Molecular Basis of Kindlin-2 Binding to Integrin-linked Kinase Pseudokinase for Regulating Cell Adhesion*

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Background: Mechanism of the kindlin-2/ILK interaction remains elusive.

Results: Kindlin-2 specifically recognizes the ILK pseudokinase domain via a leucine-rich amphipathic α-helix, and such binding is crucial for focal adhesion assembly and cell spreading.

Conclusion: The leucine-rich helix-mediated kindlin-2/ILK interaction is crucial for integrin outside-in signaling but dispensable for inside-out signaling.

Significance: The results provide significant molecular insights into kindlin-2-ILK complex-mediated cell adhesion.

Integrin-linked kinase (ILK) is a distinct intracellular adaptor essential for integrin-mediated cell-extracellular matrix adhesion, cell spreading, and migration. Acting as a major docking platform in focal adhesions, ILK engages many proteins to dynamically link integrins with the cytoskeleton, but the underlying mechanism remains elusive. Here, we have characterized the interaction of ILK with kindlin-2, a key regulator for integrin bidirectional signaling. We show that human kindlin-2 binds to human ILK with high affinity. Using systematic mapping approaches, we have identified a major ILK binding site involving a 20-residue fragment (residues 339–358) in kindlin-2. NMR-based analysis reveals a helical conformation of this fragment that utilizes its leucine-rich surface to recognize the ILK pseudokinase domain in a mode that is distinct from another ILK pseudokinase domain binding protein, α-parvin. Structure-based mutational experiments further demonstrate that the kindlin-2 binding to ILK is crucial for the kindlin-2 localization to focal adhesions and cell spreading (integrin outside-in signaling) but dispensable for the kindlin-2-mediated integrin activation (integrin inside-out signaling). These data define a specific mode of the kindlin-2/ILK interaction with mechanistic implications as to how it spatiotemporally mediates integrin signaling and cell adhesion.

The adhesion of cells to extracellular matrix is mediated primarily by a class of cell surface receptors called integrins (1). Integrins are noncovalent heterodimers (αβ) with each subunit consisting of a single transmembrane domain, a large extracellular domain of several hundred amino acids, and typically, a small cytoplasmic tail of ~20–70 residues. Integrins function via a so-called bidirectional signaling process (1, 2): (i) They are first activated through “inside-out” signaling, i.e. an agonist-induced cellular signal stimulates a change in the integrin cytoplasmic face, which propagates to the extracellular domain, activating the receptor to bind extracellular matrix ligands. (ii) Upon ligand binding, integrins then cluster and transmit signals back to the integrin cytoplasmic tails, leading to the formation of highly organized protein complexes known as focal adhesions (FAs) that connect to actin filaments (outside-in signaling). In this manner, integrins physically connect the cell exterior with the cytoskeleton, allowing for regulation of dynamic cell adhesion processes such as cell spreading, migration and survival. Integrin-linked kinase (ILK) is a central component of FAs, in which it acts as a major docking platform to engage many FA proteins. In particular, it forms a tight heterotrimer termed IPP with two adaptor proteins PINCH (particularly interesting new cysteine and histidine-rich protein) and α-parvin (3, 4). Evolutionary analysis has demonstrated that each component of the IPP complex emerged early in evolution and that the IPP complex may have been one of the first molecular machineries involved in integrin adhesion and signaling (5). ILK consists of two domains: the N-terminal ankyrin repeat domain and the C-terminal kinase-like pseudokinase domain (KLD) (6). The ILK KLD lacks structural motifs (7, 8) that are essential for ATP hydrolysis and subsequent phosphoryl transfer activity. ILK is thus a catalytically incompetent adaptor (4, 9). Genetic studies in Drosophila (10), Caenorhabditis elegans (11), and mammals (12) all support the notion of noncatalytic adaptor function of ILK. However, the structural mechanisms underlying how ILK pseudokinase acts as the adaptor to regulate the integrin adhesion remain poorly understood.

Kindlin-2 belongs to a subfamily of FERM (four-point-one, ezrin, radixin, moesin) domain-containing proteins (kindlin-1, kindlin-3, Kamila Bledzka, Jun Yang, H. Dhanuja Perera, Edward F. Plow, and Jun Qin)

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-2, and -3) comprising F0, F1, F2, and F3 subdomains. The distinct feature of the kindlin FERM domain is an insertion of a pleckstrin homology domain in the middle of the F2 subdomain (13–16). Both kindlin-2 F0 and pleckstrin homology domains were found to bind membrane phospholipids (17, 18), whereas the F3 subdomain mediates the binding to the β-integrin cytoplasmic tails and directly promotes integrin inside-out activation (19–22). Upon integrin activation, kindlin-2 is also involved in focal adhesion assembly and cell spreading (integrin outside-in signaling) (14–16). Interestingly, cell biological and genetic studies in C. elegans and mice suggested that kindlin-2 interacts with ILK and regulates integrin function (11, 20, 23). However, little is known at structural level as to how these two key integrin regulators interact and mediate integrin bidirectional signaling.

In this study, we have undertaken biochemical, biophysical, structural, and functional characterization of the human ILK/kindlin-2 interaction. Using systematic mapping approaches, we first identify a major ILK binding site, which involves a 20-residue fragment (residues 339–358) in kindlin-2 F2 subdomain. NMR-based structural analyses then reveal a helical conformation of this fragment that utilizes its conserved leucine-rich hydrophobic surface to recognize the ILK KLD. Gel filtration experiment demonstrates that ILK KLD, α-parvin, and kindlin-2 F2 subdomain co-elute as a stable ternary complex, indicating that the kindlin-2 binding site on ILK KLD is distinct from that for α-parvin. Importantly, structure-based mutational experiments reveal that kindlin-2 binding to ILK is crucial for the kindlin-2 localization to FAs and cell spreading (integrin outside-in signaling) but dispensable for the kindlin-2-mediated integrin activation (integrin inside-out signaling). These data provide important structural insights into the kindlin-2/ILK interaction and suggest a spatiotemporal pathway wherein kindlin-2 co-localizes with ILK at FAs after triggering integrin activation to dynamically regulate FA assembly and cell adhesion.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Primary and secondary antibodies were obtained from the following commercial sources: rabbit anti-ILK (Millipore), mouse anti-vinculin (Sigma), mouse anti-kindlin-2 (Millipore), Alexa 568 goat anti-rabbit (Life Technologies Inc.), Alexa 488 goat anti-mouse (Life Technologies Inc.), Alexa 633 phalloidin (Life Technologies Inc.), Alexa 568 phalloidin (Life Technologies Inc.), and goat anti-rabbit HRP (Santa Cruz Biotechnology) for confocal microscopy; rabbit anti-kindlin-2 (Sigma), rabbit anti-ILK clone 4G9 (Cell Signaling), mouse anti-β-actin (clone 8H10D10; Cell Signaling), mouse anti-ILK (EMD Chemicals, Inc.), mouse anti-GST (EMD Chemicals, Inc.), and donkey anti-mouse HRP (Santa Cruz Biotechnology) for Western blot; and anti-αlββ3 PAC-1 (BD Biosciences), mouse anti-EGFP (Clontech), and Alexa 647 goat anti-mouse IgM (Jackson ImmunoResearch) for FACS. All other chemical reagents were of analytical grade.

Plasmid Construction, Expression, and Purification—The expression plasmids for the various kindlin-2 fragments were constructed by standard molecular biology protocols. Briefly, the GST-fused various kindlin-2 constructs for co-immunoprecipitation and pulldown experiments were generated using a parallel expression system (25) by PCR using the full-length human kindlin-2 cDNA (24) as a template. The wild type F2 subdomain (residues 281–569) of kindlin-2 was subcloned into a pET15b vector (Novagen) to yield a thrombin-cleavable N-terminal hexahistidine-tagged protein. The F2 subdomain and F2 N-terminal short fragment (residues 328–367) of kindlin-2 were also subcloned into a pGEX4T-1 vector (GE Healthcare) to yield a thrombin-cleavable GST-fused protein for in vitro binding and NMR experiments. A full-length kindlin-2 construct for bacterial expression was generated in a modified PET28 vector that carries an N-terminal hexahistidine followed by SUMO tags. A GFP-fused full-length kindlin-2 construct was also generated in a pEGFP-C2 vector (Clontech) for immunofluorescence microscopy experiments as previously described (24). The amino acid substitutions in the full-length, the F2 subdomain, and the F2 short fragment of the kindlin-2 constructs were created using a QuikChange site-directed mutagenesis (Stratagene) kit with appropriate primer sets, and all the wild type and mutant DNA constructs were verified by DNA sequencing.

Recombinant hexahistidine-tagged wild type and mutant F2 subdomain proteins of kindlin-2 were expressed in Escherichia coli and purified from bacterial cell lysates by nickel affinity chromatography, followed by HiLoad 16/60 Superdex 200 gel filtration and Resource-Q anion exchange chromatography columns (all from GE Healthcare). The N-terminal hexahistidine tag was removed by a thrombin digestion. The GST-fused wild type and mutant kindlin-2 proteins were bacterially expressed and purified from the cell lysate by glutathione-Sepharose 4B resin (GE Healthcare), followed by HiLoad 16/60 Superdex 200 gel filtration chromatography column. The recombinant human ILK kinase-like KLD proteins (wild type and C346S/C422S mutant) bound to α-parvin CH2 were bacterially expressed and purified using a bicistronic co-expression system (26) as previously described (7). The recombinant hexahistidine and SUMO-tagged full-length kindlin-2 protein was bacterially expressed and purified from the bacterial cell lysate by nickel affinity chromatography followed by HiLoad 16/60 Superdex 200 chromatography. The N-terminal hexahistidine and SUMO tags were removed by a protease digestion using Ulp1 (ubl-specific protease 1). The GST-fused full-length kindlin-2 protein was bacterially expressed and purified, as previously demonstrated (27).

Size Exclusion Chromatography—The experiment was performed on either a Superose 6 10/300 GL (24 ml) or a Superdex 200 10/300 (24 ml) (both from GE Healthcare) in a running buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 5% glycerol, 2 mM DTT at a flow rate of 0.5 ml/min. For each sample, 500 μl of protein solution was loaded onto a column, and each elution profile was monitored by UV absorbance at 280 nm. The column was calibrated with a gel filtration standard protein mixture (Bio-Rad) containing thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B12 (1,350).

In Vitro GST Pulldown Binding Experiments—Each purified GST-fused kindlin-2 protein (wild type or mutant) or GST (30 μg for each) was immobilized on 40 μl of glutathione-Sephar-
ose 4B beads (GE Healthcare) and equilibrated in the pulldown binding buffer consisting of phosphate-buffered saline, 5% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, and Complete EDTA-free protease inhibitor. The purified ILK KLD (wild type)-α-parvin CH2 complex was added to each affinity bead at 15 or 50 μg, and the binding reactions were incubated at 4 °C for 3 h. The beads were washed three times using binding buffer. The bound proteins were eluted in 30 μl of 20 mM reduced glutathione in the binding buffer and analyzed by SDS-PAGE followed by Coomassie staining.

**Surface Plasmon Resonance**—All kinetic studies on the interaction between kindlin-2 (either in the full-length or its F2 subdomain) and ILK KLD(WT)-α-parvin CH2 complex were carried out on a Biacore 3000 instrument (GE Healthcare) at room temperature in the running buffer consisting of 10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.005% Surfactant P-20 (GE Healthcare). The full-length or F2 subdomain of kindlin-2 was coupled covalently to a sample flow cell on a CM5 sensor chip (GE Healthcare) using an amine coupling protocol according to the manufacturer’s protocol. Another flow cell was utilized as a reference control (blank channel) without any coupled protein. For buffer exchange and removal of insoluble material, the analyte protein (ILK KLD-α-parvin CH2 complex) was applied to a size exclusion chromatography column (Superdex 200 10/300) equilibrated in the Biacore running buffer immediately before the experiments. Serial 2-fold dilutions of the analyte from the major peak fractions of the column were prepared in the same running buffer. Purified analyte protein was injected at a flow rate of 20 μl/min over the parallel flow cells (experimental sample and control). The resonance unit differences between the experimental sample and control flow cells yielded the specific binding. Blank injections of the running buffer were also performed. All sensorgrams were corrected by performing a blank run subtraction. Regeneration of the sensor chip surface between each sample injection was carried out by pulse injections of a solution containing 10 mM HCl and 1 M NaCl. The kinetic parameters were fit to a 1:1 binding model using BIAevaluation software (GE Healthcare). Two independent sets of the kinetic measurements were used for calculation of the apparent affinity constants.

**NMR Experiments and Structure Determination of the F2 Short Fragment of Kindlin-2**—The uniformly 2H,15N-labeled F2 subdomain proteins (wild type and L353A/L357A mutant; hereinafter referred to as WT and LL/AA, respectively) of kindlin-2 were bacterially expressed in deuterated minimal medium containing [15N]ammonium chloride and purified as for the unlabeled protein. The labeled purified kindlin-2 proteins and unlabeled purified ILK KLD(C346S/C422S mutant)-α-parvin CH2 complex were dialyzed against 50 mM sodium phosphate, pH 6.7, 50 mM sodium chloride, and 2 mM sodium azide. Each NMR sample was supplemented with 10% (v/v) 2H2O. The binding interaction of ILK KLD(C346S/C422S mutant)-α-parvin CH2 to the F2 subdomain of kindlin-2 showed similarity to that of the wild type ILK KLD-α-parvin CH2 by GST pull-down binding experiments (data not shown). Thus, we utilized the ILK KLD(C346S/C422S mutant)-α-parvin CH2 for the NMR titration analysis to ensure protein solubility at higher concentrations. The two-dimensional heteronuclear single quantum coherence (HSQC) spectra of 2H,15N-labeled K2 subdomain of kindlin-2 (41 μM) in the absence or presence of the ILK KLD-α-parvin CH2 complex (82 μM) were acquired at 15 °C using Bruker Avance I/600 spectrometer (Cleveland Clinic Lerner Research Institute). The uniformly 15N-labeled N-terminal short fragment (residues 328–367) in the F2 subdomain of kindlin-2 (referred to as K2F2N) was bacterially expressed as a GST-fused protein in minimal medium. The GST fusion tag was cleaved by a thrombin digestion, and the 15N-labeled K2F2N was purified by HiLoad 16/60 Superdex 75 size exclusion chromatography column (GE Healthcare). The NMR data acquisitions of the K2F2N (45 μM) in the presence or absence of ILK KLD-α-parvin CH2 complex (91 μM) were carried out as described above. All the two-dimensional (HSQC) spectra were processed and analyzed by the program NMRpipe (28).

The ILK-binding F2 short fragment peptide of kindlin-2 (residues 339–358) was chemically synthesized and purified by HPLC in the Biotechnology Core in the Cleveland Clinic Lerner Research Institute. 1H NMR experiments of the kindlin-2 peptide (2 mM) were carried out at 15 °C in the absence or presence of 50 μM ILK KLD-α-parvin CH2 complex in 20 mM Hepes, pH 6.82, 100 mM NaCl, 2 mM DTT, 10% D2O on a Bruker 900 spectrometer. Two-dimensional total correlated spectroscopy and two-dimensional NOESY spectroscopy for the free form (control) of the kindlin-2 peptide were acquired with mixing times of 70 and 400 ms, respectively. For the complex with the ILK KLD-α-parvin CH2, two-dimensional NOESY was recorded with mixing time of 400 ms. Sequential assignments were carried out using NOESY spectra and standard procedures. A total of 267 NOEs were observed and used to calculate an ensemble of 100 structures, and the 20 structures with the lowest energies were refined using the program X-PLOR-NIH (29) and evaluated by the program Procheck-NMR (30). Structure analysis and figure preparation were carried out by the program PyMOL.

**Cell Culture and Transfection**—The human cervical cancer cell line HeLa and the human ovarian carcinoma cell line OVCAR-3 were obtained from American Type Culture Collection (Manassas, VA) and maintained in high glucose DMEM with 10% FBS and penicillin/streptomycin. A Chinese hamster ovary cell line stably expressing αIIbβ3 (CHO-A5) was maintained in DMEM/F-12 with 10% FBS and penicillin/streptomycin and 1.4 mg/ml geneticin (Invitrogen). The human ovarian carcinoma cell line (OVCAR-3) was cultured in RPMI 1640 with 20% FBS, 0.01 mg/ml insulin, and penicillin/streptomycin. The cells were subcultured by T/E (0.05% trypsin/0.53 mM EDTA) dissociation at ~80% (HeLa, CHO-A5, and OVCAR-3).

**Cell Spreading Assays**—OVCAR-3 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions with plasmids encoding the following EGFP, EGFP-kindlin-2, EGFP-kindlin-2 (LL/AA) mutant. The cells were allowed to adhere to fibronectin-coated coverslips for 12 h after transfection, and cell spreading was measured after an additional 2 h at 37 °C. After extensive washing with PBS, the adherent cells were fixed with 4% paraformaldehyde and stained with the Alexa 568 phalloidin and photographed with 40X objective. The cell areas of EGFP-positive
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cells were measured using ImageJ software. The error bars presented in the figures represent means ± S.D. of three independent experiments.

Immunofluorescence Microscopy—Cells were plated on 20 µg/ml fibrinogen or 10 µg/ml fibronectin for the times indicated, fixed, permeabilized with 0.1% Triton X-100, blocked in horse serum, and stained with the indicated antibodies overnight at 4 °C. Antibody-antibody complexes were detected by staining with Alexa-coupled secondary antibodies for 1 h, and slides were mounted using Immuno-Fluoro mounting medium (MPI Biomedicals). Stained cells were visualized with a 40× or 63× 1.4 oil objectives using a Leica TCS-NT laser scanning confocal microscope ( Imaging Core, Cleveland Clinic). Laser intensities were adjusted to eliminate cross-talk between channels, and images were collected using Leica Confocal Software (version 2.5 Build 1227). Images are representative of three independent experiments (bar size, 10 µm).

Integrin Activation Assays—Integrin αmβ3 activation was evaluated using PAC-1, an mAb specific for the activated conformation of this integrin as previously described (21). Talin-1 domain tagged with DsRed monomer or EGFP tagged kindlin-2 and its mutants were expressed in CHO-A5 cells by transient transfection using Lipofectamine 2000 (Invitrogen). PAC-1 binding to the different transfectants (EGFP and DsRed double-positive cells) was analyzed by flow cytometry after incubating the transfected cells with 10 µg/ml of anti-PAC-1 mAb in HBSS buffer containing 0.1% BSA, 0.5 mM CaCl2, 0.5 mM MgCl2 for 30 min at room temperature followed by fixation with 1% paraformaldehyde for 10 min in room temperature, washing, and incubation with 5 µg/ml Alexa 647-conjugated F(ab’2) anti-mouse IgM secondary antibody for 30 min on ice. αmβ3 expression level on the transfected cell surfaces was evaluated based on reactivity with a mAb (2G12), which reacts with αmβ3 independent of its activation state (31). PAC-1 binding was analyzed on a LSR Fortessa flow cytometer (BD Biosciences). Integrin activation was quantified as an activation index calculated using the following formula: 100 × (MFI − MFI of empty vector) / (MFI of maximal PAC-1 binding in the cells treated with 3 mM Mn2+ − MFI of empty vector), where MFI is the mean fluorescence intensity.

RESULTS

Characterization of the Human Kindlin-2/ILK Interaction—The interaction of kindlin with ILK was initially derived from a genetic analysis in C. elegans, where the N-terminal short segment (residues 1–31) of UNC-112, the C. elegans kindlin ortholog, was thought to mediate the binding to the kinase domain of PAT-4, the C. elegans ILK ortholog (11). However, human kindlin-2 F0 encompassing the corresponding the C. elegans segment (residues 1–31) failed to interact with ILK in our NMR binding experiment (data not shown). A more recent genetic study in C. elegans suggested that the binding to ILK may involve the larger N-terminal half of UNC-112 1–396, and a specific D382V mutation impaired the UNC-112 binding to PAT-4 (23). However, Asp-382 is not conserved across species, e.g. it is Ser-351 in corresponding human kindlin-2, and S351V mutation had no effect on human kindlin-2/ILK interaction (see below). To definitively map the ILK binding site in kindlin-2, we decided to perform a series of systematic in vivo and in vitro binding experiments. We first confirmed that endogenous ILK can bind kindlin-2 in mammalian cells using CHO and HeLa cells as models (Fig. 1A). Then we generated the N-terminal enhanced green fluorescent protein (EGFP)-tagged plasmids containing various deletion fragments of kindlin-2 and transfected them into CHO cells. The lysates from these transfected cells were used in immunoprecipitation experiments with the anti-GFP antibody, and bound proteins were analyzed by Western blots. Fig. 1B shows that the deletion of a middle region of kindlin-2 from residues 288–564 (corresponding to the human kindlin-2 F2 subdomain) resulted in the loss-of-binding to ILK. To confirm this finding, we performed GST pulldown experiments using various kindlin-2 fragments each fused to GST. Consistent with the co-immunoprecipitation data, the F2 subdomain encompassing residues 281–541 exhibited binding to human ILK (Fig. 1, C and D). The N-terminal F0-containing 11–143 had no binding to ILK (Fig. 1, C and D), which is consistent with our earlier NMR experiment but contrasts with the earlier genetic analysis (11).

To further confirm the direct interaction between kindlin-2 and ILK, we expressed and purified the bacterial recombinant F2 subdomain of human kindlin-2 and the human ILK KLD bound to α-parvin calponin homology domain 2 (CH2) and analyzed their binding by size exclusion chromatography experiments (Fig. 2). We note that among various F2 constructs, we have examined including residues 281–541, 288–564, and 281–569. As the F2 281–569 was the best in stability and yield, we used it for all the subsequent biochemical and structural characterizations. It is also of note that co-expression and co-purification with ILK binding partners such as α-parvin CH2 were necessary to obtain a soluble mono-dispersed form of the ILK KLD as previously demonstrated (7) because ILK KLD alone is unstable and forms soluble aggregates (data not shown). The size exclusion chromatography experiments clearly show that the F2 subdomain of kindlin-2 is capable of forming a tripartite complex with ILK KLD and α-parvin CH2, indicating that kindlin-2 and α-parvin bind to distinct sites of the ILK KLD. An NMR-based HSQC experiment of 2H,15N-labeled F2 subdomain of kindlin-2 shows a dispersed spectral pattern, indicating F2 is well folded (Fig. 3A). Upon addition of ILK KLD-α-parvin CH2 complex, kindlin-2 F2 underwent significant line broadening and some chemical shift changes (Fig. 3A), providing independent evidence that kindlin-2 F2 binds to ILK KLD. As a control, the HSQC of kindlin-2 F2 was not altered by α-parvin CH2 alone (data not shown).

To obtain quantitative binding information, we performed surface plasmon resonance experiments, which revealed the apparent binding affinity (Kd of 112 ± 5.5 nM) between kindlin-2 F2 and ILK KLD (Fig. 3B). The relatively high binding affinity is consistent with a tripartite protein complex as demonstrated by the gel filtration experiments above (Fig. 2). As a comparison, full-length kindlin-2 also binds the ILK KLD with a Kd = 150 ± 26.5 nM (Fig. 3C) similar to that of the kindlin-2 F2 subdomain. These data demonstrate that ILK KLD binds strongly to the F2 subdomain of kindlin-2, and the F2 binding site on ILK KLD does not overlap with that for α-parvin.
Mapping the ILK-binding Site in Kindlin-2—The kindlin-2 F2 subdomain comprises a unique pleckstrin homology domain inserted in the middle of the subdomain (18). The pleckstrin homology domain insertion makes kindlin-2 an atypical FERM domain-containing protein. To further delineate the ILK binding region in kindlin-2 F2, we generated various short fragments of kindlin-2 F2 fused to GST. The GST pulldown experiments showed that both the two short N-terminal segments (residues 328–367 and 328–358) retained the ability to bind directly to ILK (Fig. 4A). We then prepared 15N-labeled kindlin-2 residues 328–367 (termed K2F2N) (Fig. 4B). Fig. 4C shows that kindlin-2 residues 328–367 are largely unstructured but specifically bind to unlabeled ILK KLD/H18528/H9251-parvin CH2, with 16 kindlin-2 residues undergoing significant line-broadening and/or chemical shift perturbations including two characteristic glycine peaks. Because there are only Gly-359 and Gly-360 in the K2F2N, these data suggest that K2F2N interacts with ILK via a 16-residue segment containing these two glycines (Fig. 4A). To confirm this localization, we randomly chose six residues around these two glycine residues that might be directly involved in interactions with ILK. We generated two sets of triple mutants and a single hextuple mutant of the GST-fused K2F2N construct by replacing the selected residues with alanine. Our pulldown binding experiments clearly showed that all these kindlin-2 mutant proteins lost the ability to bind to the ILK KLD (Fig. 4D). Next, we generated single alanine substitution mutants of the GST-fused kindlin-2 F2 construct (residues 281–569). Using GST pulldown binding experiments, we found that the four kindlin-2 F2 point mutants (V346A, L350A, L353A, or L357A) but not others abolished ILK binding (Fig. 5A). Notably, S351V, which corresponds to D382V, which was shown to abolish the ILK binding in C. elegans (23), did not significantly affect ILK binding (Fig. 5A). Size exclusion chromatography experiments showed that these mutant proteins of the kindlin-2 F2 were eluted at a position similar to the wild type protein (data not shown), suggesting that these single amino acid substitutions did not affect protein folding. Using NMR, we further showed that 2H,15N-labeled HSQC spectrum of a double kindlin-2 mutant (L353A and L357A; hereinafter referred to as LL/AA) exhibited a well dispersed spectrum that is similar to the wild type but has no chemical shift perturbation and line broadening upon addition of unlabeled ILK KLD/H18528/H9251-parvin CH2 (Fig. 5B). These data strongly suggest that the double amino acid substitution (LL/AA) completely abolished the kindlin-2 binding to ILK without perturbing the overall structure.

Structure of the ILK-binding Fragment in Kindlin-2—Given that individual mutations of V346A, L350A, L353A, and L357A of kindlin-2, but not other substitutions, abolished the kindlin-2 binding to ILK KLD (Fig. 5A) and that these four hydrophobic residues are highly conserved across vertebrate kindlins.
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(Fig. 5C), we suspected that there is a conserved hydrophobic surface patch in kindlin-2 involved in ILK binding. The secondary structure prediction by the program PSIPRED (32) revealed that a potential α-helix may be formed around these ILK-binding residues of the kindlin-2 F2. Consistently, the analysis of a helical wheel projection suggested that it may comprise an amphipathic leucine-rich α-helix, in which Val-346, Leu-350, Leu-353, and Leu-357 are part of the hydrophobic surface (Fig. 5D). To experimentally confirm this prediction, we decided to determine the conformation of this ILK-binding fragment of kindlin-2 using NMR. We designed a chemically synthesized peptide encompassing kindlin-2 residues 339–358, which was based on the observation that only 16 residues in kindlin-2 (residues 328–367) were perturbed by ILK and that kindlin-2 residues 328–358 also bind to ILK, with Leu-357 being important from our mutation experiment (Fig. 5). Fig. 6 shows the expanded amide region of two-dimensional transferred NOE spectra where ILK KLD-α-parvin CH2 induced significant transferred NOEs on kindlin-2 (residues 339–358) as compared with the free form peptide (Fig. 6, A versus B). We assigned 267 NOE cross-peaks and calculated the solution structure of the kindlin-2 residues 339–358 bound to ILK (Fig. 6C and Table I), which revealed a short random coil followed by
a single amphipathic α-helix consisting of residues from 346 to 358. The two loss of ILK binding residues (Leu-353 and Leu-357) are part of the highly conserved leucine-rich hydrophobic patch in the helical structure (Fig. 6D). Based on the mutation data, we suggest that this hydrophobic patch provides a core recognition motif for the direct binding of kindlin-2 to ILK. As mentioned earlier, a recent genetic analysis of C. elegans kindlin-2 ortholog (UNC-112) demonstrated that D382V resulted in a loss of binding to a C. elegans ILK ortholog (PAT-4) (23). However, we found that the corresponding residue S351V retained the binding to human ILK (Fig. 5A). The D352A mutation in human kindlin-2 that is chemically equivalent to Asp-382 in C. elegans but spatially placed in the neighbor to Ser-351 also did not result in the loss of binding to human ILK (Fig. 5A).

Effect of the Kindlin-2/ILK Interaction on Integrin Activation—Kindlin-2 has been previously shown to play a key role in binding and activating integrin (19–21).
pressed with talin head domain (talin-H), a major integrin activator, kindlin-2 was shown to dramatically enhance the talin-H-mediated activation of platelet integrin α<sub>IIb</sub>β<sub>3</sub> (20, 21). Because ILK was also implicated in playing a role in the activation of platelet integrin α<sub>IIb</sub>β<sub>3</sub> (33–35), we wondered whether the kindlin-2/ILK interaction may impact on integrin activation. We expressed kindlin-2 (either wild type or ILK binding defective LL/AA mutant) and talin-H in CHO cells stably expressing integrin α<sub>IIb</sub>β<sub>3</sub> and measured integrin activation. Wild type kindlin-2 significantly enhanced talin-H-mediated...
FIGURE 5. Identification of the ILK-binding residues in the kindlin-2 F2 subdomain by alanine-scanning mutagenesis and GST pulldown experiment. A, Coomassie-stained SDS-PAGE gels of various single mutants of GST-fused kindlin-2 F2 subdomain in the interaction with the ILK KLD/H18528/H9251-parvin CH2 complex by pulldown assays. Either 15 μg (+) or 50 μg (+ +) of the ILK KLD/α-CH2 complex was incubated with various GST-fused kindlin-2 F2 single point mutant proteins immobilized onto the glutathione-Sepharose beads. The position of bound ILK KLD is indicated with an arrowhead in each lane. The amino acid substitution of Ser-351 by Val-351 instead of alanine residue was created because of the recent mutagenesis report using *C. elegans* kindlin-2 ortholog (UNC-112). It is of note that the mutation at Ser-351 of human kindlin-2 by Val-351 did not result in a significant loss of binding to ILK, whereas that (D382V) of *C. elegans* abolished the binding to the *C. elegans* ILK ortholog (PAT4) (see text for a detail). B, HSQC spectra of F2 subdomain mutant (L353A and L357A; LL/AA) of human kindlin-2 in the absence (black) and presence (red) of the human ILK KLD/α-CH2 complex. The HSQC spectra shows that the mutant protein of the F2 subdomain of human kindlin-2 is folded in solution yet with no chemical shift perturbation and line-broadening effect upon addition of the human ILK KLD/α-CH2, demonstrating that the mutations abolished the direct binding of kindlin-2 to ILK. C, sequence alignment of the ILK-binding minimal segment in the kindlin-2 proteins across species. Red arrowheads highlight crucial residues of kindlin-2 derived from loss of binding function in the interaction with ILK KLD. Secondary structure (β-helix) and random coil are shown as a magenta box and a line on the sequence, respectively. Conserved residues are boxed. D, a helical wheel representation of the ILK-binding amphipathic α-helix of kindlin-2. Hydrophobic residues that are responsible for binding to ILK KLD are highlighted in red boxes.

FIGURE 6. Structure determination of the ILK-binding short peptide in the F2 subdomain of kindlin-2 by solution NMR spectroscopy. A, two-dimensional 1H NOESY NMR spectra of the amide region of the ILK-binding peptide of kindlin-2 in the absence of ILK KLD/CH2. B, the 1H NOESY spectra in the presence of ILK KLD/CH2. C, stereo view of one of the 20 lowest energy structures of the ILK-binding short peptide of kindlin-2. The atomic coordinates without hydrogen atoms are shown in ball and stick representations. All the residues are labeled. D, a ribbon drawing model of the kindlin-2 peptide structure in the same orientation as that in C. Two critical ILK-binding residues (Leu-353 and Leu-357) are highlighted in ball and stick representations.
activation of integrin $\alpha_{10}\beta_3$ (Fig. 7, A and B), consistent with previous reports (20, 21). However, the ILK-binding defective mutation in kindlin-2 did not alter the integrin activating activity of kindlin-2 (Fig. 7, A and B). Additional expression of ILK in cells co-expressing kindlin-2 and talin-H also did not change the activation of integrin $\alpha_{10}\beta_3$ when compared with cells over-expressing both kindlin-2 and talin-H (Fig. 7A).

Given the previously suggested role of ILK in integrin activation (34, 35), we also examined whether ILK directly cooperates with talin-H to mediate $\alpha_{10}\beta_3$ integrin activation. Co-expression of ILK with talin-H did not enhance integrin activation beyond that observed with talin-H alone (Fig. 7A). Expression of ILK alone had no effect in integrin activation. Our results demonstrate at least that the kindlin-2/ILK interaction and ILK alone had no effect in integrin activation. Beyond that observed with talin-H alone (Fig. 7A). Expression of ILK with talin-H did not enhance integrin activation $p < 0.05$ by Student’s t test. n.s. indicates not significant TH + K2 versus TH + K2LL/AA ($p = 0.236$); TH + K2 + ILK ($p = 0.254$) by t test. B, representative Western blot with anti-GFP for detection of EGFP-fused kindlin-2 and its mutants expressed in NIH3T3 CHO cells. The first lane shows nontransfected cells. WB, Western blot.

**FIGURE 7. Kindlin-2/ILK interaction is not required for integrin activation.** A, the $\alpha_{10}\beta_3$ integrin co-activation analysis using CHO cells transfected with EGFP-fused kindlin-2 (K2) wild type, its mutants (LL/AA for the loss of ILK binding; QW/AA for the loss of integrin binding), talin head domain (TH), and ILK. The activation index was determined by the formula shown under “Experimental Procedures.” Expression levels of $\alpha_{10}\beta_3$ in the presence and absence of the various kindlin-2 with or without talin-H varied by less than $< 10\%$. The data represent the means ± S.D. of three independent experiments. * TH versus TH + K2 and TH versus TH K2LL/AA; $p < 0.05$ by Student’s t test. n.s. indicates not significant TH + K2 versus TH + K2LL/AA ($p = 0.236$); TH + K2 + ILK ($p = 0.254$) by t test. B, representative Western blot with anti-GFP for detection of EGFP-fused kindlin-2 and its mutants expressed in NIH3T3 CHO cells. The first lane shows nontransfected cells. WB, Western blot.

The ILK Binding Amphipathic $\alpha$-Helix of Kindlin-2 Is Crucial for the Kindlin-2 Localization to Focal Adhesion Sites and Regulation of Cell Spreading—Given the above finding that the kindlin-2/ILK interaction is not pivotal for integrin activation, we next asked whether the interaction is involved in integrin outside-in signaling. We first examined whether the leucine-rich amphipathic $\alpha$-helix of kindlin-2 plays a role in targeting kindlin-2 to FAs by transfecting the EGFP-kindlin-2 plasmids (wild type or LL/AA mutant) into the HeLa cells. Wild type EGFP-kindlin-2 showed extensive localization to focal adhesions as marked by vinculin staining (Fig. 8). In contrast, the kindlin-2-LL/AA mutant was observed in membrane ruffles and showed only minimal localization with focal adhesions. We then asked whether the loss-of-ILK binding of kindlin-2 can influence cell spreading. We first examined the spreading of HeLa cells on fibronectin and A5 cells on fibrinogen by overexpressing WT kindlin-2 versus kindlin-2 mutant in these cell lines because their actin filaments can be readily visualized by Alexa phalloidin staining (not shown). The cells expressing the mutant did show a slight delay in spreading as compared with the WT kindlin-2 at an early time point (15 min), but by 1 h the spreading of the cells expressing wild type and mutant kindlin-2 was similar (data not shown). We attribute this “catch-up” might be dependent on the endogenous wild type kindlin-2 in the cells expressing the kindlin-2 LL/AA mutant. To circumvent this problem, we utilized the OVCAR-3 cell line as a model system that lacks endogenous kindlin-2 expression (Fig. 9A) (36). OVCAR-3 cells were transfected with EGFP-kindlin-2 plasmids (wild type or LL/AA mutant), and cellular morphology and quantification were assessed by phase contrast microscopy. Kindlin-2 (wild type), but not the mutant, significantly enhanced cell spreading in OVCAR-3 cell lines (Fig. 9B). These data demonstrate that kindlin-2 binding to ILK is involved in FA assembly and cell spreading, hallmarks of integrin outside-in signaling.

**DISCUSSION**

Integrin bidirectional signaling is mediated by a complex network of protein-protein interactions, but the structural basis for these interactions is not fully understood. In this study, we have focused on analyzing the interaction of two major integrin regulators, kindlin-2 and ILK. We first established the

**TABLE 1**

**Structural Basis of the Kindlin-2/ILK Interaction**

| Complex          | Number of NOEs | Intraresidual | Sequential, $u - j = 1$ | Medium range, $1 < u - j < 5$ | Long range, $u - j \geq 5$ (28 intermolecular NOEs) | Root mean square deviation$^a$ | Ramachandran plot$^b$ | Coordinate precision ($\AA)^c$ |
|------------------|----------------|--------------|--------------------------|-------------------------------|----------------------------------|-------------------------------|---------------------------|---------------------------|
|                  | NOEs (no violations $> 0.5 \AA$) |              |                          |                               |                                  | $0.194 \pm 0.004$             | Most favored regions (%)   | Backbone atoms            |
|                  | Bonds ($\AA$) |              |                          |                               |                                  | $0.0110 \pm 0.0003$            | 70.0                      | 0.34 ± 0.07               |
|                  | Angles (°)    |              |                          |                               |                                  | $1.22 \pm 0.04$                | 29.2                      | All heavy atoms            |
|                  | Improvers ($) |              |                          |                               |                                  | $0.73 \pm 0.07$                | 0.8                       | 0.66 ± 0.11               |
|                  | $E_{i-j}$ (kcal/mol, based on CHARMM19 parameters) | |                          |                               |                                  | $-27.9 \pm 5.5$               |                          |                          |

$^a$ Means ± standard deviation over 20 structures with lowest energies.

$^b$ Structured region from Glu-345 to Glu-358.

$^c$ Means ± standard deviation over 20 structures with lowest energies.

$^e$ Means ± standard deviation over 20 structures with lowest energies.
human kindlin-2/ILK interaction both in vivo and in vitro and then determined structural and biochemical basis of the interaction. In particular, our results indicated that kindlin-2 utilizes its distinct F2 subdomain to bind to ILK KLD, and the binding site is clearly distinct from that for parvin because we were able to co-elute the kindlin-2 F2-ILK pseudokinase domain-α-parvin CH2 ternary complex. We further showed that kindlin-2 utilizes a conserved leucine-rich amphipathic α-helix in the F2 subdomain to recognize ILK. This is the first example of a leucine-rich helix-mediated interaction between a FERM domain and a kinase-like domain. These structural/biochemical analyses have laid down the foundation for us to design a definitive ILK binding defective kindlin-2 mutant (LL/AA). Using this mutant, we were able to dissect the role of kindlin-2/ILK inter-
action in various stages of integrin signaling, i.e. we showed that the interaction is not required for mediating integrin activation/inside-out signaling but is crucial for integrin outside-in signaling involving FA assembly and cytoskeleton reorganization (cell spreading). The former finding is surprising given the previously implicated role of both kindlin-2 (19–21) and ILK (33–35) in integrin activation. The kindlin-2-mediated integrin activation is well accepted and confirmed in a large number of studies (for review, see Refs. 14–16, 37, and 38), but the role of ILK in mediating integrin activation remains controversial. Although ILK was previously indicated to play a role in integrin αIIbβ3 activation (33–35), a more recent study demonstrated that ILK is not essential for integrin αIIbβ3-mediated platelet activation (39). Instead, it may be involved in regulating the rate of platelet activation and spreading (39). Our results demonstrate that ILK had no effect in modulating integrin αIIbβ3 activation with or without associating with kindlin-2 and/or talin. We cannot exclude the possibility that ILK may be involved in activating integrins by associating with other binding partners such as PINCH and Parvin as suggested before (35). Nevertheless, our results are consistent with vast majority of studies indicating that ILK is involved in integrin outside-in signaling (for review, see Refs. 3, 9, and 14). It is likely that upon kindlin-2-mediated integrin activation, kindlin-2 is associated with ILK to localize to FAs and regulates cytoskeleton to trigger dynamic cell adhesion such as cell spreading (Fig. 9B).

Previous cell biological studies suggested that C. elegans kindlin-2 ortholog (UNC-112) exists in a dormant form, and the binding of PAT-4/ILK to the N-terminal fragment of UNC-112/kindlin-2 may release the autoinhibited conformation (23). This is inconsistent with a structural study of a homologous mouse kindlin-3, which revealed a rather elongated conformation (40) contrasting to a typically compact autoinhibited conformation. We found that either full-length or kindlin-2 F2 subdomain binds to ILK at comparable binding affinities (110 nM versus 150 nM), suggesting an open conformation of human kindlin-2 or that the ILK binding site in human kindlin-2 is not buried. We note here that expression and purification of a N-terminal half domain (residues 1–367) of kindlin-2 resulted in a highly soluble aggregate in our gel filtration experiment, likely corresponding to the same region in UNC-112 (23) resulted in a highly soluble aggregate in our gel filtration experiment, likely because this construct truncates in the middle of F2 N-terminal half and thus destabilizes the unfolding. Consequently, this fragment fused to either maltose-binding protein or hexahistidine failed to bind ILK in our pulldown binding experiments (data not shown). Thus, the autoinhibition mechanism of kindlin-2 needs to be further investigated with caution. Nevertheless, our studies and others (20, 23) all indicate the importance of kindlin-2/ILK co-localization in mediating cell adhesion.

In summary, we have mapped a major ILK binding site in the kindlin-2 FERM domain, which utilizes a leucine-rich hydrophobic surface to recognize the ILK KLD. The binding mode is distinct from that of another ILK KLD binding protein, α-parvin, thus demonstrating how ILK KLD acts as distinct adaptor to engage different proteins and to mediate FA assembly. Mechanistically, we show that the kindlin-2 binds to ILK via a conserved helix motif, which is crucial for the kindlin-2 localization to FAs and cell spreading (integrin outside-in signaling) but dispensable for the kindlin-2-mediated integrin activation (integrin inside-out signaling). Overall, the data provide significant insights into the kindlin-2/ILK interaction in regulating integrin-mediated cell adhesion.

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