

Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites

Yongjun Zhang¹, Ka Chen¹, Yizeng Tu¹, Algirdas Velyvis², Yanwu Yang², Jun Qin² and Chuanyue Wu^{1,*}

¹Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15261, USA

²Structural Biology Program, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA

*Author for correspondence (e-mail: carywu@pitt.edu)

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Summary

PINCH, integrin-linked kinase (ILK) and calponin homology-containing ILK-binding protein (CH-ILKBP) form a ternary complex that plays crucial roles at cell-extracellular matrix adhesion sites. To understand the mechanism underlying the complex formation and recruitment to cell-adhesion sites we have undertaken a combined structural, mutational and cell biological analysis. Three-dimensional structure-based point mutations identified specific PINCH and ILK sites that mediate the complex formation. Analyses of the binding defective point mutants revealed that the assembly of the PINCH-ILK-CH-ILKBP complex is essential for their localization to cell-extracellular matrix adhesion sites. The formation of the PINCH-ILK-CH-ILKBP complex precedes integrin-mediated cell adhesion and spreading.

Furthermore, inhibition of protein kinase C, but not that of actin polymerization, inhibited the PINCH-ILK-CH-ILKBP complex formation, suggesting that the PINCH-ILK-CH-ILKBP complex likely serves as a downstream effector of protein kinase C in the cellular control of focal adhesion assembly. Finally, we provide evidence that the formation of the PINCH-ILK-CH-ILKBP complex, while necessary, is not sufficient for ILK localization to cell-extracellular matrix adhesion sites. These results provide new insights into the molecular mechanism underlying the assembly and regulation of cell-matrix adhesion structures.

Key words: Integrin-linked kinase, PINCH, CH-ILKBP, Focal adhesions, Protein kinase C

Introduction

Cell-extracellular matrix (ECM) adhesion is a fundamental process that controls, at least in part, cell behavior including shape change, migration, proliferation, differentiation and survival. Upon adhesion to ECM, a selective group of membrane and cytoplasmic proteins are recruited to the adhesion sites (e.g. focal adhesions), where they link the ECM to the actin cytoskeleton and transduce mechanical and chemical signals (Burrige and Chrzanoska-Wodnicka, 1996; Calderwood et al., 2000; Clark and Brugge, 1995; Geiger et al., 2001; Giancotti and Ruoslahti, 1999; Howe et al., 1998; Jockusch et al., 1995; Schwartz et al., 1995). Although the molecular identities of many components of the cell-ECM adhesion structures have been well described (Zamir and Geiger, 2001), how they are recruited to the cell-ECM adhesion sites, which are the primary functional sites of many of these proteins, is much less well understood.

Integrin-linked kinase (ILK) is a cytoplasmic component of cell-ECM adhesion structures that plays critical roles in the coupling of ECM to actin cytoskeleton and signaling complexes (Dedhar et al., 1999; Wu, 1999; Wu and Dedhar, 2001). Structurally, ILK comprises an N-terminal ankyrin (ANK) repeat domain, a C-terminal kinase domain that exhibits significant homology to other protein kinase catalytic domains and a pleckstrin homology (PH)-like motif that partially overlaps with the N-terminal region of the kinase

domain. ILK is capable of interacting with several other focal adhesion proteins including $\beta 1$ integrins (Hannigan et al., 1996), LIM-domain containing protein PINCH (Tu et al., 1999), calponin homology-containing protein CH-ILKBP (Tu et al., 2001) [also known as actopaxin (Nikolopoulos and Turner, 2000) or α -parvin (Olski et al., 2001)], affixin (Yamaji et al., 2001) [also known as β -parvin (Olski et al., 2001)] and paxillin (Nikolopoulos and Turner, 2001). We have previously shown that ILK binds to PINCH and CH-ILKBP simultaneously, resulting in the formation of a PINCH-ILK-CH-ILKBP ternary complex in mammalian cells (Tu et al., 2001). In mammalian systems, inhibition of the PINCH-ILK-CH-ILKBP complex formation with dominant negative inhibitors results in defects in cell shape change, migration, proliferation and ECM deposition (Guo and Wu, 2002; Zhang et al., 2002), indicating a critical role of the PINCH-ILK-CH-ILKBP complex in these processes. The interactions of ILK with PINCH and CH-ILKBP are well conserved during evolution (Mackinnon et al., 2002; Wu and Dedhar, 2001; Zervas and Brown, 2002). In genetic model systems such as *C. elegans*, mutations in any one of the components [*pinch-1/unc-97* (Hobert et al., 1999), *ilk/pat-4* (Mackinnon et al., 2002) 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 the assembly of muscle cell

attachment structures (Gettner et al., 1995; Williams and Waterston, 1994). Thus, the PINCH-ILK-CH-ILKBP complex appears to represent a functionally important and evolutionally conserved cell-ECM adhesion complex that is indispensable for the proper assembly of the actin-integrin adhesion structures.

To better understand the molecular and cellular mechanisms underlying the assembly and regulation of cell-ECM adhesion structures, we have defined the PINCH and ILK sites that mediate the assembly of the PINCH-ILK-CH-ILKBP complex, identified upstream signaling proteins that are involved in the regulation of the ILK ternary complex and investigated the mechanism underlying the localization of PINCH and ILK to cell-ECM adhesion sites.

Materials and Methods

Cells culture, antibodies and other reagents

Mouse C2C12 cells and human 293 embryonal kidney cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Rat kidney glomerular mesangial cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1× insulin-transferrin-selenium-A solution supplement. Mouse monoclonal anti-ILK and anti-CH-ILKBP antibodies and rabbit polyclonal anti-PINCH antibodies were previously described (Guo et al., 2001; Li et al., 1999; Tu et al., 2001). Mouse monoclonal anti-paxillin antibody (clone 349) was purchased from Transduction Laboratories. Polyclonal anti-GFP antibodies were from Clontech. Monoclonal anti-FLAG antibody M2 and agarose beads conjugated with anti-FLAG antibody M2 were from Sigma. Synthetic oligonucleotides were prepared by Life Technologies (Grand Island, NY). Protein kinase C inhibitors calphostin C and chelerythrine chloride were purchased from Calbiochem (CN Biosciences, San Diego, CA). Cytochalasin D was from Sigma. Media for cell culture were from Life Technologies (Grand Island, NY) or Mediatech/Cellgro^R (Herndon, VA).

Site-directed mutagenesis

A QuickChangeTM site-directed mutagenesis system (Stratagene) was used to introduce substitution mutations at positions as specified in each experiment. All point mutations were confirmed by DNA sequencing.

Construction of GFP- or FLAG-tagged PINCH and ILK expression vectors and transfection

DNA fragments encoding wild-type or mutant forms of PINCH or ILK were cloned into the *EcoRI/SalI* sites of the pEGFP-C2 vector (Clontech) or the *EcoRI/XbaI* sites of the pFLAG-CMV-2 vector (Sigma). Mouse C2C12 cells or human 293 cells were transfected with the expression vectors using LipofectAmine PLUS (Life Technologies) (Huang et al., 2000). The expression of GFP- or FLAG-tagged wild-type or mutant forms of PINCH or ILK was confirmed by western blotting.

Immunofluorescence staining

Immunofluorescence staining was performed as described (Zhang et al., 2002). Briefly, cells were plated on fibronectin-coated coverslips, fixed, and stained with mouse monoclonal antibodies as specified in each experiment. The primary mouse monoclonal antibodies were detected with a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody and observed under a fluorescence microscopy.

Cell preparation

To prepare cells that lacked ECM adhesion structures, cells were harvested by trypsinization and washed twice with normal medium. Cells were then resuspended in normal culture medium and maintained in suspension on a rocking platform for 60 minutes and then harvested by centrifugation. The cells were washed once with PBS and the pellets were lysed with 1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 100 mM NaF and protease inhibitors (lysis buffer). To prepare cells that had adhered and spread on fibronectin, cells were plated in fibronectin (10 µg/ml) coated tissue culture dishes for different periods of time. Under the condition used, cells began to spread within 30 minutes and were fully spread after two hours. Cells that adhered and spread on fibronectin were rinsed once with PBS and then lysed on the plates with the lysis buffer. In some experiments, cell monolayers were incubated in medium containing cytochalasin D or protein kinase C inhibitors calphostin C (under light) or chelerythrine chloride for 50 to 60 minutes (as specified in each experiment) prior to lysis with the lysis buffer.

Immunoprecipitation assays

Immunoprecipitation of the PINCH-ILK-CH-ILKBP complex was performed as previously described (Tu et al., 2001; Zhang et al., 2002). Briefly, lysates (500 µg) of cells that were prepared as described above were mixed with 500 µl of hybridoma culture supernatant containing monoclonal anti-CH-ILKBP antibody 1D4. 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 minutes. 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-tagged or FLAG-tagged wild-type or mutant forms of ILK or PINCH, cells expressing the GFP- or FLAG-tagged proteins were lysed as described above. To immunoprecipitate GFP-tagged proteins, cell lysates were mixed with rabbit polyclonal anti-GFP antibodies (Clontech) and the immune complexes were precipitated with UltraLink Immobilized Protein G beads. To immunoprecipitate FLAG-tagged proteins, cell lysates were mixed with agarose beads conjugated with anti-FLAG antibody M2 (Sigma). The immunoprecipitated proteins were released from the beads by boiling in SDS-PAGE sample buffer for 5 min and analyzed by western blotting with antibodies as specified in each experiment.

Results

Defining the PINCH site that mediates the interaction with ILK

We used a three-dimensional structure-based mutagenesis strategy to define the site on PINCH that mediates the interaction with ILK. Based on our previous deletion mutational studies and recently solved solution structure of the PINCH LIM1 domain (Tu et al., 1999; Velyvis et al., 2001; Zhang et al., 2002), we predicted that the ILK binding is mediated by a site containing Gln40, Phe63, Met 65 and Leu66 within the C-terminal region of the LIM1 domain (Fig. 1A). To test this experimentally, we substituted Gln40, which is located at the loop connecting the two antiparallel β-sheets within the second zinc finger of the PINCH LIM1 domain (Fig. 1A) and therefore has a minimal effect on the overall structure of the PINCH LIM1 domain, to Ala. We expressed a GFP-fusion protein containing the PINCH Q40→A point mutant,

and a GFP-fusion protein containing the wild-type PINCH as a control, in mammalian cells by DNA transfection. The expression of GFP-Q40A as well as GFP-PINCH was confirmed by western blotting with anti-GFP antibodies (Fig. 1B, lanes 1,2). To analyze the interaction with ILK, we immunoprecipitated GFP-Q40A and GFP-PINCH, respectively, from lysates of the corresponding transfectants (Fig. 1B, lanes 3,4). As expected, ILK was readily co-immunoprecipitated with GFP-PINCH (Fig. 1C, lane 3). In contrast, no ILK was co-immunoprecipitated with GFP-Q40A

(Fig. 1C, lane 4), indicating that the Q40→A point mutation effectively disrupted the interaction of PINCH with ILK.

The Q40→A point mutation that ablates the ILK binding abolishes the localization of PINCH to focal adhesions

We next tested whether the interaction with ILK is required for the localization of PINCH to focal adhesions. To do this, we stained cells expressing the GFP-tagged ILK-binding defective PINCH point mutant, and those expressing GFP-PINCH as a control, with a monoclonal anti-paxillin antibody (as a marker of focal adhesions). The results showed that, as expected, GFP-PINCH readily localized to focal adhesions (Fig. 1D,E). By contrast, the ILK-binding defective Q40→A point mutant failed to localize to focal adhesions where abundant paxillin was detected (Fig. 1F,G), suggesting that the ILK-binding is required for the localization of PINCH to focal adhesions.

Defining the ILK site that mediates the interaction with PINCH

We have previously shown that the PINCH-binding site is located within the ILK N-terminal ankyrin (ANK) repeat domain (Li et al., 1999; Tu et al., 1999). Although the details of the three-dimensional structure of the ILK ANK domain remain to be determined, sequence homology indicates that it likely adopts a fold similar to other known ANK repeat protein structures, which are cupped-handed consisting of an antiparallel beta-hairpin (fingers) and alpha-helix bundles (palm) that are perpendicular to the plane of the beta-hairpin (Venkataramani et al., 1998). Our previous deletion data has shown that the first ANK repeat is important for ILK-PINCH interaction (Li et al., 1999). Because previous structural studies of ANK repeat protein complexes suggested that ANK repeats may use their hairpin regions to interact with the target proteins (Batchelor et al., 1998; Gorina and Pavletich, 1996; Huxford et al., 1998; Jacobs and Harrison, 1998), we investigated if this occurs to ILK by substituting Asp31, which is located at the perspective hairpin region of the first ANK repeat, with Ala. We expressed a GFP-fusion protein containing the ILK point mutant (GFP-D31A), and a GFP-fusion protein containing the

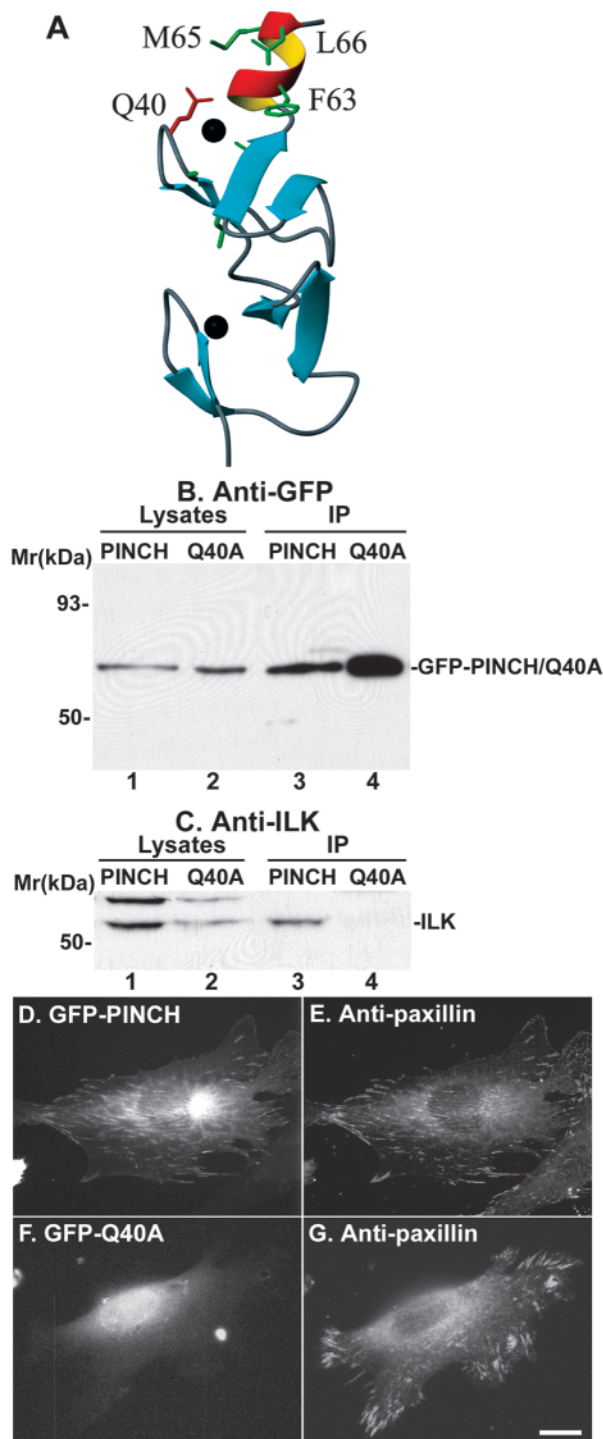


Fig. 1. The Q40→A point mutation within the LIM1 domain abolishes the ILK binding and the localization of PINCH to cell-ECM adhesion sites. (A) Three-dimensional structure of the PINCH LIM1 domain. Amino acid residues that comprise the PINCH binding site are labeled. (B,C) Complex formation with ILK. Lysates of C2C12 cells expressing GFP-tagged wild-type or mutant (Q40→A) form of PINCH were mixed with rabbit anti-GFP antibodies. The GFP-PINCH (lane 3) and GFP-Q40A (lane 4) immunoprecipitates were analyzed by western blotting with HRP-conjugated anti-GFP antibodies (B) or mouse monoclonal anti-ILK antibody 65.1 and HRP-conjugated anti-mouse IgG antibodies (C). Lanes 1-2 were loaded with cell lysates (13 µg/lane) as indicated in the figure. (D-G) Subcellular localization. C2C12 cells transfected with expression vectors encoding GFP-Q40A (D,E) or GFP-PINCH (F,G) were plated on fibronectin-coated coverslips and stained with a mouse monoclonal anti-paxillin antibody (as a marker of focal adhesions) and a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. GFP-Q40A, GFP-PINCH and paxillin were visualized under a fluorescence microscope equipped with GFP (D,F) and rhodamine (E,G) filters. The experiments were performed three times and similar results were obtained. Bar, 10 µm.

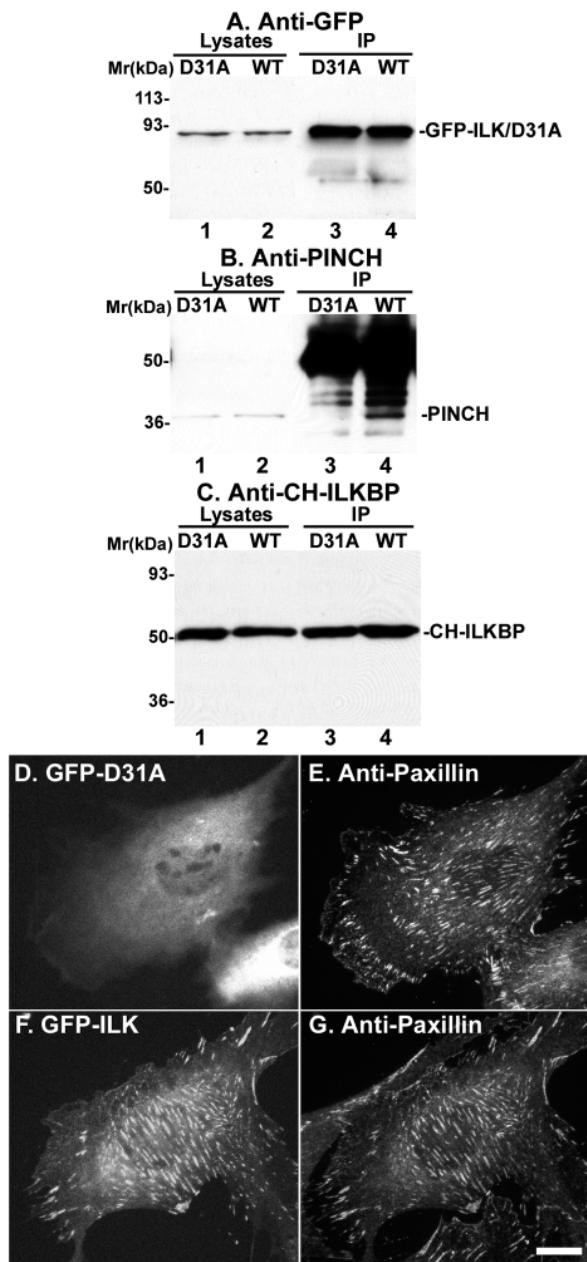


Fig. 2. The D31→A point mutation disrupts the interaction with PINCH and impairs ILK localization to focal adhesions. (A-C) PINCH binding. Lysates of C2C12 cells expressing GFP-tagged wild-type or mutant (D31→A) form of ILK were mixed with rabbit anti-GFP antibodies. The GFP-D31A (lane 3) and GFP-ILK (lane 4) immunoprecipitates were analyzed by western blotting with HRP-conjugated anti-GFP antibodies (A), rabbit anti-PINCH antibodies and HRP-conjugated anti-rabbit IgG antibodies (B), or mouse monoclonal anti-CH-ILKBP antibody 3B5 and HRP-conjugated anti-mouse IgG antibodies (C). Lanes 1 and 2 were loaded with cell lysates (10 µg/lane) as indicated in the figure. (D,G) Subcellular localization. C2C12 cells transfected with expression vectors encoding GFP-D31A (D,E) or GFP-ILK (F,G) were plated on fibronectin-coated coverslips and stained with a mouse monoclonal anti-paxillin antibody and a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. GFP-D31A, GFP-ILK and paxillin were visualized under a fluorescence microscope equipped with GFP (D,F) and rhodamine (E,G) filters. The experiments were performed twice and similar results were obtained. Bar, 10 µm.

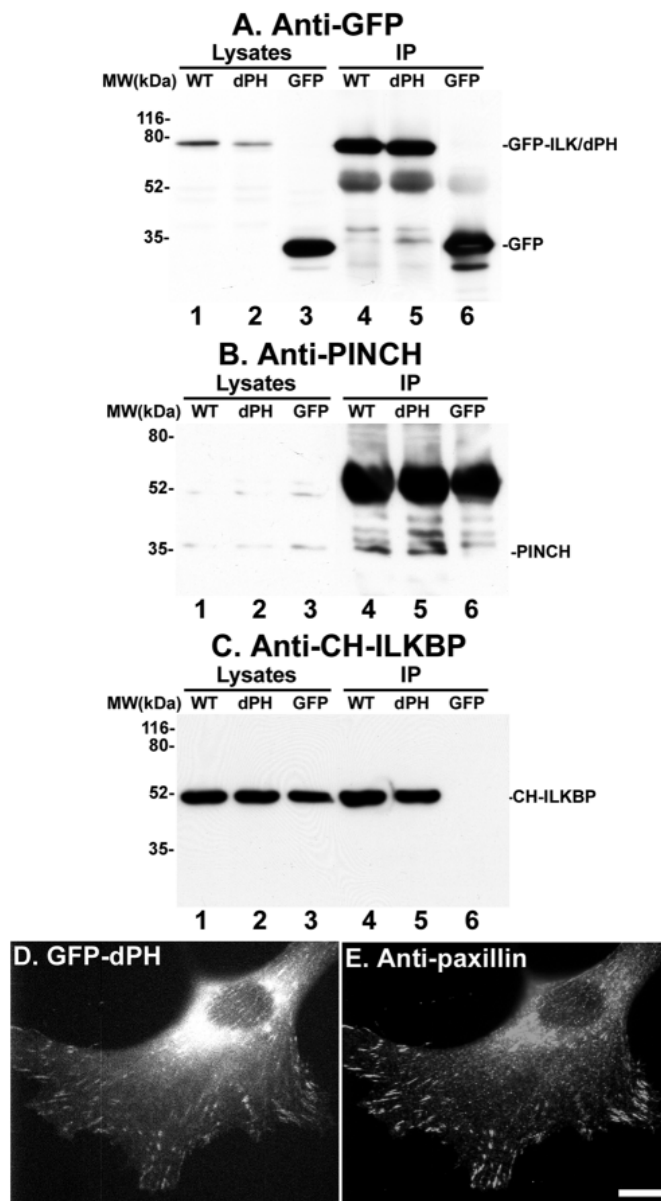


Fig. 3. The PH-like motif is not required for the ILK complex formation and localization to focal adhesions. (A-C) ILK complex formation. Lysates of C2C12 cells expressing GFP-ILK, GFP-tagged ILK mutant in which residues 180-190 within the PH-like motif were deleted (dPH) or GFP alone were mixed with rabbit anti-GFP antibodies. The GFP-ILK (lane 4), GFP-dPH (lane 5) and GFP (lane 6) immunoprecipitates were analyzed by western blotting with HRP-conjugated anti-GFP antibodies (A), rabbit anti-PINCH antibodies and HRP-conjugated anti-rabbit IgG antibodies (B), or mouse monoclonal anti-CH-ILKBP antibody 3B5 and HRP-conjugated anti-mouse IgG antibodies (C). Lanes 1-3 were loaded with cell lysates (15 µg/lane) as indicated in the figure. (D,E) Subcellular localization. C2C12 cells expressing GFP-dPH were plated on fibronectin-coated coverslips and stained with a mouse monoclonal anti-paxillin antibody and a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. GFP-dPH and paxillin were visualized under a fluorescence microscope equipped with GFP (D) and rhodamine (E) filters. The experiments were performed twice and similar results were obtained. Bar, 10 µm.

wild-type ILK as a control, in mammalian cells. The expression of GFP-D31A and GFP-ILK was confirmed by western blotting analyses of the cells (Fig. 2A, lanes 1,2). To test the PINCH-binding, we immunoprecipitated GFP-D31A (Fig. 2A, lane 3) and GFP-ILK (Fig. 2A, lane 4), respectively, from the cell lysates. Western blotting analyses of the immunoprecipitates with anti-PINCH antibodies showed that PINCH was co-immunoprecipitated with GFP-ILK (Fig. 2B, lane 4) but not with GFP-D31A (Fig. 2B, lane 3), suggesting that residue D31 is required for the interaction with PINCH. Analyses of the same immunoprecipitates with monoclonal anti-CH-ILKBP antibody 3B5 showed that, as expected, CH-ILKBP was co-immunoprecipitated with GFP-D31A (Fig. 2C, lane 3) as well as with GFP-ILK (Fig. 2C, lane 4).

The D31A point mutation that ablates the PINCH binding abolishes the localization of ILK to focal adhesions

To test whether the PINCH binding is required for the localization of ILK to cell-ECM adhesion sites, we stained the cells expressing GFP-D31A and those expressing GFP-ILK with a monoclonal anti-paxillin antibody. The GFP-tagged PINCH-binding defective ILK point mutant (Fig. 2D,E), unlike GFP-ILK (Fig. 2F,G), was unable to localize to focal adhesions. To further analyze this, we substituted Lys43, which is located twelve residues C-terminal of D31 and is predicted to be in the helix region, with Ala. The K43A point mutant, unlike D31A point mutant, readily interacted with PINCH and localized to focal adhesions (data not shown). Taken together, these results suggest that the interaction with PINCH, which involves D31, is required for the localization of ILK to cell-ECM adhesion sites.

The PH-like motif is not required for the localization of ILK to focal adhesions

ILK contains a PH-like motif (residues 180-212) that is C-

terminal to the ANK domain and partially overlaps with the N-terminal region of the C-terminal kinase domain. To test whether the PH-like motif is required for the localization of ILK to cell-ECM adhesion sites, we deleted GTLNKHSIDFK (residues 180-190) from the PH-like motif. Residues 180-190 were chosen because (1) they contain the consensus sequence GXLXX-GXXXX and therefore are an essential part of the PH-like motif and (2) they do not overlap with the C-terminal kinase domain and therefore unlikely interfere with the binding activities mediated by the C-terminal domain. We expressed a GFP-fusion protein containing the ILK deletion mutant (GFP-dPH) (Fig. 3, lane 2), GFP-ILK (Fig. 3, lane 1) as a positive control and GFP (Fig. 3, lane 3) as a negative control in mammalian cells. Co-immunoprecipitation experiments showed that GFP-dPH (lane 5), like GFP-ILK (lane 4) but unlike GFP (lane 6), bound to both PINCH (Fig. 3B) and CH-ILKBP (Fig. 3C). Consistent with the binding activities towards PINCH and CH-ILKBP, analyses of cells expressing GFP-dPH shows that it was recruited to focal adhesions (Fig. 3D,E). Thus, the PH-like motif is neither required for the assembly of the PINCH-ILK-CH-ILKBP complex nor required for the localization of ILK to focal adhesions.

Disruption of the interaction with CH-ILKBP impairs the localization of ILK to focal adhesions

To assess whether the interaction with CH-ILKBP is required for the localization of ILK to focal adhesions, we extended the region of deletion mutation into the CH-ILKBP-binding C-terminal domain of ILK. To do this, we expressed in mammalian cells a GFP fusion protein containing an ILK mutant (D215) in which residues 180-215, which include residues within the N-terminal region of the C-terminal domain, were deleted. Co-immunoprecipitation analyses showed that deletion of residues 180-215, unlike deletion of residues within the PH-like motif only (Fig. 3C, lane 5), disrupted the interaction with CH-ILKBP

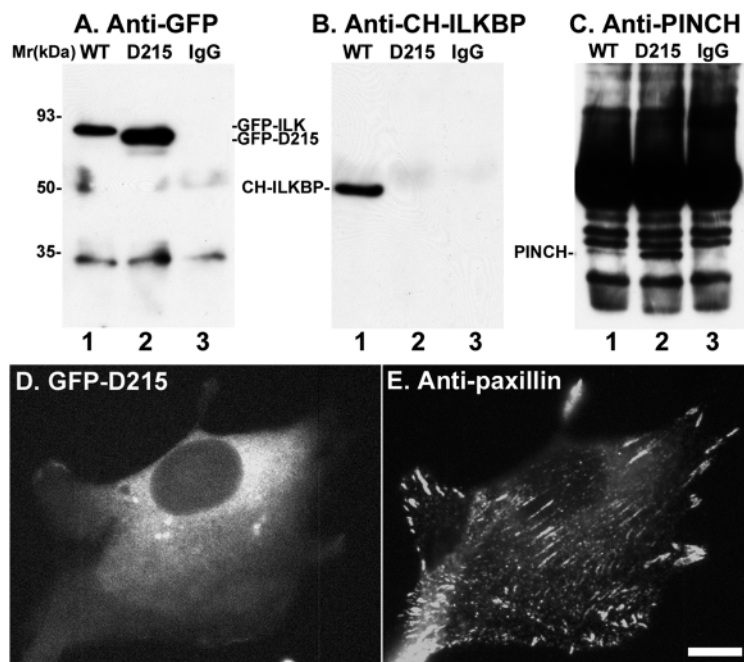


Fig. 4. Disruption of the interaction with CH-ILKBP inhibits the localization of ILK to focal adhesions. (A-C) Co-immunoprecipitation. The immunoprecipitates were prepared from C2C12 cells expressing GFP-ILK or GFP-tagged ILK mutant in which residues 180-215 were deleted (D215) with anti-GFP antibodies as described in Fig. 3. The GFP-ILK (lane 1) and GFP-D215 (lane 2) immunoprecipitates were analyzed by western blotting with HRP-conjugated anti-GFP antibodies (A), mouse monoclonal anti-CH-ILKBP antibody 3B5 and HRP-conjugated anti-mouse IgG antibodies (B), or rabbit anti-PINCH antibodies and HRP-conjugated anti-rabbit IgG antibodies (C). The sample loaded on lane 3 was prepared as those of lanes 1 and 2 except that the cell lysates were omitted (to show bands derived from rabbit IgG, which were strong when HRP-conjugated anti-rabbit IgG antibodies were used, such as in panel C). (D,E) Subcellular localization. C2C12 cells expressing GFP-D215 were plated on fibronectin-coated coverslips and stained with a mouse monoclonal anti-paxillin antibody and a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. GFP-D215 and paxillin were visualized under a fluorescence microscope equipped with GFP (D) and rhodamine (E) filters. The experiments were performed twice and similar results were obtained. Bar, 10 μ m.

(Fig. 4A,B, lane 2). GFP-D215 (Fig. 4C, lane 2), like GFP-dPH (Fig. 3B, lane 5), bound to PINCH. As expected, GFP-ILK (lane 1), but not GFP (lane 3), bound to both CH-ILKBP (Fig. 4B) and PINCH (Fig. 4C). Importantly, the CH-ILKBP binding defective GFP-D215 (Fig. 4D,E), unlike the CH-ILKBP binding GFP-dPH (Fig. 3D,E), failed to localized to focal adhesions, suggesting that the localization of ILK to focal adhesions requires not only the interaction with PINCH but also the interaction with CH-ILKBP.

The formation of the PINCH-ILK-CH-ILKBP complex precedes integrin-mediated cell adhesion and spreading
The foregoing experiments, together with our previous

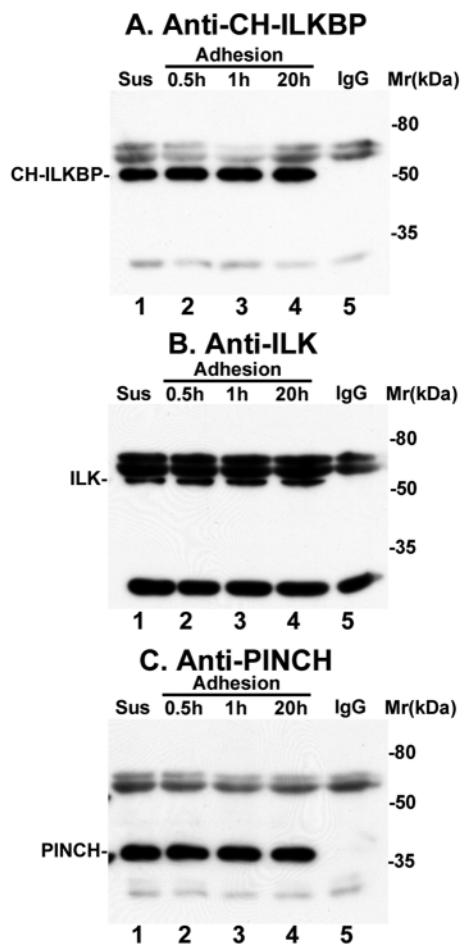


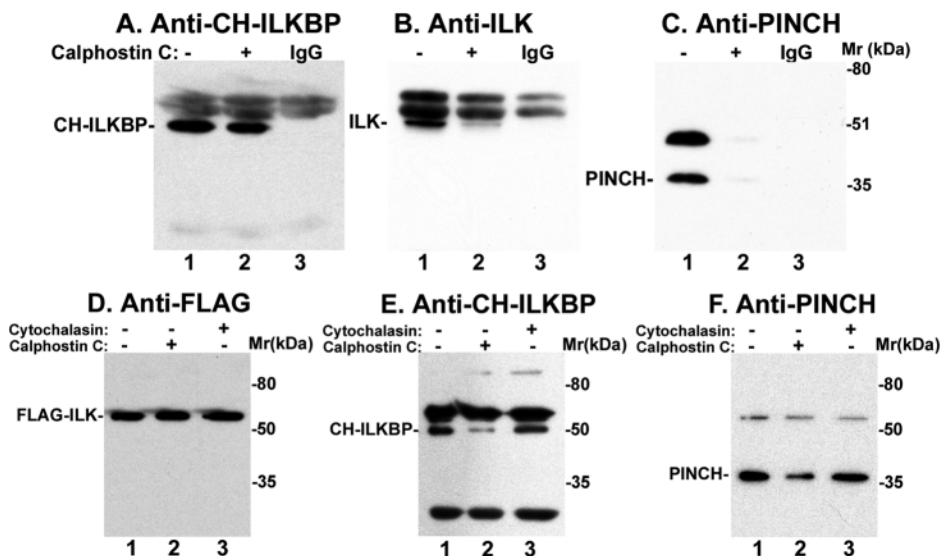
Fig. 5. The formation of the PINCH-ILK-CH-ILKBP complex precedes cell adhesion and spreading. CH-ILKBP was immunoprecipitated from C2C12 cells that were maintained in suspension (lane 1) or had adhered and spread on fibronectin for 0.5 hour (lane 2), 1 hour (lane 3) or 20 hours (lane 4) with monoclonal anti-CH-ILKBP antibody 1D4. The immunoprecipitates were analyzed by western blotting with mouse monoclonal anti-CH-ILKBP antibody 3B5 (A), mouse monoclonal anti-ILK antibody 65.1 (B) and rabbit anti-PINCH antibodies (C), respectively. Lane 5 was loaded with a sample that was prepared as those of lanes 1 and 2 except that the cell lysates were omitted. Note that PINCH and ILK formed a complex with CH-ILKBP before cells were in contact with fibronectin (lane 1). The experiments were performed three times and similar results were obtained.

observation that the interaction with ILK is required for the localization of CH-ILKBP to cell-ECM adhesion sites (Tu et al., 2001), suggest that PINCH, ILK and CH-ILKBP are interdependent in their localization to cell-ECM adhesion sites. This predicts that the formation of the PINCH-ILK-CH-ILKBP complex does not require the presence of cell-ECM contacts. To test this, we immunoprecipitated CH-ILKBP from cells that were maintained in suspension (so that they lacked any cell-ECM contacts) with monoclonal anti-CH-ILKBP antibody 1D4 (Fig. 5A, lane 1). In parallel experiments, CH-ILKBP was immunoprecipitated from cells that had adhered and spread on fibronectin-coated surface for different periods of time (Fig. 5A, lanes 2-4). Analyses of the CH-ILKBP immunoprecipitates with anti-ILK (Fig. 5B) and anti-PINCH (Fig. 5C) antibodies showed that PINCH, ILK and CH-ILKBP formed a complex in cells that lacked any cell-ECM contacts (lane 1) as well as in cells that were plated for a relatively short period of time (and therefore contained nascent focal complexes) (lane 2) or well-spread (and therefore contained mature focal adhesions and ECM contacts) (lane 4). Thus, consistent with the interdependence between PINCH, ILK and CH-ILKBP in their localization to cell-ECM adhesion sites, the formation of the PINCH-ILK-CH-ILKBP complex precedes integrin-mediated cell adhesion and spreading.

Inhibition of protein kinase C, but not that of actin polymerization, inhibits the PINCH-ILK-CH-ILKBP complex formation

It has been well described that protein kinase C plays an important role in the cellular control of assembly of cell-ECM adhesion structures (Berrier et al., 2000; Chun and Jacobson, 1993; Defilippi et al., 1997; Lewis et al., 1996; Woods and Couchman, 1992) and the protein kinase C mediated-regulation precedes integrin-mediated cell spreading (Vuori and Ruoslahti, 1993). Because the formation of the PINCH-ILK-CH-ILKBP complex is critical to cell spreading (Zhang et al., 2002) and it occurs prior to the formation of cell-ECM adhesion structures (Fig. 5), we tested whether protein kinase C is involved in the regulation of the PINCH-ILK-CH-ILKBP complex formation. Cells were treated with calphostin C, a specific protein kinase C inhibitor, and the formation of the PINCH-ILK-CH-ILKBP complex was analyzed (Fig. 6). The results showed that inhibition of protein kinase C significantly reduced the amount of ILK (Fig. 6B) and PINCH (Fig. 6C) that were in complex with CH-ILKBP (Fig. 6A). Treatment of cells with chelerythrine chloride, another inhibitor of protein kinase C, resulted in a similar reduction of the PINCH-ILK-CH-ILKBP complex (data not shown). To determine whether protein kinase C regulates the ILK ternary complex formation by regulating the ILK-CH-ILKBP interaction, the PINCH-ILK interaction or both, we transfected human 293 cells with an expression vector encoding FLAG-tagged ILK. FLAG-ILK was immunoprecipitated with a monoclonal anti-FLAG antibody (Fig. 6D, lane 1). Analyses of the FLAG-ILK immunoprecipitates with anti-CH-ILKBP and anti-PINCH antibodies showed that both CH-ILKBP (Fig. 6E, lane 1) and PINCH (Fig. 6F, lane 1) were co-immunoprecipitated with FLAG-ILK. Treatment of the cells with calphostin C significantly reduced the amount of CH-ILKBP (Fig. 6E, lane 2) and PINCH (Fig. 6F, lane 2) that were in complex with

Fig. 6. Protein kinase C regulates the formation of the PINCH-ILK-CH-ILKBP complex. (A-C) CH-ILKBP was immunoprecipitated from lysate of human mesangial cells that were treated with calphostin C (1 μ M) for 50 minutes (lane 2), or that of untreated human mesangial cells as a control (lane 1) with monoclonal anti-CH-ILKBP antibody 1D4. The immunoprecipitates were analyzed by western blotting with mouse monoclonal anti-CH-ILKBP antibody 3B5 (A), mouse monoclonal anti-ILK antibody 65.1 (B) and rabbit polyclonal anti-PINCH antibodies (C), respectively. The sample loaded on lane 3 was prepared as those of lanes 1 and 2, except that the cell lysates were omitted (to show mouse IgG bands, which were apparent in A and B). Note that treatment of cells with protein kinase C inhibitor calphostin C significantly reduced the amounts of ILK and PINCH associated with CH-ILKBP (compare lanes 1 and 2). Similar results have been obtained with other cell types including human IMR-90 fibroblasts and mouse C2C12 myoblasts (not shown in the figure). (D-F) Human 293 embryonal kidney cells expressing FLAG-ILK was incubated in normal medium (lane 1) or medium containing 0.2 μ M calphostin C (lane 2) or 0.4 μ M cytochalasin D for 60 minutes. FLAG-ILK was immunoprecipitated from the cell lysates with monoclonal anti-FLAG antibody M2 as described in Materials and Methods. The immunoprecipitates were analyzed by western blotting with mouse monoclonal anti-FLAG antibody M2 (D), mouse monoclonal anti-CH-ILKBP antibody 3B5 (E) and rabbit polyclonal anti-PINCH antibodies (F), respectively. Note that calphostin C (lane 2), but not cytochalasin D (lane 3), inhibited the interactions of FLAG-ILK with CH-ILKBP (E) and PINCH (F). The experiments were performed four times and similar results were obtained.



FLAG-ILK (Fig. 6D, lane 2), suggesting that protein kinase C is involved in the regulation of both the ILK-CH-ILKBP and the PINCH-ILK interactions. In additional experiments, we immunoprecipitated FLAG-PINCH from FLAG-PINCH transfectants that were treated with or without calphostin C and found that much less ILK and CH-ILKBP were co-immunoprecipitated with FLAG-PINCH in the presence of the protein kinase C inhibitor (data not shown). In contrast to the effect of the protein kinase C inhibitors, inhibition of actin polymerization with cytochalasin D did not inhibit the complex formation (Fig. 6D-F, lane 3), indicating that actin polymerization is not required for the formation of the ILK complex.

ILK interactions with PINCH and CH-ILKBP, although necessary, are not sufficient for mediating ILK localization to focal adhesions

To test whether ILK interactions with PINCH and CH-ILKBP are sufficient for mediating ILK localization to focal adhesions or there exist other sites that are critical to this process, we generated an ILK point mutant in which residue Phe438 at ILK C-terminus was substituted with Ala. We expressed a GFP-fusion protein containing the ILK F438A mutant in mammalian cells. GFP-F438A (Fig. 7A, lane 5), like GFP-ILK (Fig. 7A, lane 5), formed a complex with both CH-ILKBP (Fig. 7B, lanes 5,6) and PINCH (Fig. 7C, lanes 5,6). However, although the F438→A point mutation did not inhibit the ILK complex formation with PINCH and CH-ILKBP, it impaired the ability of ILK to localize to focal adhesions (Fig. 7D,E). These results indicate that the interactions of ILK with PINCH

and CH-ILKBP are necessary but not sufficient for mediating ILK localization to focal adhesions.

Discussion

How cells assemble and regulate cell-ECM adhesion complexes are important questions in cell biology. The results described in this paper shed light on the molecular mechanism underlying the assembly, localization and regulation of the PINCH-ILK-CH-ILKBP complex, a key multi-component complex of the cell-ECM adhesion structures. Based on the results described in this paper, we propose a model for the assembly and focal adhesion localization of the PINCH-ILK-CH-ILKBP complex (Fig. 8). In this model, PINCH, ILK and CH-ILKBP form a complex prior to the formation of cell-ECM adhesion structures. Upon cell-ECM adhesion, PINCH, ILK and CH-ILKBP are recruited to the adhesion sites as a complex. The formation of the PINCH-ILK-CH-ILKBP complex allows it to interact with other components of the cell-ECM adhesion structures and thereby promote efficient localization of PINCH, ILK and CH-ILKBP to these sites. This model has several important predictions. First, it predicts that any mutations in PINCH, ILK or CH-ILKBP that disrupt the complex formation will impair the ability of the mutants to localize to cell-ECM adhesion sites. Second, it predicts that while the localization to cell-ECM adhesion sites requires the formation of the PINCH-ILK-CH-ILKBP complex, the

complex formation does not require the presence of cell-ECM contacts. Third, it predicts that there exist additional interactions mediated by other sites on PINCH, ILK and CH-ILKBP that are required for the localization to cell-ECM adhesion sites. The results described in this paper are highly consistent with these predictions and together, they provide strong experimental evidence supporting this model.

What are the other interactions that are involved in the

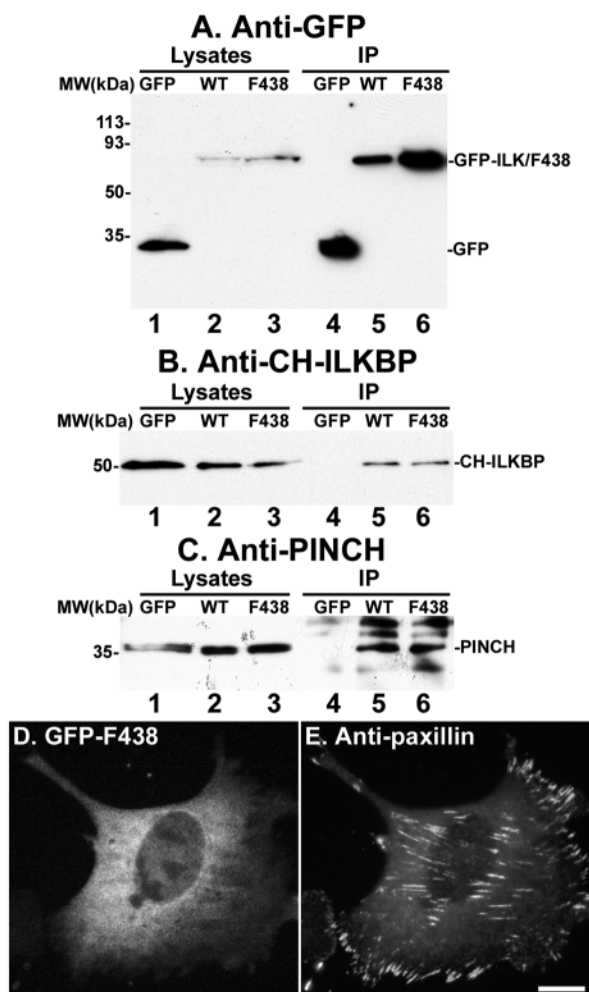


Fig. 7. The F438→A point mutation does not inhibit the interactions with PINCH and CH-ILKBP but impairs the localization of ILK to focal adhesions. (A-C) ILK complex formation. Lysates of C2C12 cells expressing GFP, GFP-ILK or GFP-ILK F438A point mutant were mixed with rabbit anti-GFP antibodies. The GFP (lane 4), GFP-ILK (lane 5), GFP-F438A (lane 6) immunoprecipitates were analyzed by western blotting with HRP-conjugated anti-GFP antibodies (A), mouse monoclonal anti-CH-ILKBP antibody 3B5 and HRP-conjugated anti-mouse IgG antibodies (B), or rabbit anti-PINCH antibodies and HRP-conjugated anti-rabbit IgG antibodies (C). Lanes 1-3 were loaded with cell lysates (10 µg/lane) as indicated in the figure. (D,E) Subcellular localization. C2C12 cells expressing GFP-F438A were plated on fibronectin coated coverslips and stained with a mouse monoclonal anti-paxillin antibody and a Rhodamine Red^{TX}-conjugated anti-mouse IgG antibody. GFP-F438A and paxillin were visualized under a fluorescence microscope equipped with GFP (D) and rhodamine (E) filters. The data shown are representative of three experiments. Bar, 10 µm.

localization of the PINCH-ILK-CH-ILKBP complex to cell-ECM adhesion sites? Our finding that a point mutation at the ILK C-terminus (F438→A) does not disrupt the ILK complex formation with PINCH and CH-ILKBP but impairs ILK localization to cell-ECM adhesion sites suggests that this site likely mediates one of the other interactions that are involved in this process. In addition to interacting with PINCH and CH-ILKBP, ILK is capable of interacting with several other focal adhesion proteins including the $\beta 1$ integrins (Hannigan et al., 1996), affixin/ β -parvin (Olski et al., 2001; Yamaji et al., 2001) and paxillin (Nikolopoulos and Turner, 2001). These proteins are strong candidates for recruiting the PINCH-ILK-CH-ILKBP complex to cell-ECM adhesion sites. In addition, there likely exist other proteins that participate in this process. Mackinnon et al., recently showed that in *C. elegans*, PAT-4/ILK requires UNC-112, a newly identified FERM domain containing ILK-binding protein (Rogalski et al., 2000), to be properly recruited to muscle attachments (Mackinnon et al., 2002). It will be interesting to test in future studies whether ILK interacts with Mig-2, a mammalian homologue of *C. elegans* UNC-112 (Rogalski et al., 2000), and if it does, whether it involves the F438 site.

It has been well established that protein kinase C plays important roles in the regulation of cell spreading, migration, proliferation and fibronectin matrix assembly (Berrier et al., 2000; Chun and Jacobson, 1993; Defilippi et al., 1997; Huang et al., 1998; Lewis et al., 1996; Miranti et al., 1999; Schlaepfer and Hunter, 1998; Schwartz et al., 1995; Somers and Mosher, 1993; Woods and Couchman, 1992). The finding that inhibition of protein kinase C down-regulates the PINCH-ILK-CH-ILKBP complex formation (Fig. 6), together with our previous findings that disruption of the PINCH-ILK-CH-ILKBP complex formation inhibits cell spreading, migration, proliferation and fibronectin matrix assembly (Guo and Wu, 2002; Zhang et al., 2002), suggest that the PINCH-ILK-CH-ILKBP complex likely serves as an important downstream effector in protein kinase C-mediated regulation of cell spreading, migration, proliferation and fibronectin matrix

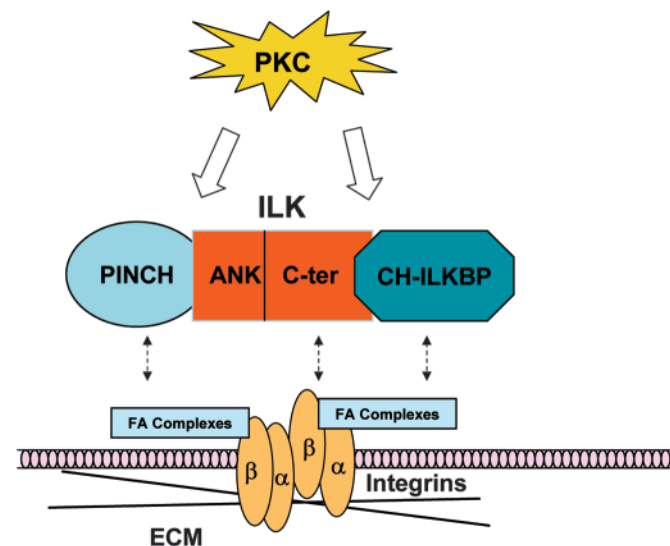


Fig. 8. A model of the assembly and focal adhesion localization of the PINCH-ILK-CH-ILKBP complex. See text for further details.

assembly. It is interesting to note that cytochalasin D, which inhibits cell spreading, migration and fibronectin matrix assembly by inhibition of actin polymerization, does not inhibit the PINCH-ILK-CH-ILKBP complex formation (Fig. 6). Thus, the formation of the PINCH-ILK-CH-ILKBP complex does not require actin polymerization, which is consistent with our observation that the formation of the PINCH-ILK-CH-ILKBP complex precedes cell adhesion and spreading. These results, together with the well documented effects of protein kinase C on actin cytoskeleton organization and recent findings that the PINCH-ILK-CH-ILKBP complex couples integrins to the actin cytoskeleton (Mackinnon et al., 2002; Tu et al., 2001; Wu, 2001; Wu and Dedhar, 2001; Zervas and Brown, 2002; Zervas et al., 2001), suggest a new pathway (Fig. 8) in which protein kinase C regulates cell adhesion and actin cytoskeleton organization by, at least in part, modulating the assembly of the PINCH-ILK-CH-ILKBP complex.

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