The EBP50-moesin interaction involves a binding site regulated by direct masking on the FERM domain

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Summary

Members of the ezrin-radixin-moesin (ERM) protein family serve as regulated microfilament-membrane crosslinking proteins that, upon activation, bind the scaffolding protein ERM-phosphoprotein of 50 kDa (EBP50). Here we report a 3.5 Å resolution diffraction analysis of a complex between the active moesin N-terminal FERM domain and a 38 residue peptide from the C terminus of EBP50. This crystallographic result, combined with sequence and structural comparisons, suggests that the C-terminal 11 residues of EBP50 binds as an α-helix at the same site occupied in the dormant monomer by the last 11 residues of the inhibitory moesin C-terminal tail. Biochemical support for this interpretation derives from in vitro studies showing that appropriate mutations in both the EBP50 tail peptide and the FERM domain reduce binding, and that a peptide representing just the C-terminal 14 residues of EBP50 also binds to moesin. Combined with the recent identification of the I-CAM-2 binding site on the ERM FERM domain (Hamada, K., Shimizu, T., Yonemura, S., Tsukita, S., and Hakoshima, T. (2003) EMBO J. 22, 502-514), this study reveals that the FERM domain contains two distinct binding sites for membrane-associated proteins. The contribution of each ligand to ERM function can now be dissected by making structure-based mutations that specifically affect the binding of each ligand.

Key words: Moesin, Ezrin, EBP50, FERM

Introduction

The ezrin-radixin-moesin (ERM) family of closely related cytoskeletal proteins provide a regulated linkage between F-actin and membrane-associated proteins (Bretscher et al., 2002; Mangeat et al., 1999; Tsukita and Yonemura, 1999). The ERM proteins consist of an N-terminal ~300-residue FERM domain in a highly extended structure consisting of one β-strand and four helical segments (αA-αD) spread over a large surface of lobes F2 and F3 (Fig. 1). While Pearson et al. (Pearson et al., 2000) identified six potential interaction sites on the FERM domain, it could not be predicted whether the masking of membrane protein binding sites was direct or indirect. Subsequently, the structures of the free active FERM domains of radixin (Hamada et al., 2000), moesin (Edwards and Keep, 2001) and ezrin (Smith et al., 2003) revealed that they differ from the dormant structure mostly by an increase in mobility and some large local conformational changes in lobes F2 and F3. Smith et al. (Smith et al., 2003) noted that the structural changes seemed most related to the loss of helices αA and αD of the C-terminal tail, and they predicted that these positions would be sites of tight binding of target proteins (Smith et al., 2003).

While the dormant and activated structures of the FERM domain are now well characterized, very little is known about how the FERM domain recognizes the many proteins with which it interacts. Membrane protein ligands for ERM proteins include the adhesion molecules CD44, CD43 and I-CAM-1,2,3 (Heiska et al., 1998; Helander et al., 1996; Serrador et al., 1997; Serrador et al., 1998; Serrador et al., 2002; Tsukita
terminal tail are labeled with the nomenclature used in this paper: three FERM domain lobes are labeled as F1, F2 and F3 (F for FERM the surface of the FERM domain (from Pearson et al., 2000). The which binds as an extended peptide inhibitor to block a large part of Fig. 1.

The overall structure of dormant moesin. The N-terminal FERM domain (cyan) is associated with the C-terminal tail (red) which binds as an extended peptide inhibitor to block a large part of the surface of the FERM domain (from Pearson et al., 2000). The three FERM domain lobes are labeled as F1, F2 and F3 (F for FERM domain), and the major secondary structural elements of the C-terminal tail are labeled with the nomenclature used in this paper: β1 for the single β-strand, and αA, αB, αC and αD for the four α-helices. Fig. 1 was created with the programs Swiss-PdbViewer (Guex and Peitsch, 1997) and Pov-Ray (http://www.povray.org).

e-terial mother liquors containing 15% PEG 4000, 100 mM Tris pH 8.0 or Hepes pH 7.0 and glycerol concentrations of 0, 5, 10 and 15%, and were frozen in a cold N2 stream. Data were collected at ~150°C at the Cornell High Energy Synchrotron Source (CHESS; Ithaca, NY) and at the Advanced Light Source (ALS; Berkeley, CA), and processed with the programs Denzo/Scalepack (Otwinowski and Minor, 1997) or MOSFLM and SCALA (Collaborative Computational Project, 1984). Five data sets extending to between 4 and 3 Å resolution were collected at CHESS and ALS. The crystals showed ~1.5% variation in the a and c axis lengths, and difference maps revealed the data sets had variable peptide occupancy. The analyses presented here are all based on the single data set that appeared to have the highest peptide occupancy (Table 1).

Molecular replacement and refinement were done using CNS (Brünger et al., 1998), with the moesin FERM domain (Pearson et al., 2000) used as the search model. The rotation function using data from 15-4 Å resolution gave a unique solution 9.7 sigma above the mean, and the translation function solution gave a correlation coefficient of 0.4. With 10% of the data used for cross-validation, rigid body refinement (R=3.5 Å resolution) brought the R/R-free values from 45.1/42.8% to 43.1/41.4%. With one molecule in the asymmetric unit, the crystals are fairly loosely packed with a solvent content of 68% (V_M=3.84). Overall scaling revealed that the diffraction was highly anisotropic with B_11=69 Å², B_22=38 Å², B_33=31 Å², B_12=−32 Å², and consistent with this, statistics indicate that reliable data only extend to ~4.5 Å in the h-direction and to 3.5 Å in the k- and l-directions (data not shown). Most attempts at individual atomic refinement caused R-free to rise, but by adjusting the weights a minimization was

| Table 1. X-ray data collection statistics |
|------------------------------------------|
| Space group | C2 |
| Unit cell (Å) | a=126.6, b=70.1, c=62.7, β=105.9 |
| Resolution range (Å) | 3.5 (3.63-3.5) |
| Unique obs | 6712 (664) |
| Completeness (%) | 99 (100) |
| Multiplicity | 3.7 (3.9) |
| R_factor (%) | 8.4 (44) |

Data collected at ~150°C at Cornell High Energy Synchrotron Source (CHESS) using λ=0.943 Å.
Moesin-EBP50 interaction

Carried out that yielded \(R/R\)-free=33.8/40.1%. For higher resolution refinements, an \(R\)-free of 40% would imply serious problems with the model, but experience shows that convergence at an \(R\)-free of 40% is reasonable for refinement against data that extend only to about 3.5/4.5 Å resolution [see figure 4A of Brunger (Brunger, 1997)].

Site-directed mutagenesis of the FERM domain

The ezrin FERM mutant was made by recovering the DNA encoding the mutated residues (N210F and T214A) from a full length ezrin mutant (D.C. and A.B., unpublished data) by digestion with \(Kpn\)I and \(Hpa\)I and ligating it into a similarly digested ezrin FERM (1-297) construct (see Reczek et al., 1997). The construct was transformed into BL21 for expression and the protein was purified according to the FERM domain purification scheme (Reczek et al., 1997), except that the cultures were grown at 29°C overnight following induction to increase the yield of soluble protein.

Binding experiments

Ezrin FERM (1-297) was coupled to cyanogen bromide-activated Sepharose 4B beads (Sigma) at 2 mg protein per ml of beads as described previously (Reczek et al., 1997). 50 ml cultures of LB were inoculated with the protease-deficient \(E. coli\) strain ER2508 carrying plasmids for the expression of the MBP-EBP50 tail fusion proteins were expressed using the pMAL-c2 expression plasmid (New England Biolabs) transformed into the protease-deficient \(E. coli\) strain ER2508.
two eluted fractions were combined and analyzed by SDS-PAGE. The peptide beads were then blocked with L-cysteine HCl, washed and stored in PBS plus 0.05% sodium azide as a 50% slurry. The same protocol without the peptide was followed to prepare control beads. For each binding experiment, 25 μl of a 50% bead slurry was washed twice with buffer A. 40 μg of ezrin FERM or ezrin mutant FERM in 40 μl buffer A was added to yield an estimated 10:1 molar ratio of bound peptide to protein. Tubes were rotated for 1 hour at room temperature, the beads were then collected and washed three times with 1 ml buffer A. Bound protein was eluted with successive washes twice with buffer A. 40 μg of moesin FERM domain is close to the position of the cryo-electron density feature, making it appear more strongly as two turns of the helix. The second electron density feature matches the position of thebound ERM C-terminal tail (Fig. 2B). The strongest electron density feature revealed two features, whose interpretation was aided by comparison with the structure of the bound ERM C-terminal tail (Fig. 2B). The second electron density feature is near a crystallographic twofold axis, making it appear more complex (Fig. 2A), but the unique density closest to the relevant FERM domain is close to the position of αA (Fig. 2B). At this resolution, the two electron density features do not have sufficient detail to assign them to specific residues of peptide P38. However, in the first case, we have been able to partly do this by noting that an important component of αD in the dormant moesin complex is the α-carboxylate group of its C-terminal residues (Pearson et al., 2000; Smith et al., 2003), which is nestled into the C-terminal tail and the shape of the density corresponds to about three turns of the helix. The second electron density feature is near a crystallographic twofold axis, making it appear more complex (Fig. 2A), but the unique density closest to the relevant FERM domain is close to the position of αA (Fig. 2B). At this resolution, the two electron density features do not have sufficient detail to assign them to specific residues of peptide P38. However, in the first case, we have been able to partly do this by noting that an important component of αD in the dormant moesin complex is the α-carboxylate group of its C-terminal residue (Pearson et al., 2000; Smith et al., 2003), which is nestled into the FERM domain and bound by Asn210, Lys212, and Ser214. Assuming that P38 recognition at this site also involves an α-carboxylate group, then the residues bound must be the C-terminal α-carboxylate of the bound helix. For testing the impact of mutations in the FERM domain, we used an N210F and T214A (N210F/T214A) double mutant of the ezrin FERM domain (originally created for another purpose; D.C. and A.B., unpublished data). As noted above, moesin residues Asn210 and Ser214 (Thr214 in ezrin) hydrogen-bond with the terminal α-carboxylate of the bound helix. In addition, Asn210 hydrogen-bonds to another carbonyl oxygen of the bound helix and stabilizes the functional conformation of the 210-214 turn in the FERM domain by hydrogen-bonding to the peptide nitrogen of residue 214. Consistent with our model for EBP50 recognition, the mutant ezrin FERM domain failed to be retained by beads on which P14 was immobilized (Fig. 3B).

**Discussion**

Taken together, the crystallographic data, the sequence

terminal ERM-association domain (C-ERMAD) with Glu244 on the FERM domain. Of additional note is the Trp348 side chain that is conserved between EBP50 and E3KARP. The ERM proteins have a Thr at the equivalent position (Fig. 2C), which is buried in the dormant moesin complex, so that a Thr substitution would seem unfavorable. However, when a Thr side chain is modeled into this position, it fits into a pocket surrounded by Phe240, Ile245, Ile257, Pro259, Ala264 and Phe267. We have insufficient clues to confidently interpret the second region of density associated with lobe F2 (see Discussion).

**Results**

Crystallographic analysis of the peptide:FERM domain complex

To explore how EBP50 binds to the moesin FERM domain, a synthetic peptide (P38) corresponding to the last 38 amino acids of EBP50, plus an additional cysteinyI residue at the N terminus, was synthesized and co-crystallized with purified moesin FERM domain (residues 1-297). Crystals of the complex grew under conditions that did not lead to crystals in the absence of peptide. The crystals had highly anisotropic density associated with lobe F2 (see Discussion). 14 residues of EBP50 are sufficient for recognition

The first biochemical approach to validate our interpretation was to test the binding of a shorter EBP50 peptide. Since the electron density indicated the key involvement of at least 11 C-terminal residues of P38, we added additional cysteinyI residues to avoid steric effects and synthesized P14, a peptide having the last 14 residues of EBP50 plus an additional Cys residue at the N terminus. The P14 peptide, covalently coupled to agarose beads, bound the wild-type ezrin FERM domain, whereas control beads lacking the peptide did not (Fig. 3A). Thus the 14 residues at the C terminus of EBP50 are sufficient for binding to the FERM domain.

Designed mutations of EBP50 and ezrin disrupt recognition

A second biochemical approach to validate our structural model was to test the effects of mutations in both EBP50 and ezrin specifically designed to disrupt the proposed interaction between the last 11 residues and F3 in the FERM domain. The EBP50 mutations were created in the context of a fusion between the maltose binding protein (MBP) and the 39 C-terminal residues of EBP50. The mutations of EBP50 targeted the two conserved hydrophobic residues, Phe355 and Leu358, that we propose are bound in complementary hydrophobic pockets in the FERM domain. The variants created were F355R, in which an arginine was substituted for Phe355, and L358Δ, in which the (C-terminal) Leu358 was deleted. Binding experiments showed that the mutations did disrupt binding: the fusion protein with wild-type EBP50 sequence was efficiently retained from a bacterial lysate by beads containing immobilized FERM domain, whereas the construct with F355R was not, and the construct with L358Δ was only poorly retained (Fig. 3B).
Fig. 3. Biochemical support that the C-terminal residues of EBP50 bind as proposed. (A) Residues F355 and L358 in the EBP50 tail are important for the interaction with the FERM domain. Bacteria containing plasmids for the expression of MBP fused to the C-terminal 39 residues of EBP50 (WT), or containing the F355R mutation (F355R) or the last residue deleted (L358A), were grown to log phase and either subjected to induced protein expression with IPTG (+) or not induced (−). Lysates were prepared and applied to beads on which the ezrin FERM domain had been immobilized. After washing, bound proteins were eluted and analyzed. The three eluates show proteins recovered from the induced lysates; essentially no material was recovered from parallel uninduced lysates (not shown). (B) The C-terminal 14 residue peptide of EBP50 binds to the FERM domain. Purified wild-type ezrin FERM domain (Wild type) or purified ezrin mutant N210F/T214A FERM domain (N210F/T214A) was mixed with beads containing covalently linked peptide (Peptide) or beads lacking the peptide (Control). The load (L), unbound (U), and bound and eluted (B) fractions were analyzed by SDS-PAGE.

The atomic coordinates and structure factors (code 1SGH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org).

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