The Structure of α-Parvin CH2-Paxillin LD1 Complex Reveals a Novel Modular Recognition for Focal Adhesion Assembly*

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α-Parvin is an essential component of focal adhesions (FAs), which are large multiprotein complexes that link the plasma membrane and actin cytoskeleton. α-Parvin contains two calponin homology (CH) domains, among which C-terminal CH2 domain binds multiple targets including paxillin LD motifs for regulating the FA network and signaling. Here we describe the solution structure of α-parvin CH2 bound to paxillin LD1. We show that although CH2 contains the canonical CH-fold, a previously defined N-terminal linker forms an α-helix that packs unexpectedly with the C-terminal helix of CH2, resulting in a novel variant of the CH domain. Importantly, such packing generates a hydrophobic surface that recognizes the Leu-rich face of paxillin-LD1, and the binding pattern differs drastically from the classical paxillin-LD binding to four-helix bundle proteins such as focal adhesion kinase. These results define a novel modular recognition mode and reveal how α-parvin associates with paxillin to mediate the FA assembly and signaling.

The adhesion of cells to the extracellular matrix is mediated by focal adhesions (FAs), which are large, dynamic protein complexes that contain integrin transmembrane receptors and many other associated proteins (1–3). FAs function by mechanically linking the extracellular matrix to the actin cytoskeleton, thereby promoting strong attachment of cells to the extracellular matrix. They also act as a signaling machinery that transmits diverse signals between extracellular matrix and actin, regulating a variety of cellular processes such as cell migration, proliferation, and differentiation. Extensive genetic and cell biology studies have led to the identification of many FA proteins that have either scaffolding or signaling properties or both (4, 5).

Interestingly, all of these proteins are found almost exclusively as multidomain proteins that engage in multiple protein-protein interactions, leading to the formation of complex FA interaction network. The spatiotemporal assembly and disassembly of this network during various cell adhesive processes has been the subject of intense studies over the past decades (1–2, 5), but the structural basis for how specific domains recognize each other to form the multiprotein FA complexes remain poorly understood.

Parvins are a family (α,β,γ) of adaptor proteins (~42 kDa) found in FAs (for review, see Refs. 6 and 7). They contain two tandem copies of calponin homology (CH) domains and have been shown to play crucial roles in mediating the FA assembly and cell adhesion regulation via their CH domains, primarily the C-terminal CH2 (Fig. 1A). However, parvin CHs have very low sequence homology to conventional CH domains, which led to their classification as a distinct subfamily of CH-containing proteins (8). Among parvins, α-parvin (also called actopaxin or CH-ILKBP) is the most extensively characterized (6). α-Parvin binds multiple proteins including paxillin, a well known FA regulator that contains five distinct N-terminal Leu-Asp (LD)-rich motifs (Fig. 1B) and four C-terminal LIM domains (9, 10). The paxillin LD1 and LD4 motifs have been shown to trigger the early formation of focal adhesions (focal complexes) by specifically interacting with α-parvin CH2 (11). On the other hand, the paxillin LD motifs are known to interact with a class of four helix bundle-containing FA proteins including FAK, vinculin, and paxillin kinase linker (PKL) (10). The paxillin LD binding to FAK FAT has been extensively characterized at the structural level (12–16). A universally conserved paxillin binding subdomain (termed PBS) for the LD binding has been thought to exist in all paxillin LD-binding proteins (17) including α-parvin CH2 (11). However, the conventional CH domain is not a four-helix bundle (18, 19), and the recognition of α-parvin CH2 by paxillin LDs to mediate specific focal complex formation remains unclear.

As part of our effort to dissect the mechanism of the supramolecular FA assembly involving diverse protein-protein recognitions, we set out to investigate the molecular details of the α-parvin CH2/paxillin LD interaction. We show here that although α-parvin CH2 largely exhibits the canonical CH-fold, its N-terminal linker forms an α-helix that packs unexpectedly with the C-terminal helix of CH2, resulting in a novel variant of the CH domain. Importantly, such packing generates a hydro-
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phobic surface that is highly conserved in the parvin family for recognizing the paxillin LD motifs. The LD binding pattern shown here is distinct from previously reported parvin LD binding patterns to the four-helix bundle protein FAK, and the LD binding site also differs from the previously defined PBS. Thus, our results define a novel mode of modular recognition and reveal how α-parvin spatially associates with paxillin to mediate the specific multiprotein complex for FA assembly and signaling.

EXPERIMENTAL PROCEDURES

Sample Preparation—DNA encoding for human α-parvin amino acid residues 222–372 and 244–372 were inserted into PET3a vector and PET15b (Novagen), respectively, transformed into Escherichia coli BL21(DE3), grown at 37 °C in minimal media to an A660 of 0.6–0.8, induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and then left to grow for 3 h. 15N- and 15N/13C-labeled proteins were obtained by growing cultures in minimal media with 1.1 g of [15N,13C]glucose as nitrogen and carbon sources, respectively. Cells were harvested by centrifugation, resuspended in 100 ml of lysis buffer (50 mM Tris–Cl, 50 mM NaCl, 1 mM EDTA, and 25 mg lysozyme, pH 8.0), then lysed by French Press twice. α-Parvin-(222–372) was purified with a DEAE column (GE Healthcare) using 50 mM Tris–Cl, pH 8.0, and a 0–1 M NaCl gradient. Fractions containing the target protein were concentrated and further purified by Superdex 75 (Amersham Biosciences) using 100 mM NaCl, 50 mM sodium phosphate buffer, pH 6.8. α-Parvin-(244–372) (α-parvin-C, see the text) with an N-terminal His tag was purified by nickel resin followed by the cleavage of the His tag and gel-filtration using Superdex G-75. Note that there is a natural Factor Xa cleavage site on 243–244 as well as the transferred NOE experiment where 1 mM LD1 was mixed with 10% 15N/13C-labeled peptide in our transferred NOE experiment and two-dimensional NOESY (mixing time 400 ms). Note that because of its small size (10 residues, ~1.0 kDa), the free LD1 peptide is like a small molecule with a fast tumbling rate, exhibiting almost no NOEs in our two-dimensional NOESY experiment (data not shown). However, when bound to CH2, we were able to see NOEs for the peptide in our transferred NOE experiment and two-dimensional filtered NOESY experiment. These NOEs, which include sequential NH–NH1+i connectivity characteristic of α-helix, must be from the bound peptide, were then used for structure calculations.

All NMR data were processed using NMRPipe (21) and analyzed by PIPP (22). For chemical shift mapping, weighted chemical shift changes were calculated using the equation: \[ \Delta \delta_{\text{obs}}(\text{HN},N) = (\Delta \delta_{\text{HN}} W_{\text{HN}})^2 + (\Delta \delta_{\text{N}} W_{\text{N}})^2 )^{1/2}, \] where \( W_{\text{HN}} = 1 \) and \( W_{\text{N}} = 0.154 \) are weighting factors based on the gyromagnetic ratios of \( ^1H \) and \( ^15N \). \( K_0 \) was calculated using the approach as previously described (23).

Paramagnetic Spin-labeling Experiment—The cysteine-specific spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate, MTSSL) was purchased from Sigma, Inc. MTSSL was attached to the synthetic N-ter-Cys-LD1 peptide using the following procedure: 1.0 mM LD1 peptide and a 10-fold excess of MTSSL were mixed and stirred for 12 h in a 4:1 (v/v) solution of 130 mM NaCl, 20 mM sodium phosphate buffer (pH 7.2), and acetonitrile. Spin-labeled LD1 was then purified.
by reverse-phase high-performance liquid chromatography and confirmed by mass spectroscopy.

Structure Calculations—Structure calculation for the \( \alpha \)-parvin-C/LD1 complex was performed using a combination of HADDOCK (23) and XPLOR-NIH (24). First, the structures of \( \alpha \)-parvin-C and LD1 were calculated separately using Xplor-NIH. The constraints included NOE-derived distances that had upper bounds of 2.5, 3.5, and 5.0 Å and the backbone \( \psi \), \( \varphi \) angles obtained from program TALOS. Although the intermolecular NOEs from the \( \alpha \)-parvin-C side can be unambiguously assigned, the majority of intermolecular NOEs involving LD1 Leu methyls are quite degenerate. However, we know that every Leu is involved in binding based on our mutagenesis data. Thus, we decided to first use the HADDOCK program to dock LD1 onto \( \alpha \)-parvin-C. HADDOCK represents high ambiguity driven protein-protein docking, which is based on experimental data such as chemical shift perturbation data from NMR titration experiments or mutagenesis data (25). Based on the chemical shift mapping data and intermolecular NOEs, we defined 10 active residues on \( \alpha \)-parvin-C: Ala249, Thr252, Leu253, His256, Asp259, Lys260, Val264, Lys266, Ile269, and Glu287, and four passive residues: Asp251, Asp255, Phe257, and Val262. Four leucines in LD1 (Leu4, Leu7, Leu8, and Leu11) were defined as active residues, whereas the other six residues were passive residues. The side chains of all LD1 residues and \( \alpha \)-parvin-C residues 244–272 were allowed for rearrangement. 1000 structures were calculate after rigid-body docking, followed by 200 structures after semiflexible simulated annealing, and 200 structures after water refinement. The final 200 structures by HADDOCK were found to converge quite well except that the bound LD1 was found in two opposite orientations (both orientations are roughly 50%). This was probably due to the ambiguous Leu constraints from LD1, which do not differentiate the two LD1 orientations. However, our spin labeling data readily eliminated one orientation because the N-terminal end of LD1 is spatially close to the linker especially Ala249 and Phe257 and the surrounding residues (supplemental Fig. S1). Using this HADDOCK-based structure, we were able to distinguish between previously ambiguous NOEs derived from the LD1 side chains and further refine the structure. Hydrogen-bond restraints were incorporated during final stage of calculations based on hydrogen-exchange data and secondary-structure elements identified from previous rounds of structure calculations. Table 1 lists the detailed structural statistics of 20 final structures with the lowest energies. The PDB code for the final 20 structures and the NMR constraints is 2K2R. The BMRB accession code for the chemical shift assignments is 15760. Molecular images were drawn using PyMOL from The PyMOL Molecular Graphics System.

RESULTS

Structural Characterization of the \( \alpha \)-Parvin/Paxillin Interaction—Previous biochemical and cell biology studies have shown that the C-terminal fragment of \( \alpha \)-parvin-(222–372) binds not only to paxillin LD1, but also to LD4 (11). Structure-based sequence analysis designated residues 222–263 to be part of a 60-residue linker between CH1 and CH2, with residues 264–372 forming CH2 (26). However, although \( \alpha \)-parvin CH2 is highly conserved in the parvin family, it has very low sequence homology to other actin-binding CH2 domains (<20% sequence identity, see Fig. 1A) and ill-defined structural bound-
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(a) and (b) to map the precise domain boundaries, we performed $^1$H-$^{15}$N heteronuclear single quantum coherence analyses of α-parvin-(222–372) in conjunction with backbone resonance assignment. Residues 222–245 were found to be unstructured, exhibiting very intense and sharp signals in the narrow 7.5–8.5 ppm region, and addition of neither paxillin LD1 nor LD4 affected this region. However, residues from position 246 onwards are structured as judged from the NMR spectrum. This was confirmed by limited protease digestion and sequencing, which revealed a compact structure starting at Asp$^{248}$, 16 residues longer than previously suggested by the CH2 domain (264–372) (26). All subsequent structural/biochemical analyses were therefore carried out on α-parvin-(244–372) (termed α-parvin-C). Chemical shift mapping analysis of LD1 binding revealed that the N and C termini of α-parvin-C were significantly affected, with the linker region 248–256 being mostly perturbed (Fig. 2A) at the $K_D \sim 1.2 \times 10^{-3}$ M (supplemental Fig. S2). A similar pattern was also observed for the LD4/α-parvin-C interaction (Fig. 2B), but binding was weaker as the extent of the chemical shift changes was significantly smaller than observed for LD1. This result is consistent with earlier biochemical analysis by which α-parvin was found to bind primarily to paxillin LD1 and secondarily to LD4 (11). However, the strong perturbation of linker region 248–256 by LDs was unexpected given that the LD binding site was indicated to reside in the so-called PBS region (residues 277–285) based on sequence analysis and mutagenesis (11). However, we noticed that the PBS region had little chemical shift perturbation upon LD1/LD4 binding (Fig. 2, A and B).

Further corroborate our finding, we performed a spin labeling experiment using a paramagnetic spin-label (MTSSL) attached to the N terminus of LD1. Consistent with the chemical shift mapping result, the spin-labeled LD1 dramatically broadened resonances in the linker 248–256 region but not the 277–285 region (supplemental Fig. S3a versus S3b).

We also evaluated the importance of leucine residues in LD1 for binding to α-parvin-C. We found that individual Leu/Ala mutations in LD1 diminished LD1 binding to α-parvin-C (supplementary Fig. S4a versus S4b), whereas Asp/Ala mutations had little effect (supplementary Fig. S4c versus S4c). Because LD motifs are known to be amphipathic helix with a distinct Leu-rich face (14–16), these data suggested that LD1 binding to α-parvin-C involves its Leu-rich face.

α-Parvin-C Exhibits an Atypical CH-fold That Is Conserved in the Parvin Family—To gain atomic insight into LD1 binding to α-parvin-C, we set out to pursue the total structure determination of the α-parvin-C-LD complex. Because LD1 and LD4 are highly homologous (Fig. 1B) and they both bind to α-parvin-C in a similar manner (Fig. 2, A versus B), we decided to focus on the structure determination of α-parvin-C in complex with LD1. A series of standard triple resonance experiments were performed to obtain resonance assignments of the complex as well as NOE distance constraints within and between $^{15}$N/$^{13}$C-labeled α-parvin-C and unlabeled LD1. A total of 1422 NOEs including 27 intermolecular NOEs were assigned. The structure is well defined as shown in Fig. 3A and Table 1. Despite low sequence identity with other known actin-binding CH2 domains, the main portion of α-parvin-C-(259–370) exhibits the canonical CH-fold, which corresponds to the previously defined CH2 boundaries, i.e. 264–372 (Fig. 1A). α-Parvin contains four main helices ($\alpha_A$, $\alpha_C$, $\alpha_D$, and $\alpha_F$) and two short helices ($\alpha_B$, $\alpha_E$) (Fig. 3B). $\alpha_C$, $\alpha_D$, and $\alpha_F$ form a three-helix bundle with the long helix $\alpha_A$ lying on top of it (Fig. 3B). However, $\alpha_A$ is much longer than the corresponding helix in other CH2 domains (Fig. 1A). Surprisingly, the assumed linker region (249–256) adopts a helical conformation (termed $\alpha_L$, Fig. 3B) and forms a helix-turn-helix with $\alpha_A$. More strikingly, $\alpha_L$ packs with the C-terminal $\alpha_F$, which results in a distinct “variation” of the CH-fold when compared with the canonical fold (Fig. 3B). Given the high sequence homology of α-parvin-C with other parvin family members (>75% identity, Fig. 1A), this spatial arrangement is likely conserved in the parvin family. Thus we have identified a novel variant of the CH-fold that may be
specific for the parvin-mediated protein-protein interactions.

**LD1/Parvin-C Interface**—LD1 in the bound state exhibits an amphipathic helical conformation that interacts primarily with the N-terminal L-turn of Parvin-C (Fig. 3B). Importantly, packing of the linker helix against the canonical CH-fold produces the novel CH-fold variant. Another view of the structure rotated ~90° horizontally (bottom).

**TABLE 1**

**Structural statistics of α-parvin-C-LD1 complex**

The statistics were based on 20 lowest energy structures calculated from 100 structures.

| Parameters | MOE distance constraints | Root mean square deviation from idealized covalent geometry | Ramachandran plot | Average root mean square deviation to the mean structure |
|------------|--------------------------|-----------------------------------------------------------|------------------|------------------------------------------------------|
| All        | 1422                     | Bonds (Å) 0.0079 ± 0.0008 | Most favored regions (%) | 0.61 ± 0.12 |
| Sequential (|i – j| = 1) | 327 (15)* | Angles (degree) 0.873 ± 0.028 | Additionally allowed regions (%) | 1.15 ± 0.21 |
| Medium (1 < |i – j| ≤ 5) | 193 | Improper (degree) 0.628 ± 0.057 | Generally allowed regions (%) | a-Parvin-C-(249–372) and LD1-(3–12). |
| Long range (|i – j| > 5) | 172 | E[1] (kcal mol^{-1}) ~509.1 ± 11.8 | Disallowed regions (%) | bConstraints in the parentheses are for LD1. |
| Intraresidue | 427 (37)* | | | |
| Intermolecular | 27 | | | |
| H-bond | 256 (24)* | | | |

a Constraints in the parentheses are for LD1.

FIGURE 3. The structure of α-parvin-C bound to LD1. A, superposition of 20 α-parvin-C/LD1 structures with lowest energies. B, ribbon diagram in the same view (top). The LD1 and the so-called “linker” α-L are colored in green and yellow, respectively. Packing of α-L against the canonical CH-fold (magenta) produces the novel CH-fold variant. Another view of the structure rotated ~90° horizontally (bottom).

FIGURE 4. Binding interface between LD1 and α-parvin-C. Note that all Leu side chains in LD1 reside on one side of the helix and interact with the hydrophobic face formed by αL, αA, and αF of α-parvin-C. The color codes are the same as described in the legend to Fig. 3B. Stereo diagram for “wall-eyed viewing” is applied.
every Leu/Ala mutation was found to disrupt the LD1 binding to α-parvin-C (supplemental Fig. S3). The previously defined linker region 249–260 makes significant contact with LD1 consistent with the chemical shift mapping and spin-labeling experiments. To further substantiate our observation, we mutated two interface residues, Thr-252 and Leu-253 in L, which engage in multiple hydrophobic contacts with LD1 in our structure. The T252A/L253A mutant failed to bind to LD1 (supplemental Fig. S4), thereby demonstrating that αL is crucial for the α-parvin/paxillin interaction.

LD1/α-Parvin Binding Topology Is Distinct from LD/FAK Complex—As mentioned above, paxillin LD motifs have been known to bind to a class of four-helix bundle proteins including the FAK FAT domain, vinculin, and PKL. Structural studies of the representative complex FAT/LD2 or LD4 have indicated that the LD binding mode is conserved in which the four-helix bundle interacts with two LD motifs (e.g. LD2 and LD4) at opposite faces (13–16) (Fig. 5A). In contrast, we found that α-parvin-C has only one unique LD binding site. Furthermore, the folding of α-parvin-C and its binding topology to LD motif are drastically different from the arrangement seen for FAT (Fig. 5, A versus B). The LD target sequence in α-parvin-C versus those for the LD-FAK complex exhibit little similarity (Fig. 5c) despite the presence of hydrophobic binding features that appear to be important for both types of interactions.

**DISCUSSION**

The main goal of this study was to understand the structural basis of α-parvin/paxillin recognition mediated by the C-terminal CH-containing fragment of the α-parvin and paxillin LD motif. Discovered nearly 18 years ago (27), the CH domain has been widely known to function as a major actin binding domain. It is present in a variety of actin-binding proteins such as α-actinin, filamin, dystrophin, and spectrin. However, a growing quantity of data shows that the CH domain also plays important roles in binding to non-actin proteins, mediating diverse signaling and regulatory processes (e.g. Refs. 11 and 28–30). Numerous CH domain structures have been determined, including those of actin-binding proteins (for review, see Ref. 8). However, no atomic structure of any CH domain-target complex has been reported to date. Our complex structure therefore provides the first atomic view of a CH domain-target complex. More importantly, it has resulted in several significant novel findings: (i) α-parvin-C was found to exhibit a distinct fold in which the N-terminal linker (based on sequence considerations alone) packs unexpectedly against the C-terminal helix of the canonical CH-fold. Importantly, this packing generates a hydrophobic surface that is crucial for recognizing the Leu-rich face of the paxillin LD motif. Our complex structure therefore defines a novel structural variant of the CH domain for the parvin family that is distinct from the CH domains of actin-binding proteins. The unique recognition surface in the present CH domain suggests that such a structural variant may have evolved for specifically interacting with non-actin targets, such as paxillin in focal adhesion assembly. (ii) The LD binding topology is distinctly different from that for the classical four-helix bundle proteins such as FAK FAT, vinculin, and PKL, suggesting that the paxillin LD motifs may engage in variable binding modes with different targets, triggering distinct signaling events. (iii) The LD1 binding site in our structure is quite different from the previously suggested LD binding site (PBS) (Lys<sup>277</sup>–Leu<sup>285</sup>, see Fig. 1A). We note that the PBS was identified based on sequence homology by which Lys<sup>277</sup>–Leu<sup>285</sup> was found to be similar to the PBS in other paxillin LD-binding proteins including FAK, vinculin, and PKL (11). Although point mutations of the PBS residues, V282G/L285R, abolished α-parvin binding to the paxillin LD motif, we note that Val<sup>282</sup> and Leu<sup>285</sup> in our structure are deeply buried in a hydrophobic core of α-parvin-C and thus their mutations into Gly or highly charged Arg likely disrupt the hydrophobic core and the structural integrity of the α-parvin-C-fold, which in turn impaired the α-parvin binding to paxillin. Recent structural studies have shown that LD
binding sites on FAK also do not conform well the PBS rule (14–16).

In addition to binding to paxillin, α-parvin-C has also been shown to bind to ILK (28) and TESK1 (31). It remains to be determined how these proteins recognize α-parvin-C. These proteins may share an overlapping binding site with paxillin or bind to completely different regions in α-parvin-C, which may allow synergistic or competition-based switch mechanisms for regulating the supramolecular FA assembly. For example, deletion of the linker helix α-L in α-parvin was shown to have little effect on ILK binding (28). In contrast, our results showed that mutation in this region (supplemental Fig. S5) abolished α-parvin-C binding to paxillin, suggesting that ILK and paxillin may bind to distinct regions in α-parvin. Paxillin LD1 has also been indicated to bind to the ILK kinase domain (32). Thus, although LD1 binds weakly to α-parvin-C (Fig. S2), it is possible that LD1 binds simultaneously to ILK and α-parvin forming a three-way ternary complex. Interestingly, our gel filtration data revealed that α-parvin CH1 is a dimer (data not shown) suggesting that α-parvin may be a dimer. Because paxillin LD4 also binds to α-parvin-C, it is possible that one paxillin molecule may contact both CH2 subunits in the dimeric α-parvin via its LD1 and LD4, respectively. Thus whereas the binary interaction of LD/CH2 is weak as indicated in Fig. 2 and supplemental Fig. S2, it may become significant as part of the supramolecular focal adhesion assembly. Similar results have been found in the case of weak interaction between FA proteins PINCH and Nck2 (33).

In summary, we have determined the structural basis of how paxillin and α-parvin specifically assemble as a critical component within FAs. Our structure provides the first atomic view of the α-parvin CH domain, providing the specificity for the paxillin/α-parvin interaction. The nature of the LD binding site suggests that a significantly revised assembly model has to be considered for α-parvin binding to paxillin and the regulation of higher order complex assembly and the formation of FAs. Because dysfunctions of paxillin or α-parvin have been implicated in numerous human disease developments (6, 10), specific structure-based manipulation of the paxillin/α-parvin interaction may be useful not only for understanding the disease pathogenesis but also for developing new ways to treat these diseases.

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