Integrin-linked kinase (ILK) is a ubiquitously expressed protein serine/threonine kinase that has been implicated in integrin-, growth factor- and Wnt-signaling pathways. In this study, we show that ILK is a constituent of cell-matrix focal adhesions. ILK was recruited to focal adhesions in all types of cells examined upon adhesion to a variety of extracellular matrix proteins. By contrast, ILK was absent in E-cadherin-mediated cell-cell adherens junctions. In previous studies, we have identified PINCH, a protein consisting of five LIM domains, as an ILK binding protein. We demonstrate in this study that the ILK-PINCH interaction requires the N-terminal-most ANK repeat (ANK1) of ILK and one (the C-terminal) of the two zinc-binding modules within the LIM1 domain of PINCH. The ILK ANK repeats domain, which is capable of interacting with PINCH in vitro, could also form a complex with PINCH in vivo. However, the efficiency of the complex formation or the stability of the complex was markedly reduced in the absence of the C-terminal domain of ILK. The PINCH binding defective ANK1 deletion ILK mutant, unlike the wild-type ILK, was unable to localize and cluster in focal adhesions, suggesting that the interaction with PINCH is necessary for focal adhesion localization and clustering of ILK. The N-terminal ANK repeats domain, however, is not sufficient for mediating focal adhesion localization of ILK, as an ILK mutant containing the ANK repeats domain but lacking the C-terminal integrin binding site failed to localize in focal adhesions. These results suggest that focal adhesions are a major subcellular compartment where ILK functions in intracellular signal transduction, and provide important evidence for a critical role of PINCH and integrins in regulating ILK cellular function.

Key words: ILK, PINCH, Focal adhesion, Integrin, β-Catenin

INTRODUCTION

Cell adhesion to extracellular matrix triggers signals leading to gene expression, proliferation, migration and morphological changes. Focal adhesions are specialized cell adhesion sites through which are transduced from extracellular matrix into intracellular compartment (for review see Burridge and Chrzanowska-Wodnicka, 1996; Jockusch et al., 1995). Upon ligand activation, transmembrane receptors for extracellular matrix such as integrins become clustered in focal adhesions that are physically connected to cytoskeleton through stable (such as actin stress fibers) and transient (such as microtubules) interactions. In addition to non-catalytic proteins such as integrins, talin, vinculin, paxillin and zyxin, protein kinases are also recruited into focal adhesions in response to cell adhesion, which leads to increased protein phosphorylation of focal adhesion components. Several protein tyrosine kinases, among which the best described are FAK and Src (Ilic et al., 1997; Parsons and Parsons, 1997; Schwartz et al., 1995), are localized in focal adhesions. In addition to tyrosine phosphorylation, serine-phosphorylation of many focal adhesion components including paxillin (Bellis et al., 1997; De Nichilo and Yamada, 1996) and FRNK (FAK-related, non-kinase) (Richardson et al., 1997) are also up-regulated upon integrin stimulation.

Integrin-linked kinase (ILK) is a ubiquitously expressed protein serine/threonine kinase that was initially identified from a yeast two-hybrid screen based on its interaction with the β1 integrin cytoplasmic domain (Hannigan et al., 1996; Dedhar and Hannigan, 1996). ILK comprises three structurally well conserved domains (Hannigan et al., 1996; Li et al., 1997; Delcommenne et al., 1998; Tu et al., 1999; Huang and Wu, 1999). The C-terminal domain contains a protein kinase catalytic site. In addition, it includes a binding site for the integrin β1 cytoplasmic domain (Hannigan et al., 1996). N-terminal to the kinase domain is a PH-like domain that likely binds PtdIns(3,4,5)P3 and participates in the regulation of the kinase activity (Delcommenne et al., 1998). The N-terminal-most domain comprises primarily four ANK repeats, which are
embryonal kidney epithelial cells and mouse C2C12 muscle myoblasts. Mouse embryo NIH3T3 cells were kindly provided by Drs Louise T. Couchman and Anne Woods (University of Alabama at Birmingham).

Cells, cDNAs and other reagents

Over-expression of ILK in integrin function, ILK has been implicated in Wnt- and growth factor-signaling pathways. Over-expression of ILK in epithelial cells activated the LIF-1/β-catenin signaling pathway (Novak et al., 1998), and inhibited E-cadherin expression (Novak et al., 1998; Wu et al., 1998). Additionally, treatment of cells with insulin transiently stimulated kinase activity of ILK through a phosphoinositide-3-OH kinase-dependent mechanism (Delcommenne et al., 1998). Finally, ILK appears to be able to directly phosphorylate PKB/AKT on serine-473, one of the two phosphorylation sites involved in the activation of PKB/AKT. ILK is also capable of directly phosphorylating GSK-3 and regulates GSK-3 kinase activity (Delcommenne et al., 1998). These studies suggest that ILK plays critical roles in cellular regulation of cell survival and proliferation, and therefore may be involved in oncogenic transformation. Indeed, overexpression of ILK in epithelial cells rendered the cells the ability to form tumor in vivo (Wu et al., 1998). Studies using transgenic mice have shown that ILK expression is regulated by erbB-2 (Xie et al., 1998), a member of the EGF receptor family. Moreover, examination of human tumor tissues has revealed that ILK is consistently overexpressed in Ewing’s sarcoma and primitive neuroectodermal tumor and thus is a marker of these tumors (Chung et al., 1998).

Although it is increasingly clear that ILK plays an important role in intracellular signal transduction triggered by a variety of stimuli, the molecular and cellular mechanisms by which ILK functions are not clear. An important question is whether ILK is a constituent of focal adhesions. Previous studies using polyclonal anti-ILK antibodies suggested that ILK is present in some integrin containing focal adhesion plaques (Hannigan et al., 1996). However, the extent to which ILK was detected in focal adhesions was not known. To address this question, we have generated a monoclonal anti-ILK antibody and examined the subcellular localization of ILK in different types of cells upon adhesion to a variety of extracellular matrix proteins. Furthermore, we have investigated the molecular basis underlying the clustering and focal adhesion localization of ILK.

MATERIALS AND METHODS

Cells, cDNAs and other reagents

Rat kidney glomerular mesangial cells and Madin-Darby canine kidney (MDCK) epithelial cells were kindly provided by Drs John Couchman and Anne Woods (University of Alabama at Birmingham). Mouse embryo NIH3T3 cells were kindly provided by Dr Louise T. Chow (University of Alabama at Birmingham). Human 293 embryonal kidney epithelial cells and mouse C2C12 muscle myoblast cells were from American Type Culture Collection (Rockville, MD). Rat IEC-18 intestinal epithelial cells, ILK transfectant ILK13 (clone A1a3), control transfectant ILK 14 (clone A2C3) and a polyclonal anti-human ILK antibody (91-5) were kindly provided by Dr Shoukat Dedhar (Jack Bell Research Center, Vancouver, Canada). Media for cell culture were from Gibco Laboratories (Grand Island, NY) or Mediatech/Cellgro® (Herndon, VA). Fetal bovine serum was from HyClone Laboratories, Inc. (Logan, UT). Mouse ILK cDNA was isolated as previously described (Li et al., 1997). Enactin, laminin and vitronectin were kindly provided by Drs Albert Chung (University of Pittsburgh) and Eric Simon (Tulane University), respectively. Type I collagen coated cover slips and culture slides were purchased from Becton Dickinson Labware (Medford, MA). Rabbit polyclonal anti-α5 integrin antibody was generated as previously described (Roman et al., 1989). Rabbit polyclonal anti-FAK antibody (A-17) was purchased from Santa Cruz Biotechnology, Inc. Mouse monoclonal anti-FLAG antibody M2 conjugated agarose beads (anti-FLAG M2 affinity gel) and monoclonal anti-FLAG antibody M5 were from Kodak. Rabbit polyclonal anti-β-catenin antibody (C2206), anti-cadherin antibody (C36/78) and anti-GST antibody were from Sigma. Restriction enzymes, DNA modifying enzymes, DNA molecular mass markers and dideoxyribonucleotide triphosphates (dNTPs) were purchased from Promega. Synthetic oligonucleotides were prepared by Gibco/BRL.

Generation and characterization of monoclonal anti-ILK antibodies

Mouse monoclonal anti-ILK antibodies were prepared using purified His-tagged full length mouse ILK recombinant protein as an antigen based on a previously described procedure (Tu et al., 1998). Hybridoma supernatants were screened for anti-ILK antibody activity by ELISA and immunoblotting using His-ILK, GST-ILK and GST (as a negative control) recombinant proteins. One monoclonal antibody (clone 65.1, IgG2b) that recognizes His-tagged ILK and GST-ILK but not GST and irrelevant His-tagged proteins was selected.

Immunoblotting

GST fusion proteins and mammalian cell extracts were separated by reducing SDS-polyacrylamide gel electrophoresis, transferred onto an Immobilon-p® membrane and probed with the monoclonal anti-ILK antibody 65.1 (2 μg/ml). The mouse primary antibody was detected with a horseradish peroxidase-conjugated anti-mouse IgG antibody (40 ng/ml) and the SuperSignal® chemiluminescent substrate (Pierce).

Expression and isolation of MBP-, GST- and His-fusion proteins

MBP, MBP-PINCH, GST, GST-ILK, GST-Nck-1 and GST-Nck-2 fusion proteins were generated and purified as previously described (Li et al., 1997; Tu et al., 1998, 1999). To generate GST fusion protein containing ILK ANK repeats, a cDNA encoding ILK amino acid residues 1-189 was inserted into the EcoRI/Xhol site of the pGEX-5x-3 vector (pGEX-ILK/1-189). The recombinant vector pGEX-ILK/1-189 was then used to transform Escherichia coli cells (DH5α). The expression of the GST-ILK fusion protein was induced with IPTG, and the GST-ILK fusion protein was purified by glutathione-Sepharose 4B affinity chromatography. To produce His-tagged fusion proteins containing ILK and various PINCH LIM domains of PINCH, cDNA sequences encoding full length mouse ILK and various human PINCH LIM domains (as specified in each experiment) were amplified by PCR and inserted into the Ndel/V BamHI site of a pET-15b vector (Novagen, Madison, WI). The recombinant vectors were then used to transform Escherichia coli BL21(DE3) cells, and the His-tagged recombinant proteins were purified with His-Bind® Resin (Novagen, Madison, WI) following the manufacturer’s protocol.

Solid phase-based binding assays

To analyze ILK-PINCH and PINCH-Nck-2 interactions, we immobilized His-tagged fusion proteins containing various PINCH or ILK domains (as specified in each experiment) onto surfaces of Reacti-Bind™ metal chelate coated 96-well plates (Pierce). This was
achieved by incubation of the plates with 100 µl/well of 0.1 µM His-tagged proteins for one hour at room temperature (with shaking), followed by washing three times with washing buffer (0.05% Tween-20 in 20 mM Tris-HCl, 150 mM NaCl, pH 8.0). The wells were then incubated with equil volume (100 µl/well) of 0.1 µM GST- or MBP-fusion proteins (as specified in each experiment) for one hour at room temperature. After washing four times with washing buffer, the wells were incubated with a rabbit anti-GST antibody (1 µg/ml, Sigma) or a rabbit anti-MBP antibody (1: 1000 dilution, New England Biolabs, Inc.) at 37°C for one hour to detect bound GST- or MBP-fusion proteins. The wells were then washed four times with washing buffer, and incubated with alkaline-phosphate-conjugated goat anti-rabbit IgG (60 ng/ml, Jackson Immuno Research). After rinsing four times with washing buffer and twice with TBS (20 mM Tris-HCl, 150 mM NaCl, pH 8.0), bound alkaline phosphate conjugate was detected colorimetrically with p-nitrophenyl phosphate at 405 nm using an ELISA microplate reader.

Construction and transfection of wild-type and mutant forms of FLAG-tagged ILK
To generated FLAG-ILK expression vectors, cDNAs encoding the full length ILK, the ANK1-deletion mutant (residues 66-452) and the N-terminal ANK repeats domain (residues 1-230) were inserted into the EcoRI/XbaI and EcoRI/SalI sites, respectively, of mammalian expression vector pFLAG-CMV-2 (Kodak). Chinese hamster ovary (CHO) cells were cultured in α-MEM medium supplemented with 10% FBS. The cells were transfected with the pFLAG-ILK vectors using LipofectAMINE reagent (Life Technologies). Mock transfectants were generated by transfection of the cells with pFLAG-CMV-2 lacking ILK sequence. Expression of FLAG-ILK proteins was confirmed by immunofluorescence staining of the cells and immunoblotting with a mouse monoclonal anti-FLAG antibody (M5). Under the experimental conditions used, the transfection efficiency for the full length ILK, the ANK1-deletion mutant (residues 66-452) and the N-terminal ANK repeats domain (residues 1-230), was approximately 20%, 70% and 10%, respectively.

Co-immunoprecipitation
CHO cells were transfected with the pFLAG-ILK vectors encoding the full length ILK, the ANK1-deletion mutant (residues 66-452), or the N-terminal ANK repeats domain (residues 1-230) as described above. Forty eight hours after transfection, the cells were lysed with 1% (v/v) Triton X-100 in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 10 µM leupeptin, 0.2 mM AEBSP, 1 µg/ml aprotinin and 1 µM pepstatin. The cell lysates (360 µg) were incubated with monoclonal anti-FLAG antibody M2 conjugated agarose beads (anti-FLAG M2 affinity gel, Kodak) (50 µl) at 4°C for three hours. At the end of incubation, the beads were washed four times with PBS containing 1 M NaCl and then mixed with SDS-PAGE reducing sample buffer (35 µl). PINCH and the FLAG-tagged wild-type and mutant forms of ILK co-precipitated with the anti-FLAG antibody M2 conjugated agarose beads were detected by immunoblotting with a polyclonal anti-PINCH antibody and a mouse monoclonal anti-FLAG antibody (M5), respectively.

Immunofluorescence staining
Cells were plated in wells of Lab-Tek 8-chamber culture slides (Nunc, Inc.) or coverslips that were precoated with 20 µg/ml fibronectin or other extracellular matrix proteins as specified in each experiment and incubated in a 37°C incubator under a 5% CO2-95% air atmosphere to allow cells to adhere and spread. Cells were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and stained with mouse monoclonal and rabbit polyclonal antibodies as specified in each experiment. After rinsing, the bound mouse IgG and rabbit IgG were detected with a fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG antibody (minimal cross-reaction with human, bovine, rabbit and swine serum proteins; Jackson ImmunoResearch Laboratories, Inc; 28 mg/ml) and a Rhodamine RedX-conjugated AffiniPure goat anti-rabbit IgG antibody (minimal cross-reaction with human, mouse and rat serum proteins, Jackson ImmunoResearch Laboratories, Inc; 15 µg/ml), respectively. Stained cells were observed under a fluorescence microscope equipped with rhodamine and FITC filters. In control experiments, no cross-reactivity between the mouse monoclonal antibodies and the Rhodamine RedX-conjugated AffiniPure goat anti-rabbit IgG antibody or that between the rabbit polyclonal antibodies and the fluorescein (FITC)-conjugated AffiniPure goat anti-mouse IgG antibody was observed.

To confirm the specificity of ILK immunofluorescence staining, mAb 65.1 (0.03 µM) was incubated with GST-ILK (0.15 µM), or GST-Nck-1 (0.15 µM) as a control, at 37°C for one hour and then used for immunofluorescence staining. The mouse monoclonal antibody was detected with a Cy3-conjugated AffiniPure goat anti-mouse IgG antibody (2.1 µg/ml; Jackson ImmunoResearch Laboratories, Inc).

RESULTS
ILK is a component of cell-matrix focal adhesions in a wide variety of cells
To allow specific detection of ILK proteins in cells, we have generated a monoclonal anti-ILK antibody (clone 65.1, IgG2b). The monoclonal anti-ILK antibody specifically recognized GST-ILK (Fig. 1A, lane 1) and His-tagged ILK (data not shown) but not GST (Fig. 1A, lane 2) or irrelevant His-tagged fusion proteins (data not shown). To test whether mAb 65.1 also recognizes mammalian ILK proteins, we probed lysates from a variety of mammalian cells, including rat kidney mesangial cells, human IMR90 fibroblasts, CHO cells, human embryonal kidney epithelial 293 cells, mouse NIH3T3 fibroblasts, ILK transfectant ILK13 cells (clone A1a3) (Hannigan et al., 1996), control transfectant ILK 14 cells (clone A2C3) (Hannigan et al., 1996) and rat IEC-18 intestinal epithelial cells, with mAb 65.1. The results showed that mAb 65.1 recognized a major protein band with an apparent molecular mass equivalent to that of ILK (59 kDa) in all cell lysates tested (Fig. 1B, lanes 1-8). Moreover, the 59 kDa band detected by mAb 65.1 in the ILK-overexpressing ILK13 transfecants (Fig. 1B, lane 6) is much stronger than that in the ILK14 control transfectants (Fig. 1B, lane 7) or the parental IEC18 cells (Fig. 1B, lane 8). Taken together, these results demonstrate that mAb 65.1 recognizes an epitope present in both bacterially expressed recombinant ILK proteins and mammalian ILK proteins. Interestingly, one or two protein bands with molecular masses higher than 59 kDa, which could represent either SDS-resistant ILK-containing protein complexes or ILK variants, were also detected in several cell lines including ILK13 transfectants (Fig. 1B, lane 6), although little, if any, of the higher molecular mass protein bands were detected in the ILK14 control transfectants (Fig. 1B, lane 7) or the parental IEC18 cells (Fig. 1B, lane 8). Among the cells analyzed, the level of ILK in the epithelial cells (293 and IEC18) is noticeably lower than that in the fibroblasts (IMR90, CHO and NIH3T3) or the smooth muscle-like mesangial cells (Fig. 1B).

To test whether ILK localizes in focal adhesions, we plated rat mesangial cells on fibronectin coated surface and stained them with mAb 65.1. Abundant ILK proteins were detected in
Fig. 1. Immunoblotting analysis of monoclonal anti-ILK antibody. (A) Recombinant ILK protein. GST-ILK (lane 1, 10 ng/lane) and GST (lane 2, 10 ng/lane) were separated by reducing SDS-polyacrylamide gel electrophoresis and immunoblotted with monoclonal anti-ILK antibody 65.1. (B) Mammalian cell extracts. Rat kidney mesangial cells (lane 1), human IMR90 fibroblasts (lane 2), CHO cells (lane 3), human embryonal kidney epithelial 293 cells (lane 4), mouse NIH3T3 fibroblasts (lane 5), ILK transfectant ILK13 (lane 6), control transfectants ILK 14 (lane 7) and rat IEC-18 intestinal epithelial cells (lane 8) were extracted with 1% (w/v) SDS in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 10 μM leupeptin, 0.2 mM AEBSF, 1 μg/ml aprotinin and 1 μM pepstatin. Equal amounts of the SDS cell extracts (30 μg of protein/lane) were separated by reducing SDS-polyacrylamide gel electrophoresis and immunoblotted with monoclonal anti-ILK antibody 65.1.

focal adhesions (Fig. 2A,B,D). Co-staining of the cells with phalloidin (Fig. 2C) or an anti-α5β1 integrin antibody (Fig. 2E) demonstrated that ILK was concentrated at the ends of actin filament bundles (Fig. 2B and C) and co-clustered with α5β1 integrins (Fig. 2D and E). In addition to prominent focal adhesion localization, variable amount of ILK was also detected in the cytoplasm (Fig. 2A,B,D,G). To confirm the specificity of the ILK staining, mAb 65.1 was incubated with GST-ILK, or GST-Nck-1 as a control, and then applied to the cells. Incubation of mAb 65.1 with GST-ILK (Fig. 2F), but not GST-Nck-1 (Fig. 2G), eliminated the mAb 65.1 immunofluorescence staining. Taken together, these results suggest that ILK is a constituent of integrin-mediated cell-matrix focal adhesions.

We next analyzed focal adhesion localization of ILK in cells that were plated on different extracellular matrix proteins including laminin, vitronectin, type I collagen and entactin. Rat mesangial cells adhered well to laminin, vitronectin, type I collagen, entactin and fibronectin. Within two hours of plating, extensive spreading occurred in cells plated on laminin, vitronectin, type I collagen, and fibronectin. The cells spread to a much smaller extent on entactin. Staining of the cells with mAb 65.1 showed that ILK was co-localized with FAK in focal adhesions regardless of the particular matrix protein used or the extent of cell spreading (Fig. 3). In addition to primary mesangial cells, we have analyzed subcellular localization of ILK in many established cell lines including human 293 cells, mouse C2C12 cells and NIH 3T3 cells. In all the cell lines analyzed, ILK was readily detected in focal adhesions. We

Fig. 2. Subcellular localization of ILK in primary mesangial cells. Rat kidney mesangial cells were suspended in RPMI-1640 culture medium containing 20% FBS and plated on fibronectin coated Lab-Tek 8-chamber culture slides for 3.5 hours (A-E) or 18 hours (F and G), fixed and analyzed by immunofluorescence staining. (A) Singular staining with mouse anti-ILK monoclonal antibody 65.1. (B and C) Double staining with mouse anti-ILK monoclonal antibody 65.1 (B) and rhodamine-labeled phalloidin (C). (D and E) Double staining with mouse anti-ILK monoclonal antibody 65.1 (D) and a rabbit polyclonal anti-α5 integrin antibody (E). (F and G) Mouse anti-ILK monoclonal antibody 65.1 was incubated with GST-ILK (F) or control protein GST-Nck-1 (G) and then used for immunofluorescence staining of the cells. Bar, 5 μm.
conclude from these studies that ILK is recruited to cell-matrix focal adhesions in response to a variety of extracellular matrix proteins.

**ILK is not localized to epithelial cell-cell adhesions**

Previous studies have shown that overexpression of ILK in epithelial cells decreased cellular level of E-cadherin, promoted nuclear translocation of β-catenin and disrupted cell-cell adhesions (Novak et al., 1998; Wu et al., 1998). This prompted us to examine whether ILK is also localized to epithelial cell-cell adhesions that are mediated by E-cadherin complexes. To do this, we stained rat intestinal epithelial cells that had formed cell-cell contacts with monoclonal anti-ILK antibody 65.1 and a rabbit polyclonal anti-β-catenin antibody as described in Materials and Methods. Bar, 10 μm.

**PINCH amino acid residues 11 to 38 and the first ANK repeat of ILK are required for the ILK-PINCH interaction**

We next sought to investigate the molecular basis underlying the focal adhesion localization of ILK. Our recent studies have identified PINCH, an adaptor protein comprising five LIM domains, as an ILK binding protein (Tu et al., 1999). We have previously mapped a major ILK binding site to the LIM1 (residues 1-70) of PINCH (Tu et al., 1999). LIM domains are cysteine-rich sequences consisting of two distinct zinc-binding subdomains (zinc fingers) which are potentially involved in...
independent protein-protein interactions (Schmeichel and Beckerle, 1997). To facilitate studies aimed at testing whether the PINCH-ILK interaction is involved in the focal adhesion targeting of ILK, we have defined the structural elements required for the ILK-PINCH interaction. Consistent with our previous binding results, a His-tagged fusion protein containing PINCH LIM1-4 domains readily interacted with GST fusion protein containing the ANK repeats of ILK (Fig. 5). Furthermore, His-tagged PINCH fusion proteins in which the amino-terminal 10 residues (including the highly conserved metal-coordinating Cys10 residue located within the N-terminal zinc-binding subdomain) of LIM1 were deleted exhibited significant ILK binding activity (Fig. 5), suggesting that the N-terminal zinc-binding subdomain is not required for the ILK binding. Deletion of the amino-terminal 38 residues of LIM1, which include the highly conserved metal-coordinating Cys38 residue located within the C-terminal zinc-binding subdomain, abolished the ILK binding (Fig. 5). In control experiments, all three forms of PINCH interacted with Nck-2, which is known to interact with the LIM4 domain of PINCH, and none of them interacted with GST (Fig. 5), confirming the specificity of the binding assay. These results demonstrate that PINCH amino acid residues 11 to 38, but not residues 1-10, within the LIM1 domain are required for interaction with ILK.

The N-terminal domain of ILK comprises primarily four ANK repeats. We previously showed in yeast two-hybrid assays that deletion of the two N-terminal ANK repeats (residues 1-95) abrogated the PINCH binding activity. To determine whether the N-terminal-most ANK repeat (ANK1) is required for the PINCH binding, we analyzed the PINCH binding activity of an ILK mutant (ΔANK1) in which ANK1 is deleted in a solid phase based binding assay (Fig. 6A). Unlike the full length ILK, the ANK1 deletion ILK mutant failed to interact with PINCH (Fig. 6A). Thus, ANK1 is required for ILK interaction with PINCH.

**FLAG-tagged ILK is capable of localizing in focal adhesions**

To test whether ecotopically expressed ILK is capable of localizing in focal adhesions, we transfected CHO cells with a pFLAG-ILK expression vector encoding an epitope (FLAG)-tagged ILK and analyzed the transfectants by immunofluorescence staining with a mouse monoclonal anti-FLAG antibody. Under the experimental condition used, approximately 20% of the cells were transfected. Strong immunofluorescence staining was observed in the pFLAG-ILK transfectants (Fig. 6B) but not the mock transfectants (Fig. 6D), although the latter cells could be readily detected with an anti-FAK antibody (Fig. 6E). A large amount of the ecotopically expressed FLAG-tagged ILK proteins was present rather diffusely in the cells. Noticeably, a fraction of the ecotopically expressed FLAG-tagged ILK proteins was recruited into focal adhesions (Fig. 6B) where clusters of FAK were also detected (Fig. 6E). Thus, the FLAG-tagged ILK is capable of localizing in focal adhesions.

**The PINCH binding defective ILK mutant fails to localize in focal adhesions**

We next analyzed the ability of the PINCH binding defective ILK mutant (ΔANK1) to localize in focal adhesions. To do this, we transfected CHO cells with a pFLAG expression vector that encodes the ILK mutant in which ANK1 is deleted (pFLAG-ΔANK1). Immunofluorescence staining with a monoclonal anti-FLAG antibody (M5) showed that a high percentage (approximately 70%) of the cells were transfected under the experimental condition used. The ILK (ΔANK1) transfectants were then analyzed by double immunofluorescence staining with the mouse monoclonal anti-FLAG antibody and a rabbit polyclonal anti-FAK antibody. In contrast to wild-type ILK, clusters of the PINCH binding defective ILK mutant were not detected in focal adhesions (Fig. 6F) where clusters of FAK were detected (Fig. 6G). Thus, ablation of the PINCH-binding activity eliminated the ability of ILK to localize and cluster in focal adhesions, suggesting that the PINCH-ILK interaction is necessary for focal adhesion localization and clustering of ILK.

**The N-terminal ANK repeats domain is not sufficient for mediating focal adhesion localization of ILK**

To test whether the N-terminal ANK repeats domain is sufficient for mediating focal adhesion localization of ILK, we expressed a FLAG-tagged ILK N-terminal fragment containing an intact ANK repeats domain in CHO cells. Immunofluorescence staining with anti-FLAG antibody M5 showed that approximately 10% of the cells were expressing the FLAG-tagged ILK N-terminal fragment. Double staining of the transfectants that were plated on fibronectin with the anti-FLAG and anti-FAK antibodies showed that the ILK N-terminal fragment containing the ANK repeats domain failed
to localize in focal adhesions (Fig. 6H) where clusters of FAK were detected (Fig. 6I). Thus, although the ANK repeats domain is necessary for focal adhesion localization of ILK, it is not sufficient for mediating focal adhesion localization of ILK.

Wild-type ILK protein forms a stable complex with PINCH in mammalian cells (Tu et al., 1999). To test whether the ANK repeats domain of ILK, which is capable of interacting with PINCH in vitro (Fig. 5) and in yeast cells (Tu et al., 1999), could form a complex with PINCH in mammalian cells, we immunoprecipitated the FLAG-tagged wild-type or mutant forms of ILK from lysates (Fig. 7A, lanes 2-4) of CHO cells expressing the different forms of ILK proteins with monoclonal anti-FLAG antibody M2 conjugated agarose beads. Lysates of the mock transfectants (Fig. 7A, lane 1) were used as a negative control in parallel experiments. The presence of the FLAG-tagged wild-type or mutant forms of ILK proteins in the immunoprecipitates from the corresponding ILK transfectants but not the mock transfectants was confirmed by immunoblotting with a monoclonal anti-FLAG antibody (Fig. 7A, lanes 5-8). Immunoblotting analysis of the same immunoprecipitates with an anti-PINCH antibody showed that, as expected, PINCH was readily co-precipitated with the FLAG-tagged wild-type ILK (Fig. 7B, lane 6). By contrast, a much smaller amount of PINCH was co-precipitated with the ANK repeats domain of ILK (Fig. 7B, lane 8) and no PINCH was co-precipitated with the PINCH binding defective ANK1-deletion ILK mutant (Fig. 7B, lane 7) despite the presence of abundant ILK mutants in the immunoprecipitates (Fig. 7A, lanes 7 and 8). In control experiments, no PINCH was detected in the precipitates derived from the mock transfectants (Fig. 7B, lane 5) although similar amount of PINCH was expressed by the mock transfectants (Fig. 7B, lane 1) and the ILK transfectants (Fig. 7B, lanes 2-4). We concluded from these experiments that (1) the ANK repeats domain of ILK is required for the formation protein complexes containing PINCH and ILK in vivo and (2) although the ILK ANK repeats domain alone is capable of associating with PINCH in vivo, the efficiency of the complex formation or the stability of the complex is markedly reduced in the absence of the C-terminal domain of ILK.

**Fig. 6.** The PINCH-binding ANK repeats domain is necessary but not sufficient for focal adhesion localization of ILK. (A) Deletion of the first ANK repeat of ILK eliminates the interaction with PINCH. The binding of MBP-PINCH and MBP (as a negative control) to His-tagged fusion proteins containing full length ILK or a mutant form of ILK (residues 66-452) in which the first ANK repeat is deleted was determined using a solid phase based assay as described in Materials and Methods. The numbers in parentheses denote ILK amino acid residues. (B-I) Subcellular localization of wild-type and mutant forms of FLAG-tagged ILK proteins CHO cells were plated on fibronectin coated coverslips and transfected with a pFLAG-ILK vector that encodes full length ILK or a mock control vector pFLAG-CMV-2 (D and E), a pFLAG expression vector that encodes the ILK mutant (residues 66-452) in which the first ANK repeat is deleted (F and G) or a pFLAG expression vector that encodes the ANK repeats-containing N-terminal fragment of ILK (residues 1-230) (H and I). Two days after transfection, the cells were double stained with a mouse monoclonal anti-FLAG antibody (B, D, F and H) and a rabbit polyclonal anti-FAK antibody (C, E, G and I) as described in Materials and Methods. Bar, 5 μm.
DISCUSSION

In this study, we have shown that ILK, an important regulator of cell morphology and cell cycle progression, is recruited to cell-matrix focal adhesions but not epithelial cell-cell adhesions. The focal adhesion localization of ILK is observed in all types of cells examined including epithelial cells, fibroblasts, and kidney glomerular mesangial cells in response to a variety of extracellular matrix proteins. Additionally, we have defined the structure basis of the ILK-PINCH interaction and provided evidence indicating that the PINCH binding ANK repeats domain is necessary but not sufficient for focal adhesion localization of ILK.

Focal adhesions represent specialized subcellular structures that mediate cell adhesion and signal transduction between extracellular matrix and intracellular compartment (Burrage and Chrzanowska-Wodnicka, 1996; Jockusch et al., 1995). Over the last two and a half decades, more than twenty focal adhesion components have been identified. Among them more than a dozen appear to be universal focal adhesion constituents, which include integrins, talin, vinculin, paxillin, CRP, zyxin, Src and FAK. Tyrosine- and serine-phosphorylation at focal adhesions are regulated in response to cell adhesion or other stimuli and they play critical roles in signal transduction through focal adhesions. Several focal adhesion protein tyrosine kinases including Src and FAK have been well characterized. They are responsible for tyrosine phosphorylation of a number of focal adhesion proteins that are involved in cellular control of migration, proliferation and apoptosis (Ilic et al., 1997; Parsons and Parsons, 1997; Schwartz et al., 1995). A number of focal adhesion components including FAK (Yamakita et al., 1999) and paxillin (Bellis et al., 1997; De Nichilo and Yamada, 1996) are phosphorylated not only at tyrosine but also at serine residues. Moreover, several major focal adhesion components including talin (Beckerle, 1990; Pavalko et al., 1995), vinculin (Beckerle, 1990; Pavalko et al., 1995) and integrins (Barreuther and Grabel, 1996; Freed et al., 1989; Hogervorst et al., 1993; Reszka et al., 1992) are phosphorylated primarily at serine and threonine residues. The observation that ILK is a focal adhesion component strongly suggests that ILK could function as one of the major kinases responsible for serine and threonine phosphorylation in focal adhesions during integrin-mediated signal transduction. In this regard, it is particularly interesting to note that the kinase activity of ILK is tightly regulated by cell adhesion to fibronectin (Delcommenne et al., 1998), and ILK is capable of phosphorylating a peptide representing β1 integrin cytoplasmic domain in vitro (Hannigan et al., 1996). Another protein kinase that has been implicated in serine/threonine phosphorylation at focal adhesions is protein kinase C. It is worth noting, however, that these two serine/threonine kinases appear to be regulated through distinct mechanisms. Protein kinase C (α and δ) is typically found in small or nascent focal adhesions (Jaken et al., 1989; Woods and Couchman, 1992; Barry and Critchley, 1994). Furthermore, in contrast to ILK, protein kinase Cα interacts with and is activated by syndecan-4 (Oh et al., 1997a,b), another transmembrane component of focal adhesions. Thus, there appear to exist at least two parallel serine/threonine phosphorylation mechanisms at focal adhesions, one involves integrin/ILK and the other involves syndecan-4/protein kinase Cα.

The data shown in this report that ILK is a focal adhesion component provide important cellular evidence for a role of ILK in cell-matrix adhesion and signaling. This is consistent with previous yeast two-hybrid binding studies showing that ILK interacts with the β1 integrin cytoplasmic domain and suggests that focal adhesions are a major subcellular compartment where ILK functions. Recent genetic studies by Mackinnon and Williams indicate that a null mutation in C. elegans ILK homologue gene (pat-4) causes a Pat phenotype (paralyzed and arrested elongation at the two-fold stage) that resembles the phenotype of integrin α and β subunit mutants pat-2 (B. Williams and H. Waterston, personal communications) and pat-3 (Gettner et al., 1995), respectively.
Other known Pat genes include other intracellular or extracellular focal adhesion components deb-1 (vinculin) (Barstead and Waterston, 1991) and unc-52 (perlecan) (Rogalski et al., 1993).

Cells in epithelial tissues form both cell-matrix adhesions and cell-cell adhesions. It has been well described that β1 integrins are present in both epithelial cell-matrix adhesions and cell-cell adhesions. However, the organization and conformation of β1 integrins in epithelial cell-cell adhesions appear to be different from those in cell-matrix adhesions. For example, Braga et al. (1998) have demonstrated that β1 integrins localize but are not particularly concentrated in cell-cell adhesions in keratinocytes. Moreover, unlike β1 integrins in the cell-matrix adhesions, the β1 integrins in the cell-cell adhesions were not recognized by monoclonal antibodies that preferentially recognize ligand-occupied β1 integrins (Bishop et al., 1998; Kim and Yamada, 1997). Our observation described in this report that ILK is recruited to epithelial cell-matrix focal adhesions but not cell-cell adhesions raises an interesting possibility that ILK may preferentially interact with the ligand-occupied β1 integrins and thus specifically transduces signals from the ligand-activated integrins. In this regard, it is worth noting that ILK was initially identified from a yeast two-hybrid screen based on its interaction with a chimeric polypeptide containing the β1 integrin cytoplasmic domain in the absence of a normally suppressive α cytoplasmic domain. Although the conformation of the chimeric polypeptide containing the β1 integrin cytoplasmic domain expressed by the yeast cells is not known, such a chimeric polypeptide could potentially mimic the ligand-occupied β1 integrins. Using an IL-2 α-chain/β1 tail chimeric molecule, LaFlamme et al. have demonstrated that the β1 integrin cytoplasmic domain is sufficient for focal adhesion localization (LaFlamme et al., 1992) and can function as a dominant-negative inhibitor of integrin-mediated cell adhesion, spreading and fibronectin matrix assembly (LaFlamme et al., 1994), presumably by competing with endogenous ligand-occupied β1 integrins. The possibility that ILK may preferentially interact with the ligand occupied β1 integrins is also supported, although not proven, by the observation that the kinase activity of ILK is stimulated upon cell adhesion to fibronectin (Delcommenne et al., 1998).

A proper balance between epithelial cell-cell and cell-matrix interactions is critical for normal control of epithelial differentiation, proliferation, survival and tissue integrity, and disruption of this balance results in pathological conditions including cancer. Previous studies have suggested that ILK plays important regulatory roles in both epithelial cell-matrix and cell-cell adhesions and overexpression of ILK in epithelial cells shifts the balance towards the cell-matrix interactions (Hannigan et al., 1996; Wu et al., 1998; Novak et al., 1998). While the localization of ILK in focal adhesions supports a direct role of ILK in regulation of cell-matrix interactions, the apparent absence of ILK in epithelial cell-cell adherens junctions suggests that ILK influences cell-cell interactions through an indirect mechanism. Although the precise mechanism remains to be established, available experimental data suggest that it likely involves down-regulation of E-cadherin expression (Wu et al., 1998) and nuclear translocation of β-catenin (Novak et al., 1998).

In addition to demonstrating that ILK is a constituent of focal adhesions, we have investigated the molecular basis underlying the focal adhesion localization of ILK by mutational analyses. The interaction between ILK and the integrins appears to play a role in focal adhesion targeting of ILK, as an ILK mutant that contains both the N-terminal ANK repeats and the PH-like phospholipid binding domain but lacks the C-terminal integrin-binding domain failed to localize in focal adhesions. The ILK-integrin interaction, however, is apparently not sufficient for focal adhesion localization of ILK, as an ANK1-deletion ILK mutant that contains intact integrin-binding domain failed to localize in focal adhesions. Because PINCH is the only protein that is known to interact with the ANK repeats of ILK (the only known defect of the ANK1-deletion ILK mutant is in the interaction with PINCH) and PINCH is capable of localizing to cell-matrix adhesions sites (Tu et al., 1999; Hobert et al., 1999), the simplest and most likely explanation of our data is that focal adhesion localization of ILK requires the PINCH-ILK interaction. Alternative explanations are possible. For example, a focal adhesion component other than PINCH could bind to the ANK repeats of ILK and thus facilitates the focal adhesion localization of ILK. However, because there is currently no experimental evidence for the presence of an ILK ANK repeats-binding protein other than PINCH, the alternative, although possible in principle, is less likely. In further supporting a critical role of PINCH in focal adhesion localization of ILK, we have found that the ILK ANK repeats-containing N-terminal fragment, unlike the wild-type ILK, does not efficiently form a stable complex with PINCH in mammalian cells. Thus, the failure of the ILK ANK repeats-containing N-terminal fragment to localize to focal adhesions could result from the defect in the formation of a stable complex with PINCH in cells. Based on results from this and other studies, we propose a model for focal adhesion localization of ILK. In this model, ILK is targeted to focal adhesions through a direct interaction with integrins, which are known enriched in focal adhesions. The initial association of ILK with integrins, however, is transient or labile. Stable association of ILK with integrins in focal adhesions is achieved through multiple interactions mediated by PINCH, which binds to the ANK repeats of ILK via the N-terminal-most LIM1 domain and presumably other focal adhesion components via the C-terminal LIM domains. This model suggests that PINCH functions as a scaffold protein regulating focal adhesion localization of ILK and thus implies a critical role of PINCH in ILK function. Recently, Hobert and his colleagues have demonstrated that UNC-97, a C. elegans PINCH homologue, is required for assembly and stability of focal adhesion-like muscle attachment structures in C. elegans (Hobert et al., 1999). Furthermore, suppression of unc-97 (C-PINC) expression resulted in a Pat phenotype that is identical to loss of function phenotype of pat-4 (C-ILK) and βpar-integrin (Hobert et al., 1999). Our results that the PINCH-binding ANK repeats are required for focal adhesion localization of ILK suggest that the Pat phenotype exhibited in the UNC-97 (C-PINC) deficient mutants could result from a failure of focal adhesion localization of Pat-4 (C-ILK).

Focal adhesions appear to be a site that is particularly rich in LIM domains. In addition to PINCH (Tu et al., 1999; Hobert et al., 1999), it has been well established that LIM-containing proteins paxillin (Turner et al., 1990), zyxin (Beckerle, 1997) and CRP (Sadler et al., 1992) localize in focal adhesions.
REFERENCES


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