Solution Structure of the Focal Adhesion Adaptor PINCH LIM1 Domain and Characterization of Its Interaction with the Integrin-linked Kinase Ankyrin Repeat Domain*

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PINCH is a recently identified adaptor protein that comprises an array of five LIM domains. PINCH functions through LIM-mediated protein-protein interactions that are involved in cell adhesion, growth, and differentiation. The LIM1 domain of PINCH interacts with integrin-linked kinase (ILK), thereby mediating focal adhesions via a specific integrin/ILK signaling pathway. We have solved the NMR structure of the PINCH LIM1 domain and characterized its binding to ILK. LIM1 contains two contiguous zinc fingers of the CCHC and CCCH types and adopts a global fold similar to that of functionally distinct LIM domains from cysteine-rich protein and cysteine-rich intestinal protein families with CCHC and CCCCC zinc finger types. Gel-filtration and NMR experiments demonstrated a 1:1 complex between PINCH LIM1 and the ankyrin repeat domain of ILK. A chemical shift mapping experiment identified regions in PINCH LIM1 that are important for interaction with ILK. Comparison of surface features between PINCH LIM1 and other functionally different LIM domains indicated that the LIM motif might have a highly variable mode in recognizing various target proteins.

Proteins often function through domains or recurring motifs. The LIM domain is a common protein-protein interaction motif that was originally described in the products of the lin-11, isl-1, and mec-3 genes and hence given the acronym “LIM” (1, 2). The domain consists of a loosely conserved cysteine-rich consensus sequence (CX_6–15CX_2–3(H/D/C))/CX_16–21CX_2–3(H/D/C) that encodes two separate zinc fingers (shown in underlined and boldface type, respectively) (3–5). Frequently occurring as an array of one to five copies, the double zinc finger LIM domains have been found in a variety of proteins with diverse functions, either alone or associated with other functional domains (5). Based on sequence similarity, LIM-containing proteins are classified into three groups (5): Group 1 includes the LHX (LIM homeodomain protein), LMO, and LiMK (LIM kinase) subfamilies; Group 2 contains the CRP and CRIP subfamilies; and Group 3 is heterogeneous, and the sequences in this group are very divergent from those in Groups 1 and 2. Although genetic and biochemical studies have shown that LIM domains can interact with diverse target proteins, the molecular basis of how the domains confer specificity and/or coordinate with other functional domains remains elusive. Structural studies geared toward understanding the LIM functions so far have been performed on avian CRPs, related mammalian intestinal protein (CRIP), and a fragment of Lasp-1 protein (6–12), which belong to Group 2 of the LIM subfamilies with CCHC and CCCCC zinc finger types. These studies revealed a conserved fold of LIM domains in which two zinc ions orchestrate the formation of separate zinc fingers that stack together through a shared hydrophobic core. Each finger consists of two orthogonally packed antiparallel β-sheets, and the C-terminal CCCC module is terminated by a α-helix. The conserved tetrahedral zinc coordination and hydrophobic core appear to determine the overall fold of LIM domains, whereas other variable regions in the domains may confer the specificities for binding diverse target proteins. The exact mode of LIM domain binding to various target proteins remains essentially uncharacterized.

PINCH (particularly interesting gene Cys-His protein) is a widely expressed adaptor protein comprising five LIM domains that belong to Group 3 of the LIM protein subfamilies (5). Originally identified from screening of a human cDNA library with antibodies recognizing senescent erythrocytes (13), PINCH has become increasingly interesting because of its involvement in mediating integrin signaling (14, 15). Specifically, PINCH interacts with a membrane-proximal integrin-linked kinase (ILK), which colocalizes with β-integrin in the focal adhesion plaques (16, 17). PINCH-ILK interaction is essential for the focal adhesion localization of ILK (17) and integrin signaling as evidenced by genetic studies in which loss of PINCH function in Caenorhabditis elegans resulted in a paralized, arrested elongation at two-fold phenotype resembling those of integrin-null mutants (18). It has recently been shown by deletion experiments that PINCH-ILK interaction is mediated by the LIM1 domain of PINCH and the ankyrin
repeat domain of ILK (16). Interestingly, the PINCH-ILK interaction also connects ILK to Nck-2, an SH2/SH3-containing adaptor protein that interacts with components of growth factor and small GTPase signaling pathways (19). Such a connection is mediated through the interaction between the LIM4 domain of PINCH and the third SH3 domain of Nck-2. These findings reveal that PINCH mediates multiple signaling pathways by using different LIM domains. More important, the multiple LIM-mediated interactions through a single protein (PINCH) facilitate communications between different signaling pathways, i.e. integrin/ILK signaling and growth factor signaling.

In this study, we have determined the solution structure of the PINCH LIM1 domain and characterized its binding to ILK. The structure not only serves as a starting template for molecular elucidation of PINCH-ILK-mediated integrin signaling, but also sheds light upon the questions of the fold and binding mode of functionally different LIM domains. Although the PINCH LIM1 domain has a more divergent sequence than those of the CRP and CRIP families (Fig. 1), we show here that it adopts a conserved LIM fold. On the other hand, detailed comparison of surface features in the PINCH LIM1 structure and those of the CRP and CRIP families indicates that the binding mode of LIM domains may be highly variable. Gel-filtration and NMR experiments show that PINCH LIM1 and the ILK ankyrin repeat domain form a tight 1:1 complex, providing biophysical evidence of the specific PINCH-ILK interaction that is critical for mediating focal adhesions and integrin signaling. Further chemical shift mapping analysis has revealed the regions in PINCH LIM1 that may be important in binding to the ILK ankyrin repeat domain.

**EXPERIMENTAL PROCEDURES**

**Purification and NMR Sample Preparation of PINCH LIM1**—Expression plasmid pHCL-C2x, encoding maltose-binding protein fused to the N terminus of residues 1–70 of human PINCH protein via Factor Xa-cleavable linker, was used for preparation of the NMR sample. Residues 1–70 contain the entire LIM1 domain. Due to cloning artifacts, the C terminus of LIM1 had three additional residues (WIL), whereas the N terminus contained four (ISEF). Escherichia coli BL21(DE3) cells harboring plasmid were grown in LB medium or in M9 minimal medium in the presence of 100 μg/ml ampicillin. For isotope labeling, M9 minimal medium contained 1.1 g/liter [15N]NH4Cl and unlabeled or 3 g/liter 13C-labeled glucose. Three liters of cultures were induced at A600 = 0.5 for 4 h at 37°C with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were lysed with a French press, and cleared lysates were fractionated on a DEAE-Sepharose column (50 mM Tris-HCl, pH 8.0, NaCl gradient of 0.0–0.8 M). Maltose-binding protein-
Structure of the PINCH LIM1 Domain

Table I
Statistics of experimental data and structure calculations

| Parameter                  | SA<sup>a</sup> ensemble<sup>b</sup> | Minimized average |
|----------------------------|-------------------------------------|-------------------|
| r.m.s.d. from experimental distance restraints (Å) |                                    |                   |
| All (1143)                 | 0.047 ± 0.005                      | 0.045             |
| Intrarresidue, i = j (467) | 0.033 ± 0.007                      | 0.034             |
| Sequential, i − j = 1 (264) | 0.043 ± 0.007                      | 0.039             |
| Medium range, 1 < | j | j | < 6 (160) | 0.070 ± 0.016   | 0.059 |
| Long range, | i | j | ≥ 6 (214) | 0.052 ± 0.006   | 0.054 |
| Hydrogen bond (38)         | 0.061 ± 0.009                      | 0.075             |
| r.m.s.d. from experimental dihedral restraints (*) (32) | 0.83 ± 0.22                      | 0.63              |
| r.m.s.d. from idealized covalent geometry |                                    |                   |
| Bonds (Å)                  | 0.0032 ± 0.0001                    | 0.0035            |
| Angles (*)                 | 0.72 ± 0.04                        | 0.81              |
| Improper (*)               | 0.35 ± 0.02                        | 0.47              |
| $E_{LJ}$ (kcal/mol)<sup>c</sup> | −292.4 ± 8.6               | −285.5            |
| Ramachandran plot<sup>d</sup> |                                    |                   |
| Most favored regions (%)   | 62.5                                | 61.1              |
| Additionally and generally allowed regions (%) | 35.3                             | 37.1             |
| Disallowed regions (%)     | 2.2                                 | 1.8               |
| Coordinate precision<sup>e</sup> |                                    |                   |
| r.m.s.d. of backbone atoms to the mean (Å) | 0.63                             |                   |
| r.m.s.d. of all heavy atoms to the mean (Å) | 1.30                             |                   |

<sup>a</sup> SA, simulated annealing; r.m.s.d., root mean square deviation.
<sup>b</sup> Mean ± S.E. where applicable.
<sup>c</sup> Lennard-Jones potential energy function, calculated with CHARMM19 (27) empirical energy parameters.
<sup>d</sup> Residues 7–67. Total of 1350 other than Gly and Pro residues in an ensemble of 25 simulated annealing structures; 54 residues in the minimized average structure.
<sup>e</sup> Residues 7–67.

LIM1-containing fractions were concentrated, and the buffer was exchanged to optimize for cleavage (50 mM Tris-HCl, 100 mM NaCl, and 3.5 mM CaCl<sub>2</sub>, pH 8.0) and subjected to Factor Xa treatment (Novagen). LIM1 was further purified on a Superdex 75 gel-filtration column. Fractions containing LIM1 were pooled and concentrated. LIM1 was further purified on a Superdex 75 gel-filtration column. Fractions containing LIM1 were pooled and concentrated.

**Purification of the ILK Ankyrin Repeat Domain and Its Complex with the PINCH LIM1 Domain—Residues 1–189 of mouse ILK (99% identical to human ILK) were fused to GST in pGEX-5X-3 (Amersham Pharmacia Biotech), and the construct was expressed by growing 4 liters of culture in LB medium. The induction of GST-ankyrin was performed at room temperature with 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside. Cells were lysed with 50 mM Tris-HCl and 1 mM phenylmethylsulfonyl fluoride at pH 8, and the protein was purified as described previously (16), yielding 50–100 mg/liter of culture. The cleavage of the GST fusion protein by Factor Xa was problematic due to some secondary proteolysis. The cleavage was better in the presence of LIM1, which tightly binds to ILK ankyrin. Therefore, 100 mg of unlabeled GST-ankyrin was mixed with a slightly excess amount of $^{15}$N-labeled LIM1 (18 mg) for Factor Xa cleavage (300 units of Factor Xa from Novagen); the reaction (10 ml) was stopped after 4 h with 1 mM phenylmethylsulfonyl fluoride; and the mixtures were loaded onto a Superdex 75 gel-filtration column to separate the complex from GST (dimer with a molecular mass of 60 kDa) and other impurities. The fractions containing LIM1-ankyrin complex were collected and concentrated to 0.5 ml for NMR experiments (carried out at pH 6.5 with 50 mM phosphate buffer and 100 mM NaCl). Precipitation occurred during the concentration process, and the final sample concentration was ~0.3 mM.

**NMR Experiments and Resonance Assignments of LIM1—All NMR experiments described below for resonance assignments and structural analysis were carried out as reviewed in Refs. 20 and 21. These experiments were performed at 25 °C on a Varian Inova 500-MHz spectrometer equipped with a triple resonance probe and shielded 2-gradient unit. Spectra were processed with mmPipe (22) and visualized with PIPP (23). HNCACB and CBCA(CO)NH experiments provided assignments for theHN, N, Ca, and Cβ resonances of residues 6–68. Residues 1–5 and residues from the vector appeared to be unstructured, could not be assigned, and thus were excluded from further analysis and structure calculations. Side chains were assigned with a combination of HCCH total correlation spectroscopy, HNHA, C(CO)NH, and H(CO)NH. Aromatic side chains were assigned with a combination of two-dimensional total correlation spectroscopy (24), two-dimensional NOESY (24) performed on an unlabeled sample in $^2$H<sub>2</sub>O, and three-dimensional $^{15}$N/$^{13}$C-edited NOESY (25). Three-dimensional $^{15}$N/$^{13}$C-edited NOESY was also used to confirm assignments across proline gaps and to assign residues Pro<sup>90</sup> and Cys<sup>70</sup>. Inspection of H-$\gamma$-H-$\beta$ and HN-$\gamma$-$\beta$ NOE cross-peaks to intraresidue H-$\alpha$ and HN enabled us to make stereospecific assignments of the $\gamma$-methyls of Val<sup>24</sup> and seven H-$\beta$ protons.

**Distance and Angle Restraints—Distance restraints were derived from a three-dimensional $^{15}$N/$^{13}$C NOESY (25) experiment (100-ms mixing time). A two-dimensional NOESY experiment was performed on the unlabeled sample in 100% $^2$H<sub>2</sub>O (150-ms mixing time) to obtain NOEs involving aromatic side chains. 1105 NOE distance restraints and 8 $\chi_i$ angle constraints from stereospecific assignments were obtained from these spectra. An HNHA spectrum provided 24 $^\text{JHNHN}$ coupling constants that were converted to $\phi$ angle constraints. Hydrogen bond distance constraints were derived from NOE analysis and a hydrogen/deuterium exchange experiment. Hydrogen bond constraints were only used during final structure refinement and included only residues comprising secondary structure elements.

**Structure Calculations—A simulated annealing protocol (26) was used that employs quadratic potentials for covalent geometry, flat bottom quadratic potentials for experimental distance, and dihedral angle restraints and a quadratic repulsion term for van der Waals non-bonded
interactions. CHARMM19 (27) force field was used for protein covalent geometry. For zinc, the bond lengths Zn–S and Zn–N were set to 2.30 and 2.00 Å, respectively (28); tetrahedral coordination was enforced by weak force constant (40 kcal mol\(^{-2}\) rad\(^{-2}\)) (29); Zn–N–C angles were the same as H–N–C in protonated imidazole; and an improper kept zinc in the plane of the imidazole ring. NOEs were grouped into strong (1.8–2.7 Å), medium (1.8–3.3 Å), and weak (1.8–5.0 Å). NOEs from equivalent or non-stereospecifically assigned atoms were treated with \(r^{-6}\) summation and without correction for multiplicity. Upper bound corrections for such NOEs were performed according to the recommendations of Ref. 30. The presence of zinc necessitated minor modifications of the protocol since convergence with standard procedure was low, we believe due to covalent bonding of zinc. First, we used a standard simulated annealing protocol to generate 50 structures of protein in the absence of zinc. We selected the 25 lowest total energy structures, some of which contained significant distortions from the final structure. We then added zinc atoms to these structures, which generated 125 simulated annealing structures (every zinc-free structure parented five zinc-containing structures). Out of 125 structures obtained in this manner, we selected 25 structures with the lowest total energy as our final set. The calculation statistics for this set is shown in Table I. All the structure calculations were performed with X-PLOR Version 3.851 (31). A Ramachandran plot was calculated with the program Procheck-NMR to examine the quality of the structures (32). Surface potentials were

**Fig. 4.** Surface features of PINCH LIM1 and comparison with other LIM domains. A, hydrophobic features of the average minimized structure of the PINCH LIM1 surface. The view in middle panel corresponds to the slightly rotated view in Fig. 2. The indicated residues comprise hydrophobic patches (green) discussed under “Results and Discussion.” The three panels are related by 90° rotation in page plane as shown for both A and B. B, electrostatic features of the average minimized structure of the PINCH LIM1 surface. The bar below shows color mapping of the electrostatic energy spectrum from −10 to +10 in \(k_B T\) units. The potential was calculated with the program Grasp. C, hydrophobic features of three representative LIM domains in orientations similar to the middle panel of A. The indicated residues are conserved counterparts of the residues of PINCH LIM1 that compose patch 3 near the C terminus.
calculated (Poisson-Boltzmann solver) and displayed in Grasp (33). Structures were visualized, and figures were prepared with InsightII (MSI, Inc.) and MolMol (34).

RESULTS AND DISCUSSION

Secondary Structure—The set of the 25 lowest energy simulated annealing structures of the PINCH LIM1 domain is displayed in Fig. 2A. Structural statistics and NMR constraints are summarized in Table I. The secondary structure elements of PINCH LIM1 are arranged in the order of $\beta_1$-$\beta_2$-$\beta_3$-$\beta_4$-$\alpha$, which is consistent with the sequential NOE patterns (24), hydrogen/deuterium exchange data, and $^{13}$C-$\omega$/$\beta$ chemical shift data (35). Long range $\alpha$-$\alpha$, $\alpha$-$\beta$, and $\beta$-$\beta$-HN, and $\alpha$-$\alpha$-HN; strong sequential $\alpha$-$\beta$ NOE connectivities; and slow amide exchange data revealed that Thr 9–Cys 10 and Gly15–Gly16 constitute $\beta$-sheet 1, whereas Ile 23–Ser 26 and Glu 29–His 32 form $\beta$-sheet 2. $\beta$-Sheet 3 is composed of Val 37–Cys 38 and Gln 43–Gln 44, and $\beta$-sheet 4 contains Tyr 51–Phe 53 and Arg 56–Cys 59. Similar to the previously reported LIM domain structures of the CRP and CRIP families, rubredoxin-type turns (6) connect strands of $\beta$-sheet 1 versus $\beta$-sheet 2 and $\beta$-sheet 3 versus $\beta$-sheet 4 for finger 2, and the finger 2 is terminated by an $\alpha$-helix (Fig. 2B). It is of interest to note that, in all LIM domain structures determined so far, $\beta$-sheets 1 and 3 are shorter than $\beta$-sheets 2 and 4. The edge of $\beta$-sheet 1 contacts the edge of $\beta$-sheet 2; however, these elements do not merge into a contiguous structure. The amide proton of Glu 11 at the edge of $\beta$-sheet 1 has a slow hydrogen/deuterium exchange rate, apparently due to participation in a hydrogen bond with the carbonyl oxygen of Lys 30 as reflected during structure calculations; yet the tertiary structure of LIM1 shows that $\beta$-sheets 1 and 2 are nearly orthogonal at this site and that the Glu 11 HN–Lys 30 oxygen hydrogen bond can be viewed as a pivot point. In comparison, $\beta$-sheets 3 and 4 in finger 2 are less

Tertiary Structure of PINCH LIM1 and Its Comparison with Other LIM Structures—The overall fold of LIM1 (Fig. 2B) is similar to those of previously solved LIM structures from the CRP and CRIP families (Fig. 1). Sequence alignment (Fig. 1) shows that zinc coordination residues are the same as those from CRP and CRIP, except that zinc finger 2 has a CCCH module instead of a CCCC module. Thr 9–Cys 35 form zinc finger 1 with Cys 10, Cys 13, His 32, and Cys 35 coordinated to zinc (CCHC module), whereas Cys 38–His 61 form zinc finger 2 with Cys 38, Cys 41, Cys 59, and His 61 coordinated to zinc (CCCH module). Each finger of PINCH LIM1 consists of two antiparallel $\beta$-sheets, i.e. $\beta$-sheet 1 versus $\beta$-sheet 2 for finger 1 and $\beta$-sheet 3 versus $\beta$-sheet 4 for finger 2, and the finger 2 is terminated by an $\alpha$-helix (Fig. 2B). It is of interest to note that, in all LIM domain structures determined so far, $\beta$-sheets 1 and 3 are shorter than $\beta$-sheets 2 and 4. The edge of $\beta$-sheet 1 contacts the edge of $\beta$-sheet 2; however, these elements do not merge into a contiguous structure. The amide proton of Glu 11 at the edge of $\beta$-sheet 1 has a slow hydrogen/deuterium exchange rate, apparently due to participation in a hydrogen bond with the carbonyl oxygen of Lys 30 in $\beta$-sheet 2 as reflected during structure calculations; yet the tertiary structure of LIM1 shows that $\beta$-sheets 1 and 2 are nearly orthogonal at this site and that the Glu 11 HN–Lys 30 oxygen hydrogen bond can be viewed as a pivot point. In comparison, $\beta$-sheets 3 and 4 in finger 2 are less

FIG. 5. NMR spectra of PINCH LIM1 and its interaction with the ILK ankyrin repeat domain. Shown is an overlay of the $^1$H–$^{15}$N HSQC spectra of PINCH LIM1 in its free form (black) and in a complex with the ankyrin domain of ILK (light gray). Residue labels correspond to free PINCH LIM1.
orthogonal to each other and appear to partly merge into a more extensive four-stranded $\beta$-structure.

Among the LIM domains with known structures, CRP2 LIM2 possesses the most similar sequence to PINCH LIM1: 33% identity and 49% similarity. However, sequence alignment based on the BLAST program revealed that zinc finger 1 of PINCH LIM1 contains a single amino acid insertion at Asn$^{27}$ compared with qCRP2 LIM2 and other LIM domains with known structures (Fig. 1). Hence, we superimposed the minimized average structure of PINCH LIM1 with that of qCRP2 LIM2 by omitting Asn$^{27}$, which yielded a root mean square deviation of 2.7 Å. To confirm that Asn$^{27}$ is indeed the insertion point, we superimposed the first 30 structured residues of qCRP2 LIM2 with the homologous 31-residue stretch of PINCH LIM1 while systematically omitting one residue of the latter. A distinct root mean square deviation minimum was found to occur in the region of Pro$^{19}$–Ile$^{23}$ ($\pm$ 0.2 Å), which connects $\beta$-sheets 1 and 2, suggesting that the insertion point should be in this linker region. Superposition of PINCH LIM1 with qCRP2 LIM2 by sequentially omitting one residue from Pro$^{19}$–Ile$^{23}$ yielded a root mean square deviation of 2.3–2.4 Å. On the other hand, superposition of PINCH LIM1 with more sequence-divergent LIM domains such as CRIP and chicken CRP1 LIM1 yielded larger root mean square deviations (3.8 and 3.3 Å, respectively). Although individual zinc fingers of these LIM domains superimpose well with those of PINCH LIM1, there is significant twisting between the two zinc fingers of the CRIP domains superimpose well with those of PINCH LIM1, and highly divergent CRIP proteins revealed that this one of them near the N terminus (Fig. 4A). The variable orientations between the two zinc fingers of different LIM domains are consistent with the intradomain flexibility of the two zinc fingers as indicated by the $^{15}$N backbone dynamics studies of qCRP2 LIM1 compared with qCRP2 LIM2 (10, 12). It remains to be established how such intradomain mobility might contribute to the LIM-mediated protein-protein interactions.

Hydrophobic residues that form the core of the PINCH LIM1 structure are well conserved. The aromatic ring of Phe$^{17}$ is buried and acts as a nucleus of zinc finger 1, whereas Ile$^{23}$ and Leu$^{30}$ partly pack against it. The two zinc fingers are packed through a central hydrophobic core involving the completely buried rings of Tyr$^{31}$, Phe$^{36}$, and Phe$^{50}$. Other residues such as the Val$^{24}$, Leu$^{49}$ and Lys$^{57}$ methylene side chains form the periphery of the core and are partly exposed (Fig. 3A). Interestingly, Lys$^{57}$ in PINCH LIM1 is substituted for the hydrophobic Ile/Val/Pro residue at an equivalent position in other LIM domains with known structures (6–12). The lack of a hydrophobic residue at this position appears to result in greater stacking of Tyr$^{31}$ and Phe$^{36}$ rings in PINCH LIM1 (Fig. 3A) than of the corresponding residues in other LIM domain structures. The total surface of two rings that needs to be covered is 18 Å$^2$ less compared with qCRP2 LIM1 and 69 Å$^2$ less compared with qCRP2 LIM2 structures (note that qCRP2 LIM2 contains a bulkier tryptophan at an equivalent position to Thr$^{31}$ of PINCH LIM1; see Fig. 1). On the other hand, Lys$^{57}$ still plays a similar role to purely hydrophobic residues: its methylenes pack against the edge of the Tyr$^{31}$ ring (Fig. 3A), whereas its primary amino group points toward the surface (see below).

Surface Features of PINCH LIM1 and Other LIM Domains—Analysis of the PINCH LIM1 structure revealed that only 4 out of 13 aromatic residues are completely buried in the hydrophobic core. These are Phe$^{17}$, Tyr$^{31}$, Phe$^{36}$, and Phe$^{50}$. Of the rest, the two histidines coordinate to Zn$^{2+}$, whereas the remaining two tyrosines and five phenylalanines are at the surface or at least partly exposed to the surface. Phe$^{67}$ is disordered, as is the side chain of Phe$^{45}$. Other aromatic rings compose parts of surface hydrophobic patches. There are three distinct hydrophobic regions. The methyls of Leu$^{29}$, Ile$^{35}$, and Ala$^6$ compose one of them near the N terminus (Fig. 4A), and the second hydrophobic patch comprises the exposed Phe$^{42}$ ring and Val$^{57}$. Val$^{57}$ lies at the bottom of a depression between the tips of $\beta$-sheets 1 and 3. The Phe$^{42}$ ring packs underneath in parallel to the plane of $\beta$-sheet 3. As a consequence, the bulge is created, and what would be a shallow depression becomes deeper groove (Fig. 4A). The third hydrophobic patch is at the opposite end of patch 1, where the aromatic rings of Tyr$^{51}$, Phe$^{53}$, and Tyr$^{58}$ protrude upwards from $\beta$-sheet 4 and arrange themselves in a nearly coplanar manner (Fig. 3B). The aromatic ring of Phe$^{63}$ covers part of this platform, as it stacks against the tyrosine rings. The other side of the Phe$^{63}$ ring contacts the Leu$^{66}$ side chain. Therefore, this hydrophobic surface is rather extensive, including the Leu$^{66}$ aliphatic group, the Phe$^{63}$ aromatic ring, and several edges of aromatic residues Tyr$^{51}$, Phe$^{53}$, Tyr$^{58}$, and Phe$^{57}$ (ring-disordered) (Figs. 3B and 4A). Examination of the surface features in the most homologous qCRP2 LIM2, qCRP2 LIM1, and highly divergent CRIP proteins revealed that this patch is conserved, involving mostly the C-terminal helix (Fig. 4C), what may be important for LIM-target protein interactions (see below). The size and hydrophobic residues in this patch vary significantly, which may confer specificity for diverse target proteins.

The overall surface of PINCH LIM1 is predominantly negative since negatively charged residues are more prevalent than the positively charged ones (eight Glu + one Asp residue versus three Lys + two Arg residues). However, it was surprising to find that the charges are distributed asymmetrically. As Fig. 4B illustrates, negative charges dominate on one face of the molecule. The most prominent positively charged region is located on the opposite side of the molecule right under the Phe$^{42}$ bulge. It is created by Arg$^{12}$ guanidines and Lys$^{57}$ amino moieties being close to each other with the inclusion of Glu$^{26}$, which apparently forms a salt bridge with Arg$^{12}$. Hence, the Phe$^{42}$ region contains both a hydrophobic prominence and a mosaic of charged spots (Fig. 4, compare A and B). It is not clear if this site contributes to the protein-protein interactions.

Characterization of PINCH LIM1 Binding to the ILK Ankyrin Repeat Domain—It has been shown by deletion exper-
ments that PINCH LIM1 interacts with the ankyrin repeat domain of ILK (16). In this report, we have characterized the binding of PINCH LIM1 to the ILK ankyrin repeat domain using gel-filtration and NMR experiments. Whereas PINCH LIM1 (residues 1–70) and ILK ankyrin (residues 1–189) eluted at ~8 and ~20 kDa individually, the equimolar mixture eluted at ~30 kDa (data not shown), indicating a tight 1:1 complex. Fig. 5 shows the two-dimensional 1H-15N HSQC spectrum of free 15N-labeled PINCH LIM1 in free form and 15N-labeled PINCH LIM1 in complex with the ILK ankyrin repeat domain (unlabeled). Fig. 6 shows the plot of the change in 1H and 15N chemical shifts of LIM1 upon binding to the ILK ankyrin repeat domain as a function of residue number. Although resonance assignments of the complex are not available, in certain cases (e.g. glycine residues), the proximity of the peaks of free and complexed forms renders the assignment obvious. In other instances, we used the distance to the closest unassigned neighbor in the spectrum of the complex (36). Most of the residues in zinc finger 1 appeared to be unperturbed or only slightly perturbed, whereas many residues in zinc finger 2 underwent larger chemical shift changes as compared with zinc finger 1; in particular, residues in the C-terminal helix of zinc finger 2 experienced substantial changes. Fig. 7 provides the three-dimensional mapping of the corresponding changes in LIM1, showing that zinc finger 2 is mainly involved in interaction with the ILK ankyrin repeat domain. This is consistent with the above surface analysis that the third hydrophobic patch involving the C-terminal helix is likely the major protein recognition site. Mutational analysis also supports the notion that the second zinc finger of LIM1 is involved in interacting with ILK (17). Some conformational change may occur in LIM1 upon binding to the ILK ankyrin repeat domain, as indicated by the large chemical shift changes of some residues. More accurate identification of the LIM1-binding site and how the domain undergoes conformational change upon binding to ILK require the complete structure determination of the PINCH LIM1-ILK ankyrin complex, which is in progress in our laboratory.

In summary, we have determined the NMR structure of the focal adhesion protein PINCH LIM1 and characterized its binding to ILK. Despite the loose consensus sequence of the LIM motif, PINCH LIM1 adopts a fold similar to other functionally different LIM domain structures. Analysis of the surface features revealed a hydrophobic surface at zinc finger 2 involving the C-terminal helix that appears to be critical for LIM-target protein interactions, as indicated by the chemical shift mapping of the HSQC spectrum of the PINCH LIM1-ILK ankyrin complex. The variation in this surface between the PINCH LIM1 structure and those from the CRP and CRIP families suggests a highly variable mode for the LIM domains in recognizing various target proteins.

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