizing to cell-ECM contact sites, suggesting that the ILK-binding is required for this process. Importantly, the PINCH-2-ILK and PINCH-1-ILK interactions are mutually exclusive. Overexpression of PINCH-2 significantly inhibited the PINCH-1-ILK interaction and reduced cell spreading and migration. These results identify a novel nuclear and focal adhesion protein that associates with ILK and reveal an important role of PINCH-2 in the regulation of the PINCH-1-ILK interaction, cell shape change and migration.
Introduction

Cell-extracellular matrix (ECM) adhesion is a fundamental process that controls a variety of cellular processes including cell shape changes and migration. Cell-ECM interactions are mediated by a selective group of membrane and cytoplasmic proteins at the ECM contact sites (e.g., focal adhesions), through which the ECM is physically linked to the actin cytoskeleton and signals are transduced (1-8). Recent biochemical, cell biological and genetic studies have implicated integrin-linked kinase (ILK) as a key component of the cell-ECM adhesion structures (9-11). Structurally, ILK comprises an N-terminal ankyrin (ANK) repeat domain, a pleckstrin homology (PH)-like motif and a C-terminal kinase domain that exhibits significant homology to other protein kinase catalytic domains. One of the major functions of ILK is to mediate multiple protein-protein interactions at cell-ECM adhesion sites. In previous studies, we have found that ILK binds to PINCH-1 (12), a widely expressed focal adhesion protein consisting of five LIM domains. The ILK-PINCH-1 interaction is mediated by the N-terminal ANK domain of ILK and the second zinc finger of the N-terminal-most LIM domain (LIM1) of PINCH-1 (13). In addition to interacting with PINCH-1, ILK is capable of interacting with several other focal adhesion proteins including β1 integrins (14), CH-ILKBP (15) (also known as actopaxin (16) or α-parvin (17)), affixin (18) (also known as β-parvin (17)) and paxillin (19) through its C-terminal domain. Recently, we have shown that ILK binds to both PINCH-1 and CH-ILKBP simultaneously, resulting in the formation of a PINCH-1-ILK-CH-ILKBP complex in mammalian cells (15).

Recent biochemical, cell biological and genetic studies have provided strong evidence for an important role of the PINCH-1-ILK-CH-ILKBP complex in integrin-mediated cell-ECM
interactions (9-11,20). For example, inhibition of the PINCH-1-ILK-CH-ILKBP complex formation in mammalian cells by overexpression of dominant negative PINCH-1 or ILK mutants significantly impaired cell shape change and migration (21). In genetic model systems such as *C. elegans*, mutations in *pinch-1/unc-97* (22), *ilk/pat-4* (23) or *ch-ilkbp/pat-6* (Lin and Williams, 40th ASCB Annual Meeting, 2000, Abstract 2666) all resulted in a Pat phenotype similar to that of *β-integrin/pat-3* or *α-integrin/pat-2*, which is characterized by defects in dense body and M-line (similar to cell-ECM adhesion sites in mammalian cells) assembly (24,25).

While it is increasingly clear that the PINCH-1-ILK-CH-ILKBP complex plays an important role in the coupling of the ECM to the actin cytoskeleton, how cells regulate the assembly and functions of the PINCH-1-ILK-CH-ILKBP complex was not known. Search of the genomic sequence of *C. elegans* has revealed that there exists a gene (termed *pin-2*) encoding a protein that is structurally related to PINCH-1/UNC-97 (22). A partial sequence of a putative PINCH-1-related human protein (termed as PINCH-2) has been predicted based on cDNA sequence analyses of human EST clones (22). It was not known, however, whether PINCH-2 is co-expressed with PINCH-1 in human cells. Furthermore, neither the molecular activities nor the cellular functions of PINCH-2 were known. In this study, we have cloned and characterized PINCH-2. Our results show that PINCH-2 localizes to both cell-ECM adhesion sites and the nucleus and provide strong evidence for an important role of PINCH-2 in the regulation of the PINCH-1-ILK-CH-ILKBP complex formation, cell shape change and migration.
Experimental Procedures

Cells, antibodies and other reagents

Human embryonal kidney 293 cells, human rhabdomyosarcoma (RD) cells and mouse C2C12 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Rat embryo fibroblasts (REF-52) were cultured in MEM supplemented with 10% fetal bovine serum. Primary human mesangial cells were from Clonetics (San Diego, CA) and cultured in MsGTM™ BulletKit medium (Clonetics). Mouse monoclonal anti-ILK and anti-CH-ILKBP antibodies were described previously (13,15,26). Rabbit polyclonal anti-GFP antibodies, mouse monoclonal anti-FLAG antibodies (M2 and M5) and M2 conjugated agarose beads were from Clontech and Sigma, respectively. Rhodamine Red™- or FITC-conjugated goat anti-mouse IgG antibody (minimal cross-reaction with human, bovine, rabbit and swine serum proteins), Rhodamine Red™- or FITC-conjugated goat anti-rabbit IgG antibody (minimal cross-reaction with human, mouse and rat serum proteins) and horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Media for cell culture were from Life Technologies (Grand Island, NY) or Mediatech/Cellgro® (Herndon, VA).

Cloning of human PINCH-2 cDNA by reverse transcription PCR

The full length PINCH-2 cDNA was cloned from human RD cells by reverse transcription PCR. Poly A+ RNA was isolated from the RD cells using Oligotex mRNA kits (Qiagen) following the manufacture’s protocol. Reverse transcription PCR was carried out using First-Strand cDNA Synthesis kits (Amersham Pharmacia Biotech) with PINCH-2 primers (5’-ATAGATCTGATGACGGGAAGCAATATGTCGGACGCC-3’ and 5’-ACTGTCGACTCAAGGCAGTTGAGGTCTGTGGCCCTT-3’) and PINCH-1 primers (5’-
GCAGTCTATTGGGGAACGCGCCCTGGCCAG-3' and 5'-GCAGCCTTGTGACGTATTTTCTTCAGCTCC-3'), respectively. The sequences of the PCR products were determined by automated DNA sequencing. To test the specificities of the PINCH-2 primers and PINCH-1 primers, we carried out PCR using reaction mixtures containing (1) the PINCH-2 primers + PINCH-2 cDNA, (2) the PINCH-2 primers + PINCH-1 cDNA, (3) the PINCH-2 primers + an irrelevant cDNA, (4) the PINCH-1 primers + PINCH-1 cDNA, (5) the PINCH-1 primers + PINCH-2 cDNA, and (6) the PINCH-1 primers + an irrelevant cDNA. No PCR products were synthesized in any of the reactions containing mismatched PCR primers and templates (see Results).

PINCH-2 mammalian expression vector construction and DNA transfection

DNA fragments encoding the full length or mutant forms of PINCH-2 (as specified in each experiment) were generated by PCR and cloned into the EcoRI/SalI sites of the pFLAG-CMV-2 vector (Sigma). DNA fragments encoding the FLAG-tagged full length or mutant forms of PINCH-2 were cloned into the pEGFP-C2 expression vector (Clontech). To express the full length or mutant forms of PINCH-2 in mammalian cells, cells were transfected with the FLAG or GFP expression vectors encoding the full length or mutant forms of PINCH-2 using LipofectAmine PLUS (Life Technologies) as described (15,21). The expression of the FLAG- and/or GFP-tagged full length or mutant forms of PINCH-2 in the transfectants was confirmed by Western blotting with anti-FLAG and anti-GFP antibodies.
**Adenoviral expression vector construction and infection**

The adenoviral expression vector encoding FLAG-tagged full length human PINCH-2 was generated following a previously described protocol (21). Briefly, human PINCH-2 cDNA was cloned into the *SalI/Xbal* sites of the pAdTrack-CMV shuttle vector. The shuttle vector plasmid was linearized with *PmeI*, purified and mixed with supercoiled pADEsay-1. The vectors were transferred into *E.coli* BJ5183 by electroporation. The bacteria were placed in 1 ml of LB Broth, Lennox (Fisher, Pittsburgh, PA) at 37°C for 1 hour. The bacteria were then inoculated onto agar containing LB Broth supplemented with 50 µg/ml of kanamycin. After 16-20 hour growth, colonies were picked and grown in 2 ml of LB Broth containing 50 µg/ml of kanamycin. Clones were screened by digestions with restriction endonucleases *PacI* and *BamHI*. The positive plasmids were transformed into DH10B cells by electroporation for large-scale amplification. The plasmid DNA was digested with *PacI*, ethanol-precipitated and was used to transfect 293 cells with 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 β-galactosidase was kindly provided by Drs. Tong-Chuan He and Bert Vogelstein (Howard Hughes Medical Institute, the Johns Hopkins Oncology Center, Baltimore, MD). The recombinant adenoviral expressing vectors were used to infect REF-52 cells. The infection efficiency was monitored by the expression of GFP encoded by the viral vectors and typically reached 80-90% within 48 hours. The expression of the FLAG-PINCH-2 in the infected cells was confirmed by Western blotting with monoclonal anti-FLAG antibody M5.
**Generation and purification of anti-PINCH-2 antibodies**

Polyclonal anti-PINCH-2 antisera were generated by immunizing rabbits with a KLH conjugated peptide containing PINCH-2 sequence AQPKATDLNSA. To purify anti-PINCH-2 antibodies, rabbit anti-PINCH-2 antisera were passed through an affinity column containing agarose beads coupled with the PINCH-2 peptide (1 mg peptide/ml beads). After collecting the flow through fractions, the column was washed with PBS. Anti-PINCH-2 antibodies were eluted from the affinity column with 100 mM glycine buffer (pH 2.9) and dialyzed against PBS.

**Immunofluorescent staining**

Immunofluorescent staining was performed as described (15,21). Briefly, cells (as specified in each experiment) were plated in complete medium on fibronectin-coated cover slips or Lab-Tek 8-chamber culture slides and incubated at 37°C under a 5% CO2-95% air atmosphere for at least 6 hours. The cells were fixed with 3.7% paraformaldehyde in PBS, and stained with affinity purified rabbit polyclonal antibodies and/or mouse monoclonal antibodies as specified in each experiment. The primary antibodies were detected with secondary Rhodamine Red^TX^-conjugated anti-rabbit IgG antibodies and FITC-conjugated anti-mouse IgG antibodies, respectively. In some experiments, cells were dually stained with affinity purified rabbit anti-PINCH-2 antibodies and Tetramethyl Rhodamine-labeled phalloidin (to visualize the actin cytoskeleton). The rabbit anti-PINCH-2 antibodies were detected with FITC-conjugated anti-rabbit IgG antibodies in these experiments.
**Immunoprecipitation**

Cells (as specified in each experiment) were cultured in complete medium in 60 mm or 100 mm culture plates. Cell monolayers were rinsed twice with PBS 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 Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 100 mM NaF and protease inhibitors. The protocol for immunoprecipitation with monoclonal anti-CH-ILKBP antibody or affinity purified rabbit anti-PINCH-2 antibodies was described previously (15,21). Briefly, cell lysates (500 µg) were mixed with 500 µl of hybridoma culture supernatant containing monoclonal anti-CH-ILKBP antibody 1D4 or 20 µg affinity purified rabbit anti-PINCH-2 antibodies. Control immunoprecipitates were prepared by substitution of the 1D4 supernatant with equal volume of unconditioned culture supernatant or by substitution of the affinity purified rabbit anti-PINCH-2 antibodies with equal amount of irrelevant rabbit IgG. The samples were incubated for 3 hours, mixed with 40 µl of UltraLink Immobilized Protein G (Pierce) and then incubated for an additional 1.5 hours. 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 antibodies to CH-ILKBP, ILK, PINCH-1 or PINCH-2 as specified in each experiment.

For immunoprecipitation of FLAG-tagged full length or mutant forms of PINCH-2, cells expressing the FLAG-tagged PINCH-2 proteins and control cells lacking the FLAG-tagged PINCH-2 proteins were lysed as described above. The cell lysates (500 µg) were mixed with 40 µl anti-FLAG antibody M2 conjugated agarose beads (Sigma). The immunoprecipitated FLAG-tagged PINCH-2 proteins and the associated proteins were released from the beads by boiling in
60 µl of SDS-PAGE sample buffer for 5 min and analyzed by Western blotting (20 µl/lane) with antibodies as specified in each experiment.

**Cell spreading**

Cells (as specified in each experiment) were plated in Opti-MEM I serum free medium (Life Technologies) in wells of 96-well plates that were coated with 0.1 µg/ml fibronectin. The plates were incubated at 37°C under a 5% CO2-95% air atmosphere and the cell morphology were observed under an Olympus IX70 fluorescence microscope equipped with an Hoffman Modulation Contrast system. The images were recorded with a DVC-1310C Magnafire™ digital camera (Optronics). Unspread cells were defined as round cells while spread cells were defined as cells with extended processes as described (15,21,27,28). The percentage of cells adopting spread morphology was quantified by analyzing at least 300 cells from three randomly selected fields (>100 cells/field) (15,21,27,28).

**Cell migration**

Cell migration was assessed by the ability of the cells to migrate into a cell-free area as previously described (21,29,30). Briefly, cells were plated in complete media on 24-well plates and grown for 24 hours to reach confluence. The monolayers were then wounded by scratching with a plastic pipette tip. After washing, the cells were incubated in complete media 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

Cloning and expression of PINCH-2

To clone the full length cDNA of human PINCH-2, we carried out reverse transcription PCR on poly A⁺ RNA isolated from human RD cells using primers for a putative open reading frame (DDBJ/EMBL/GenBank accession number AK022470). A major cDNA with a size of approximate 1 kb was obtained (Fig. 1A, lane 5). In control experiments, no DNA was amplified with the same primers when the template was omitted (Fig. 1A, lane 6) or when PINCH-1 cDNA (Fig. 1A, lane 2) or an irrelevant cDNA (Fig. 1A, lane 3) was used as a template, confirming the specificity of the reactions. The cDNA obtained from the human RD cells was sequenced. It encodes a protein encompassing the previously described partial PINCH-2 sequence (22). The full length human PINCH-2 consists of five LIM domains, each of which contains a sequence motif CxxCₓ₁₆₋₁₈C/HxxC/HxxCxxCₓ₁₆₋₁₈CxxC/H/D (the full length nucleotide sequence and the deduced protein sequence of PINCH-2 derived from the human RD cells have been deposited into GenBank (accession no. AF484961)). BLAST search of human genome database indicates that the gene encoding PINCH-2 is located within the human chromosome 2q14.3 region. Human PINCH-2 is 82% identical to human PINCH-1 at the amino acid sequence level. Analyses of DNA sequences suggest that they are not splicing variants but are instead encoded by two different genes. To test whether PINCH-2 and PINCH-1 are co-expressed in human cells, we carried out reverse transcription PCR on poly A⁺ RNA from human RD cells using primers specific for PINCH-1. A major cDNA with the predicted PINCH-1 size was amplified (Fig. 1B, lane 2). In control experiments, no DNA was amplified with the PINCH-1 primers when the template was omitted (Fig. 1B, lane 6), or when an irrelevant cDNA (Fig. 1B, lane 3) or PINCH-2 cDNA (Fig. 1B, lane 4), which was readily amplified with the PINCH-2 primers
(Fig. 1A, lane 4), was used as a template. Taken together, these results suggest that PINCH-2 and PINCH-1 transcripts are co-expressed in human RD cells.

To facilitate studies on PINCH-2, we generated an anti-PINCH-2 antibody by immunizing rabbits with a peptide containing a specific PINCH-2 sequence. Western blotting analyses of lysates of CHO cells transfected with expression vectors encoding GFP- and FLAG-tagged human PINCH-2 or PINCH-1 showed that the rabbit anti-PINCH-2 antiserum recognized the recombinant human PINCH-2 (Fig. 2A, lane 2) but not the PINCH-1 protein (Fig. 2A, lane 1). In control experiments, no protein from the CHO transfectants was recognized by pre-immune rabbit serum under the same condition (Fig. 2B). The expression of the GFP- and FLAG-tagged recombinant human PINCH-2 (Fig. 2C, lane 2) and PINCH-1 (Fig. 2C, lane 1) proteins in the corresponding transfectants was confirmed by Western blotting with an anti-FLAG antibody. To test whether the rabbit anti-PINCH-2 antiserum recognizes endogenous human PINCH-2 protein, we probed lysates of human WI-38 cells by Western blotting with the anti-PINCH-2 antiserum. The results show that the anti-PINCH-2 antiserum recognized a protein band with an apparent molecular weight similar to the calculated molecular weight of human PINCH-2 protein (38,917.03) (Fig. 2D, lane 1). Binding of the antibody to the ~39 kDa endogenous protein was completely blocked by incubation of the antiserum with the PINCH-2 peptide (Fig. 2D, lane 2), indicating that the ~39 kDa band represents the endogenous human PINCH-2 protein. Several additional protein bands with higher or lower molecular weights were also detected (Fig. 2D, lane 1). However, they unlikely represent proteins specifically recognized by the anti-PINCH-2 antibody, as they were not blocked by the PINCH-2 peptide (Fig. 2D, lane 2). To confirm that the anti-PINCH-2 antiserum recognizes PINCH-2 but not
PINCH-1, we re-probed the membrane that had been probed with the anti-PINCH-2 antiserum (Fig. 2D, lane 1) with an antibody recognizing PINCH-1. The result showed that PINCH-1 protein, which migrated faster than PINCH-2 (the calculated molecular weight of PINCH-1 = 37,251.32) and was not recognized by the anti-PINCH-2 antiserum (Fig. 2D, lane 1), was readily detected with the anti-PINCH-1 antibody in the same cell lysates (Fig. 2D, lane 3). These results confirm the specificity of the anti-PINCH-2 antiserum. Furthermore, they show that PINCH-2 and PINCH-1 are co-expressed in human WI-38 cells. Western blotting analyses of other human cell types including RD cells, A-431 epidermoid carcinoma cells, IMR-90 lung cells, HT-1080 fibrosarcoma cells, embryonal kidney 293 cells and primary glomerular mesangial cells revealed that PINCH-2 is also co-expressed with PINCH-1 in these cells (data not shown).

**PINCH-2 localizes to both focal adhesions and the nucleus**

We next analyzed the subcellular localization of PINCH-2. To do this, we purified the anti-PINCH-2 antibodies with an affinity column containing the PINCH-2 peptide (Fig. 3A). The affinity-purified antibodies specifically recognized PINCH-2 (Fig. 3A, lane 2), whereas the flow through fractions failed to recognize PINCH-2 (Fig. 3A, lane 3). Immunofluorescent staining of human cells with the affinity-purified anti-PINCH-2 antibody showed that PINCH-2 localizes to both focal adhesions and the nucleus (Fig. 3B). No focal adhesion or nuclear staining was observed with the flow through fraction (Fig. 3C), confirming the specificity of the PINCH-2 immunofluorescent staining. Double staining of the cells with the affinity-purified rabbit anti-PINCH-2 antibodies and mouse anti-ILK antibody 65.1 showed that PINCH-2 is co-clustered with ILK in focal adhesions (Fig. 3, D and E). Interestingly, although abundant PINCH-2 was detected in the nucleus (Fig. 3D), ILK was absent in the nucleus (Fig. 3E). As
expected, the clusters of PINCH-2 at the focal adhesions (Fig. 3F), which are docking sites for actin stress fibers at the cell-extracellular matrix adhesion sites, are co-aligned with actin filaments (Fig. 3G).

PINCH-2 associates with ILK in mammalian cells

To test whether PINCH-2 forms a complex with ILK, we immunoprecipitated CH-ILKBP and ILK from human cell lysates with a monoclonal anti-CH-ILKBP antibody. Western blotting analyses of the anti-CH-ILKBP immunoprecipitates with antibodies to CH-ILKBP, ILK, PINCH-1 and PINCH-2 revealed that both PINCH-2 (Fig. 4D, lane 2) and PINCH-1 (Fig. 4C, lane 2) were co-immunoprecipitated with ILK (Fig. 4B, lane 2) and CH-ILKBP (Fig. 4A, lane 2). In control experiments, no specific proteins (CH-ILKBP, ILK, PINCH-1 or PINCH-2) were precipitated from the lysates when the anti-CH-ILKBP hybridoma supernatant was substituted with unconditioned culture supernatant (data not shown). To confirm that PINCH-2 forms a complex with ILK, we immunoprecipitated PINCH-2 from cell lysates with the affinity-purified anti-PINCH-2 antibody. Analyses of the anti-PINCH-2 immunoprecipitates with monoclonal anti-ILK antibody showed that ILK (Fig. 4F, lane 2) was readily co-immunoprecipitated with PINCH-2 (Fig. 4E, lane 2). In control experiments, no ILK was detected in the control immunoprecipitates obtained with irrelevant rabbit IgG (Fig. 4F, lane 3). These experiments demonstrate that PINCH-2, like PINCH-1 (15), forms a complex with ILK in human cells.

The N-terminal-most LIM domain (LIM1) of PINCH-2 mediates the association with ILK

We next sought to identify the domain of PINCH-2 that mediates the interaction with ILK. Because an ILK binding site is located within the PINCH-1 LIM1 domain (12,13,31), we
hypothesized that the PINCH-2 LIM1 domain, which contains a sequence almost identical to that of the PINCH-1 ILK-binding sequence, mediates the interaction with ILK. To test this experimentally, we expressed GFP- and FLAG-tagged LIM1-deletion mutant of PINCH-2, and the wild type and the LIM5-deletion mutant of PINCH-2 as controls, in mammalian cells. The expression of the GFP- and FLAG-tagged wild type (Fig. 5A, lane 2), the LIM5-deletion mutant of PINCH-2 (Fig. 5A, lane 3), the LIM1-deletion mutant of PINCH-2 (Fig. 5A, lane 4) or GFP alone as a negative control (Fig. 5A, lane 1) was confirmed by Western blotting with anti-GFP antibodies. The GFP- and FLAG-tagged wild type or mutant forms of PINCH-2 (Fig. 5A, lanes 6-8), but not GFP (Fig. 5A, lane 5), were immunoprecipitated with monoclonal anti-FLAG antibody (M2) conjugated beads. Western blotting analyses of the immunoprecipitates showed that ILK was co-immunoprecipitated with GFP-FLAG-PINCH-2 (Fig. 5B, lane 6) or the LIM5-deletion mutant (Fig. 5B, lane 7) but not the LIM1-deletion PINCH-2 mutant (Fig. 5B, lane 8), indicating that the LIM1 domain of PINCH-2 indeed mediates the interaction with ILK. Probing the immunoprecipitates with a monoclonal antibody recognizing CH-ILKBP, which is known to interact with the C-terminal domain of ILK (15), showed that CH-ILKBP was co-immunoprecipitated in the presence of ILK (Fig. 5C, lanes 6 and 7) but not in the absence of ILK (Fig. 5C, lane 8). In control experiments, neither ILK (Fig. 5B, lane 5) nor CH-ILKBP (Fig. 5C, lane 5) was detected in immunoprecipitates from the GFP control transfectants, confirming the specificity of the co-immunoprecipitation assays.

The association with ILK is required for the localization of PINCH-2 to focal adhesions

Because PINCH-2 associates with ILK (Figs. 4 and 5) and the two proteins are co-clustered at focal adhesions (Fig. 3), we tested whether the association with ILK is required for
the localization of PINCH-2 to focal adhesions. To do this, we analyzed the subcellular localization of GFP- and FLAG-tagged wild type PINCH-2, the ILK-binding defective LIM1-deletion mutant of PINCH-2, and the ILK-binding competent LIM5-deletion mutant of PINCH-2 in mammalian cells. Consistent with the results obtained with the affinity purified anti-PINCH-2 antibodies (Fig. 3), the GFP- and FLAG-tagged wild type PINCH-2 localized to focal adhesions (Fig. 6A), where ILK was clustered (Fig. 6B). The ILK-binding competent LIM5-deletion mutant, like the wild type PINCH-2, was also able to localize to focal adhesions, albeit the efficiency appeared to be lower (Fig. 6, E and F). By marked contrast, the ILK-binding defective LIM1 deletion mutant of PINCH-2 failed to localize to the ILK-rich focal adhesions (Fig. 6, C and D), indicating that localization of PINCH-2 to focal adhesions requires the association with ILK.

*Overexpression of PINCH-2 inhibits the PINCH-1-ILK interaction*

Because PINCH-2, like PINCH-1, forms a complex with ILK, we tested whether PINCH-2 plays a role in the regulation of the PINCH-1-ILK-CH-ILKBP complex formation. To do this, we transfected mammalian cells with an expression vector encoding FLAG-PINCH-2, or a FLAG vector lacking PINCH-2 sequence as a control, and analyzed the formation of the ILK complexes by co-immunoprecipitation with a monoclonal anti-CH-ILKBP antibody. Equal amount of ILK was co-immunoprecipitated with CH-ILKBP from both the FLAG-PINCH-2 and the vector control transfectants (Fig. 7, A and B), indicating that the ILK-CH-ILKBP interaction is not impaired by overexpression of PINCH-2. Western blotting analyses of the same immunoprecipitates with anti-PINCH-1 antibodies showed that the amount of PINCH-1 associated with ILK, however, was significantly reduced in cells overexpressing FLAG-PINCH-
2 (Fig. 7C, compare lanes 1 and 2). In control experiments, FLAG-PINCH-2 was readily detected in immunoprecipitates from cells overexpressing FLAG-PINCH-2 (Fig. 7D, lane 2) but not in those from the control cells (Fig. 7D, lane 1), confirming that FLAG-PINCH-2 forms a complex with ILK and CH-ILKBP. To further analyze this, we immunoprecipitated FLAG-PINCH-2 from the FLAG-PINCH-2 expressing cells (Fig. 8A, lane 1). Western blotting analyses of the FLAG-PINCH-2 immunoprecipitates showed that ILK was co-immunoprecipitated with FLAG-PINCH-2 (Fig. 8B, lane 1). No PINCH-1, however, was co-immunoprecipitated with ILK and FLAG-PINCH-2 (Fig. 8C, lane 1), despite the presence of abundant PINCH-1 in the cell lysates (Fig. 8C, lane 3). In control experiments, no ILK (Fig. 8B, lane 2) was precipitated with the anti-FLAG antibody in the absence of FLAG-PINCH-2 (Fig. 8A, lane 2), confirming the specificity of the co-immunoprecipitation. Taken together, these results suggest that the binding of FLAG-PINCH-2 to ILK prevents the PINCH-1 binding to ILK, resulting in a reduction of the complex formation between PINCH-1 and ILK.

Overexpression of PINCH-2 inhibits cell spreading and migration

We have previously shown that the interaction between PINCH-1 and ILK is critically involved in cell shape change and migration (21). To test whether PINCH-2 is involved in the regulation of cell shape change, we transiently transfected human embryonal kidney 293 cells, a cell line that is known for its high transfection efficiency, with an expression vector that encodes FLAG-PINCH-2 or a FLAG vector that lacks PINCH-2 sequence as a control. The expression of FLAG-PINCH-2 in the FLAG-PINCH-2 transfectants (Fig. 9A, lane 3) but not in the parental 293 (Fig. 9A, lane 1) or the control vector transfectants (Fig. 9A, lane 2) was confirmed by Western blotting with a monoclonal anti-FLAG antibody. To assess cell spreading, we plated
the parental 293 cells, the FLAG-PINCH-2 transfectants and the control transfectants on fibronectin-coated surface. Majorities of the parental 293 cells and the control transfectants exhibited spread morphology within 30 minutes of plating (Fig. 9, B and C). By contrast, most of the FLAG-PINCH-2 transfectants remained round (Fig. 9, B and C), suggesting that overexpression of PINCH-2 inhibits the spreading of 293 cells. Despite our efforts, we were unable to obtain stable 293 clones overexpressing FLAG-PINCH-2. To facilitate further functional studies on PINCH-2, we generated a recombinant adenovirus encoding FLAG-PINCH-2. Rat embryo fibroblasts (REF-52) were infected with the FLAG-PINCH-2 adenovirus or an adenovirus encoding β-galactosidase as a control. Under the experimental condition used, the viral infection efficiency was approximately 80-90%. The expression of FLAG-PINCH-2 in the FLAG-PINCH-2 adenoviral infected REF-52 cells (Fig. 10A, lane 3) but not in the control adenoviral infected REF-52 cells (Fig. 10A, lane 2) or uninfected REF-52 cells (Fig. 10A, lane 1) was confirmed by Western blotting. Consistent with the results obtained with 293 cells, overexpression of FLAG-PINCH-2 significantly inhibited the spreading of REF-52 cells (Fig. 10B). To analyze the effect of PINCH-2 overexpression on cell motility, we wound monolayers of REF-52 cells overexpressing FLAG-PINCH-2 or those of the control cells and measured the migration of the cells into the cell-free area as previously described (21,29,30). The results showed that cells overexpressing FLAG-PINCH-2 migrated much slower than the control cells (Fig. 10, C and D). Thus, consistent with an inhibitory effect on cell spreading, overexpression of FALG-PINCH-2 significantly reduces cell migration.
Discussion

In this study, we have cloned and characterized PINCH-2, a new member of the PINCH family. Our results have shown that (1) PINCH-2 is co-expressed with PINCH-1 in a variety of human cells, (2) PINCH-2 localizes to both cell-ECM contact sites and the nucleus, (3) PINCH-2 forms a complex with ILK in mammalian cells and the LIM1 domain of PINCH-2 mediates the complex formation, (4) the PINCH-2 LIM1 domain, which mediates the association with ILK, is required for the localization of PINCH-2 to cell-ECM contact sites, (5) the PINCH-2-ILK and PINCH-1-ILK interactions are mutually exclusive, and (6) overexpression of PINCH-2 inhibits the PINCH-1-ILK interaction and reduces cell spreading and migration. These results identify a new component of the cell-ECM adhesion structure and suggest an important role of PINCH-2 in the cellular control of the PINCH-1-ILK-CH-ILKBP complex formation, cell shape change and migration.

In previous studies, we have shown that ILK, through interactions mediated by its N- and C-terminal domains respectively, forms a ternary complex with PINCH-1 and CH-ILKBP in mammalian cells (15). The PINCH-1-ILK-CH-ILKBP complex likely represents an evolutionally conserved and functionally important complex that is involved in the coupling of ECM to the actin cytoskeleton at cell-ECM adhesion sites (11). In invertebrate organisms such as *C. elegans*, PINCH-1/UNC-97 (22), ILK/PAT-4 (23) and CH-ILKBP/PAT-6 (Lin and Williams, 40th ASCB Annual Meeting, 2000, Abstract 2666) are co-expressed in body wall muscle cells. Null-mutations or suppression of expression of any one of the three proteins (PINCH-1/UNC-97, ILK/PAT-4 or CH-ILKBP/PAT-6) all result in a Pat phenotype similar to that of β-integrin/pat-3 or α-integrin/pat-2, which is characterized by defects in dense body and
M-line assembly (24,25). In mammalian systems, PINCH-1 (12,32), ILK (14,19,26,33,34) and CH-ILKBP (15-17) are expressed in many different types of tissues and cells. We have detected the PINCH-1-ILK-CH-ILKBP complex in all types of mammalian cells that we have analyzed, which include both primary cells such as human and rat mesangial cells and established cell lines such as mouse C2C12 myoblasts, rat embryo fibroblasts, Chinese hamster ovary cells, human 293 kidney cells, and human WI-38 and IMR-90 lung fibroblasts (unpublished observations). Consistent with the genetic studies in invertebrate systems, disruption of the PINCH-1-ILK-CH-ILKBP complex in mammalian cells with dominant negative forms of PINCH-1 or ILK impairs cell spreading and migration (21). The importance of the PINCH-1-ILK-CH-ILKBP complex in the cellular control of cell shape change and motility and the ability of the dominant negative forms of PINCH-1 or ILK to inhibit the PINCH-1-ILK-CH-ILKBP complex formation beg the question as to whether there exist cellular proteins that can compete with the binding of PINCH-1 to ILK and thereby regulate the assembly of the PINCH-1-ILK-CH-ILKBP complex. The findings described in this report suggest that PINCH-2 likely functions as a naturally occurring regulator of the PINCH-1-ILK-CH-ILKBP complex. First, PINCH-2 is co-expressed with PINCH-1 in human cells. Second, PINCH-2, like PINCH-1, can form a complex with ILK. Third, the complex formation of ILK with PINCH-2 excludes the ability of ILK to form a complex with PINCH-1. Finally, overexpression of PINCH-2 results in a significant decrease of the amount of PINCH-1 associated with ILK. Thus, cells could regulate the formation of the PINCH-1-ILK-CH-ILKBP complex and consequently, the functions of the PINCH-1-ILK-CH-ILKBP complex, by controlling the cellular level of PINCH-2.
An important functional consequence of overexpression of PINCH-2 is that it significantly impairs cell spreading and migration. This is remarkably similar to that of overexpression of the ILK ANK fragment or the PINCH-1 fragment containing the LIM1 domain (21), two dominant negative inhibitors of the PINCH-1-ILK interaction, suggesting that the inhibitory effect of overexpression of PINCH-2 on cell spreading and migration is caused by, at least in part, down-regulation of the PINCH-1-ILK-CH-ILKBP complex. The results described in this paper, however, do not exclude the possibility that PINCH-2 could participate in other events that regulate cell shape change and migration. Cell shape change and migration are complex processes involving coordinated regulation of multiple protein-protein interactions at cell-ECM adhesion sites. A common feature of many components of the cell-ECM adhesion structures is that they interact with multiple partners at the adhesion sites (35). The finding that PINCH-2 localizes to cell-ECM adhesion sites, together with the fact that PINCH-2 contains five LIM domains and only one of them (LIM1) is involved in the association with ILK, suggest that PINCH-2 could potentially interact with other component of the cell-ECM adhesion structures. Identification and characterization of other binding partners of PINCH-2 will be an important goal of future studies.

In addition to showing that PINCH-2 localizes to cell-ECM adhesion sites and regulates the formation of the PINCH-1-ILK-CH-ILKBP complex, cell spreading and migration, we have found that a substantial amount of PINCH-2 is present in the nucleus. Nix and Beckerle have demonstrated that zyxin, a focal adhesion protein with three LIM domains, shuttles between the cytoplasm and the nucleus (36). Yang et al. have shown that Hic-5, a paxillin-related focal adhesion protein with four LIM domains, also localizes to nuclei (37). Thus, PINCH-2 appears
to join zyxin and Hic-5 as a member of the LIM protein group that localize to both focal adhesions and the nucleus. It is interesting to note, however, that at steady state, zyxin is typically undetectable in the nucleus by indirect immunofluorescent staining due to the presence of a nuclear export signal (36,38). The immunofluorescent staining of nuclear Hic-5 was also weak (37) or undetectable (39), albeit the nuclear signal enhanced after high-salt and detergent extraction to remove cytoplasm- and focal adhesion-localized Hic-5 (37). In contrast, PINCH-2 was readily detected at steady state in the nuclei by indirect immunofluorescent staining (Fig. 3).

What is the molecular basis that controls the distribution of PINCH-2 to the two different subcellular compartments? The findings that PINCH-2 is co-clustered with ILK at focal adhesions (Fig. 3) and that the mutation dissociating the PINCH-2-ILK complex impairs the focal adhesion localization of PINCH-2 (Fig. 6) suggest that the association with ILK is required for this process. While it is clear that the LIM1 domain is involved in the focal adhesion localization of PINCH-2, the sites that are involved in the nuclear transport remain to be defined. Inspection of the PINCH-2 sequence reveals that in the C-terminal region there exists a stretch of positively charged residues and an overlapping leucine-rich sequence (LELKKRLKKS): that could potentially be involved in the nuclear export. It is interesting to note in this regard that the PINCH-2 C-terminal deletion mutant (ΔLIM5), which lacks the putative leucine-rich nuclear export signal, accumulated in the nucleus (Fig. 6E), suggesting that the leucine-rich sequence could be involved in the nuclear export of PINCH-2. Additional studies, however, are needed to further define the sites that are involved in the nuclear transport of PINCH-2, as GFP-ΔLIM5 can also localize to focal adhesions (albeit with lower efficiency).
It has been well documented that cell-ECM adhesion is pivotal to the regulation of gene expression and cell cycle progression (5,40-42). Proteins that localize to both the nucleus and cell-ECM contact sites likely actively participate in this process. For example, c-Abl, a nonreceptor tyrosin kinase that localizes to both the nucleus and cell-ECM contact sites (43,44), is involved in the cell cycle-dependent and DNA damage-induced gene expression (45). The finding that abundant PINCH-2 is present in the nucleus suggest that, in addition to regulating the PINCH-1-ILK interaction, cell spreading and migration, PINCH-2 likely participates in the regulation of nuclear processes. Furthermore, the distribution of PINCH-2 in both the nuclei and focal adhesions raises an interesting possibility that PINCH-2 could be involved in linking the nuclear processes to cell shape changes. For example, it has been well described that adherent cells undergo extensive shape change during cell cycle. During M phase, mitosis, in which nuclear envelope breaks down, is always (at least in normal cells) followed by cell rounding up and cytokinesis. Given the dual localization of PINCH-2 in both the nucleus and focal adhesions and its role in the regulation of the PINCH-1-ILK-CH-ILKBP complex and cell spreading, it will be of considerable interest to test whether PINCH-2 participates in the cellular control of cytokinesis and other processes that involve coordinated changes of cell shape.

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Footnotes

Abbreviations used in this paper: ANK, ankyrin; ASCB, American Society for Cell Biology; CH, calponin homology; CH-ILKBP, calponin homology domain-containing ILK-binding protein; ECM, extracellular matrix; ILK, integrin-linked kinase; RD, rhabdomyosarcoma; RT-PCR, reverse transcription PCR.

Y. Zhang and K. Chen contributed equally to this work.
Figure Legends

**Figure 1. RT-PCR cloning of PINCH-2**

Human PINCH-2 cDNA was cloned from human RD cells by RT-PCR as described in Experimental Procedures. PCR was carried out using PINCH-2 (A) or PINCH-1 (B) specific primers. Lane 5 was loaded with RT-PCR products from the RD cells. To test the specificities of the PINCH-2 and PINCH-1 primers, PCR was carried out using PINCH-1 cDNA (lane 2), an irrelevant cDNA (lane 3) or PINCH-2 cDNA (lane 4) as templates. Lane 6, PCR was carried out in the absence of templates. Lane 1 was loaded with DNA ladder (Life Technologies) as indicated in the figure.

**Figure 2. PINCH-2 expression in human cells**

(A-C) Lysates (10 µg proteins/lane) of CHO cells expressing GFP-FLAG-PINCH-1 (lane 1) or GFP-FLAG-PINCH-2 (lane 2) were analyzed by Western blotting with a rabbit anti-PINCH-2 antiserum (1:1000 dilution)(A), pre-immune rabbit serum (1:1000) as a negative control (B) and monoclonal anti-FLAG antibody M2 (1 µg/ml) as a positive control (C), respectively. (D) Lysates (25 µg proteins/lane) of human WI-38 cells were analyzed by Western blotting with rabbit anti-PINCH-2 antiserum (1:500 dilution) (lane 1) or anti-PINCH-2 antiserum (1:500 dilution) that was neutralized with the PINCH-2 peptide antigen (20 µg/ml) (lane 2). Lane 3, the membrane that was used in lane 1 was re-probed (without stripping) with a rabbit polyclonal anti-PINCH-1 antibody.
Figure 3. Subcellular localization of PINCH-2

(A) Affinity purification of anti-PINCH-2 antibodies. Lysates (25 μg proteins/lane) of human WI-38 lung cells were analyzed by Western blotting with rabbit anti-PINCH-2 antiserum (1:500 dilution)(lane 1), the affinity purified anti-PINCH-2 antibodies (1 μg/ml) (lane 2) or the flow through fraction (1:100 dilution) from the PINCH-2 peptide affinity column that was depleted of anti-PINCH-2 activities (lane 3). (B-G) Immunofluorescent staining. Human IMR-90 lung cells were stained with the affinity purified anti-PINCH-2 antibodies (10 μg/ml) (B), the flow through fraction (1:25 dilution) as a control (C), or dually stained with the affinity purified anti-PINCH-2 antibodies (D) and mouse monoclonal anti-ILK antibody 65.1 (E), or the affinity purified anti-PINCH-2 antibodies (F) and tetramethyl Rhodamine-labeled phalloidin (G). Bar, 10 μm. Similar results have been obtained with WI-38 cells.

Figure 4. Association of PINCH-2 with ILK

(A-D) Lysates of human mesangial cells were mixed with anti-CH-ILKBP antibody 1D4 and immunoprecipitation was carried out as described in Experimental Procedures. The anti-CH-ILKBP immunoprecipitates (lane 2) and mesangial cell lysates (lane 1) were analyzed by Western blotting with mouse monoclonal anti-CH-ILKBP antibody 3B5 (A), mouse monoclonal anti-ILK antibody 65.1 (B), rabbit polyclonal anti-PINCH-1 (C) and rabbit polyclonal anti-PINCH-1 antibodies (D), respectively. (E and F) Lysates of WI-38 cells were mixed with affinity purified rabbit anti-PINCH-2 antibodies and immunoprecipitation was carried out as described in Experimental Procedures. The cell lysates (lane 1), anti-PINCH-2 immunoprecipitates (lane 2) and control immunoprecipitates obtained with irrelevant rabbit IgG
(lane 3) were analyzed by Western blotting with rabbit anti-PINCH-2 antiserum (E) and monoclonal anti-ILK antibody 65.1 (F), respectively.

Figure 5. The LIM1 domain of PINCH-2 mediates the association with ILK

C2C12 cells expressing GFP (lane 5) or GFP-FLAG-fusion proteins containing the full length PINCH-2 (lane 6), the LIM5-deletion mutant of PINCH-2 (residues 1-254) (lane 7) or the LIM1-deletion mutant of PINCH-2 (residues 69-341) (lane 8) were analyzed by immunoprecipitation with anti-FLAG antibody M2 as described in Experimental Procedures. The immunoprecipitates were analyzed by Western blotting with rabbit anti-GFP antibody (A), mouse anti-ILK antibody 65.1 (B), and mouse anti-CH-ILKBP antibody 3B5 respectively. Lanes 1-4 were loaded with lysates (20 µg proteins/lane) of C2C12 cells expressing GFP or GFP-FLAG-tagged full length or mutant forms of PINCH-2 as indicated. The sample in lane 9 was prepared as those of lanes 5-8 except that cell lysates were omitted (to show mouse Ig bands from the anti-FLAG antibody M2 conjugated beads, which were detected by HRP-conjugated anti-mouse IgG antibodies in panels B and C but not by HRP-conjugated anti-rabbit IgG antibodies in panel A).

Figure 6. The ILK binding LIM1 domain is required for the localization of PINCH-2 to cell-matrix adhesion sites

C2C12 cells expressing GFP-FLAG-tagged PINCH-2 (A and B), the LIM1-deletion mutant (C and D), or the LIM5-deletion mutant (E and F) were plated on fibronectin-coated culture slides. The cells were stained with monoclonal anti-ILK antibody 65.1 and Rhodamine RedTX-conjugated anti-mouse IgG antibodies. GFP-PINCH-2 (A), GFP-LIM2-5 (C), GFP-
LIM1-4 (E), and ILK (B, D and F) were visualized under a fluorescence microscope equipped with GFP (A, C and E) and rhodamine (B, D and F) filters. Bar, 10 \( \mu \text{m} \).

**Figure 7. Overexpression of PINCH-2 inhibits the PINCH-1-ILK interaction**

CH-ILKBP was immunoprecipitated from lysates of C2C12 transfectants overexpressing FLAG-PINCH-2 (lane 2) or the control vector transfectants (lane 1) with anti-CH-ILKBP antibody 1D4. The samples were analyzed by Western blotting with anti-CH-ILKBP antibody 3B5 (A), anti-ILK antibody 65.1 (B), anti-PINCH-1 antibody (C) and anti-FLAG antibody (D), respectively. Lane 3 was loaded with 1D4 IgG (to show mouse Ig bands). Note that much less PINCH-1 bound to ILK in cells overexpressing FLAG-PINCH-2.

**Figure 8. PINCH-1 does not bind to ILK that associates with FLAG-PINCH-2**

FLAG-PINCH-2 was immunoprecipitated from lysates of FLAG-PINCH-2 expressing C2C12 cells with mouse anti-FLAG antibody B2 (lane 1). Lane 2, anti-FLAG immunoprecipitates from the vector control cells. Lanes 3 and 4, lysates from the FLAG-PINCH-2 expressing C2C12 cells and the control cells. The samples were analyzed by Western blotting with mouse anti-FLAG antibody M5 (A), anti-ILK antibody 65.1 (B) and rabbit anti-PINCH-1 antibody (C), respectively. Note that no PINCH-1 (lane 1, C) bound to ILK (lane 1, B) that associated with FLAG-PINCH-2 (lane 1, A).

**Figure 9. Overexpression of PINCH-2 reduces cell spreading**

(A) Expression of FLAG-PINCH-2 in 293 cells. Lysates (20 \( \mu \text{g/lane} \)) of 293 cells (lane 1), the control FLAG vector transfectants (lane 2) and the FLAG-PINCH-2 transfectants of 293
cells (lane 3) were analyzed by Western blotting with anti-FLAG antibody M5. (B) 293 cells (parental), the FLAG vector control 293 transfectants (vector) and 293 transfectants overexpressing FLAG-PINCH-2 (PINCH-2) were seeded in fibronectin-coated plates and allowed to spread for 25 minutes. Bar, 20 µm. The percentages of cells adopting spread morphology were quantified by analyzing more than 300 cells from three randomly selected fields (C). Data represent means ± SD.

**Figure 10. Overexpression of PINCH-RP inhibits cell migration**

(A) Expression of FLAG-PINCH-2 in REF-52 cells. Lysates (20 µg/lane) of REF-52 cells (lane 1), REF-52 cells infected with the control β-galactosidase adenovirus (lane 2) and REF-52 cells infected with the FLAG-PINCH-2 adenovirus (lane 3) were analyzed by Western blotting with anti-FLAG antibody M5. (B) Cell spreading. REF-52 cells, REF-52 cells infected with the control β-galactosidase adenovirus and REF-52 cells infected with the FLAG-PINCH-2 adenovirus were seeded in fibronectin-coated plates and allowed to spread for 30 minutes. The percentages of cells adopting spread morphology were quantified by analyzing more than 300 cells from three randomly selected fields (B). Data represent means ± SD. (C and D) cell migration. Monolayers of REF-52 cells infected with the FLAG-PINCH-2 adenovirus (PINCH-2) and those infected with the control β-galactosidase adenovirus (Control) were wounded at 0 hour, and the cells were allowed to migrate into the cell-free area for 16 hours (C). Bar, 150 µm. Distances traveled by the cells at the acellular front (D) were calculated as described in Experimental Procedures. Data represent means ± SD of distances traveled by the cells in three different segments of the wound.
Figure 3
Figure 4
Figure 5
Figure 7

Figure 8
Figure 9
Figure 10
Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading and migration
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