Structural Basis for Paxillin Binding and Focal Adhesion Targeting of β-Parvin

Amy L. Stiegler, Kyle M. Draheim, Xiaofeng Li, Naomi E. Chayen, David A. Calderwood, and Titus J. Boggon

Background: β-Parvin is a cytoplasmic adaptor protein that localizes to focal adhesions.

Results: A direct interaction between β-parvin and paxillin is revealed by biochemistry and crystallography.

Conclusion: Proper β-parvin localization to focal adhesions requires both the paxillin and integrin-linked kinase binding sites.

Significance: Identification of β-parvin binding partners suggests the mechanism of focal adhesion targeting.

β-Parvin is a cytoplasmic adaptor protein that localizes to focal adhesions where it interacts with integrin-linked kinase and is involved in linking integrin receptors to the cytoskeleton. It has been reported that despite high sequence similarity to α-parvin, β-parvin does not bind paxillin, suggesting distinct interactions and cellular functions for these two closely related parvins. Here, we reveal that β-parvin binds directly and specifically to leucine-aspartic acid repeat (LD) motifs in paxillin via its C-terminal calponin homology (CH2) domain. We present the co-crystal structure of β-parvin CH2 domain in complex with paxillin LD1 motif to 2.9 Å resolution and find that the interaction is similar to that previously observed between α-parvin and paxillin LD1. We also present crystal structures of unbound β-parvin CH2 domain at 2.1 Å and 2.0 Å resolution that show significant conformational flexibility in the N-terminal α-helix, suggesting an induced fit upon paxillin binding. We find that β-parvin has specificity for the LD1, LD2, and LD4 motifs of paxillin, with K_D values determined to 27, 42, and 73 μM, respectively, by surface plasmon resonance. Furthermore, we show that proper localization of β-parvin to focal adhesions requires both the paxillin and integrin-linked kinase binding sites and that paxillin is important for early targeting of β-parvin. These studies provide the first molecular details of β-parvin binding to paxillin and help define the requirements for β-parvin localization to focal adhesions.

The parvins (α, β, and γ) are a family of calponin homology (CH) domain-containing adaptor proteins that localize to focal adhesions, where they form complexes with proteins that connect integrin adhesion receptors and the actin cytoskeleton. Each parvin can bind directly to integrin-linked kinase (ILK), which in turn, binds the cytoplasmic adaptor protein PINCH to form the trimeric ILK-PINCH-parvin (IPP) complex (see Fig. 1A) (1–4). The IPP complex forms in the cytoplasm prior to cell adhesion and localizes to focal adhesions. This complex is critical for focal adhesion formation (5). Although ILK binding to integrin β-tails (6) provides one mechanism for targeting the IPP complex to sites of integrin-mediated cell adhesion, a more comprehensive picture of recruitment to focal adhesions requires better understanding of the protein-protein interactions of each component of the IPP complex.

β-parvin (affixin) was first identified as an ILK-binding protein (3) and by homology searching for CH domains (7). β-Parvin expression is enriched in heart and skeletal muscle and is up-regulated late in development (3, 8), whereas the other well studied parvin family member, α-parvin, is ubiquitously expressed throughout development (3, 8). There is overlap of expression in some tissues (9). The loss of α-parvin expression causes embryonic lethality (10), yet mice deficient in β-parvin are viable and fertile, with up-regulated α-parvin expression that may functionally compensate for loss of β-parvin (11). Similarly, knockdown of β-parvin in cells leads to up-regulation of α-parvin expression and vice versa (9). Nonetheless, α- and β-parvin do not have completely redundant functions (12). For example, β-parvin is reported to bind α-actinin (13) and the guanine nucleotide exchange factor α-PIX/ARHGEF6 (14) and is proapoptotic, whereas α-parvin is implicated in Rac1 inhibition and protection of cells from apoptosis (9).

Both α-parvin and γ-parvin directly bind the scaffolding protein paxillin, potentially impacting focal adhesion localization of their respective IPP complexes (1, 15–18). Paxillin is a critical scaffolding protein that facilitates recruitment of multiple proteins to focal adhesions (19). The N terminus contains five leucine-aspartic acid repeat (LD) motifs (Fig. 1A) (20), which act as protein-protein interaction modules. Specific LD motifs are responsible for binding focal adhesion kinase (21–23), proline-
Paxillin Binding and Focal Adhesion Targeting of β-Parvin

The parvin proteins have been described to contain three specific regions: an N-terminal disordered region and tandem calponin homology domains termed CH1 and CH2 (7). Tandem CH domains in other proteins such as filamin and α-actinin comprise an F-actin binding domain; however, the CH domains in parvin lack many of the canonical actin-binding residues (7, 27). The parvin CH2 domain is implicated in ILK binding for all three parvins (2, 3, 18) and paxillin binding for α-parvin and γ-parvin (1, 18). Surprisingly, despite high sequence homology to α-parvin in the CH2 domain and co-localization with paxillin in focal adhesions (3), previous studies indicate that β-parvin does not bind parvin (13).

Here, we present biochemical, biophysical, and crystallographic data that strongly support a direct interaction between β-parvin and paxillin LD motifs. We determine the co-crystal structure of the β-parvin CH2 domain in complex with the paxillin LD1 motif. We also determine crystal structures of β-parvin alone. Together, these structural data provide a detailed understanding of the β-parvin interaction with paxillin and suggest an induced fit in β-parvin upon paxillin binding. The localization of β-parvin to focal adhesions is investigated, and we dissect the impact on targeting of its interactions with both paxillin and ILK. Our results provide significant insight into the mechanism of recruitment of β-parvin to focal adhesions.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Codon-optimized synthetic cDNA (GenScript; Piscataway, NJ) encoding human β-parvin (UniProt no. Q9HBI1) full-length (residues 1–364) or CH2 domain (residues 235–364) was subcloned into a modified pCVDuet vector (Novagen). β-Parvin proteins were expressed as N-terminal hexahistidine (His6)-tagged fusions in E. coli and purified by nickel affinity (HisTrap), anion exchange (Superdex) chromatography. When applicable, the His tag was removed with tobacco etch virus protease. Point mutations were introduced with Quikchange mutagenesis (Agilent). Human paxillin LD motifs (LD1, residues 1–14; LD2, residues 142–155; LD3, residues 214–227; LD4, residues 263–276; LD5, residues 297–310) were generated by insertion of synthetic oligonucleotides 3′ to the GST coding and protease recognition sequence of pGEX-6p-1 (GE Healthcare) by Quikchange mutagenesis (Agilent). Human paxillin LD motifs were expressed in E. coli and purified on glutathione 4B beads (GE Healthcare), eluted with 10 mM reduced glutathione, and further purified by size exclusion chromatography (Superdex 200). For pulldown assays, purified β-parvin and paxillin proteins were reapplied to beads. A synthetic peptide corresponding to human paxillin LD1 (residues 1–20) was purchased from Tufts University Core Facility.

Pulldown Assays—GST-LD motif proteins on glutathione 4B beads were preblocked in 1% BSA. CHO cell lysates were prepared in buffer X (1 mM Na4VO4, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM NaCl, 150 mM sucrose, 10 mM Pipes, pH 6.8) plus 0.5% Triton X-100, 0.1% deoxycholate, and EDTA-free protease inhibitor (Roche Applied Science) and incubated with β-parvin-coated beads. Beads were washed in buffer X plus 0.05% Triton X-100. Bound proteins were eluted, resolved by SDS-PAGE, and transferred to nitrocellulose for probing with anti-paxillin (clone 349, BD Transduction Laboratories no. 610051) or anti-ILK antibodies (Cell Signaling no. 3862). Equal amounts of β-parvin proteins on beads were verified by Coomassie staining.

FIGURE 1. β-Parvin binds directly to LD motifs of paxillin. A, schematic diagram of IPP complex and paxillin binding to β-parvin. ILK ankyrin repeat domain (ARD) binds PINCH1 or PINCH2, and the ILK pseudokinase domain binds α-, β-, or γ-parvin via the CH2 domain. Parvin CH2 also binds paxillin LD1, LD2, and LD4 motifs. B, full-length (FL) β-parvin binds paxillin LD1, LD2, and LD4 directly in GST pulldown experiments. GST alone (GST) serves as negative control. C, β-Parvin binding to LD1 is disrupted in a double mutant LD1 L7R/L8R. Binding of full-length α-parvin to paxillin LD motifs (bottom panel) is included for comparison. All proteins are visualized by Coomassie Blue staining. D, sequence alignment of human paxillin LD motifs (numbered according to human sequence) and the consensus LD motif sequence. The numbering scheme for the LD motif residues is based on Ref. 15.
**Paxillin Binding and Focal Adhesion Targeting of β-Parvin**

**Table 1**

| Data Collection                       | Apo-β-parvin CH2 (PDB code 4EDL)* | Apo-β-parvin CH2 (helix-swapped) (PDB code 4EDM) | β-parvin CH2/paxillin LD1 (PDB code 4EDN) |
|---------------------------------------|-----------------------------------|-------------------------------------------------|------------------------------------------|
| X-ray source                          | NSLS X25                          | NSLS X6A                                        | NSLS X25                                 |
| Wavelength (Å)                        | 1.1                               | 1.0781                                          | 1.1                                      |
| Space group                           | P21                               | P22,2,2;                                        | C2                                       |
| Cell dimensions                       | $a = 80.9, b = 52.6, c = 82.3$ Å;  | $a = 49.4, b = 67.4, c = 105.7$ Å;               | $a = 330.7, b = 55.8, c = 95.6$ Å;       |
|                                       | $α = 90, β = 101.5, γ = 90^\circ$ | $α = 90, β = 90, γ = 90^\circ$                 | $α = 90, β = 97.4, γ = 90^\circ$       |
| Resolution range (Å)$^b$              | 50.0–2.1 (2.18–2.1)               | 50.0–2.0 (2.07–2.0)                             | 50.0–2.9 (2.30–2.9)                      |
| Unique reflections                    | 40,131                            | 24,058                                          | 38,942                                   |
| Redundancy                            | 2.9 (2.5)                         | 7.8 (5.6)                                       | 6.2 (6.0)                                |
| Completeness (%)$^b$                  | 99.3 (97.3)                       | 98.2 (92.5)                                     | 97.9 (100.0)                             |
| $R_{	ext{int}}$ (%)$^b$              | 6.5 (50.1)                        | 8.2 (68.0)                                      | 7.8 (74.2)                               |
| Wilson B-factor                       | 14.4 (2.0)                        | 25.9 (1.9)                                      | 19.8 (2.1)                               |
| Wilson B-factor                       | 32.1                              | 40.1                                            | 85.2                                     |

**Refinement**

| Resolution range$^b$                  | 50.0–2.1 (2.15–2.10)              | 50.0–2.0 (2.05–2.0)                             | 50.0–2.9 (2.975–2.9)                     |
| Total no. (total)                     | 6593                              | 2262                                            | 11,011                                   |
| Protein/peptide                      | 6291                              | 2118                                            | 10,293 (675)                            |
| Water                                 | 234                               | 116                                             | 38                                       |
| Other solvent                         | 68 (17 molecules)                 | 28 (7 molecules)                                | 5 (1 ion)                               |
| Reflections (total)$^b$               | 39,650 (2604)                     | 23,924 (1491)                                   | 38,837 (2616)                           |
| Free (no.)$^b$                        | 1990 (135)                        | 1228 (71)                                       | 1948 (154)                              |
| Free (%)$^b$                          | 5.0 (4.9)                         | 5.1 (4.8)                                       | 5.0 (5.9)                               |
| R factors                             |                                   |                                                 |                                          |
| $R_{	ext{work}}$ (%)$^b$             | 21.5 (28.3)                       | 21.2 (34.4)                                     | 22.8 (30.8)                             |
| $R_{	ext{free}}$ (%)$^b$             | 26.6 (34.3)                       | 25.3 (31.1)                                     | 26.5 (36.5)                             |
| Average B factors                     |                                   |                                                 |                                          |
| Overall                               | 51.9                              | 50.1                                            | 94.8                                     |
| Protein/peptide                      | 51.9/47.6                         | 47.6/112.7                                      | 91.4/112.7                              |
| Water/other solvent                   | 49.4/58.1                         | 56.7/69.6                                       | 62.3/95.0                               |

**Model statistics**

| Ramachandran plot (%)                | 98.3/1.7                          | 98.4/1.6                                        | 98.0/2.0                                |
| MolProbity                           | 0                                  | 0                                               | 0                                        |
| Score                                 | 1.54                              | 1.42                                            | 1.25                                     |
| Percentile                           | 97th                              | 98th                                            | 100th                                    |
| r.m.s.d.                              |                                   |                                                 |                                          |
| Lengths (Å)                          | 0.01                              | 0.013                                           | 0.004                                    |
| Angles                                | 1.3°                              | 1.4°                                            | 0.8°                                     |
| B-factors (main/side)                | 2.0/2.8                           | 2.0/2.8                                         | 2.0/2.3                                  |

*PDB indicates Protein Data Bank.

$^b$ Values in parentheses are for the highest resolution shell.

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Surface Plasmon Resonance—Anti-GST antibody (GE Healthcare; GST capture kit) was coupled to a CM5 sensor chip, and paxillin GST-LD motif fusion proteins were captured to the anti-GST surface, with GST alone captured to the reference cell. β-Parvin CH2 and α-parvin CH2 proteins were prepared as 2- or 3-fold dilution series in running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM EDTA) and injected at 25°C. Binding responses were measured on a Biacore T100 optical biosensor (GE Healthcare) and double-referenced against the signal from the GST reference cell and buffer-only injections. Three independent experiments (including duplicates) with different GST-LD surface densities were performed, and the $K_D$ was calculated with the Biacore T100 Evaluation software.

Crystallization and Structure Solution—Initial crystallization hits were obtained from sparse matrix and grid screens (Qiagen). Apo-β-parvin CH2 P2$_1$ crystals were grown at room temperature by vapor diffusion in hanging drops with precipitant conditions of 18% PEG 4000, 0.1 mM sodium citrate, and 3% (v/v) isopropanol. A 2.1 Å data set was collected at beamline X25 at NSLS BNL. Apo-β-parvin CH2 P2$_2$,2$_2$ crystals were grown at room temperature by microbatch (28) under 100% paraffin oil in crystallization conditions containing 12% (w/v) PEG 550 monomethyl ether and 100 mM Tris, pH 7.5. A 2.0 Å data set was collected at beamline X6A at NSLS BNL. Co-crystals (C2) of β-parvin CH2 with paxillin LD1 motif peptide were grown at room temperature by vapor diffusion in hanging drops with a 1:2:1 molar ratio of peptide:protein with precipitant conditions containing 2.1 M ammonium sulfate and 10 mM Tris, pH 8.5. A 2.9 Å data set was collected at beamline X25 at NSLS BNL. For all three crystal forms, diffraction data were processed with HKL2000 (29). Each structure was determined by molecular replacement using Phaser (30), with the apo-α-parvin CH2 structure (Protein Data Bank code 2VZC) (15) used as a search model. Data collection and refinement statistics are included in Table 1. Analysis of the P2$_1$ apo-reflection data with phenix.xtriage (31) revealed the presence of pseudotranslational symmetry, with a Patterson function off-origin peak at (0.5, 0.0, 0.0) with a height of 76.6% of the origin peak; thus, Zanuda (32) was used to aid in correction of the origin assignment. Automatic model building was performed in ARP/wARP (33) or Buccaneer (34), manual model building was done in Coot (35), and refinement was done in Refmac5 (36) using TLS, NCS, and/or jelly-body refinement. TLS groups were determined using the TLSMD server (37). Good electron density is observed throughout all three structures (data not shown). The paxillin LD1 motif peptides were modeled into unbiased positive $F_{\text{obs}} - F_{\text{calc}}$ electron density maps after the β-parvin CH2...
molecules were refined to convergence. Peptide direction was verified using Coot and by careful analyses of refined models. The register of the peptide was unambiguous based on positive side chain density near the C\(_\beta\)/H9252 position after refinement of a polyalanine peptide.

**Localization Assays**—Chinese hamster ovary (CHO) cells or paxillin-deficient primary mouse embryonic fibroblasts (38) were transfected using polyethylenimine (Sigma) with N-terminally eGFP-tagged \(\beta\)-parvin wild-type, F299D, V256Q mutants, or eGFP alone (pEGFP-C3, Novagen). Full-length paxillin in pcDNA3.1 (39) was transfected in the paxillin-null cells using polyethylenimine (Sigma). 20 h after transfection, cells were detached and replated on coverslips coated with 10 \(\mu\)g/ml fibronectin. Early (6–8 h post replating) and late (24–26 h after replating) time points were taken; cells were simultaneously fixed and permeabilized in 4% paraformaldehyde in PBS, pH 7.4, with 0.1% Triton X-100 for 30 min. Coverslips were washed with PBS containing 0.2% BSA, 0.1% Triton X-100, and 50 mM NH\(_4\)Cl and then incubated with anti-vinculin antibody (Sigma) or anti-paxillin antibody (BD Biosciences) followed by Alexa Fluor 568 anti-mouse secondary antibody, and washed in PBS. Coverslips were mounted using ProLongGold (Invitrogen). Images were acquired using Nikon Eclipse Ti-S with a 100X objective. A Pearson correlation coefficient comparing

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**FIGURE 2. Crystal structure of \(\beta\)-parvin CH2 domain.**

A, sequence alignment of \(\beta\)-parvin CH2 with \(\alpha\)-parvin CH2. Residue numbering for \(\beta\)-parvin and \(\alpha\)-parvin (human) is shown. Secondary structure assignment is depicted above the sequences (determined by DSSP (51)). The figure was created in Aline (52). Residues responsible for paxillin binding are indicated with magenta arrowheads; those that bind ILK are indicated with green arrowheads. B, ribbon diagram of a representative chain of \(\beta\)-parvin CH2 (blue) from the P2, crystal form superposed with the crystal structure of \(\alpha\)-parvin CH2 (gray) (15). The helical assignments and N and C termini are shown. C, details of the \(\alpha\N and \(\alpha\G intramolecular interface, with \(\alpha\N adopting an \(\alpha\)-helical conformation (chain B). Side chains identity of residues involved in the interface are shown. D, \(\alpha\N adopts a 3/10 helix (chain A). E, helix-swapped dimer of \(\beta\)-parvin CH2 in the P2, 2, 2, crystal structure. The two chains are colored blue (chain B) and green (chain A).
the signals of eGFP with vinculin was calculated using the JACoP plugin for ImageJ (40). A minimum of 10 cells were analyzed for each condition to obtain and average Pearson correlation coefficient. We verified by immunoblot that GFP-\(\text{H925}\)-parvin proteins are full-length (data not shown).

RESULTS

\(\text{H925}\)-Parvin Binds Directly to Paxillin LD Motifs—To test whether \(\text{H925}\)-parvin directly binds paxillin, we employed a GST-LD pulldown assay similar to that used previously for \(\text{H925}\)-parvin (1) and other proteins, e.g. CCM3 (25, 26), focal adhesion kinase, vinculin, and GIT1 (21). Recombinant \(\text{H925}\)-parvin protein was expressed and purified, and binding to paxillin GST-LD fusion proteins assessed. \(\text{H925}\)-Parvin protein binds directly to paxillin LD1, LD2, and LD4; however, no binding is detected to LD3 or LD5 (Fig. 1B). The binding profile of \(\text{H925}\)-parvin to LD1, LD2, and LD4 parallels that of \(\text{H925}\)-parvin (Fig. 1B) (15). We also show by pulldown analysis that the CH2 domain of \(\text{H925}\)-parvin is sufficient to bind paxillin LD1, LD2, and LD4 (Fig. 1C). Thus, we conclude that \(\text{H925}\)-parvin binds paxillin LD-motifs directly via its CH2 domain. We next asked whether \(\text{H925}\)-parvin binding utilizes the typical protein binding surface on LD motifs, which adopt an \(\alpha\)-helical conformation and present a hydrophobic stripe comprised of residues at the 0, 3, and 7 positions (Fig. 1D) to bind hydrophobic surfaces on LD-binding proteins. We show that a double mutant GST-LD1

**FIGURE 3. Alignment of \(\text{H925}\)-parvin CH2.** Secondary structure elements \(\alpha\)-helices (cylinder) and 3/10 helices (ribbon) are shown above the sequence and labeled, determined by DSSP (51). Paxillin-binding residues (magenta) and CH2-binding residues (green) are indicated with arrowheads. The sequence alignment for human \(\text{H925}\)-parvin CH2 is shown. Consensus sequence for \(\text{H925}\)-parvin CH2 is included, as are aligned sequences from the single parvin in Drosophila melanogaster and Caenorhabditis elegans, \(\text{H925}\)-parvin and \(\text{H925}\)-parvin, and filamin B. Identical residues are highlighted in yellow. Latin names (listed in alignment), UniProt accession no. (except where otherwise noted) and common names are as follows: Homo sapiens (Q9HBI1, human), Mus musculus (Q3UGT9, mouse), Rattus norvegicus (D3ZKG5, rat), Macaca mulatta (F6Y5C6, Rhesus macaque), Bos taurus (A6QLQ6, bovine), Sus scrofa (NCBI XP_003481567.1, pig), Equus caballus (F6XV19, horse), Canis familiaris (F1PSS5, dog), Monodelphis domestica (F7B4W5, opossum), Cavia porcellus (H0VUI1, guinea pig) Heterocephalus glaber (G5BKH3, naked mole rat), Callithrix jacchus (F6T6X4, marmoset), Gallus gallus (E1C891, chicken), Xenopus tropicalis (Q6GLA6, frog), Xenopus laevis (GeneID 734573, African clawed frog), Danio rerio (Q6PC42, zebrafish), Caligus rogercresseyi (C1BRG2, sea louse), Tetraodon nigroviridis (Q4RSJ9, pufferfish), Camponotus floridanus (E2AFR6, Florida carpenter ant), Culex quinquefasciatus (B0W9D1, mosquito), Drosophila melanogaster (Q9WD0, fruit fly), Caenorhabditis elegans (O16785), and filamin B (Homo sapiens, O75369). Alignments were performed by ClustalW (53); interacting residues were defined by PISA server (42); and the figure was made in Aline (52).

the signals of eGFP with vinculin was calculated using the JACoP plugin for ImageJ (40). A minimum of 10 cells were analyzed for each condition to obtain and average Pearson correlation coefficient. We verified by immunoblot that GFP-\(\text{H925}\)-parvin proteins are full-length (data not shown).

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with mutations at the +3 and +4 positions (L7R/L8R) is unable to bind β-parvin (Fig. 1B).

Overall Structure of β-Parvin CH2 Domain—To investigate the molecular basis for β-parvin interaction with paxillin LD motifs, we determined crystal structures of β-parvin CH2 alone and in complex with a paxillin-derived LD1 motif peptide. We obtained two crystal forms of apo-β-parvin CH2, and determined these structures at 2.1 and 2.0 Å resolution. The 2.1 Å P21 crystal form has six molecules in the asymmetric unit, and the 2.0 Å P212121 crystal has two molecules in the asymmetric unit (Table 1). We determined the structure of the β-parvin CH2 domain in complex with paxillin LD1 peptide to 2.9 Å resolution by co-crystallization. This structure contains 10 copies of β-parvin CH2 in the asymmetric unit (Table 1). These three crystal structures represent the first atomic level descriptions of β-parvin.

Overall, our crystal structures reveal eight α-helices in the β-parvin CH2 domain, four of which comprise the common CH domain core; αC and αG arranged in parallel, sandwiched on either side by αA and αE. The protein core is capped by shorter helices (αB, αD, and αF) in an overall arrangement that is conserved among other CH domain folds (Fig. 2, A and B) (27). β-Parvin CH2 also contains an additional N-terminal helix, αN, comprising residues 240–249. This is similar to the N-terminal helix observed in α-parvin CH2 and is atypical among CH domains (15, 17). In β-parvin CH2, αN interacts with αA and αG via an extensive intramolecular interface comprising both van der Waals contacts (Phe-242, Leu-245, Phe-246 in αN; Lys-252, Leu-253, Val-256 in αA; Leu-346 and Leu-350 in αG) and electrostatic interactions (Asp-240 with Arg-351 and Asp-243 with Arg-351). These residues are highly conserved (Figs. 2C and 3). The primary sequences of β-parvin and α-parvin CH2 domains differ at only 14 residues with no gaps (Fig. 2A), and their structures superpose very well with root mean square deviation values ranging between 0.6 and 1.3 Å over 127 equivalent Ca positions (Fig. 2B). Using the Dali server (41), the closest non-parvin structural neighbor to β-parvin CH2 is the CH1 domain of filamin B, which lacks αN (root mean square deviation is 1.7 Å over 110 equivalent Ca positions; 22% identity) (Fig. 3).

Structure of Apo-β-parvin CH2 Domain—We determined two crystal structures of apo-β-parvin CH2 domain. In the asymmetric units of our apo-β-parvin CH2 structures, there are eight β-parvin chains: six in the P21 crystal form and two in the P212121 crystal form. The largest differences between these chains are in the conformation and position of αN. In the P21 crystal form, helix αN is found both as an α- and a 3/10 helix (Fig. 2, C and D). In the P212121 crystal form, it engages in intermolecular helix-swapping (Fig. 2E), which we do not expect to represent a favorable conformation because β-parvin CH2 behaves as a monomer in solution (data not shown). Thus, the β-parvin αN helix possesses inherent conformational flexibility when not in complex with a binding partner.

Co-crystal Structure of β-Parvin CH2 Domain in Complex with Paxillin LD1 Peptide—We also determined the co-crystal structure of β-parvin CH2 domain in complex with the LD1 motif of paxillin. We find that paxillin LD motifs bind a conserved hydrophobic patch on the surface of β-parvin CH2.

**FIGURE 4. Co-crystal structure of β-parvin CH2 bound to paxillin LD1 motif.** A, structural overview of β-parvin CH2 (green) in complex with paxillin LD1 motif peptide (magenta). B, specific interaction of β-parvin CH2/LD1 binding, with molecules colored as in A. Side chains at the interface are shown and labeled, with Val-256 at the center of the interface in yellow. C, surface electrostatic potential for β-parvin CH2 shows that paxillin LD1 binds a largely hydrophobic binding site. D, final refined 2Fo − Fc electron density map for paxillin LD1 (chain K) contoured to 2σ (cyan) and 1σ (blue). E, surface conservation of paxillin binding site on β-parvin (by CONSURF) (54). F, β-parvin V256Q mutant is unable to bind paxillin LD motifs. GST pulldown assays examine the direct binding of purified full-length (FL) β-parvin wild-type (WT; top) or V256Q mutant (bottom) proteins to paxillin LD motif fusion proteins (GST-LD) or GST alone (GST) as negative control. Proteins are visualized by Coomassie Blue staining.
domain that is juxtaposed between helices αN, αA, and αG (Fig. 4, A and B). The paxillin LD1 peptide adopts a helical conformation, with four residues (Leu-4, Leu-7, Leu-8, and Leu-11) forming a hydrophobic surface that binds the hydrophobic patch of -parvin CH2 (comprising αN residues Ala-241, Phe-242, Thr-244, Leu-245, and Ala-249; αA residues, Lys-252, Val-255, Val-256, Ser-259, Leu-260; and αG residues Tyr-354 and Phe-357) (Fig. 4, B and C). Hydrogen bonding is observed between Asp-10 (in LD1) and Tyr-354/Lys-361 (in -parvin) (Fig. 4B). Analysis of 20 -parvin sequences (Fig. 3) shows that the paxillin binding site is extremely well conserved (Fig. 4E). The interface buries a total surface area of ~990 Å² (on average 450 Å² on CH2 and 540 Å² on LD1, determined with Pisa (42)). As α-parvin CH2 domain is reported to bind paxillin LD motifs in both “forward” and “reverse” orientations (15), we analyzed the direction of binding for -parvin CH2 with paxillin LD1. In our structure, paxillin LD1 is observed in the forward direction in six of the seven modeled peptides. This is the same direction as paxillin LD1 binding to α-parvin. In the seventh LD1 peptide, we observed a perpendicular orientation of paxillin LD1 and additional contacts with three neighboring -parvin CH2 domains (data not shown), which we interpreted as a crystal packing artifact. This crystal structure therefore provides a clear description of the molecular basis for paxillin recognition by β-parvin.

To verify the binding site for paxillin LD motifs, we introduced a single point mutation in -parvin at Val-256. This result is shown in Figure 5. 

**FIGURE 5. β-Parvin binds paxillin LD1 motif via an induced fit interaction.** A, structural comparison of β-parvin CH2 (green) bound to paxillin LD1 (magenta) with β-parvin CH2 alone (gray). Close-up view shows the details of the conformational change in αN upon binding of paxillin LD1. B, superposition of eight chains of β-parvin CH2 from our two apo-crystal structures shows conformational flexibility in αN in the absence of a binding partner. C, superposition of 10 chains of β-parvin CH2 from our co-crystal structure with paxillin LD1 motif peptide (gray). The common conformation of αN in all 10 copies indicates that an induced fit occurs on paxillin LD motif binding.

**TABLE 2**  
Dissociation constants (K_d) of parvin binding to paxillin LD motifs measured by SPR

| Paxillin LD motifs | K_d (μM) | α-Parvin CH2 | β-Parvin V256Q |
|-------------------|---------|-------------|---------------|
| LD1               | 27 ± 1  | 53 ± 2      | N.D.          |
| LD2               | 42 ± 1  | 76 ± 4      | N.D.          |
| LD4               | 73 ± 7  | 55 ± 2      | N.D.          |

K_d values (±S.D.) measured from average of three independent experiments with different surface densities of GST-LDs.  
N.D. Not detected. No binding of β-parvin CH2 V256Q to LD1, LD2, or LD4 was detected.

**FIGURE 6. Binding of β-parvin to paxillin and ILK are independent.** β-Parvin binds both paxillin and ILK from cell lysates, and the binding can be selectively disrupted by mutation in β-parvin. Ni²⁺ beads coated with His-tagged β-parvin CH2 WT or mutants F299D or V256Q protein were used in pulldowns with lysates from CHO cells. β-Parvin coating of beads is visualized with Coomassie Blue staining, whereas bound paxillin and ILK are detected by immunoblotting with anti-paxillin or anti-ILK antibodies. Uncoated Ni²⁺-agarose beads (Ni²⁺) were included as negative control.
idue is located in αA at the center of the conserved hydrophobic binding patch and is glutamine in the CH domains of filamin (Fig. 3) and α-actinin, which do not bind paxillin (1). Mutation of Val-256 to glutamine (V256Q) disrupts β-parvin binding to paxillin LD1, LD2, and LD4 motifs (Fig. 4F). This indicates that paxillin LD1, LD2, and LD4 all bind the same location on

**FIGURE 7. Disruption of the paxillin or ILK binding sites alters localization of β-parvin to focal adhesions.** A, CHO cells transfected with expression vectors encoding GFP β-parvin (green), WT, or mutants V256Q or F299D were plated on fibronectin-coated coverslips and stained with anti-vinculin antibody (red) as a marker for focal adhesions for either 8 h (top, early) or 24 h (bottom, late) after plating. Merged images show co-localization. β-Parvin V256Q, which is deficient in paxillin binding, shows delayed localization to focal adhesions, whereas β-parvin F299D, a mutant that lacks ILK binding, is deficient in focal adhesion localization for both time points. Scale bar, 10 μm. B, Pearson correlation coefficients of eGFP colocalization with vinculin for empty vector (eGFP), WT, F299D and V256Q eGFP β-parvin constructs. Results are mean ± S.E. for 10 random profiles from at least 10 cells. Statistical significance is indicated above the bars. *p values were calculated using the Student’s t test. An asterisk indicates p value < 0.005.
Paxillin Binding and Focal Adhesion Targeting of β-Parvin

A

Paxillin /- MEF  Paxillin /- MEF + Paxillin

β-parvin-WT eGFP control  β-parvin-WT eGFP control

GFP-β-parvin

Paxillin

Merge

Early

Vinculin

Merge

Late

B

Paxillin Coefficient GFP & Vinculin

Early

Late

Late+Plac

Early+Plac

β-parvin WT

GFP
β-parvin CH2 domain. Because a single point mutation in the CH2 domain of full-length β-parvin is sufficient to disrupt binding, we conclude that β-parvin contains a single paxillin LD motif binding site.

**Induced Fit in β-Parvin on Binding Paxillin LD Motifs**—The overall structure of the β-parvin CH2 domain core (helices αA through αG, residues 250–364) is very similar between the 18 chains that we have modeled, both in the absence and presence of bound LD1 (root mean square deviation is 0.5 to 1.1 Å over 115 equivalent Cα positions calculated with Superpose (Fig. 5A) (43)). Interestingly, we note that although helix αN is conformationally divergent in the apo-β-parvin structures, it is stabilized to an invariant conformation when bound to the paxillin LD1 motif (Fig. 5, B and C). Upon LD binding, αN is converted to an α-helix (residues Ala-241–His-248), shifts translationally toward αA/αG, and rotates about its helical axis so that Phe-242 and Leu-245 line the bottom of the hydrophobic surface (Fig. 5A). The conformational heterogeneity of helix αN in the apo-β-parvin CH2 domain structures indicates that helix αN is not intrinsic to CH2 domain folding for β-parvin and may suggest that the relative orientation of CH1 and CH2 domains could be impacted upon CH2 binding to LD motifs.

**Binding Affinities of β-Parvin CH2 for Paxillin LD1, LD2, and LD4**—We next used surface plasmon resonance to measure the solution binding affinities of paxillin LD motifs for β-parvin (Table 2). By surface plasmon resonance, we find that the highest affinity ligand of β-parvin CH2 is LD1, measured at 27 μM, while binding to LD2 is 42 μM (a 1.5-fold decrease; \( p = 0.00001 \)) and binding to LD4 is 73 μM (a near 3-fold decrease compared with LD1; \( p = 0.0002 \)). The differences in affinities of β-parvin among LD1, LD2, and LD4 may reflect a preference for binding specific paxillin LD motifs in the cell. β-parvin CH2-V256Q used in pulldown assays (Fig. 4F) does not interact with LD1, LD2, or LD4 by surface plasmon resonance (Table 2). We also measured binding affinities of α-parvin CH2 to paxillin LD motifs. These are comparable with β-parvin CH2 and similar to values published previously (\( K_d \) in the 30–200 μM range) (15). Furthermore, the affinities of LD motifs for other paxillin binding proteins, e.g. focal adhesion kinase (1–9 μM) (26, 44–46), CCM3 (17–39 μM) (26), and Pyk2 (45 μM) (44) are similar to β-parvin CH2, indicating that β-parvin is a paxillin binding protein.

**β-Parvin Independently Binds Both Paxillin and ILK**—To confirm that β-parvin CH2 binds full-length endogenous paxillin from cell lysates, we conducted pulldown experiments. We find that wild-type β-parvin CH2 pulls down paxillin but that β-parvin CH2 V256Q mutant, which is deficient in binding LD1, LD2, and LD4 (Fig. 4F), does not bind paxillin from cell lysates (Fig. 6). Because ILK has also been reported to bind paxillin (16, 47), loss of endogenous paxillin pulldown by β-parvin mutant V256Q could be due to loss of ILK binding; however, ILK is pulled down from cell lysates by both wild-type and V256Q mutant β-parvin (Fig. 6). Thus, the β-parvin CH2 V256Q mutant is defective in direct paxillin binding. This supports our conclusion that β-parvin binds paxillin via a CH2-LD motif interaction.

We next sought to confirm that the paxillin and ILK binding sites in β-parvin are independent in a manner similar to that predicted for α-parvin (16, 48). Therefore, guided by sequence alignment (Fig. 2A) and homology modeling with the structure of α-parvin in complex with ILK (48), we introduced a single point mutant in β-parvin CH2, F299D, corresponding to α-parvin Phe-307, which is deeply buried in the ILK interface (data not shown) (48). We find that β-parvin CH2 mutant F299D is deficient in ILK binding but retains the ability to bind paxillin (Fig. 6). We therefore conclude that the binding sites on β-parvin CH2 for paxillin and ILK are independent and that these interactions can be selectively disrupted by point mutation in β-parvin CH2.

**Proper Localization of β-Parvin to Focal Adhesions Requires Both the Paxillin- and ILK-binding Sites**—The CH2 domain of β-parvin mediates its localization to focal adhesions (2, 3). β-Parvin binds both paxillin (Figs. 1 and 4) and ILK (Fig. 6) (3), proteins that facilitate localization of binding partners to focal adhesions (for example, focal adhesion kinase (49), and PINCH (50)). Therefore, we asked whether focal adhesion targeting of β-parvin is facilitated by the paxillin-binding site, the ILK-binding site, or both. We expressed wild-type β-parvin, paxillin-binding mutant V256Q, or ILK-binding mutant F299D as GFP fusions in CHO cells, detected β-parvin localization to focal adhesions by fluorescence microscopy, and quantified β-parvin targeting as Pearson correlation coefficients (\( r \)) for colocalization with the focal adhesion marker vinculin (a value of +1 indicates perfect correlation, 0 indicates no correlation, −1 indicates inverse correlation). As expected based on previous studies (3), wild-type GFP β-parvin strongly targets to focal adhesions, evident in the early post-plating time point and remaining in the late (Fig. 7, A and B; early \( r = 0.60 \); late \( r = 0.73 \)). In contrast, localization of paxillin-binding defective mutant β-parvin V256Q is markedly affected: at early time points, β-parvin V256Q localization to focal adhesions is weak (\( r = 0.28 \)) yet is almost restored for the late time point (Fig. 7, A and B; \( r = 0.58 \)). The delay in focal adhesion targeting of paxillin binding defective mutant β-parvin V256Q suggests that the paxillin binding site is important for early localization but not required for ultimate targeting of β-parvin to focal adhesions. We next asked whether the ILK binding site is critical for focal adhesion targeting of β-parvin and observed that unlike wild-type, the F299D mutant β-parvin fails to target to focal adhesions at both early and late time points (Fig. 7, A and B; \( r = 0.14 \) and 0.11, respectively). These values are not different from GFP alone (\( r = 0.14 \) and 0.15). These data indicate that the LD binding site in β-parvin is important for early targeting to focal adhesions and that the ILK binding site is required.

**FIGURE 8. Paxillin is important for early targeting of β-parvin to focal adhesions.** A, left, paxillin (Pax)-deficient mouse embryonic fibroblasts (38) were transfected with GFP β-parvin (green) constructs, plated on fibronectin, and stained with either anti-paxillin (top) or anti-vinculin antibody (red, middle and bottom) 6 h (top, early) or 24 h (bottom, late) after plating. Right, paxillin-deficient fibroblasts were co-transfected with GFP β-parvin and paxillin expression constructs. Scale bar, 10 μM. B, Pearson correlation coefficient of eGFP colocalization with vinculin for empty vector (eGFP) and WT β-parvin constructs. An asterisk indicates \( p \) value < 0.005.
Paxillin Binding and Focal Adhesion Targeting of β-Parvin

We next asked whether paxillin binding facilitates targeting of β-parvin to focal adhesions. We transfected GFP-β-parvin into paxillin-deficient fibroblasts (38) and find that in the absence of paxillin, wild-type β-parvin targeting is impaired at early time points (r = 0.36; Fig. 8, A and B); however, when paxillin expression is restored in these cells, early localization more closely correlates with focal adhesions (r = 0.64; Fig. 8, A and B). Additionally, as predicted based on our results in CHO cells (Fig. 7), wild-type β-parvin localizes properly to late focal adhesions both in the absence of paxillin and when paxillin expression is restored (Fig. 8, A and B). Therefore, paxillin facilitates the early targeting of β-parvin to focal adhesions. To ensure that GFP-β-parvin is localizing to paxillin containing focal adhesions in the reconstituted cell, we also stained for paxillin. Expectedly, wild-type β-parvin co-localizes with paxillin in the reconstituted cell at both the early (Fig. 8A) and late (data not shown) time points. We also confirm that parental cells lack paxillin (Fig. 8A). Also, as expected based on our previous data (Fig. 7), localization of β-parvin LD-binding mutant V256Q to early focal adhesions in both paxillin-deficient and paxillin-rescued cells is impaired yet is normal at later time points, whereas targeting of ILK-binding mutant β-parvin F299D is disrupted at both early and late time points in both cell types (data not shown). Our results indicate that paxillin binding is important for early recruitment of β-parvin to focal adhesions.

DISCUSSION

In this study, we have used biochemical, biophysical, and crystallographic techniques to demonstrate for the first time that β-parvin directly binds paxillin and provide evidence that this interaction is important for early recruitment of β-parvin to focal adhesions. We discovered a single binding site for paxillin that is analogous to the paxillin binding site in α-parvin. This binding surface is extremely well conserved through evolution and among parvin family members (Fig. 3), suggesting that paxillin binding is a conserved function shared among the parvins.

Our studies provide a framework to understand the mechanism of β-parvin targeting to focal adhesions. We demonstrate that both the LD and ILK binding sites are required for proper focal adhesion localization of β-parvin and that ILK is an obligate binding partner for β-parvin to localize to focal adhesions. Our results, however, also show that a paxillin binding mutant is delayed in its localization, indicating that, in addition to ILK binding, paxillin binding contributes to targeting β-parvin to early focal adhesions. Indeed, in the absence of paxillin, β-parvin targeting to early focal adhesions is also impaired (Fig. 8). These results point toward the conclusion that interaction of β-parvin with paxillin is important for early recruitment to focal adhesions. We cannot, however, rule out that β-parvin binds another protein via its LD binding site because β-parvin targeting to focal adhesions in paxillin-deficient cells is weak but not absent (Fig. 8). LD motifs are present in the paxillin-related proteins leupaxin and Hic-5 (20), raising the possibility that β-parvin may bind these proteins directly via its paxillin binding site. Paxillin LD1, LD2, and LD4 motifs are highly similar in sequence to those in Hic-5 and paxillin (20), and α-parvin reportedly binds Hic-5 (1) and has been postulated to bind leupaxin (15). Hic-5 is up-regulated in paxillin-null cells and is localized to focal adhesions (38), consistent with the notion that β-parvin may bind Hic-5 directly in addition to paxillin.

We propose a model by which paxillin and ILK facilitate distinct temporal aspects of β-parvin targeting to focal adhesions. Prior to cell adhesion, β-parvin binds ILK as part of the IPP complex, which targets to early focal adhesions. During this initial recruitment stage, the LD binding site of β-parvin engages paxillin in focal adhesions, which helps to stabilize β-parvin at these sites. Thus, β-parvin localization is dynamically regulated at least in part by binding both paxillin and ILK. The significance of defining β-parvin as a paxillin binding protein has implications in other aspects of β-parvin signaling. For example, only β-parvin is reported to bind α-actinin (13) and α-PIX/ARHGEF6 (14). Thus, it will be important to determine whether these or other binding activities of β-parvin are affected by paxillin binding and whether paxillin-mediated early focal adhesion targeting of β-parvin affects its role in regulating the actin cytoskeleton (through binding α-actinin) or Rac1 signaling (through binding PIX).

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