Ezrin is a member of the ERM (ezrin, radixin, moesin) family of proteins that cross-link the actin cytoskeleton to the plasma membrane and also may function in signaling cascades that regulate the assembly of actin stress fibers. Here, we report a crystal structure for the free (activated) FERM domain (residues 2–297) of recombinant human ezrin at 2.3 Å resolution. Structural comparison among the dormant moesin FERM domain structure and the three known active FERM domain structures (radixin, moesin, and now ezrin) allows the clear definition of regions that undergo structural changes during activation. The key regions affected are residues 135–150 and 155–180 in lobe F2 and residues 210–214 and 235–267 in lobe F3. Furthermore, we show that a large increase in the mobilities of lobes F2 and F3 accompanies activation, suggesting that their integrity is compromised. This leads us to propose a new concept that we refer to as keystone interactions. Keystone interactions occur when one protein (or protein part) contributes residues that allow another protein to complete folding, meaning that it becomes an integral part of the structure and would rarely dissociate. Such interactions are well suited for long-lived cytoskeletal protein interactions. The keystone interactions concept leads us to predict two specific docking sites within lobes F2 and F3 that are likely to bind target proteins.

The ERM1 (ezrin (1,2)/radixin (3,4)/moesin (5)) family of proteins serve as regulated cross-linkers between the actin cytoskeleton and the plasma membrane (reviewed in Refs. 6 and 7). Ezrin, radixin, and moesin are found in vertebrates as highly similar paralogs (~75% sequence identity) that differ in their primary tissue distributions but probably have a large degree of functional equivalence. In addition to binding to actin filaments and membrane proteins like CD44 (8) and ICAM2 (9), members of the ERM family appear to function in cell signaling as they interact with several signaling molecules, including the 85-kDa regulatory subunit of PI3-kinase (designated p85) (10), the multifunctional regulator, RhoGDI (11), EBP50 (12), and the tumor suppressor, hamartin (13). At least some of these various binding activities are regulated by a masking/unmasking phenomenon (14), and it is becoming apparent that such a mechanism is a theme common to many cytoskeletal proteins such as vinculin (15, 16), Wiscott-Aldrich syndrome protein (17), and formins (18). Our structural and functional studies of the ERM proteins are aimed at elucidating the details of this type of regulation.

The ~65-kDa ERM proteins consist of three functional domains: an amino-terminal 300-residue FERM domain (band four-point one, ezrin, radixin, moesin homology domains) that is responsible for binding to membrane proteins and many signaling proteins (reviewed in Ref. 7), a central 200-residue putative coiled-coil region that when phosphorylated on Tyr-353 contributes to an interaction with p85 (10), and a carboxy-terminal 100-residue auto-inhibitory carboxyl-terminal tail domain (also known as the C-ERMAD) that contains the F-actin binding site (19, 14). In resting cells, ERM proteins are in a dormant state characterized by an intramolecular association of the FERM and tail domains. This dormant state has no known binding partners except perhaps the regulatory subunit of protein kinase-A (20), although additional partners cannot be ruled out. One pathway of activation (i.e. release of the FERM-tail interaction) of the ERM proteins appears to be triggered by phosphorylation of a specific threonine in the tail domain (Thr-558 in moesin) by the Rho-associated kinase (21) and the protein kinase C-ß (22, 23) and/or the association with the phosphotyrosine lipid, PIP_2 (24, 25).

Our structural understanding of ERM function was given a solid foundation with the determination of the crystal structure for a dormant moesin FERM-tail complex (26). It revealed that the FERM domain is a clover-shaped molecule consisting of three structural domains (lobes F1, F2, and F3). Residues 2–82 (lobe F1) possess a ubiquitin-like fold, residues 83–195 (lobe F2) fold into a topology like that of acyl-CoA-binding protein (27), and residues 196–297 (lobe F3) adopt the pleckstrin homology/phosphotyrosine binding fold found in a broad range of signaling molecules, including dynamin, Sos, and Shc (28–30). In the dormant molecule, the carboxy-terminal tail binds as an extended peptide covering a large surface of lobes F2 and F3 of the FERM domain. It was speculated that the tail domain could cause dormancy by either directly blocking partner protein interactions or by sequestering the FERM domain from other signaling molecules.
Binding sites and/or by inducing conformational changes in the FERM domain that alter (and inactivate) binding sites (26). Subsequently, structures reported for the active FERM domains of radixin with and without bound inositol (1, 4, 5)-triphosphate (IP₃) (31) and of moesin (32) have given some insight into the structural aspects of activation. The structures of unmasked radixin with or without IP₃ (2.8 Å resolution) were highly similar to each other, and comparison to dormant moesin showed local changes in three regions, 138–150, 160–178, and 243–280. The structure of activated moesin (2.7 Å resolution) also revealed shifts in these three regions (32). However, the moesin analysis was complicated by crystal packing interactions that caused large shifts in lobes F1 and -3 and concluded that consistent shifts due to activation were limited to residues 166–170 and 260–264.

Here we present the 2.3 Å resolution crystal structure of the activated FERM domain of ezrin. This represents the first structure for the protein ezrin. Combining this structure with the other two active ERM structures gives us an enhanced ability to identify those structural changes that are due to activation as opposed to sequence differences or crystal packing interactions. Moreover, we go on to document that mobility changes are an important aspect of activation that has not been previously described. Furthermore, our analysis of the active (radixin, moesin, and ezrin) and the dormant (moesin) ERM structures reveals that the tail domain contributes residues that allow the FERM domain to complete its folding, a concept that we refer to as a keystone interaction.

**EXPERIMENTAL PROCEDURES**

**Protein Crystallization and Data Collection**—The crystallization of and data collection for the FERM domain of human ezrin (residues 1–297) have been described previously (33). Briefly, the crystals were grown by the hanging drop method using a reservoir solution containing 10–15% (w/v) monomethyl-ether PEG 2000 (Fluka), 15% glycerol (w/v), 10% 2-propanol (w/v), 0.1 M Na-Hepes, (pH 8.1), and they belong to the space group P2₁ with cell constants a = 48.5 Å, b = 112.8 Å, c = 66.3 Å, β = 102.3°. Two molecules occupy the asymmetric unit. The crystallographic R-factor was 30.1% after rigid body refinement using data from 2.7 to 2.3 Å. At this stage the 2Fₐ – Fₑ electron density was of sufficient quality to guide the construction of the ezrin FERM molecule using the program O (35). Refinement using amplitudes as the maximum likelihood target involved manual rebuilding, simulated annealing, and conjugated gradient minimization without NCS restraints. An overall anisotropic B-factor correction with a low resolution cutoff of 6 Å yielded Bₐ = 15 Å², Bₑ = 22 Å², Bₐ = 6 Å², and Bₑ = 8 Å². Waters were placed initially using the CNS water pick program and later by manual inspection. Simulated annealing omit maps were also used occasionally to check the quality of the model. The final model includes 2 chains of ezrin (A and B), each with residues 2–297 of ezrin, and water molecules. The crystallographic R-factor is 22.3%, and R-free is 27.6% (Table I).

### Table I

Statistics for x-ray structure determination

|                           |                  |
|---------------------------|------------------|
| Space group               | P₂₁              |
| Unit cell parameters (Å)  | a = 48.49, b = 112.80, c = 66.301, β = 102.32° |
| Total measured reflections| 183710           |
| Unique measured reflections| 27498            |
| Completeness (%)          | 89.3 (77.2)      |
| Resolution range (Å)      | 26.0–2.3         |
| Rₚₐₓ (%)                  | 7.2 (25.6)       |
| R-factor (%)              | 22.9 (39.9)      |
| R-free (%)                | 28.2 (36.4)      |
| Bond lengths r.m.s (Å)    | 0.011            |
| Bond angles r.m.s (°)     | 2.20             |
| Average B-factor (Å⁻¹)    | 53               |
| Number of water molecules | 314              |

* Multiplicity corrected Rₚₓ as defined by Diederichs and Karplus (52).

† Deviations from ideality.

Unusual geometries in well ordered regions of the structure include cis-prolines at residues 75 and 297. Because of weak side chain electron density, residues Ser-148, Arg-151, and Lys-162 were modeled as alanine in both molecules in the asymmetric unit.

**Structural Comparisons**—FERM domain structures used for comparison included ezrin chains A and B (from this work), radixin (PDB code 1GE7), radixin bound to IP₃ (PDB code 1GE6), moesin (PDB code 1E5W), and the dormant moesin bound to the carboxyl-terminal tail domain (PDB code 1EF1). Overlays were accomplished using the programs LSQMAN (36) and DOMOV (37). The overlays reveal that cis-Pro-75 is modeled as a trans-Pro in the radixin structures; we suspect that this is an error and note that it has not influenced our analyses.

**RESULTS**

**Structural Overview**—Crystals of the FERM domain (residues 1–297) of ezrin diffract to 2.3 Å and have two molecules in the asymmetric unit. The structure was solved by molecular replacement, and refinement has led to a model that consists of residues 2 to 297 for each of two chains and 314 water molecules (Table I). The two molecules in the asymmetric unit A and B are in approximately equivalent environments because the non-crystallographic symmetry is nearly a perfect centering operation. Consistent with this, molecules A and B are very similar with a root mean square deviation of C₆ₐ-atoms of 0.4 Å, and all descriptions will refer to molecule A unless specifically noted. The FERM domain is a clover-shaped molecule (Fig. 1A) consisting of three distinct lobes and is globally similar to structures reported previously for ERM proteins (26, 31, 32), merlin (38, 39), and the band-4.1 protein (40). This basic structure has already been well described and will not be elaborated here.

**Mobility Properties of the FERM Domain**—In contrast, the mobility properties of the FERM domain have not yet been described, although such information is available from protein crystallography in the form of atomic B-factors (also known as temperature factors). Knowledge of mobility along the chain in the activated and dormant structures is likely to provide insight into the mechanism of activation. We first examine the mobility properties in each of the published FERM domains, and then in the next section we compare them to those of dormant moesin to gain insight into the activation process. B-factors are related to the amplitude of motion of the atoms so that in the absence of large model errors, higher B-factors indicate a higher disorder of the atom in question (41). As seen visually in Fig. 1A and graphically in Fig. 1B, the B-factors...
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Fig. 1. Overall structure and mobility of ezrin. A, a ribbon diagram of the ezrin FERM domain and a semitransparent molecular surface illustrates the compact globular cloverleaf-shaped structure possessing three lobes: lobe F1 (residues 2–82), lobe F2 (residues 83–195), and lobe F3 (residues 196–297). The ribbon is colored with respect to temperature factor ramped from cold-blue to hot-red for B-factors from $\leq 20$ to $\leq 80$ Å$^2$, respectively. B, temperature-factor plot as a function of residue number for activated FERM domains of ezrin (red), radixin (cyan), and moesin (green), and the dormant FERM domain of moesin (violet). All ribbon figures were prepared with the programs Bobscript and Raster3D (49–51).
FIG. 2. Overlay of the FERM domains of the ERM family members. Superposition of the FERM domains of ezrin (red), radixin (cyan), and active moesin (green) onto dormant moesin (violet) bound to the inhibitory carboxyl-terminal tail (ghostly yellow). To be most informative, these overlays have been done as described in Fig. 3B.

have Asn-74 and Gln-77, whereas moesin has Ser-74 and Leu-77.

For lobe F2 (residues 88–199), the lobe rotation is again small, ranging from 1.2–1.5°. However, major local changes (up to 3-Å shifts) occur for residues 132–151 and 155–177 (Fig. 3B). As seen in Fig. 4A, these changes involve coordinated shifts of the last turn of helix B and the following loop and of all of helix C (maximal at its amino terminus). A look at side chain positions reveals some clear ways that these movements associated with activation can compensate for the removal of helix A of the carboxyl-terminal tail. The three most notable cases are the shift of Trp-175 to fill the pocket vacated by Leu-525, a flip of the side chain of Arg-171 to help fill the space vacated by Leu-529, and a shift in the side chain of Trp-168, as well as the whole of residues 160–162, to fill space vacated by residues 533 to 537 (Fig. 4A). Residues 132–151 pack onto the backside of lobe F2 helix C, and their motion appears to be due to being pulled along by the shifting helix C. In addition to many non-specific contacts in this core, a key side chain connecting helix C to this chain segment is the buried His-176 whose side chain hydrogen bonds to Tyr-146-OH and Gly-135-O.

For lobe F3, the rigid body rotation is again small, only 0.7–1.4°. Notable local changes in the conformation of lobe F3 include the small shifts of residues 211–213 and the extensive shifts of residues 240–267, which have a maximal shift of nearly 6 Å at residue 261. These movements can be described as mainly a collapsing of the β1-β2 hairpin and the β-meander subdomain (β5, -6, -7) into the space occupied by helix D of the carboxyl-terminal tail (Fig. 4B). As with lobe F2, it is possible to describe some clear ways that these movements can be seen to compensate for the removal of helix D of the carboxyl-terminal tail. The movement of the β-meander seems tied to a shift of Phe-267 to fill the space that had been occupied by Phe-574 and so Pro-265 and Asp-266 can fill the space occupied by Ile-571. Similarly, the side chain of Lys-237 flips directions so that it fills the space occupied by Met-577. Interestingly, the change of Lys-237 seems correlated with flips of the side chains of Glu-229 and Asn-231 (Fig. 4B).

Mobility Changes because of Activation—A broad comparison of the mobilities of the activated structures with those of dormant moesin reveals that the overall mobility of lobe F1 remains similar during activation, but the mobilities of lobes F2 and F3 as a whole increase significantly (Fig. 5). This results in an inversion such that the lobe F1 goes from being the least well ordered lobe to being the most well ordered lobe. The transition occurs near residue 88 (see Fig. 1B), which is about halfway through the linker between lobes F1 and F2 (26).
main, but we must leave open the question of how much their mobility increases upon activation.

Inspection of the dormant moesin complex structure (26) indicates that the increases in mobility near residues 212 and 265 can be directly traced to the removal of helix D of the carboxyl-terminal tail. In the first case, residues 210, 211, 212, and 214 all make H-bonds to residues 574–577 (the last four residues of the inhibitory tail), and Leu-216 is buried by the side chain of Met-577 in the complex (see Fig. 6B). In the second case, the key seems to be that Phe-267 of /beta/2-strand 7 was well buried by helix D, but with helix D absent Phe-267 is no longer pinned in place, which increases the lever arm around which the loop near residue 260 can fluctuate.

DISCUSSION

The crystal structure of ezrin presented here completes the picture, so that a structure is now available for the FERM domains of each of the three ERM proteins. Also, the 2.3-Å resolution of this structure provides details such as solvent structure, not visible in the lower resolution structures of activated moesin (2.7 Å) or radixin (2.8 Å). Because the FERM domain fold is already well characterized, one major value of this structure derives from the opportunity to compare all the ERM structures to differentiate which structural changes are because of activation and which are because of crystal packing.
interactions or sequence differences among the ERM proteins.
A second major value of this work is that the analyses of B-factors reveals that, in addition to conformational changes, FERM domain activation involves large increases in the flexibility of lobes F2 and F3. This increase in flexibility has important bearing on the energetics and specificity of ligand binding (see below).

**The Extent and Origins of Conformational Changes**—The use of the quantitative variation in the activated structures as a reference for defining what changes are significant is powerful in that it allows a quantitative, unambiguous delineation of a set of residues that show significant motion (Fig. 3B). However, it is worth noting that because of the limited resolution of the structures and possible variations among activated structures due to crystal packing interactions and intrinsic chain mobility, we do not expect the analysis to reliably reveal changes in structure much smaller than about 1 Å, even though such changes must exist.

Our analysis confirms the validity of the conformational changes due to activation that were proposed based on the activated radixin structure (31) as opposed to the much more limited set of changes highlighted in the analysis of the activated moesin structure (32). These changes are all local in nature, involving virtually no relative movements of the lobes as a whole. This is in line with the expectation of Pearson et al. (26) that the relative positions of the three FERM domain lobes are rather well fixed. However, it must be borne in mind that the >5° lobe rotations induced by a crystal packing interaction in the moesin structure show that significant motions can occur upon the binding of an appropriate ligand.

But what triggers these changes? Both the conformational and mobility changes associated with the activation of ERM proteins appear to be concretely related to the loss of interactions with parts of the autoinhibitory carboxyl-terminal tail, in particular helix A and the AB loop (Fig. 4A), helix D (Fig. 4B), and possibly strand β1 (see Fig. 2). The effect of the loss of strand β1 of the carboxyl-terminal tail is not clear because the residues in contact with that strand (e.g. Ile-245, Ile-248) do move but their movement can already be related to the loss of helix D. The loss of other parts of the carboxyl-terminal tail are not visibly associated with conformational changes even though they cover about half the surface area buried by the carboxyl-terminal tail. Indeed, of 57 FERM domain residues highlighted by Pearson et al. (Fig. 4 of Ref. 26) as involved in carboxyl-terminal tail binding, only 28 of them are in the regions that change upon activation.

**The Role of IP₃ Binding in Activation**—It was proposed by Hamada et al. (31), based on the radixin-IP₃ complex, that IP₃ binding pushes helix A to widen the cleft between lobes F1 and F3 and that this movement in helix A induces the other shifts in lobe F3 and leads to activation. The similarity of activated ezrin to activated radixin (with or without IP₃) strengthens the argument presented by Edwards and Keep (32) that the movements of lobe F3 are not a direct outcome of IP₃ binding but are simply related to the loss of the carboxyl-terminal tail. It could be that IP₃ would also force these changes to occur (and thus stimulate activation), but there is no evidence that that is true. At a more fundamental level, one important outstanding question regarding the relevance of the IP₃-radixin complex is whether IP₃ can substitute for PIP₂ in activation experiments. We are not aware of experiments that answer this question, and if IP₃ does not serve as an activator then results about its binding site may not be informative about how PIP₂ activates ERM proteins. In this regard, the mutagenesis results of
Barret et al. (44), though not conclusive in themselves, support the idea that activation by PIP_2 may be more complex than the crystallographically defined IP_3 binding site would imply.

Helices A and D of the Carboxyl-terminal Tail as Keystone Binding Partners—As we see it, the high degree of disordering that occurs upon activation indicates that lobes F2 and F3 of the FERM domain can be viewed as incompletely folded domains; that is, lobes F2 and F3 need residues from the carboxyl-terminal tail (or some other source) to be contributed to their hydrophobic cores so they can complete their folding (Fig. 6). In the case of lobe F2, Leu-525 and Leu-529 of helix A insert into the core to complete the fold, and in the case of lobe F3, Ile-571, Phe-574, and Met-577 of helix D insert to do the same. This type of interaction suggests that the carboxyl-terminal tail residues and the associated helices that hold them serve to complete and stabilize the structures of lobes F2 and F3 much like a keystone completes and stabilizes the structure of an arch. Although it was not called a keystone interaction, a classic example in which an incompletely folded protein requires an external piece to complete its folding is provided by bovine trypsinogen (45). As synthesized, the inactive protease precursor trypsinogen has a highly mobile incompletely folded “activation domain,” and upon cleavage at Ile-16, the new amino terminus tucks inside the protein, as a keystone, to complete and stabilize the structures of lobes F2 and F3 much like a keystone completes and stabilizes the structure of an arch. Although it was not called a keystone interaction, a classic example in which an incompletely folded protein requires an external piece to complete its folding is provided by bovine trypsinogen (45). 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