Crystal Structure of the FERM Domain of Focal Adhesion Kinase*

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to focal adhesions in adherent cells. Through phosphorylation of proteins assembled at the cytoplasmic tails of integrins, FAK promotes signaling events that modulate cellular growth, survival, and migration. The amino-terminal region of FAK contains a region of sequence homology with band 4.1 and ezrin/radixin/moesin (ERM) proteins termed a FERM domain. FERM domains are found in a variety of signaling and cytoskeletal proteins and are thought to mediate intermolecular interactions with partner proteins and phospholipids at the plasma membrane and intramolecular regulatory interactions. Here we report two crystal structures of an NH2-terminal fragment of avian FAK containing the FERM domain and a portion of the regulatory linker that connects the FERM and kinase domains. The tertiary folds of the three subdomains (F1, F2, and F3) are similar to those of known FERM structures despite low sequence conservation. Differences in the sequence and relative orientation of the F3 subdomain alters the nature of the interdomain interface, and the phosphoinositide binding site found in ERM family FERM domains is not present in FAK. A putative protein interaction site on the F3 lobe is masked by the proximal region of the linker. Additionally, in one structure the adjacent Src SH3 and SH2 binding sites in the linker associate with the surfaces of the F3 and F1 lobes, respectively. These structural features suggest the possibility that protein interactions of the FAK FERM domain can be regulated by binding of Src kinases to the linker segment.

The adhesion of cells to the extracellular matrix is mediated by the integrin family of cell surface receptors (1). Extracellular matrix engagement triggers conformational changes and clustering of integrins leading to the remodeling of protein complexes at focal adhesions (2). These intracellular complexes contain actin-cytoskeletal proteins and signaling molecules that regulate a number of processes including cell migration, proliferation, and differentiation (3). Focal adhesion kinase (FAK)3 is present at focal adhesions and becomes both tyrosine-phosphorylated and activated in response to integrin signaling (4–6). As a potential mediator of integrin signaling, it is important to understand how FAK is activated and regulated.

FAK and the related tyrosine kinase Pyk2 (also referred to as CAK-β, RAFTK, or CADTK) comprise a subfamily of non-receptor protein-tyrosine kinases defined by a central tyrosine kinase domain flanked by an amino-terminal FERM domain and a carboxyl-terminal domain. The latter domain contains a proline-rich region that interacts with other signaling molecules and a distal focal adhesion targeting domain (Fig. 1A). Autophosphorylation of FAK at Tyr397 within a linker region between the FERM and kinase domain creates a binding site for SH2-containing proteins such as Src family kinases, the p85 subunit of phosphatidylinositol 3-kinase, and Grb7 (7–11). An adjacent RXXPXXP motif serves as a binding site for the Src family SH3 domain and contributes to the efficient recruitment of Src family kinases to FAK (12, 13). Subsequent phosphorylation of tyrosines (Tyr570 and Tyr577) within the kinase domain of FAK by the associated Src family kinase leads to the formation of an active signaling complex (14). Through phosphorylation of additional proteins assembled at the cytoplasmic tails of integrins, FAK and Src promote signaling events that modulate cellular growth, survival, and migration (6, 15, 16).

The amino terminus of FAK and Pyk2 share a region of homology with membrane-cytoskeletal linker proteins ezrin, radixin, and moesin (referred to collectively as ERM proteins) (17). The band 4.1 and ERM homology domain (FERM domain) is a module of ~300 amino acids found in a number of proteins that are membrane targeted (18). FERM domains of ERM proteins interact directly with the cytoplasmic regions of transmembrane receptors (19–21). In addition, association of the FERM domain with the phosphoinositide phosphatidylinositol 4,5-P2 has been demonstrated both structurally and with vesicle co-sedimentation (22–24). Mutagenesis of the basic residues within the FERM domain that mediate the phosphoinositide interaction alters the subcellular distribution of the protein (24, 25). These findings suggest that both protein–protein and protein–lipid interactions may be responsible for the efficient localization of FERM-containing proteins to membrane targets.

In addition to a general role as a membrane localization module, the FERM domain may serve additional regulatory functions. Among the 518 protein kinases annotated within the human genome, FERM domains are not a common accessory domain and are only found within FAK and Janus kinase families (26). The association of Janus kinases with the cytoplasmic tails of cytokine receptors is dependent on the FERM domain (27–30). Mutations in the Janus kinase 3 FERM domain inhibit receptor binding and may abrogate kinase activity (31).

To date, several interactions involving the FERM domain of FAK have been described including those with the cytoplasmic tails of inte-
FAK FERM Domain Structure

To better understand the function of the FERM domain of FAK, and as a starting point for dissecting its inter- and intramolecular interactions, we have determined its structure in two crystal forms at resolutions of 2.5 Å and 2.35 Å. The tri-lobed architecture of ERM family FERM domains is preserved in FAK (22, 43–47), but a unique orientation of the F3 subdomain alters the global conformation of the domain as compared with all previously studied FERM domains. The structures show that the phosphatidylinositol 4,5-P2 binding site found in ERM family FERM domains is not preserved in FAK. Additionally, the structures reveal that the FERM-kinase linker segment masks a potential protein interaction site on the F3 lobe; the corresponding site in the talin and radixin FERM domains has been shown to bind the cytoplasmic tails of β integrins and ICAM-2, respectively (21, 48). We hypothesize that this masking interaction could be released by binding of Src family proteins to their adjacent docking sites in the linker.

EXPERIMENTAL PROCEDURES

Materials—Restriction and modifying enzymes were purchased from New England Biolabs. Oligonucleotides were from Sigma; B834 Escherichia coli cells were from Novagen. Seleno-L-methionine was from Sigma; metal chelating, ion exchange, and gel filtration columns were from Amersham Biosciences.

Protein Expression and Purification—The FERM domain of avian FAK was amplified by PCR from cDNA (Swiss-Prot accession number Q00944). DNA fragments encoding amino acids 1–405, 31–405, and 31–399 were each ligated into a modified pET vector containing an FAK was amplified by PCR from cDNA (Swiss-Prot accession number P47117) and cloned into the pET vector. Electroelution of FAK and kinase domains was confirmed by DNA sequencing. The expression of recombinant protein in E. coli BL21(DE3) cells was induced at an optical density (OD600) of 0.8 by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside and grown for an additional 12–16 h at 18 °C. The cells were harvested and the pellets were stored frozen at −70 °C. Cell pellets were thawed in buffer A (20 mM Tris, pH 8.0, 200 mM NaCl, 5 mM imidazole, 5 mM β-mercaptoethanol) with the addition of 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM benzamidine, 10 mg/ml lysozyme, and 1 mg/ml DNase. Cell membranes were disrupted by sonication on ice and supernatants obtained following a 30-min centrifugation at 50,000 × g were loaded onto a Ni-chelating column. Protein was eluted with buffer A containing 200 mM imidazole. FERM domain-containing fractions were pooled and the His6 tag was cleaved by incubation with 1 μg of tobacco etch virus protease per mg of protein overnight at 4 °C. The concentration of NaCl was reduced to 50 mM by dialysis, and the protein was loaded onto a HiTrap Q column and eluted with buffer A containing 500 mM NaCl. The FERM domain was concentrated and loaded onto a Superdex 75 size exclusion column previously equilibrated in buffer A containing 100 mM NaCl. The eluted FERM domain of FAK was greater than 95% pure as estimated by SDS-PAGE and was concentrated to 6–10 mg/ml and stored at −70 °C.

Selenomethionine (SeMet)-substituted protein was prepared using the methionine auxotroph B834(DE3) strain. Cells were grown in M9 minimal media supplemented with SeMet (40 μg/ml). The subsequent steps for SeMet-labeled FERM domain proceeded as described for the native protein.

Cryotallyzation and Data Collection—Protein crystals of FAK (residues 31–399 of avian FAK) were obtained by the hanging drop method using 6–10 mg/ml protein mixed in a 1:1 volume ratio with precipitant solution containing 100 mM Tris, pH 8.0, 16–22% polyethylene glycol 4000, 20% glycerol, 200 mM NaCl, 100 mM CsCl, 10 mM dithiothreitol. Crystals grew over 1–2 weeks at room temperature reaching maximum dimensions of 40 × 100 × 20 μm. Diffraction data were recorded to 2.5-Å resolution from a single, frozen native crystal with a monoclinic space group, P21, and unit cell parameters a = 44.2, b = 146.2, c = 68.9 Å, β = 96.4°. Multiple anomalous diffraction data on a single, frozen, SeMet-derivatized FAK crystal was collected at the NSLS X12C beamline to 2.9-Å resolution (see Table 1 for details). Crystals of FAK (residues 31–405) were grown in 22.5% polyethylene glycol 4000, 0.1 M citrate, pH 5.5, 0.2 M ammonium acetate, 20% glycerol, and in the presence of 1.2 Molar ratio of inositol (1,4,5)P3. The phosphoinositide head group did not affect crystallization and is not present in the crystal structure. Diffraction data from a single frozen crystal with an orthorhombic space group (unit cell a = 50.54, b = 123.99, c = 133.93 Å) was collected at APS (14-ID-B) to 2.35-Å maximum resolution.

Structure Determination—The program SOLVE was used to determine the position of 10 selenium atoms from two copies of the FERM domain in the asymmetric unit in the FAK structure. The initial electron density map was improved by solvent flattening and phase-extension to 2.5-Å resolution using program DM (49). A partial model was built automatically using the program ARP/wARP (50). The structure was completed and refined with several rounds of manual rebuilding using the program O and atomic refinement with CNS. The model was assessed using PROCHECK (51) and waters were added by CNS. The refined model contains residues 33–363 of FAK, whereas residues 31–32 and 364–399 were not visible in the electron density maps and presumed to be disordered.

The structure of FAK was phased by molecular replacement using the previously determined FAK structure as the search model and the program Phaser (52). Difference electron density maps revealed that the ordered portion of one of the two molecules in the asymmetric unit extended to residue 375, and also revealed density corresponding to residues 394–403 and 394–401 in the linker regions of the two molecules. These regions were built and the FAK structure was refined as described above for the FAK structure.

RESULTS

Domain Mapping, Crystallization, and Structure Determination—The amino-terminal fragment of FAK (residues 1–405) containing the predicted FERM domain and autophosphorylation site Tyr979 was expressed in E. coli and purified. Limited proteolysis with the protease trypsin and amino-terminal sequencing of the resulting polypeptide identified a cleavage event that removed the NH2-terminal 28 residues of FAK (data not shown). Analysis of the FERM region of FAK and PYK2 from a number of species revealed that sequence conservation initiated at residues 31–399 (numbering refers to avian FAK). A truncated protein containing residues 31–399 (referred to here as FAK) was generated and produced monoclinic crystals that diffracted x-rays to better than 2.5-Å resolution.
FAK FERM Domain Structure

The FAK structure reveals the three-lobed architecture characteristic of the archetypal FERM domains of ERM family members ezrin (47), radixin (22), and moesin (43) (Fig. 1B). The three subdomains (labeled F1, F2, and F3) intimately associate with one another to form a compact structure with the overall shape of a cloverleaf. Each of the three lobes of the FERM domain bears striking similarity to otherwise unrelated single-domain protein structures as previously noted (43). The F1 lobe spans residues 33–127 and exhibits a ubiquitin-like fold (53) consisting of a five-stranded β-sheet capped by an α-helix. The F2 subdomain (residues 128–253) is all α-helical and contains a core 4-helix bundle similar to that found in the acyl-CoA-binding protein (54). The F3 lobe (residues 254–352) is a β-sandwich capped by a COOH-terminal α-helix that very closely resembles a pleckstrin homology domain. The pleckstrin homology domain shares the same three-dimensional fold with other modular signaling domains including phosphotyrosine binding and EVH1 domains in addition to the F3 lobe of the FERM domain (55). It has evolved distinct binding sites for diverse protein and phospholipid ligands (56). Both phosphotyrosine binding domains and the F3 lobe of some FERM domains bind peptide ligands in a groove formed between one edge of the β-sandwich and the COOH-terminal α-helix. Interestingly, this groove is occupied in the FAK FERM domain by residues just COOH-terminal to the FERM domain in the FERM-kinase linker segment (Fig. 4). This interaction is observed in both FAK399 and FAK405 and is discussed more fully in a following section.

The two protein molecules in the asymmetric unit are quite similar. They superimpose with a root mean square (r.m.s.) deviation of 0.9 Å over 330 amino acids. The only significant structural deviations in the two molecules are found in loop regions (the loop connecting α2 and α3 in the F2 lobe, the loop between strands β3 and β4, and the loop between strands β5 and β6 loop in the F3 lobe). Similarly, the FAK405 structure superimposes well with that of FAK399 (r.m.s. deviation of 1.2 Å for 324 equivalent residues). Both structures reveal a similar non-crystallographic dimer interface, but the contact between the two molecules is small (it buries ~400 Å² of surface area on each monomer) and is therefore not suggestive of a biologically relevant dimer. Also, the isolated FERM domain elutes in size-exclusion chromatography at a volume consistent with a monomeric protein (data not shown). However, FAK self-association is thought to play a role in its catalytic regulation and we cannot exclude the possibility that the FERM-FERM interaction observed in the crystal lattice is part of a larger interaction in the intact kinase.

Comparison with the Radixin FERM—The FAK FERM domain is rather distantly related to other FERM domains. For example, it shares only 12–15% sequence identity with ERM family members (Fig. 1C).
This considerable sequence divergence is reflected in significant structural changes in the FAK FERM domain as compared with other FERM domain structures studied to date. As expected, the overall fold of the FERM domain is preserved, but marked differences are observed both in the structures of the individual lobes and in their relative orientations within the domain as a whole (Fig. 2). In contrast, the FERM domains of merlin and ERM family members much more closely resemble one another (they superimpose with overall root mean square deviations on the order of 1 Å or less). We compare the FAK FERM domain with that of radixin, a well studied ERM family member.

The 64-residue core of the F1 subdomain superimposes with that of radixin with an r.m.s. deviation of 1.4 Å (Fig. 2A). The second helix in the F1 subdomain (F1-α2) is approximately two turns longer in FAK than in ERM family members. Additionally, the β1-β2 and α2-β3 loop regions are longer, and contain 310 helices not found in other FERM structures. These insertions account for the 12 additional residues within the F1 domain of FAK as compared with the structurally aligned FERM domain of radixin (Fig. 1C). Structural similarity with the F2 subdomain of radixin is limited to helices α1 to α4; the 78 residues in this 4-helix core superimpose with an r.m.s. deviation of 1.6 Å. The FAK F2 lobe includes four structural elements that are not seen in other FERM structures; these include three segments of 310 helix and a 9-residue helix α2', all of which are inserted between core helices α2 and α3 (Figs. 1C and 2A). The F3 lobes of FAK and radixin superimpose with an r.m.s. deviation of 1.5 Å over 73 residues for 6 of the 7 β-strands and the α1 helix. The relatively modest structural differences between the F3 subdomains of FAK and radixin include a shift in the position of the α3-α4 loop, a 310 helix within β4-β5 turn in FAK, and a 7-residue insertion in the loop connecting strands β3 and β6.

Perhaps the most striking structural difference between the FAK FERM domain and other intact FERM structures determined to date is the relative orientation of the subdomains. In particular, the position of the F3 lobe with respect to the F1 and F2 lobes is markedly different in FAK. The FAK FERM domain is shown superimposed with the structurally related radixin and merlin FERM domains in Fig. 2B. The FAK F3 subdomain is rotated and translated such that its α1 helix is positioned 3.5–4 Å closer to the F1 lobe and its β3 strand is ∼9 Å closer to the F1 lobe than the equivalent regions in radixin and merlin. This difference is
much greater than the shift described in comparison of the active and inactive-tail bound structures of moesin and ezrin (47, 57). The net effect of this rearrangement is a significant difference in the interfaces of F3 with both F1 and F2 as compared with other intact FERM structures. The F3 lobe is less intimately associated with the F2 lobe in FAK (a buried surface area of 749 Å² as compared with 990 Å² of contact in radixin). In contrast, the F1–F3 interface is more intimate (compare 1207 Å² buried in FAK with 1004 Å² in radixin). Interacting residues between the F1 and F3 domains of FAK include His99 with Glu338, Arg127 with Asp342, and Asn339 with Val95 and Trp97. This novel positioning of F3 against F1 is the same in the two independent FAK molecules in the crystallographic asymmetric unit and also observed in the FAK405 structure, which has different crystal packing contacts.

The divergent structure of the FAK FERM domain also indicates a divergence in function. The FERM domains of ERM proteins bind phosphoinositide phosphatidylinositol 4,5-P2; this interaction is important for localization of the ERM proteins in cells (24). The radixin FERM domain has been crystallized in complex with inositol 1,4,5-trisphosphate (the soluble head group of phosphatidylinositol 4,5-P2) (22). The IP3 head group binds radixin in a shallow, basic cleft between the F1 and F3 domains (Fig. 3B). The FAK FERM domain clearly cannot bind phosphoinositides in the analogous position (Fig. 3A and C). In FAK, the F1–F3 cleft is different in both structure and amino acid composition. The interface between the F1 and F3 domains of FAK is narrow relative to other FERM structures because of the divergent position of the F3 lobe as described above. Additionally, the presence of acidic residues and the lack of basic residues within this cleft is expected to preclude phosphoinositide coordination at this site in the FERM domain of FAK (Fig. 3, A and C).

The FERM-Kinase Linker Segment—In human FAK, the FERM domain ends at residue 352, and the kinase domain begins at approximately residue 415 (58). The intervening 60-residue linker segment is important for regulation of FAK function, as it contains the Tyr397 phosphorylation site (which when phosphorylated is bound by the Src SH2 domain) and also the binding site for the Src SH3 domain. The linker is largely disordered in FAK399, however, in both the FAK399 and FAK405 structures the first 10 residues of the linker bind in the cleft between the F1 and F3 lobes and in the putative peptide recognition groove on the F3 lobe (Fig. 4). In the longer FAK405 structure, the ordered region extends to Lys375 and encompasses the Src SH3 docking site. Additionally, a discontinuous segment of clear density is observed for residues 394--
Arg361 in the linker and the side chains of Asp101 and His89 in the F1 lobe include a salt-bridge hydrogen bond network involving both the F1 lobe and the F3 binding groove (Fig. 4A). Contacts with the F1 lobe include a salt-bridge hydrogen network involving residues Arg65 in the F1 lobe and the side chain of Arg361 in the linker, and the side chains of Asp101 and His89 in the F1 lobe, and also a hydrogen bond between the carbonyl of Thr62 of F1 and the side chain of Arg465 in the F1 lobe. The linker interaction with the F3 lobe is stabilized by hydrophobic interactions of Phe358 to Ile360 with the β5 strand in the F3 binding groove (Fig. 4C). In the FAK405 structure, the linker region beyond Pro362 no longer contacts the FERM domain. However, in one of the two molecules in the FAK405 structures, the ordered region extends through the Src SH3 binding site (residues 368–375), which interacts with the surface of the F3 lobe (Fig. 5A and B). The RALPSIP sequence (the key residues of the RXXPXXP Src SH3 binding motif are underlined) adopts a polyproline type II helical conformation and packs against the exposed β-sheet of the F3 lobe. Contacts with the F3 subdomain include 5 backbone-mediated hydrogen bond interactions including two with the side chains of Gln303 and Gln317, and hydrophobic interactions involving Leu370 and Ile373 (Fig. 5B). Interestingly, polyproline motifs bind to EVH1 domains of Ena/Vasp proteins, which also share the pleckstrin homology domain fold of the F3 lobe, in a topologically similar location (59). The orientation and specific interactions of the proline motif in the present structure, however, is not the same as that observed in EVH1 complexes.

The segment containing the phosphorylation site Tyr397 packs against the F1 subdomain of both FERM molecules (Fig. 5A and C). This polypeptide consists of residues 394–403 and forms a 3_10 helix and short β-strand that extends the anti-parallel β-sheet formed by the β1 and β2 strands of F1. This interface buries a total of 1047 Å² and comprises 7 backbone-mediated hydrogen bonds with only Thr62 of F1 making a specific side chain interaction and two side chain-mediated interactions of Gln403 with Ser54 and His41 (Fig. 5C). There are no previously identified examples of this surface of a FERM domain making a protein-protein interaction. The side chain of Tyr397 is surface exposed and in close proximity to Lys70. The last ordered residue of the COOH-terminal segment, Glu405, is near Lys38, His41, His75, and the clef region between the F1 and F2 subunits. Note that it is not possible to ascertain whether the interaction of this segment is intramolecular or whether it occurs between adjacent molecules in the crystal lattice because we do not observe density for the preceding 20 residues. Either connectivity is crystallographically plausible, but the fact that this construct is monomeric in solution suggests that the interaction is intramolecular.

DISCUSSION

The F3 binding groove, occupied by the NH₂-terminal portion of the linker segment in the present structure, is a well-characterized interaction site on FERM domains. In ERM family proteins, this groove is occupied by part of the COOH-terminal actin-binding tail, which makes intramolecular interactions with the FERM domain (43). Upon activation, this autoinhibitory conformation is thought to be released, allowing interaction with the cytoplasmic tails of cognate cell-surface receptors (60). Indeed, the tail region of ICAM-2 was recently demonstrated to bind at this site in a crystal structure of radixin complexed with an ICAM-2 peptide (21). The peptide forms a β-sheet interaction with the β5 strand of the F3 lobe, thereby extending the β-sheet (Fig. 257).
domain, as seen, for example, in the insulin receptor substrate-1 phosphotyrosine binding domain (61). Although the linker segment in FAK binds to the F3 lobe in the same location, its mode of binding is quite different from that in the structures noted above. The linker segment binds in the opposite orientation, thus forming a parallel rather than anti-parallel β-sheet interaction with strand β5 (Fig. 4).

The significance of the interaction of the NH2-terminal portion of the linker with the F3 lobe is unclear. It is possible that this portion of the linker segment is simply an extension of the fold of the FAK FERM domain itself, and therefore represents a structural divergence between FAK and other FERM domains. Alternatively, it is tempting to speculate that it represents a regulatory interaction, masking all or part of the binding site of an interacting protein. Like talin, FAK may associate with β-integrin tails. If it is a regulatory interaction, the structure suggests that it might be released by binding of a Src family protein to the SH3 and/or SH2 binding sites. Indeed, binding of a Src SH3 domain to the F3 groove by steric effects. The phosphorylation of Tyr397 is a key regulatory event in the activation of FAK and scaffolding properties that recruit SH2-domain containing proteins such as Src family kinases, phosphatidylinositol 3'-kinases, and Grb family adaptor proteins (62).

Phosphorylation and subsequent binding to the Tyr397 site will also require its dissociation from the surface of the FERM domain.

Although structurally unrelated, the interactions of the linker segment with the FERM domain are reminiscent of the intramolecular interactions between the SH3 domain and the SH2 kinase linker segment in Src family kinases. It is tempting to speculate that one or both of the linker contacts may additionally participate in autoinhibitory interactions with the kinase, in analogy with Src kinases where the SH3 and linker together bind the N-lobe of the kinase (63). In FAK, association of SH2-containing proteins may be an important step in the activation of the kinase by facilitating disassembly of the FERM-linker and or FERM/kinase intramolecular interactions. In loose analogy with Src kinases, phosphorylation of Tyr397 and subsequent recruitment of a Src family kinase to the linker segment might be expected to stabilize an open and active conformation of FAK. The present structure will facilitate mutagenesis studies designed to test these possibilities and to further dissect the intramolecular interaction of the FERM domain with the kinase domain.

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