Structural Basis for the Interaction between Pyk2-FAT Domain and Leupaxin LD Repeats

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Supporting Information

ABSTRACT: Proline-rich tyrosine kinase 2 (Pyk2) is a nonreceptor tyrosine kinase and belongs to the focal adhesion kinase (FAK) family. Like FAK, the C-terminal focal adhesion-targeting (FAT) domain of Pyk2 binds to paxillin, a scaffold protein in focal adhesions; however, the interaction between the FAT domain of Pyk2 and paxillin is dynamic and unstable. Leupaxin is another member in the paxillin family and was suggested to be the native binding partner of Pyk2; Pyk2 gene expression is strongly correlated with that of leupaxin in many tissues including primary breast cancer. Here, we report that leupaxin interacts with Pyk2-FAT. Leupaxin has four leucine-aspartate (LD) motifs. The first and third LD motifs of leupaxin preferably target the two LD-binding sites on the Pyk2-FAT domain, respectively. Moreover, the full-length leupaxin binds to Pyk2-FAT as a stable one-to-one complex. Together, we propose that there is an underlying selectivity between leupaxin and paxillin for Pyk2, which may influence the differing behavior of the two proteins at focal adhesion sites.

Proline-rich tyrosine kinase 2 (Pyk2) is a nonreceptor tyrosine kinase that belongs to the focal adhesion kinase (FAK) family.1−4 Recently, several studies have demonstrated that Pyk2 is highly expressed in a variety of human tumors and may serve as a novel biomarker with prognostic significance in neuroglioma, breast cancer, and hepatocellular carcinoma.1,5−7 FAK family proteins have a large N-terminal FERM domain, a centrally located kinase domain, and a C-terminal focal adhesion targeting (FAT) domain.9 Despite the structural similarities between FAK and Pyk2 kinases, FAK is ubiquitously expressed, whereas Pyk2 shows more tissue-specific expression.1,3,10−12 Pyk2 is highly expressed in endothelium, central nervous system, and hematopoietic lineages.13−16 Previous studies in several cell types have shown that expression of endogenous Pyk2 was observed when FAK levels are low,17−19 suggesting that a compensatory role of Pyk2 can be acquired by cells to maintain the regulatory function of FAK during cell adhesion and migration. However, FAK does not compensate for Pyk2 in Pyk2-deficient B cells and macrophages.20 Therefore, it appears that FAK and Pyk2 differ from each other in regulating cellular functions and signaling pathways.

The FAT domain of FAK binds to paxillin, a major scaffold protein in focal adhesions;21−25 this interaction is required to recruit FAK to form robust focal adhesions.26 The C-terminal half of paxillin has four well-folded LIM domains that are used to target focal adhesions. The N-terminal region is generally disordered in the apo state and contains four or five LD motifs linked by unstructured loops in a “beads on a string” fashion. These LD motifs interact with other FA proteins, including the FAT domain of FAK and Pyk2.22 We27 and others28−32 have determined the structure of the FAT domain of FAK by NMR and X-ray crystallography studies. The structure of the FAT domain is a four-helix bundle. Two potential LD peptide binding sites were found on the surface of the FAT domain: one spanning helices H1 and H4 (H1/H4 site) and one spanning helices H2 and H3 (H2/H3 site).27−30 By conducting a detailed, systematic study using NMR and other biophysical approaches, we showed that the LD2 and LD4 motifs of a paxillin molecule bind simultaneously to a single FAT domain; the LD2 motif binds at the H1/H4 site and the LD4 motif binds at the H2/H3 site.33 Like FAK, Pyk2 also has a C-terminal FAT domain that forms a four-helix bundle (H1−H4).28,33−36 However, our recent studies showed that the binding mechanism between Pyk2 and FAK for paxillin LD2 and LD4 motifs is different; in Pyk2, paxillin LD2 and LD4 motifs compete equally for the...
high-affinity binding site H2/H3 and undergo a conformational switching mechanism. In other words, paxillin forms a much more stable complex with the FAT domain of FAK than with the FAT domain of Pyk2. Indeed, paxillin binds more tightly to the FAT domain of FAK than the FAT domain of Pyk2. Such observation led us to speculate that paxillin is not the native binding partner of Pyk2 and that the FAT domain of Pyk2 should form a stable complex with its native binding partner.

Leupaxin was initially identified as a leukocyte-specific isoform of paxillin and shares the highest sequence similarity with paxillin of any paxillin family member. It is preferentially expressed in macrophages, osteoclasts, and hematopoietic cells. Previous studies have demonstrated that Pyk2 associates with leupaxin in lymphoid cells, where these proteins may form a cell type-specific signaling complex. Furthermore, the association of these two proteins can modulate cell migration, adhesion, and motility in prostate cancer. Like paxillin, leupaxin is composed of multiple functional modules, including leucine–aspartate (LD) motifs and LIM domains, suggesting that leupaxin also serves as a molecular adaptor at focal adhesions. Phylogenetic tree and sequence analysis suggest that, unlike paxillin, leupaxin has four LD repeats (LD1, LD3, LD4, and LD5) at its N-terminus. Leupaxin does not contain an equivalent paxillin LD2 motif. Hence, it has been speculated that in the absence of the LD2 motif a potential interaction between leupaxin and FAK would be relatively weak.

In this study, we examined how the interaction between leupaxin and Pyk2. We determined how the LD motifs of leupaxin bind to Pyk2-FAT using various biophysical methods, including isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR). We also present two crystal structures of Pyk2-FAT complexes with high-affinity leupaxin LD1 and LD4 peptides and its stable 1:1 interaction with full-length leupaxin. Considering our studies along with previous reports, we identify an underlying selectivity difference between leupaxin and paxillin in the way they associate with Pyk2 that directly affects their complex stability and may ultimately influence the behavior of Pyk2 at focal adhesion sites.

Experimental Procedures

Analysis of GEO Profiling. Publicly available data sets that assayed breast cancer primary tumors (GSE1276) and normal breast cells (GSE20437) were downloaded. MAS 5.0 signal data was log start transformed by the following formula: \( \log_2(\text{signal} + 20) \). It was then plotted and correlated. Data transformations, scatterplots, linear fits, and Pearson correlations were generated using STATA/MP 11.2. Typically, a correlation sums the gene expression differences between any two genes across multiple arrays and then divides that sum by the total number of array experiments examined to give the Pearson’s \( r \) value, which measures the degree of association between variables, range from –1 to 1.

Cloning, Expression, and Purification of the FAT Domain of Pyk2. The FAT domain of human Pyk2 (residues 871–1005) was expressed as an N-terminal His-tag fusion in Escherichia coli cells and purified as described. The Pyk2-FAT protein concentration was measured using a standard Coomassie (Bradford) protein assay. The final protein buffer used for all NMR experiments, biochemical studies, and X-ray crystallization was 20 mM MES, pH 6.2.

Cloning, Expression, and Purification of Leupaxin1–105. The coding sequence of leupaxin1–105 (human leupaxin, residues 1–105; Figure S13A) was cloned into the pET-28 expression vector (Novagen). Protein was then expressed as an N-terminal His-tag fusion in E. coli Rosetta2 (DE3) pLysS cells (Novagen). Cells were initially grown at 37 °C and induced with 1 mM IPTG at 18 °C overnight. Protein was purified using nickel-column affinity chromatography followed by HPLC (Figure S13B,C). To avoid nonspecific proteolysis by thrombin, the N-terminal His-tag was not cleaved during purification. The \( 15^N \)-labeled and \( 13C/15N \)-labeled samples were prepared by growing the cells in MOPS-buffered media containing \( 15^N \)-labeled and \( [13C6] \) glucose (3 g/L). All biochemical and structural studies of leupaxin1–105 were performed in 20 mM MES buffer, pH 6.2.

Cloning, Expression, and Purification of Leupaxin1–151. The coding sequence of leupaxin1–151 (human leupaxin, residues 1–151) was cloned into the pET-28 expression vector (Novagen). The expression and purification procedure of the leupaxin1–151 construct was similar to those for the leupaxin1–105 construct.

Cloning, Expression, and Purification of Pyk2-FAT-LD1 Constructs. Pyk2-FAT-LD1 fusion constructs were generated using Pyk2-FAT as a template. Double-stranded DNA corresponding to the “GGS-LD1” sequence (CCTGCA-GGGGGCGGACTGGAGAcTGGATGCTTACT-GGAAGACTGGAGCCTAGCTAAGGATTACGATTAG) was obtained from Integrated DNA Technologies and cloned using the PosI restriction enzyme site downstream of the existing Pyk2-FAT construct in the pET28a vector. Site-directed mutagenesis was performed to remove the stop codon at the end of the Pyk2-FAT sequence and to add additional residues. The expression and purification procedures for Pyk2-FAT-LD1 were similar to those for the Pyk2-FAT construct.

Synthesis of Leupaxin Peptide Mimics LD1, LD3, LD4, and LD5. Leupaxin derived peptides leupaxin-LD1 (human leupaxin, residues 1–20), leupaxin-LD3 (human leupaxin, residues 36–56), leupaxin-LD4 (human leupaxin, residues 86–104), and leupaxin-LD5 (human leupaxin, residues 125–150) were chemically synthesized and purified by high-pressure liquid chromatography (HPLC) at the Hartwell Center of Bioinformatics and Biotechnology of St. Jude Children’s Research Hospital. The length of the leupaxin-LD1, leupaxin-LD3, leupaxin-LD4, and leupaxin-LD5 peptides used was based on our previous paxillin binding studies with Pyk2, FAK, and GIT1. All peptide stocks were prepared at a concentration of 5 mM in 20 mM MES, pH 6.2.

Isothermal Titration Calorimetry (ITC). ITC experiments were performed using a Microcal ITC200 instrument (Microcal). Sample buffer conditions for ITC studies were the same as those for NMR studies. The sample cell of the calorimeter was loaded with 100 \( \mu \)M Pyk2-FAT in 20 mM MES, pH 6.2. The syringe was loaded with leupaxin-LD1, leupaxin-LD3, leupaxin-LD4, and leupaxin-LD5 peptides and leupaxin1–105 (1000 \( \mu \)M leupaxin-LD1, leupaxin-LD3, leupaxin-LD4, or leupaxin-LD5; 500 \( \mu \)M leupaxin1–105) for titrations in the same buffer. All solutions were degassed for 10 min. For leupaxin-LD3, leupaxin-LD5, and leupaxin1–105 binding to Pyk2-FAT, titrations were performed at 25 °C with injection volumes of 2 \( \mu \)L and a spacing of 120 s. However, for leupaxin-LD1 and leupaxin-LD4 binding to Pyk2-FAT, titrations were performed at 15 °C with injection volumes of 2 \( \mu \)L and a spacing of 120 s. These low temperature experiments for the leupaxin-LD1 and leupaxin-LD4 peptides were performed to observe the second weak binding site by ITC, which we could not achieve when
Table 1. Thermodynamic Parameters for the Binding of Pyk2-FAT to Leupaxin LD1, LD3, LD4, and LD5 Peptides and Leupaxin Obtained by ITC

| Peptide   | Sites | Kd (μM)  | ΔG (kcal/mol) | ΔH (kcal/mol) | −TΔS (kcal/mol) |
|-----------|-------|----------|---------------|---------------|----------------|
| LD1       | first | 2.8 ± 1.0 | −7.6 ± 0.3    | −2.0 ± 0.2    | −5.6 ± 0.4     |
|           | second| 52.0 ± 26.0| −5.3 ± 0.7    | 1.6 ± 0.3     | −7.0 ± 0.5     |
| LD3       | first | 2.2 ± 1.0 | −7.6 ± 0.3    | −3.0 ± 0.2    | −4.6 ± 0.6     |
|           | second| 33.0 ± 7.2 | −6.0 ± 0.1    | 0.5 ± 0.3     | −6.5 ± 0.4     |
| LD4       | first | 6.0 ± 0.2 | 5.9 ± 0.2     | −2.5 ± 0.5    | −3.4 ± 0.05    |
|           | second| 1.0 ± 0.2  | 6.5 ± 0.2     | −2.7 ± 0.5    | −3.4 ± 0.05    |
| LD5       | first | 6.0 ± 0.2 | 6.5 ± 0.2     | −2.7 ± 0.5    | −3.4 ± 0.05    |
|           | second| 1.0 ± 0.2  | 6.5 ± 0.2     | −2.7 ± 0.5    | −3.4 ± 0.05    |

The peptide sequences are shown in Figure S3A. Not Determined.
leupaxin-LD4 peptide mixture (1:1) was titrated with 15N-labeled 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2. Similarly, a leupaxin-LD1 and leupaxin-LD4 peptides to Pyk2-FAT-LD1 was performed by adding similar ratios. NMR titration of leupaxin-LD1 and leupaxin-LD4 peptides to Pyk2-FAT by adding the following protein/peptide ratios: 1:0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2.4, and 1:4. Pyk2-FAT titration to leupaxin-LD1 and leupaxin-LD4 were obtained based on HNCA, CBCA(CO)-HN, HNCA, and HN(CO)CA experiments.

For CSP analysis, NMR titration of leupaxin-LD1 and leupaxin-LD4 peptides to Pyk2-FAT was performed by adding the following protein/peptide ratios: 1:0.4, 1:0.8, 1:1.2, 1:1.6, 1:2.4, and 1:4. Pyk2-FAT titration to leupaxin-LD1 was also performed by adding similar ratios. NMR titration of leupaxin-LD1 and leupaxin-LD4 peptides to Pyk2-FAT-LD1 was performed by adding the following protein/peptide ratios: 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2. Similarly, a leupaxin-LD1 and leupaxin-LD4 peptide mixture (1:1) was titrated to 15N-labeled Pyk2-FAT by adding the following protein/peptide ratios: 1:0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2. A series of 1H–15N-HSQC and 1H–15N-TROSY spectra were collected on Bruker 600 and 800 MHz spectrometers, and data was analyzed using CARA 1.8.4. 1H and 15N chemical shift values for the displaced peaks in 1H–15N-HSQC and 1H–15N-TROSY titration experiments were determined for each of the successive titration points using CARA. To determine the per-residue chemical shift perturbation upon binding and account for differences in spectral widths between 15N and 1H resonances, weighted average chemical shift differences, Δav(HN) were calculated for the backbone amide 1H and 15N resonances using the following equation: Δav(NH) = [(ΔH2+ (ΔN/5))/2]/1/2, where ΔH and ΔN are chemical-shift differences for 1H and 15N, respectively.

**RESULTS**

**Pyk2 Gene Expression Profile.** It was shown that both Pyk2 and leupaxin exhibit elevated expression levels in prostate cancer, where they associate and affect cell adhesion and migration.37,38 We questioned whether such a correlation might also be found in other systems. Because it is also known that Pyk2 is highly expressed in breast cancer cells, we therefore investigated whether this elevated Pyk2 gene expression similarly correlates with leupaxin in breast cancer as well. We decided to analyze the Pyk2 gene expression profiles of primary breast tumors and normal breast cells using the GEO database.40 We downloaded raw data from primary breast tumors (GSE1276) and normal breast cells (GSE20437). Pearson correlations of Pyk2 with paxillin and leupaxin were examined and compared with those of FAK. In normal breast cells, we found that leupaxin negatively correlated with Pyk2 gene expression (ρ = −0.44), consistent with the notion that leupaxin is not preferentially expressed in these cells. In primary breast tumors, we found that Pyk2 expression correlated more strongly with leupaxin than paxillin, with ρ values of 0.46 and 0.20, respectively (Figure S1). Moreover, we detected a negative Pearson correlation for FAK expression with leupaxin (ρ = −0.09) and a positive correlation for paxillin (ρ = 0.52) in primary breast cancer cells. These results suggest that Pyk2 gene expression is correlated with leupaxin and that FAK gene expression is correlated with paxillin in primary breast cancer tumors. Such tight correlations among the four proteins is in agreement with the notion that paxillin is the native binding partner of FAK and that leupaxin is the native binding partner of Pyk2.37,55

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**Table 2. Data Collection and Refinement Statistics**

|                      | Pyk2-FAT/leupaxin-LD1 | Pyk2-FAT/leupaxin-LD4 | Pyk2-FAT-LD1/leupaxin-LD4 |
|----------------------|-----------------------|-----------------------|--------------------------|
| **Data Collection**  |                       |                       |                          |
| space group          | P2_2_2_1              | P2_2_2               | P2_1                     |
| **Cell Dimensions**  |                       |                       |                          |
| a, b, c (Å)          | 27.5, 78.1, 165.4     | 138.0, 30.5, 40.6    | 52.1, 79.2, 53.2         |
| a, b, c (deg)        | 90.0, 90.0, 90.0      | 90.0, 90.0, 90.0     | 90.0, 117.6, 90.0        |
| resolution (Å)       | 2.5 (2.59–2.50) b     | 1.8 (1.86–1.80)      | 2.0 (2.12–2.0)           |
| R<sub>ref</sub> (%)  | 9.0 (5.6)             | 5.0 (36.8)           | 3.9 (42.8)               |
| I/α                  | 20.9 (2.2)            | 46.9 (3.5)           | 18.6 (2.4)               |
| completeness (%)     | 98.4 (91.7)           | 95.9 (76.2)          | 95.5 (77.3)              |
| redundancy (%)       | 7.5 (5.0)             | 8.9 (6.7)            | 4.2 (3.2)                |
| **Refinement**       |                       |                       |                          |
| resolution (Å)       | 25.0–2.5              | 35.0–1.8             | 30.0–2.0                 |
| no. reflections      | 12 885                | 16 103               | 24 569                   |
| R<sub>work</sub>/R<sub>free</sub> | 22.4/26.6            | 20.8/23.1            | 20.4/25.0                |
| No. Atoms            |                       |                       |                          |
| protein              | 2045                  | 1039                 | 2247                     |
| peptide              | 385                   | 106                  | 261                      |
| water                | 12                    | 67                   | 71                       |
| B-factors            |                       |                       |                          |
| protein              | 48.5                  | 38.4                 | 58.9                     |
| peptide              | 66.7                  | 46.0                 | 78.5                     |
| water                | 46.2                  | 39.2                 | 53.5                     |
| RMS Deviations       |                       |                       |                          |
| bond lengths (Å)     | 0.009                 | 0.007                | 0.007                    |
| bond angles (deg)    | 1.1                   | 1.0                  | 1.0                      |

*Each data set was collected from a single crystal. Values in parentheses are for the highest resolution shell.*
Leupaxin LD Motifs Have Preferential Binding for Pyk2-FAT. We next decided to investigate the interaction between Pyk2 and leupaxin. Leupaxin has four LD motifs in its N-terminus, all of which can potentially interact with the Pyk2-FAT domain. On the basis of phylogenetic tree and sequence alignment analysis of leupaxin and paxillin using ClustalW, we defined the nomenclature of leupaxin’s LD motifs based on the LD motifs of paxillin (Figure S2) and thus named leupaxin’s four LD motifs LD1, LD3, LD4, and LD5. As shown in Figure S2, although there is no leupaxin LD motif that is equivalent to the paxillin LD2 motif, the paxillin LD2 motif shares a stronger phylogenetic relationship with LD1 of leupaxin than with other LD motifs of leupaxin.

To examine the association between Pyk2 and leupaxin, we first generated four peptides corresponding to the four LD motifs of leupaxin, leupaxin-LD1 peptide (residues 1–20), leupaxin-LD3 peptide (residues 36–56), leupaxin-LD4 peptide (residues 86–104), and leupaxin-LD5 peptide (residues 125–150), and measured the binding affinity of the peptides to the Pyk2-FAT domain using ITC (Figure S3). We found that both leupaxin-LD1 and leupaxin-LD4 peptides bound to the Pyk2-FAT domain with a 2 to 1 stoichiometry; however, leupaxin-
LD3 and leupaxin-LD5 peptides did not bind to the Pyk2-FAT domain. The binding data for both leupaxin-LD1 and leupaxin-LD4 could be fitted to a sequential two-site binding model (Table 1). For leupaxin-LD1, the binding affinity ($K_D$) was 2.8 μM to the first site and 52.0 μM to the second; similarly, for the leupaxin-LD4 peptide, the binding affinity was 2.2 μM to the first site and 33.0 μM to the second site.

**Crystal Structural Study of Pyk2-FAT in Complex with Leupaxin-LD1 Peptide.** To further investigate the molecular basis for leupaxin LD motif recognition by Pyk2-FAT, we co-crystallized Pyk2-FAT with the leupaxin-LD1 and leupaxin-LD4 peptides. A detailed analysis of Pyk2-FAT binding to leupaxin-LD1 and leupaxin-LD4 was performed, along with comparisons to previously reported Pyk2-FAT complexes with paxillin-LD2 and paxillin-LD4. The leupaxin-LD1 and leupaxin-LD4 complex structures were determined at 2.5 and 1.8 Å, respectively. Data collection and refinement statistics are shown in Table 2, and final simulated annealing omit density for the peptides is shown in Figures 2A,C and 3A. Both leupaxin-LD1 and leupaxin-LD4 peptides form amphipathic helices upon interaction with the four-helix bundle of Pyk2-FAT (Figure 1).

In the Pyk2-FAT/leupaxin-LD1 crystal structure, Pyk2-FAT binds two solvent-exposed leupaxin-LD1 peptides via its H1/H4 and H2/H3 surfaces. At the H1/H4 interface, we observed electron density for the first 15 residues of the 20-residue leupaxin-LD1 peptide (Met1–Thr15), with residues Glu2–Arg13 forming an amphipathic helix (Figure 2B). As predicted from previous FAK and Pyk2 structural studies with paxillin’s LD motifs, hydrophobic interactions involving the leupaxin-LD1 core motif (1LDXLXXL13) are important for complex stability. The side chains of conserved leucines 4, 7, 8, and 11 of leupaxin-LD1 make hydrophobic contacts with the side chains of Tyr881, Met885, Leu892, Ala984, Val888, Lys988, and Leu991 of Pyk2-FAT in a manner similar to what we and others have observed for structurally equivalent residues of paxillin-LD2 and paxillin-LD4 peptides in the previously reported Pyk2-FAT/paxillin-LD2 (pdb code: 4R32) and Pyk2-FAT/paxillin-LD4 complexes (PDB codes: 3GM1 and 3U3F). These leucines are invariant among the LD1, LD2, and LD4 motifs of paxillin and leupaxin, with the exception of Leu8 in leupaxin, which is equivalent to Met270 of paxillin LD4. The association of Pyk2-FAT and leupaxin-LD1 is further strengthened by interactions with amino acids immediately flanking the core leupaxin-LD1 motif sequence. Unique to leupaxin-LD1, N-terminal Met1 fills a shallow cavity on the H1/H4 surface created by Tyr881, Leu991, Lys988, Arg875, and Asp995. Also, the overall binding affinity and α-helical stability of the N-terminus of leupaxin-LD1 are likely strengthened by an intermolecular hydrogen bond between the amide moiety of Leu4 and the hydroxyl moiety of Tyr881 of Pyk2-FAT. In addition, Glu3 forms a hydrogen bond with Arg889. Although the side chain of the equivalent glutamate in paxillin-LD2 and paxillin-LD4 was not visible in our previously determined Pyk2-FAT/petide structures (4R32 and 3U3F), modeling suggests that the interaction is structurally conserved. The Pyk2-FAT/paxillin-LD4 structure (3GM1) does not provide insight into this interaction because Glu265 of LD4 and Arg889 of Pyk2-FAT lie on opposite sides of an unrelated Pyk2-FAT molecule in the asymmetric unit. Finally, at the C-terminus of the bound leupaxin-LD1, Ser14 is observed in van der Waals contact with Lys911, with its side chain hydroxyl moiety forming an internolecular hydrogen bond to the carbonyl oxygen of Leu11.

For comparison, a detailed structural analysis of leupaxin-LD1 binding at H2/H3 of Pyk2-FAT was also performed (Figure 2D). Electron density for the first 13 residues of the 20-residue leupaxin-LD1 peptide was observed (Met1–Arg13), wherein residues from Glu2 to Glu12 form an amphipathic helix on the H2/H3 surface (Figure 2C,D). Within the core of the leupaxin-LD1 motif, the same types of interactions were observed as those noted for H1/H4 binding. Specifically, side chains of conserved leucines 4, 7, and 71 form hydrophobic interactions with Leu917, Ile921, Leu950, Ala951, Ile954, and Val910 of Pyk2-FAT. Also, Leu8 is in van der Waals contact with Gly914 and Arg918. In addition, the highly conserved aspartate that defines the LD motif, Asp5, forms a hydrogen bond with Arg18. These core motif interactions are indeed conserved among Pyk2-FAT complex structures with paxillin-LD2 and paxillin-LD4 peptides. Compared to paxillin-LD2 and paxillin-LD4, however, leupaxin-LD1 interactions with Pyk2-
Electron density for the leupaxin-LD4, like paxillin-LD4, binds strongest to H2/H3. FAT/paxillin-LD4 (3GM1 and 3U3F). Rather, it is likely that H4 site in a manner similar to what was observed for Pyk2-structural basis to suggest leupaxin-LD4 cannot bind the H1/H4 site. Therefore, there is no binding site. Furthermore, leupaxin-LD4 shares a strong 33.0 μ but it is highly suggestive that H2/H3 is a stronger a leupaxin-LD4 to bind the H1/H4 site of Pyk2-FAT in solution, H1/H4 site. This packing does not demonstrate an inability of bound at the H2/H3 site, wherein crystal packing blocked the Å Pyk2-FAT/leupaxin-LD4 structure contains leupaxin-LD4 side chain of Glu12 was disordered and not included in the LD1 might also contribute to binding in solution; although the side chain of Glu12 was disordered and not included in the final structure, rotamer analysis places it in proximity to form a hydrogen bond with Lys911. Finally, comparison of leupaxin-LD1 binding at both Pyk2-FAT surfaces suggests that leupaxin-LD1 may bind tighter to the H1/H4 site than to the H2/H3 site.

**Leupaxin-LD4 Binding at H2/H3.** As a further probe of LD motif binding, detailed X-ray structural analysis of leupaxin-LD4 binding to Pyk2-FAT was performed. Despite adding a 4 M excess of leupaxin-LD4 peptide to the cocryrstallization mixture, the stable complex that readily crystallized contained only one molecule of leupaxin-LD4 peptide bound to Pyk2-FAT. This result was somewhat unexpected as our ITC results indicate a 1:2 stoichiometry in solution with H2/H3 compared to those with H1/H4. Only Met1 was observed to interact with the H2/H3 surface. Specifically, Met1 makes a hydrophobic contact with Ile921 and van der Waals interactions with Arg918, Gly922, and Asp925 from helix H2. Modeling suggests that Glu12 near the C-terminus of leupaxin-LD1 might also contribute to binding in solution; although the side chain of Glu12 was disordered and not included in the final structure, rotamer analysis places it in proximity to form a hydrogen bond with Lys911. Finally, comparison of leupaxin-LD1 binding at both Pyk2-FAT surfaces suggests that leupaxin-LD1 may bind tighter to the H1/H4 site than to the H2/H3 site.

![Figure 4](image_url). NMR analysis for binding of Pyk2-FAT to the high-affinity leupaxin-LD1 and leupaxin-LD4 peptides. (A, B) Superposition of 1H−15N-HSQC spectra of Pyk2-FAT with leupaxin-LD1 and leupaxin-LD4 peptides added at different molar ratios is shown in black (1:0), blue (1:4 leupaxin-LD1), and magenta (1:4 leupaxin-LD4). (C) Histogram outlining the magnitude of the average chemical shift perturbation (CSP) of the 15N and 1H backbone amide resonances of Pyk2-FAT upon titration with leupaxin-LD1 and leupaxin-LD4 peptides. Red and black bars indicate chemical shift changes at 4 equiv of leupaxin-LD1 and leupaxin-LD4 peptides, respectively. Residues that show significant differences in the magnitude of CSP between additions of 4 equiv of leupaxin-LD1 and leupaxin-LD4 peptides are labeled. Helices 1−4 (H1−H4) are shown in gray.

from the solvent-exposed leupaxin-LD4 peptide was observed, with residues Ser88−Leu100 forming an amphipathic helix (Figure 3). The consensus core leupaxin-LD4 motif (93LDXL-LXXL100) displays the same structurally conserved interactions as those described earlier for leupaxin-LD1, paxillin-LD2, and paxillin-LD4. These involve hydrophobic contacts with conserved leucines 93, 96, and 100 and semiconserved Met97, as well as a hydrogen bond between the highly conserved Asp94 and Arg918 of Pyk2-FAT. Hydrophobic contacts are mediated by side chains from both α helices H2 and H3 of Pyk2-FAT; Val907, Val910, Lys911, Leu915, Leu917, and Ile921 from helix H2 and Leu950, Ala951, Ile954, and Met957 from helix H3 contribute to binding (Figure 3B).

Interactions between Pyk2-FAT H2/H3 and residues flanking the consensus core of leupaxin-LD4 motif further enhance complex formation (Figure 3B). Although N-terminal residue Lys86 is not involved in direct binding, it may enhance the helical stability of the leupaxin-LD4 peptide at the H2/H3 surface by mediating an intramolecular hydrogen bond to Asp94 while also packing against the helical turn formed by Ala90 and Ala91 of leupaxin-LD4. Interestingly, Lys86 is unique to leupaxin-LD4. Thr87 may also provide helical stability, and it contributes to peptide binding directly; the side chain hydroxyl moiety makes an intramolecular hydrogen bond to the amide moiety of Ala90, effectively serving as an N-terminal α-helical cap, while also forming a hydrogen bond to the side chain carboxylate of Asp925 from Pyk2-FAT. These dual roles for Thr87 may be present in paxillin-LD4 as it contains Ser260 at the equivalent position in the sequence alignment. Paxillin-LD2 does not have a Thr87 equivalent but, instead, contains a glycine residue. Ala89, common among LD4 of leupaxin and paxillin only, is in hydrophobic contact with Ile921 and is in van der Waals contact with Glu943, Lys944, and Asn947. Ala90 of LD4, which is structurally equivalent to...
were measured by adding unlabeled leupaxin-LD1 and for either leupaxin-LD1 or leupaxin-LD4 bound to Pyk2-FAT, we performed detailed binding studies using NMR. CSPs leupaxin-LD1 and leupaxin-LD4 in their association with Pyk2-FAT. Furthermore, we observed that both paxillin LD2 and LD4 motifs compete equally for H2/H3. The CSP differences observed for leupaxin’s LD motifs may result from direct interaction of leupaxin-LD1 and leupaxin-LD4 peptides at H1/H4 and H2/H3 sites, respectively. Therefore, our NMR studies suggest that Pyk2-FAT/leupaxin likely forms a stable complex.

**Full-Length Leupaxin Binding to Pyk2-FAT.** The results of the peptide-binding experiments demonstrate that leupaxin-LD1 and leupaxin-LD4 can dock to two distinct sites on the Pyk2-FAT domain. In order to explore full-length leupaxin interaction with Pyk2-FAT, we expressed a leupaxin construct comprising LD1–LD5 motifs (leupaxin1–151). We then determined the stoichiometry of the Pyk2-FAT/leupaxin1–151 complex in solution using SEC-MALS. The complex resolved as a single peak with a molecular mass of 29.5 kDa, indicating the existence of a 1:1 complex of Pyk2-FAT to leupaxin1–151 (theoretical mass of 33.6 kDa) (Figure S4).

We then used ITC to study the association of Pyk2-FAT with a truncated leupaxin construct comprising LD1–LD4 motifs (leupaxin1–105) since leupaxin-LD5 peptide had no detectable binding for Pyk2-FAT (Table 1). Representative plots for each titration are shown in Figure S5, and a summary of the thermodynamic parameters is given in Table 1. The data best fit to a single-site model assuming 1:1 stoichiometry with a $K_d$ of approximately 0.6 μM. This apparent 1:1 complex formation is consistent with the stoichiometry observed for the Pyk2-FAT/leupaxin1–105 complex by SEC-MALS. Interestingly, the binding affinity of Pyk2-FAT for leupaxin1–105 is approximately 4–5-fold higher than that for leupaxin-LD1 and leupaxin-LD4 peptides alone.

To further probe this interaction, we performed an NMR CSP experiment. During NMR titration of unlabeled leupaxin1–105 to 15N-labeled Pyk2-FAT (Pyk2-FAT/leupaxin1–105 ratios of 1:0.4, 1:0.8, 1:1.2, and 1:2.4), most of the amide resonances from the H1/H4 and H2/H3 sites disappeared below the limit of detection. This peak disappearance is due to an intermediate slow exchange regime, indicating tight peptide binding at the H1/H4 and H2/H3 sites. Upon addition of excess leupaxin1–105 to Pyk2-FAT (1:2), most of the resonances reappeared in the spectrum, suggesting the formation of a stable complex between Pyk2-FAT and leupaxin1–105 (Figure S6). However, we observed limited NOEs, hindering structure determination. Although the backbone resonances of this complex could be assigned, the lack of strong NOEs may be due to the slower tumbling of the large Pyk2-FAT/leupaxin1–105 complex. To examine whether leupaxin1–105 binding to Pyk2-FAT forms a stable complex, 1H–15N-TROSY spectra of Pyk2-FAT bound to leupaxin1–105 and Pyk2-FAT bound to a leupaxin-LD1 and leupaxin-LD4 mixture (1:1) were overlaid (Figure S7). The spectra overlay extremely well, suggesting that Pyk2-FAT binding to
leupaxin$^{1-105}$ closely resembles Pyk2-FAT binding to a 1:1 mixture of leupaxin-LD1 and leupaxin-LD4 peptides (Pyk2-FAT/leupaxin-LD1 + leupaxin-LD4) in solution. This experiment also confirms that leupaxin$^{1-105}$ loops and LD3 motif residues are not involved in binding.

We also performed reverse NMR titration by adding unlabeled Pyk2-FAT to 15N-labeled leupaxin$^{1-105}$. During titration, most of the amide resonances from the LD1 and LD4 motifs disappeared below the limit of detection due to a slow exchange interaction with Pyk2-FAT, which is indicative of tight binding. At a 1:2.4 ratio of leupaxin$^{1-105}$ to Pyk2-FAT, some of the peaks reappear in the spectra. However, they are weak in intensity, presumably due to the slow tumbling of the large complex, which precluded backbone assignment and structure determination of the complex (Figure 5). The large loop regions that connect the LD motifs may contribute to increasing the effective overall volume of the complex, further reducing the expected tumbling relative to a globular complex of the same molecular mass. Interestingly, during titration, we observed that residues residing in the loops between LD motifs exhibit chemical shift perturbation (Figure 5E). These loop residue peak shifts may be attributed to indirect effects arising from structural rearrangements of loops upon binding of the LD1 and LD4 motifs of leupaxin to Pyk2-FAT.

**Leupaxin-LD1 and Leupaxin-LD4 Binding to Pyk2-FAT.** To further assess the structure of the Pyk2-FAT domain in complex with both LD1 and LD4 motifs simultaneously, we designed a fusion construct by linking the leupaxin-LD1 motif at the C-terminus of Pyk2-FAT. Critical to the design, the optimized linker should not interfere with the interaction between the attached LD1 motif and the H1/H4 site of Pyk2-FAT. For this purpose, we generated three fusion constructs with various linker lengths. Among these constructs, the fusion protein containing an 8-residue linker (GGSGGGGG) exhibited the best quality$^1$H–$^{15}$N-HSQC spectrum compared to that with a 6-residue (GGSGGG) or 11-residue (GGSGGGGGG) linker (Figure S8), indicating that the LD1 motif of leupaxin within this fusion protein bound to the H1/H4 binding site of the Pyk2-FAT solidly. We term this fusion protein Pyk2-FAT-LD1.
With the Pyk2-FAT-LD1 construct in hand, we first asked whether there is any preferential association between leupaxin’s LD1 and LD4 motifs to the open H2/H3 site of the Pyk2-FAT in the fusion construct. To address this question, we first measured binding affinities of both leupaxin-LD1 and leupaxin-LD4 peptides to Pyk2-FAT-LD1 respectively by ITC. Representative titrations are shown in Figure S9, and the thermodynamic parameters of binding are listed in Table S1. As expected, the experimental data for leupaxin-LD4 peptide binding to Pyk2-FAT-LD1 best fit to a one-site binding model, with an estimated $K_D \sim 2 \mu M$. However, leupaxin-LD1 peptide binding to Pyk2-FAT-LD1 appears to be very weak compared to leupaxin-LD4 peptide, and thus a reliable $K_D$ measurement could not be obtained.

We also used CSP to study the interaction between leupaxin-LD1 and leupaxin-LD4 peptides with Pyk2-FAT-LD1. NH backbone resonances of Pyk2-FAT-LD1 were assigned based on the three-dimensional HNCO, HNCA, CBCA(CO)NH, HN(CA)NH, and CBCA(CO)CA spectra. Representative assignments are shown in Figure 6. Biochemistry 2016, 55, 1332–1345.
and HNCAB spectra. We then collected $^1$H−$^{15}$N-HSQC during the titration of leupaxin-LD1 and leupaxin-LD4 peptides to $^{15}$N-labeled Pyk2-FAT-LD1 (Pyk2-FAT-LD1/LD1 or LD4 ratios of 1:0, 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2). During NMR titration of LD4 to Pyk2-FAT-LD1, most of the amide resonances from the H2/H3 site undergo intermediate slow exchange, indicating tight peptide binding at the H2/H3 region (Figure S10A). However, during the titration of leupaxin-LD1 peptide to Pyk2-FAT-LD1, most of the amide resonances from the H2/H3 binding site undergo fast exchange, indicating weak peptide binding at the H2/H3 region (Figure S10B).

Although both ITC and CSP data for leupaxin-LD1 and leupaxin-LD4 peptide binding to Pyk2-FAT-LD1 supported the notion that H2/H3 is the preferential binding site for leupaxin-LD4 in the context of the native Pyk2-FAT/leupaxin complex, an overlay of the $^1$H−$^{15}$N-TROSY spectra for the Pyk2-FAT-LD1/leupaxin-LD4 complex and Pyk2-FAT/leupaxin$^{1−105}$ shows that they are indeed very similar (Figure 6A,B).

Furthermore, an overlay of the $^1$H−$^{15}$N-TROSY spectra for the Pyk2-FAT-LD1/leupaxin-LD4 complex and the spectrum of Pyk2-FAT bound to a 1:1 mixture of leupaxin-LD1 and leupaxin-LD4 peptides (Pyk2-FAT/leupaxin-LD1 + leupaxin-LD4) is also very similar (Figure 6C,D). We therefore conclude that the leupaxin-LD4 peptide likely binds to Pyk2-FAT-LD1 in the same way as the LD4 motif of full-length leupaxin.

We next exploited the Pyk2-FAT-LD1 fusion construct for cocryrstallization with leupaxin-LD4 peptide because this complex is a close approximate of the full-length leupaxin/Pyk2-FAT complex. The cocystal structure of Pyk2-FAT-LD1 bound to leupaxin-LD4 peptide was determined at 2.0 Å resolution (Figure 7A). Data collection and refinement statistics are shown in Table 2, and final simulated annealing omit density for peptides is shown in Figure 7B for LD4 and Figure S11A for LD1. Two copies of the protein/peptide complex were observed in the asymmetric unit, corresponding to a 1:1 association of Pyk2-FAT to leupaxin-LD4. As expected, the fusion LD1 motif of leupaxin binds at the H1/H4 surface, whereas LD4 binds at the H2/H3 site. At the H1/H4 interface, electron density was observed for residues Met1−Arg13 of the C-terminal-linked leupaxin LD1 motif. Most of the interactions between Pyk2-FAT and linked LD1 residues were similar to those observed for Pyk2-FAT bound to leupaxin-LD1 peptide (Figure S11B). Interestingly, at the H2/H3 interface of Pyk2-FAT-LD1, we observed electron density for the entire leupaxin-LD4 peptide (Figure 7B). This included three additional residues not observed in the Pyk2-FAT/leupaxin-LD4 structure (Figures 7C and S11C). Likely stabilized by crystal packing, these residues form an extra helical turn at the C-terminus. In addition to extending the helix−helix interface, these residues make key interactions with the H2/H3 site of Pyk2-FAT-LD1. Met103 makes hydrophobic contact with Met957 and van der Waals interactions with Arg958 and Gln961. Also, Gln104 of leupaxin-LD4 makes van der Waals contact with Val907 and a hydrogen bond with Lys911 of Pyk2-FAT (Figure 7C).

**Discussion**

Pyk2 overexpression has been associated with tumor progression in several cancers. Furthermore, studies have shown that Pyk2 and leupaxin, a member of the paxillin family of proteins, form a functional complex in human cancers. Like paxillin, leupaxin also contains LD motifs in its N-terminal region, and using biochemical, biophysical, and crystallographic techniques, we have demonstrated that two of the four leupaxin LD motifs directly bind to Pyk2. Previous reports showed that the Pyk2-FAT domain utilizes its H1/H4 and H2/H3 sites for the interaction with paxillin’s LD2 and LD4 motifs. However, there is no selectivity difference among the peptides; both bind with a 5-fold preference for the H2/H3 site ($K_d$: paxillin-LD2 = 6.9 μM; paxillin-LD4 = 8.0 μM) relative to the H1/H4 site ($K_d$: paxillin-LD2 = 35.2 μM; paxillin-LD4 = 46.3 μM). Furthermore, LD2 and LD4 exhibit roughly the same affinity for H2/H3, and this nonselective behavior likely contributes to the dynamic nature of the full-length paxillin/Pyk2-FAT complex. However, using NMR titrations, here we found that leupaxin’s LD1 and LD4 motifs exhibited a strong preference for the H1/H4 and H2/H3 sites of Pyk2-FAT, respectively (Figure 4). We propose that this is likely due to differences in specific residues that mainly reside outside of their common core LD motifs. Indeed, our structural analysis reveals that residues N-terminal to the core leupaxin LD4 motif (86KTSA89) enhance the affinity for the H2/H3 site relative to the LD1 motif of leupaxin that is devoid of these key residues at its N-terminus. In contrast, we reason that these additional residues at the N-terminus of the LD4 motif may sterically hinder binding at H1/H4 and render weak association relative to leupaxin-LD1 (Figure S12). Furthermore, in the context of full-length leupaxin, we propose that such a steric occlusion would likely be even more pronounced and that leupaxin-LD1 may represent the optimal helical length for Pyk2-FAT binding at the H1/H4 site of Pyk2.

To further examine the interaction between leupaxin and Pyk2, we expressed leupaxin$^{1−105}$ (comprising the LD1-LD3-LD4 region) and performed binding studies with Pyk2-FAT. Our ITC results show that there is a 4−5-fold increase in the binding affinity of leupaxin$^{1−105}$ for Pyk2-FAT relative to that of free leupaxin LD motifs (Table 1). This binding affinity of leupaxin$^{1−105}$ for Pyk2-FAT is 3-fold higher than that of paxillin133−290 (which comprises LD2-LD3-LD4 motifs) to Pyk2-FAT. In addition, our comprehensive structural and biophysical studies confirm that Pyk2-FAT and leupaxin$^{1−105}$ form a stable 1:1 complex in solution. Indeed, our extensive NMR studies support the hypothesis that leupaxin binds Pyk2-FAT in a concerted fashion. This discrete binding mechanism is in striking contrast to what we previously observed for Pyk2-FAT and paxillin133−290 where complex formation, mediated by paxillin’s LD2 and LD4 motifs, is highly dynamic and composed of two equally competing conformations. On the other hand, in this study, we show that leupaxin-LD1 binds to H1/H4 of Pyk2-FAT about 13-fold stronger than paxillin-LD2 does and 17-fold stronger than paxillin-LD4 does. Likewise, leupaxin-LD4 binds H2/H3 about 3−4-fold stronger than paxillin-LD2 or paxillin-LD4 does. Furthermore, both leupaxin-LD1 and leupaxin-LD4 are able to discriminate the two LD-binding sites of Pyk2-FAT, exhibiting about 15- and 19-fold higher affinities, respectively, at their preferred Pyk2-FAT binding site. The binding specificities of both LD motifs of leupaxin likely contribute to the stable interaction between leupaxin and Pyk2.

Pyk2 is a homologue of FAK; it can functionally replace FAK in certain biological processes where FAK is limited. However, the two proteins are not exactly the same. Structural studies of the FAT domains of the proteins in complex with paxillin showed that there are clear differences between the two proteins: Paxillin forms a stable complex with the FAK-FAT domain, whereas the interaction between paxillin and the Pyk2-FAT domain is very dynamic, likely due to the nearly equal...
binding affinities of paxillin-LD2 and paxillin-LD4 to the H2/ H3 site of Pyk2-FAT. This competition does not exist in the FAK-FAT/paxillin complex; paxillin-LD2 has a severely reduced ability to bind H2/H3 of FAK-FAT due to the sequence differences between FAK-FAT and Pyk2-FAT, especially the residues comprising H3 within the H2/H3 binding site. Like FAK and Pyk2, leupaxin is a functionally distinct homologue of paxillin. Leupaxin was identified as the binding partner of Pyk2. Indeed, in many cells, including the breast cancer cells examined in this study, gene expression of Pyk2 and leupaxin is closely correlated. Like paxillin, leupaxin uses its N-terminal LD motifs to interact with Pyk2. However, unlike paxillin, in this study, we found that the leupaxin complex formed with Pyk2 is very stable; the interaction between leupaxin and Pyk2 strikingly resembles the interaction between paxillin and FAK.

Therefore, our study not only confirms further con- 

cerns between FAK-FAT and Pyk2-FAT, but also sheds light on the complexity and specificity of supramolecular focal adhesion assemblies involving diverse protein—protein recognition events.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.1b01274.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 4XEF for the Pyk2-FAT/Leupaxin-LD1 complex, 4XEK for the Pyk2-FAT/Leupaxin-LD4 complex, and 4XEV for the Pyk2-FAT-LD1/Leupaxin-LD4 complex.

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Funding

This work was supported by NIH grants GM100999 and CA21765 (Cancer Center Support Grant) and by Research to Prevent Blindness. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Drs. Christy R. R. Grace and Weixing Zhang for NMR assistance and Drs. Patrick Rodrigues and Robert Cassell for peptide synthesis. X-ray diffraction data were collected at Southeast Regional Collaborative Access Team (SER-CAT) beamlines 22-ID and 22-BM at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html.

ABBREVIATIONS

Pyk2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; FA, focal adhesion; GEO, Gene Expression Omnibus; ITC, isothermal titration calorimetry; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser enhancement; TROSY, transient relaxation optimized spectroscopy; FAT, focal adhesion targeting; SE, sedimentation equilibrium; CSP, chemical shift perturbation; CARA, computer aided resonance assignment; MES, 4-morpholineethanesulfonic acid; SER-CAT, Southeast Regional Collaborative Access Team

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