Interactome Analysis Reveals Ezrin Can Adopt Multiple Conformational States*5

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Background: Ezrin is a conformationally regulated microfilament-membrane linker restricted to the apical aspect of epithelial cells. Conformational States

Results: Quantitative mass spectrometry identified proteins that associate with different ezrin conformations; the interactors include novel apical microvilli-associated proteins, with classes perceiving ezrin’s conformation differentially.

Conclusion: Different proteins selectively associate with three distinct conformational states of ezrin.

Significance: This study extends the conformational activation model of ezrin and identifies new interacting partners.

Ezrin, a member of the ezrin-radixin-moesin family (ERM), is an essential regulator of the structure of microvilli on the apical aspect of epithelial cells. Ezrin provides a linkage between membrane-associated proteins and F-actin, oscillating between active/open and inactive/closed states, and is regulated in part by phosphorylation of a C-terminal threonine. In the open state, ezrin can bind a number of ligands, but in the closed state the ligand-binding sites are inaccessible. In vitro analysis has proposed that there may be a third hyperactivated form of ezrin. To gain a better understanding of ezrin, we conducted an unbiased proteomic analysis of ezrin-binding proteins in an epithelial cell line, Jeg-3. We refined our list of interactors by comparing the interactomes using quantitative mass spectrometry between wild-type ezrin, closed ezrin, open ezrin, and hyperactivated ezrin. The analysis reveals several novel interactors confirmed by their localization to microvilli, as well as a significant class of proteins that bind closed ezrin. Taken together, the data indicate that ezrin can exist in three different conformational states, and different ligands “perceive” ezrin conformational states differently.

The establishment and maintenance of cell polarity are carried out through the asymmetrical distribution of macromolecules. An important contributor to the structure of microvilli on the apical aspect of epithelial cells is the F-actin-binding protein ezrin. Ezrin, the founding member of the conserved ezrin-radixin-moesin (ERM) protein family, was originally isolated from the intestinal brush border (1) and later shown to play essential roles in cell polarity throughout animal development (2). Ezrin adopts a highly polarized distribution in epithelial cells where it is found both in the cytoplasm and specifically associated with the microvillar plasma membrane. Consistently, it is essential for the normal morphology of microvilli in vivo (3–6) and in cultured cells (6, 7).

In their inactive state, ERMs undergo an intramolecular head-to-tail association, masking binding sites for both plasma membrane-associated proteins on their N-terminal four-point-one ezrin-radixin-moesin (FERM) domain and the F-actin-binding site in the C-terminal tail. The appearance of ezrin in its active state on the microvillar plasma membrane requires direct interaction with the membrane phospholipid PIP(4,5)P2 through its N-terminal FERM domain (8–12) followed by phosphorylation on a conserved C-terminal threonine (Thr-567 in ezrin (7, 8, 13)). In epithelial cells, kinase and phosphatase activities drive constant, dynamic interconversion between membrane-bound phosphorylated ezrin and cytoplasmic dormant, unphosphorylated ezrin, with each state having a half-life of 1–2 min (7, 14, 15).

Although ezrin is generally considered to simply oscillate between open/active or closed/inactive states, there are likely to be varying degrees of ezrin openness, reflecting the existence of multiple conformational states. Notably, in vitro analysis has suggested that phosphorylation of the C-terminal threonine in ezrin creates a partial but not fully open state (16). Thus, we explored this possibility by examining two forms of open ezrin in our analysis.

Upon reaching the plasma membrane, ERM proteins engage a number of membrane-associated factors through the N-terminal FERM domain. Numerous binding partners of mammalian ERMs have been identified (Table 1). In most of these interactions, the interacting protein has been proposed to be the effector as opposed to being the regulator of ERMs. Conversely, one of these, the scaffolding ERM-binding phosphoprotein 50 (EBP50, also known as NHERF1 or SLC9A3R1), has been shown to regulate the ERM-dependent formation of microvilli (17–19). However, transmembrane ERM-binding proteins have also been proposed to play a role in ERM recruitment or clustering in the apical domain leading to the formation of microvilli (20), although no such protein has yet been identified in epithelial cells. Moreover, in vitro analyses show that although the surfaces on the FERM domain for EBP50, PIP(4,5)P2, and transmembrane proteins are distinct (10,
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21–25), there is likely to be a complex interplay among all of these ligands (26). Thus, multiple regulatory ERM binding partners might be identified by an unbiased proteomic screen for ERM-binding proteins in epithelial cells.

Here, we report the first global analysis of ezrin binding partners in an epithelial cell line. We first established a reversible cross-linking strategy that preserves the transient interaction between ezrin and its strongest known interactor, EB5P0. We then used mass spectrometry to determine the ezrin interactome under these optimized conditions. Next, we examined how the ezrin interactome changes depending on its conformational state, and we documented the changes in response to ezrin conformational shift. The analysis reveals many novel components of microvilli that discriminate between different open forms, as well as an unexpected category of proteins binding to the closed form of ezrin.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids for stable or transient transfection of ezrin-iFLAG and variants (in pQCXIP, Clontech) have been previously described (7). The "K4N" mutation (22) was generated by inverse PCR (using primers 5′-CCC ATC GAC AAC AAC GCA CCT GAC TTT GTG TTT TAT GCC CCA C-3′ and 5′-TTT AAT GAC AAA GGT ATT GTC ATT GAA AGA GAT GTT CCT GAT TTC ACT CC-3′). To clone TACTSTD2, BASP1, SLC1A5, FAM129B, and EPCAM, Jeg-3 RNA was first extracted using the RNeasy kit (Qiagen) and then reverse-transcribed using the SuperScript III reverse transcriptase poly(dT) primer (Invitrogen). The open reading frames were cloned from the cDNA using PCR (with the following primers: TACTSTD2, 5′-GTT CGA GGA TCC ATG GCT CGG GGC CCC GGC-3′ and 5′-TCC AAC AAG TCA GCT CAA GCT CGG TTC CGG TCC GTC TCT TCA CCA TTC CC-3′; BASP1, 5′-GTT CGA GGA TCC ACC ATG GGA GGC AGG CTC AGC ACG AAG AAG-3′ and 5′-TCC AAC AAG TCA GCT TCT TCA GAC GTT TTA TAC CAC GGT TGT TCT GTC GG-3′; SLC1A5, 5′-GTG GCC GAT CCT CCT CGA GAC-3′ and 5′-GTT CGA GGA TCC ACC ATG GGA GGC AGG CTC AGC ACG AAG AAG-3′) and inserted into pcDNA-DEST47 (Invitrogen); EPCAM, cloned from Jeg-3 cDNA by PCR (using primers 5′-GTC GAA AGC TGG GTA GAA CTC AGT CTG CAC CCC TGC GTC CAC GC-3′ and 5′-GGG GAC CAC TTC TTA GTA CAA AAG AGC AGG CCT TAT GGC GGA GCT GTC GTC CAC GC-3′ and 5′-GGG GAC CAC TTC TTA GTA CAA AAG AGC AGG CCT TAT GGC GGA GCT GTC GTC CAC GC-3′ and 5′-GGG GAC CAC TTC TTA GTA CAA AAG AGC AGG CCT TAT GGC GGA GCT GTC GTC CAC GC-3′ and 5′-GGG GAC CAC TTC TTA GTA CAA AAG AGC AGG CCT TAT GGC GGA GCT GTC GTC CAC GC-3′). Antibodies—FLAG antibody and resin were from Sigma. Antibody against ezrin was CPCT-Ezrin-1; moesin was CPCT-Moesin-1, and β-dystroglycan (DAG1) was MANDAG2, all from the Developmental Studies Hybridoma Bank. Antibody against an epitope present in all ERMs was from Cell Signaling Technologies. Antibodies against radixin and EB5P0 have been described (7, 28). Antibody against FHOD-1 was from Abcam. Antibody against Myc was 9E10 from Roche Applied Science. Antibody against GFP was from Santa Cruz Biotechnology. Antibody against HA was HA.11 from Covance. Antibody against TACTSTD2 was GA733.1 (immunofluorescence) from Abcam or H-85 from Santa Cruz Biotechnology (Western blot).

Cell Culture, Transfection, and Stable Cell Line Creation—Jeg-3 cells were obtained from the American Type Culture Collection and cultured in minimal Eagle’s medium supplemented with GlutaMAX-1 (Invitrogen), penicillin/streptomycin (Invitrogen), and 10% FBS (Invitrogen) and maintained at 37 °C and 5% CO2.

All transient transfections were by polyethyleneimine (PolyPlus) as described previously (17). Following transfection, cells were grown for ~18 h prior to use. Stable cell lines expressing ezrin-iFLAG variants were transfected as described previously (7) and then batch-selected using puromycin (2 μg/ml). Stable cell lines expressing BASP-1-GFP and SLC1A5-GFP were generated by transfection with the appropriate plasmid, grown in G418 (400 μg/ml) for 2 weeks, and ring-cloned and pooled at least three GFP-expressing colonies.

SILAC3 Labeling—Jeg-3 cells stably transfected with pQCXIP and derivatives and then selected for at least 2 weeks were grown for at least 2 additional weeks in SILAC minimal Eagle’s medium (Thermo) containing 0.1 mg/ml normal lysine and arginine ("light") or 15N2lysine and 15N4arginine ("heavy," Sigma) containing 10% dialyzed FBS (Invitrogen), penicillin/streptomycin (Invitrogen), and puromycin (2 μg/ml). Incorporation was verified by mixing lysate preparations from heavy and light cells (1:1) followed by mass spectrometry. Expected morphology and ezrin-iFLAG localization after labeling was verified by immunofluorescence (data not shown).

Dithiobis(succinimidyl Propionate) (DSP) Cross-linking—Cells grown to ~80% confluence were washed three times with PBS and treated with 1.25 mm DSP (Thermo) at 37 °C for 2 min. The cells were then removed to room temperature and washed three times with TBS. They were incubated in the last TBS wash for 15–20 min prior to use. Expected morphology and ezrin-iFLAG localization after labeling were verified by immunofluorescence (data not shown).

3 The abbreviations used are: SILAC, stable isotope labeling in cell culture; MLS, microvilli-like structure; DSP, dithiobis(succinimidyl propionate); Pi(4,5)P2, phosphatidylinositol 4,5-biphosphate.
**Immunoprecipitation and Mass Spectrometry**—For SILAC mass spectrometry, ~4 x 10^7 DSP-treated heavy and the same number of light cells were independently scraped into cold IP buffer (25 mM Tris, pH 7.4, 5% glycerol, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 8.7 mg/ml para-nitrophenyl phosphate, 0.5% Triton X-100, 0.1 μM calyculin A, protease inhibitor table from Roche Applied Science), incubated for 2–3 h in the cold using 20 μl of FLAG M2 affinity gel (Sigma), and then washed four times in IP wash buffer (25 mM Tris, pH 7.4, 5% glycerol, 150 mM NaCl, 50 mM NaF, 0.2% Triton X-100). Gel was then eluted by boiling in 50 mM Tris, pH 8.0, containing 1% SDS, reduced, and alkylated. Proteins were precipitated with 50:49.9:0.1, acetone/ethanol/acetic acid, reconstituted in urea solution, and trypsin-digested overnight. Peptides were then purified on a C-18 column (Waters), dried, and reconstituted in 80% acetonitrile and 1% formic acid and fractionated by hydrophilic interaction chromatography. Fractions were dried, reconstituted in 0.1% trifluoroacetic acid, and analyzed by LC-MS/MS using an Orbitrap XL mass spectrometer (Thermo). Database search and peptide quantification of heavy/light isotope ratios were performed as described previously (29). Criteria for selection of true interactors are presented under “Results.”

For all other immunoprecipitations, ~8 x 10^6 cells transfected with 7 μg of appropriate plasmid(s) were lysed in IP buffer, immunoprecipitated with 4 μl of FLAG M2 affinity gel for 2–3 h, washed four times with IP wash buffer, eluted by addition of 3 x FLAG peptide to 200 μg/ml, and incubated for 10 min at room temperature. Soluble material was separated in a spin column (Sigma), denatured by boiling in Laemmli buffer, and resolved by SDS-PAGE.

**Immunofluorescence**—Cells grown on glass coverslips were fixed in 3.7% formaldehyde/PBS for 15 min at room temperature. Cells were then washed with PBS and blocked with IF buffer (PBS + 0.5% BSA + 0.5% goat serum + 0.1% Triton X-100) for 10 min. Primary and secondary antibodies were then applied in IF buffer with 1% FBS. After antibody addition, the coverslips were washed three times in PBS, mounted in Vectashield (Vector Laboratories), imaged using a CSU-X spinning disk microscope (Intelligent Imaging Innovations) with a spherical aberration correction device, 63 x 1.4 NA objective on an inverted microscope (Leica), and acquired with a QuantEM EMCCD camera using Slidebook software (Intelligent Imaging Innovations). Maximum intensity projections were assembled in Slidebook and exported to Adobe Illustrator software. For clarity, side projections were vertically expanded 3-fold using Illustrator.

**RNA Interference**—Jeg-3 cells were transfected with 30 nm of siRNA for 72 h prior to use by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. siRNAs were from Ambion, Dharmacon, or Integrated DNA Technologies as follows: GL2 luciferase (17), ezrin (7), radixin (5’-UAGAGAUGUUUCUGAGGAAdUdU-3’), TACSTD2 (1-1, 5’-GUUAAAGAGUUAGAUUGGAAT-3’ and 2-5’, GCUGUUAAAGAGUUAGAUUGGAAT-3’), and DAG1 (5’-CCCUGAGCCUCUUAAdTdT-3’).

**RESULTS**

**Cross-linking Prior to Immunoprecipitation Is Required to Recover the Ezrin-EBP50 Interaction**—Since its discovery in 1983 (1), numerous proteins have been shown to interact with ezrin (Table 1). Among the most tenacious in vitro interaction partner is EBP50 (SLC9A3R1), a scaffolding protein that binds with very high affinity through its C-terminal region to the FERM domain of ezrin (28). The binding site for EBP50 on the FERM domain is masked in the closed, inactive form of ezrin (21, 28, 30). Our goal was to perform an unbiased screen for proteins present in epithelial cells that bind to active ezrin *in vivo*, and EBP50 was used as a positive control to establish optimal conditions.

Our strategy centers around the generation of cell lines expressing various mutants of ezrin representing various conformational states and then analyzing the bound proteins that are co-recovered with ezrin. As reported recently, we have placed a FLAG epitope into an internal region of ezrin that is poorly conserved in ERM proteins (7). Using this tagged version designated ezrin-iFLAG, we have generated stable Jeg-3 chorionic epithelial cell lines that express wild-type ezrin (ezrin-iFLAG), a variant with the activating T567E phosphomimetic mutation (ezrin-T567E-iFLAG), an inactive variant with the nonphosphorylatable T567A mutation (ezrin-T567A-iFLAG), and a variant containing a truncation of the C terminus that compromises the normal FERM/C-terminal tail interaction to expose the fully masked FERM domain of ezrin (ezrin(1–583)-iFLAG). The level of the ezrin-iFLAG variant expression in these cells is about the same as endogenous ezrin (7). As expected, ezrin-iFLAG localization was strictly apical, co-localizing with another ERM member radixin, whereas ezrin-T567A-iFLAG is cytoplasmic, and as reported recently, ezrin-T567E-iFLAG hyperlocalizes to the plasma membrane and localizes to the apical and basolateral domains of epithelial cells (Fig. 1A) (7).

As about 50% of the ezrin-iFLAG is phosphorylated *in vivo* (7), we were initially surprised to find that immunoprecipitation of ezrin-iFLAG failed to recover detectable EBP50. By contrast, the construct with an unmasked FERM domain due to the truncation of the final two residues, ezrin(i–583)-iFLAG, demonstrated recovery of EBP50 efficiently (Fig. 1B). To see if the interaction of EBP50 with ezrin-iFLAG can be stabilized, we explored the use of the reversible cross-linking reagent DSP. Cells were subjected to different concentrations of cross-linker for 2 min, and the recovery of EBP50 with ezrin-iFLAG was monitored. These results showed that brief cross-linking with 1.25 mM DSP allowed for modest recovery of EBP50 with ezrin-iFLAG and vice versa (Fig. 1B). To explore how the ezrin/EBP50 interaction was affected by ezrin Thr-567 phosphosite mutations, we detected EBP50 in unphosphorylatable ezrin-T567A-iFLAG or phosphomimetic ezrin-T567E-iFLAG immunoprecipitations (Fig. 1C). The modest recovery of EBP50 was significantly reduced by mutation to T567A but was unaffected by mutation to T567E (Fig. 1C). We obtained similar results by immunoprecipitating EBP50 and detecting ezrin-iFLAG phosphomutants (Fig. 1D). Thus, the DSP-preserved interaction between EBP50 and ezrin is dependent on the availability of Thr-567 for phos-
phorylation and can be greatly increased by fully unmasking the FERM domain of ezrin in ezrin-(1–583)-iFLAG (Fig. 1C).

Use of SILAC-Mass Spectrometry to Identify the Ezrin Interactome—To use mass spectrometry to identify proteins other than EBP50 that bind to wild-type ezrin, the ezrin-iFLAG cells were subject to SILAC relative to a reference culture expressing just the empty vector control. The control cells were subjected to SILAC relative to a reference culture other than EBP50 that bind to wild-type ezrin, the ezrin-iFLAG samples, background material can be minimized and proteins enriched in the ezrin-iFLAG sample identified (Fig. 2A).

In each mass spectrometry run, 7,000–13,000 high confidence peptides (representing 2,000–3,500 unique proteins) were detected with most binding the FLAG antibody beads as background. To identify ezrin-iFLAG-bound proteins, we computationally selected those present at a heavy/light or “Xpress” ratio of greater than 3.6:1, and then to ensure reproducibility, we further selected only those that were detected in wild-type ezrin-iFLAG immunoprecipitations in at least 5/6 mass spectrometry experiments (biological replicates). These were considered as true interactors (Fig. 2B; supplemental Table 1). Of the 38 reproducibly interacting proteins, 9 were expected as either these proteins or their orthologs had been previously implicated in binding ERMs. These included the other ERM protein in these cells, radixin (“RDX,” see Ref. 31), previously implicated in binding ERMs. These included the kinases LOK, SCYL3 (34), and syndecan-4 (“SDC4”), an adhesion molecule that contains the minimal syndecan ERM-binding motif first identified in syndecan-2 (35, 36). Additionally, the kinases LOK and SLK were identified, which were shown to be the major C-terminal threonine kinases for ERMs in *Drosophila* and

| Interaction partner | Selected refs. | Proposed binding mode | Proposed function |
|---------------------|---------------|-----------------------|-------------------|
| Aquaporin-0         | 50            | Unknown               | Unknown           |
| ARHGAP-18/conundrum | 33            | FERM domain           | Active RhoA level |
| Bitesize            | 51            | FERM domain (Jobe F3) | Adherens junction morphology |
| CD46                | 52            | Unknown               | Melanoma cell line migration through control of ERM-RhoGDI interaction |
| CD44                | 9, 20, 53, 57–62 | FERM domain         | Migration speed and directionality; lymphocyte polarization; microvilli length and number |
| CLIC3/4/5           | 32, 42        | C-terminal region of ezrin | Microvilli formation in RPE cells (CLIC4) |
| Dbl                 | 48, 63–67     | FERM domain with Dbl PH | Active Cdc42 level |
| DCC                 | 68, 69        | FERM domain           | Netrin-induced axon guidance |
|Dlg1                | 70, 71        | FERM domain           | Immune synapse formation |
| SLC9A3R1/2          | 17, 28, 21, 28 | FERM domain         | Microvilli formation; clustering of various receptors |
| (EBP50/E3KARP)      | 30, 49, 72    |                       |                   |
| Eps8/L1a            | 73            | Coiled-coil domain NPXY motif with Eps8 SH3 | Microvillus length and spacing |
| Fes                 | 74            | Coiled-coil domain NPXY motif with Fes SH3 | Cell spreading and migration in response to HGF |
| ICAM-1/2/-3         | 24, 25, 75–79 | FERM domain           | Polarization of ICAMs to the uropod in migrating lymphocytes; microvilli length and number |
| L1CAM               | 80, 81        | FERM domain           | Neurite outgrowth |
| Myo1Bae             | 82            | Unknown               | Unknown           |
| NHE-1               | 83, 84        | FERM domain           | Migration speed and directionality |
| NHE-3               | 85            | FERM domain           | NHE-3 surface distribution in OK cells |
| SCYL3/PACE-1        | 34            | C-terminal region of ezrin | Unknown |
| Palladin            | 86, 87        | Ezrin (278–585) with palladin | Unknown |
| PDZD8               | 88            | Unknown               | Glutamylated microtubule abundance and HSV-1 viral entry |
| PLEKHG6             | 89            | FERM domain with PLEKHG6 | Macropinocytosis |
| Podocalyxin         | 90            | Unknown               | Unknown           |
| PSGL-1              | 78, 91–95     | FERM domain           | Lymphocyte rolling on P-selectin |
| Rab11               | 96            | Unknown               | Collective cell migration |
| Ras                 | 97            | FERM domain           | Active Ras level |
| RhoGDI              | 9, 66, 98     | FERM domain           | Active Rho GTPase level |
| S100P               | 99, 100       | FERM domain           | Cellular calcium response |
| SAP97               | 101           | Unknown               | Unknown           |
| SOS                 | 107           | FERM domain with SOS DH/PH | Active Ras level |
| SYND2/Syndecan-2    | 35, 36        | FERM domain           | Unknown |
| Vps11/HOPS          | 102           | Coiled-coil domain    | Endocytosis |
| WWOX                | 103           | Coiled-coil domain NPXY motif with WWOX SH3 | Secretion in gastric parietal cells |
| WWP-1               | 104           | Coiled-coil domain NPXY motif with WWP-1 SH3 | Cell migration in response to HGF |
| ZAP-70              | 55            | Unknown               | Immune synapse formation |
| ITGβ8/β4 integrin   | 105           | FERM domain           | Filopodia formation in C2C12 cells co-transfected with active Cdc42 |
| DAG1/β-Dystroglycan  | 41, 48        | FERM domain           |                                         |
mammalian epithelial and immune cells (7, 37–39). Consistent with ezrin localization at the plasma membrane and the nature of established interaction partners, 18/38 (47%) of the interaction partners were known or predicted to be transmembrane or membrane-associated proteins (supplemental Table 1).

**Interactome of Phosphorylated Versus Unphosphorylated Ezrin**—To identify which of these interactors bind preferentially to phosphorylated ezrin, we made use of SILAC comparing Jeg-3 cells expressing the nonphosphorylatable ezrin-T567A-iFLAG (labeled light) versus wild-type ezrin-iFLAG (labeled heavy; Fig. 3A). Because ezrin-iFLAG can be phosphorylated in vivo, this approach allowed us to identify proteins that selectively associate with active, phosphorylated ezrin and, conversely, ones that preferentially associate with inactive ezrin-T567A-iFLAG. The degree of enrichment for each candidate was normalized to the degree of enrichment of ezrin peptides. Biological replicates were performed, and correlation analysis confirmed that the enrichment statistics were reproducible (Fig. 3B). As another approach to identify proteins that distinguish the activity state of ezrin, we also undertook the SILAC procedure with cells expressing ezrin-iFLAG (labeled light), of which about 50% should be in the active state, and cells expressing ezrin-T567E-iFLAG (labeled heavy), a mimic for the fully phosphorylated form. Again, correlation analysis between biological duplicates confirmed that enrichment statistics were reproducible (Fig. 3C). Moreover, most of the proteins identified in the two approaches were the same and were quite well correlated (Fig. 3D). The results of this analysis are presented in Fig. 3E (left), where a consensus of proteins (from these and subsequent experiments, listed by Uniprot identifier) that bind more active ezrin is shown on the left. These data reveal that binding of many interactors to ezrin was enhanced for wild-type versus ezrin-T567A, and approximately two-thirds of these were further enhanced by the phosphomimetic ezrin-T567E mutation. Among these proteins, as expected, is radixin (RDx), the other ERM protein present in these cells, EBP50 (“SLC9A3R1”), β-dystroglycan (“DAG1”), CLIC4, and the kinases LOK (“STK10”) and SLK. In addition, about a dozen new proteins were identified.

We also identified a subclass of proteins that were recovered at approximately similar levels with inactive ezrin-T567A versus wild-type ezrin, yet they were more strongly identified as binding wild-type ezrin versus ezrin-T567E (Fig. 3E, right). We designated these proteins as interactors with closed ezrin. These include the F-actin nucleating formin FHOD-1, the Ras GTPase activating protein RASA1, and SCYL3 (also known as PACE-1 for protein-associating with the carboxyl-terminal domain of ezrin, see Ref. 34). We believe this is the first report of such proteins, which constitute a significant fraction of all ezrin-binding proteins.

**Interactome Shows Enhanced Binding to Fully Unmasked Ezrin FERM Domain**—The analysis above identified proteins that preferentially associate with phosphorylated versus unphosphorylated ezrin. In the current understanding of ezrin regulation, the phosphorylated form is regarded as fully active. However, it is clear from the results presented in Fig. 1 that EBP50 is much more efficiently recovered with an ezrin construct when the FERM domain is completely unmasked ezrin(1–583)-iFLAG, than
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**A.** Vector (Light) Ezrin-iFlag (Heavy)

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**B.**

| Uniprot ID | Uniprot Description | Xpress Ratio, Ezrin-iFlag: Vec | Number of experiments observed | Number of peptides in 6 experiments | Reference for known or predicted interaction |
|-----------|---------------------|---------------------------------|------------------------------|-----------------------------------|---------------------------------------------|
| CLIC4     | Chloride intracellular channel protein 4 | 39.5 | 6/6 | 15 | (32,42) |
| EZR       | Ezrin               | 18.3 | 6/6 | 1974 | | |
| RDX       | Radixin             | 14.1 | 6/6 | 201 | | |
| EGFR      | Epidermal growth factor receptor | 12.3 | 6/6 | 38 | | |
| FHOD1     | FH1/FH2 domain-containing protein 1 | 11.9 | 6/6 | 209 | | |
| ITGAV     | Integrin alpha-V    | 10.9 | 6/6 | 83 | | |
| SLC1A5     | Neutral amino acid transporter B(0) | 10.6 | 6/6 | 37 | | |
| TACSTD2   | Tumor-associated calcium signal transducer 2 | 9.3 | 6/6 | 31 | | |
| UCK2      | Uridine-cytidine kinase 2 | 6.5 | 6/6 | 15 | | |
| SDC4      | Syndecan-4         | 7.9 | 5/6 | 14 | (35,36) |
| RASA1     | Ras GTPase-activating protein 1 | 7.8 | 5/6 | 45 | | |
| DAG1      | Dystroglycan       | 7.8 | 6/6 | 51 | (41,48) |
| REEP5     | Receptor expression-enhancing protein 5 | 7.8 | 5/6 | 23 | | |
| BASP1     | Brain acid soluble protein 1 | 7.4 | 5/6 | 16 | | |
| UCKL1     | Uridine-cytidine kinase-like 1 | 6.9 | 6/6 | 48 | | |
| SLC9A3R1  | Na(+)/H(+) exchange regulatory cofactor NHE-RF1 | 6.7 | 6/6 | 87 | (15,18,21,26,28,30,49,72) |
| EPCAM     | Epithelial cell adhesion molecule | 6.7 | 5/6 | 14 | | |
| BCR       | Breakpoint cluster region protein | 6.6 | 6/6 | 74 | | |
| ARHGAP18  | Rho GTPase-activating protein 18 | 6.4 | 6/6 | 26 | (33) |
| SCYL3     | Protein-associating with the carboxy-terminal domain of ezrin | 6.4 | 6/6 | 34 | (34) |
| SLC4A7    | Sodium bicarbonate cotransporter 3 | 6.3 | 5/6 | 26 | | |
| MYO9B     | Unconventional myosin-9b | 6.1 | 6/6 | 112 | | |
| SERPINB6  | Serpin B6 | 5.9 | 6/6 | 17 | | |
| PACSIN3   | Protein kinase C and casein kinase substrate in neurons 3 | 5.7 | 6/6 | 16 | | |
| ITGB1     | Integrin beta-1 | 5.8 | 6/6 | 31 | | |
| ATP11C    | Probable phospholipid-transporting ATPase IG | 5.5 | 6/6 | 39 | | |
| UPRT      | Uric acid phosphoribosyltransferase | 5.4 | 6/6 | 23 | | |
| GAN       | Gagoxin | 5.3 | 6/6 | 20 | | |
| PP1P5K2   | Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 2 | 4.7 | 5/6 | 25 | | |
| LDHA/B    | L-lactate dehydrogenase | 4.7 | 6/6 | 163 | | |
| F11R      | Junctional adhesion molecule | 4.4 | 6/6 | 36 | | |
| NUP33     | Nuclear pore complex protein Nup93 | 4.2 | 6/6 | 69 | | |
| CLIC3     | Chloride intracellular channel protein 3 | 4.2 | 6/6 | 72 | (32) |
| STK10     | Serine/threonine-protein kinase 10 | 4.2 | 6/6 | 51 | (7,39) |
| PGAM1     | Phosphoglycerate mutase 1 | 4.0 | 6/6 | 24 | | |
| UBXN1     | UBX domain-containing protein 1 | 3.9 | 6/6 | 17 | | |
| SLK       | STE20-like serine/threonine-protein kinase | 3.8 | 6/6 | 27 | (7) |
| FAM128B   | Nbn-like protein 1 | 3.6 | 5/6 | 30 | | |

**FIGURE 2. Identification of 38 high confidence interactors of ezrin.** A, schematic of SILAC experiment. Jeg-3 cells stably transfected with vector control or expressing ezrin-iFLAG were differentially labeled in SILAC medium, independently subjected to cross-linking, and subjected to FLAG immunoprecipitation (IP). The immunoprecipitates were combined, trypsin-digested, and subjected to mass spectrometry, where heavy-to-light ratio was determined for each recovered proteins (plotted along the horizontal axis) is shown. Proteins above the dashed line were considered interactors. (Most proteins enriched in the light sample are antibody fragments.) B, proteins with a heavy-to-light Xpress ratio greater than 3.6 for which 14 or more peptides were identified reproducibly in at least 5/6 experiments are listed. C, analysis of fraction of interactors containing transmembrane domains or shown to be membrane-associated (supplemental Table 1, columns U and V).

with either wild-type ezrin or ezrin-T567E. We therefore wanted to examine in an unbiased manner how general this type of interaction is.

We again performed SILAC to compare proteins recovered from cells expressing ezrin-iFLAG (light) with those recovered from cells expressing ezrin(1–583)-iFLAG (heavy). We found essentially the same set of proteins we did with ezrin-T567E-iFLAG but with a stronger preference for ezrin(1–583)-iFLAG (Fig. 3F). A notable exception was EBP50, being vastly more efficiently recovered with ezrin(1–583)-iFLAG, which is discussed further below.

**Biochemical and Localization Validation of the Mass Spectrometry Results**—Our proteomics approach identified three classes of ezrin interactors as follows: those that had a preference for phosphorylated ezrin (and ezrin-T567E), EBP50 (SLC9A3R1), which is recovered much more efficiently on unmasked ezrin than with phosphorylated ezrin, and an unexpected group of proteins that bind preferentially to inactive
ezrin-T567A. To see if these classes of interactors could be
confirmed by analysis of selected candidates, we immuno-
precipitated ezrin from cells expressing the different variants and
then Western-blotted for specific binding partners.

A close correspondence with the mass spectrometry data was
found for EBP50 (SLC9A3R1), DAG1, and FHOD1 (FH1/FH2
domain-containing protein 1, a formin protein, see Fig. 4A).
As we do not have reagents to test most of the other identified
interactors, we expressed tagged versions in Jeg-3 cells and
examined their recovery with co-expressed ezrin-iFLAG vari-
ants. For example, TACSTD2-myc shows a similar pattern of
recovery as was found with the proteomics analysis (Fig. 4B).
We summarize the results of this analysis in Fig. 4C.

We were surprised to recover a formin with ezrin, so we
examined whether the interaction was affected by its interac-
tion state. Many formins are negatively regulated by a head-to-
tail intramolecular interaction involving a region known as
the diaphanous association domain (DAD) (40). Equivalent
FIGURE 4. Biochemical validation of SILAC experiments. A, stable cell lines expressing empty vector control or indicated ezrin-iFLAG variants were subjected to cross-linking FLAG immunoprecipitation (IP) followed by Western blot (WB) for the indicated interactor. Predicted yield relative to wild-type ezrin-iFLAG is computed from mass spectrometry (spec) data in supplemental Table 1. B, parental Jeg-3 cell line was co-transfected to express empty vector or indicated ezrin-iFLAG variant along with TACSTD2-myc and then subjected to cross-linking FLAG immunoprecipitation and Myc Western blot. Predicted yield relative to wild-type ezrin-iFLAG is computed from mass spectrometry data in supplemental Table 1. C, summary of mass spectrometry interactors confirmed in low throughput methods. Black lines indicate proteins recovered and confirmed through low throughput co-immunoprecipitation followed by Western blotting experiments. Green text highlights proteins expected to bind directly with ERM proteins in vitro by others. D, cells transiently transfected to express empty vector or indicated ezrin-iFLAG variant and co-transfected to express full-length or hyperactivated (∆DAD) GFP-FHOD-1 were subjected to FLAG immunoprecipitation, and the immunoprecipitates were resolved and Western-blotted for FLAG and GFP. FHOD-1 binds to wild-type or closed ezrin but not truncated and hyperactivated ezrin. The K4N mutant (K253N,K254N,K262N,K263N) of ezrin is completely closed in cells due to a mutation in basic residues that bind to the immunoprecipitates were resolved and Western-blotted for FLAG and GFP. FHOD-1 binds to wild-type or closed ezrin but not truncated and hyperactivated ezrin. The K4N mutant (K253N,K254N,K262N,K263N) of ezrin is completely closed in cells due to a mutation in basic residues that bind to membrane PI(4,5)P2 (10, 22).

The proteins DAG1 and CLIC4 have been shown previously to localize in microvilli (32, 41–43), so these serve as positive controls. Indeed, endogenous β-dystroglycan and transiently expressed GFP-CLIC4 both co-localize precisely with ezrin in the apical microvilli of Jeg-3 cells in maximum intensity projections through the entire cell or projections through a cross-section (Fig. 5, A and B). We then tested some of the other candidates, and as predicted, TACSTD2-myc, BASP1-GFP, and SLC1A5-GFP were all preferentially localized in microvilli (Fig. 5, C–E). Expression of proteins showing a preference for inactive ezrin, SCYL3-myc, and GFP-FHOD1 was not enriched in microvilli but was found diffusely in the cytoplasm or microvilli.

amounts of GFP-FHOD1 or GFP-FHOD1ΔDAD (lacking the DAD domain) were selectively recovered with inactive forms of ezrin, indicating no preference for the activity state of the formin. No interaction was found with hyperactive ezrin(1–583)-iFLAG (Fig. 4D).

Another avenue to test the validity of the proteomic analysis is based on subcellular localization. Because active ezrin is found specifically in microvilli, candidates that show a preference for active ezrin might be expected to be enriched in microvilli. Conversely, those proteins that show a preference for inactive ezrin might be expected to be found predominantly in the cytoplasm, like ezrin-T567A-iFLAG (Fig. 1A).
enriched on basolateral membranes (Fig. 5, F and G). A summary of all the candidates examined is shown in Fig. 5H. Thus, both the co-immunoprecipitation and localization results strongly support the proteomic data.

Function of Novel Ezrin Interaction Partners—Ezrin is known to interact with many proteins (Table 1), and in this study we have documented yet more. Ezrin is important for the presence of microvilli on Jeg-3 cells (7), so we asked if altering the expression of ezrin-interacting proteins had any effect on microvilli. Jeg-3 epithelial cells express both ezrin and radixin. When ezrin levels are reduced 80% by siRNA treatment, about half the cells lose microvilli. The number of cells with microvilli can be further reduced to 20% by simultaneously knocking down radixin, supporting a critical role for ERM proteins in maintaining microvilli in Jeg-3 cells (Fig, 6, A and B). We next tested the effect of knocking down two of our candidates, DAG1 and TACSTD2. Both endogenous proteins were found to localize in microvilli (Figs. 5A and 6C). Although reduction of the levels of β-dystroglycan has no discernible effect, reduction of TACSTD2 reduced the number of cells with microvilli modestly (Fig. 6, D–F).

The overexpression of activated ERM proteins or ERM-binding proteins in fibroblastic cells has been shown to cause an increase in the number of microvilli-like structures (MLSs) (20, 44). We therefore examined the effect of overexpression of our candidates, DAG1, TACSTD2, BASP1, and ATP11C (which must be co-overexpressed with its chaperone Cdc50A for plasma membrane enrichment, see Ref. 27), and CLIC4 in COS7 fibroblastic cells. Overexpression of wild-type or activated ezrin but not the more closed ezrin mutant, T567A,
caused an elevation in the percentage of cells containing MLSs as indicated by F-actin and moesin staining (data not shown) as expected (quantified in Fig. 7B). Like moesin, ezrin was also enriched in these structures (data not shown). In the case of our candidates, DAG1, TACSTD2, and CLIC4 each caused an elevation in the percentage of cells containing MLSs (Fig. 7, A and B). By contrast, the overexpression of DAG1ΔE, a mutant previously shown to be incapable of binding to ezrin (41), BASP1, or ATP11C (expressed with its chaperone as above) had no effect (Fig. 7B). Western blotting showed that each transfected protein was present at the expected size (Fig. 7C). The combined analysis suggests that some of our interactors are capable of promoting the formation of dorsal microvilli-like structures in fibroblastic cells. Taken together with our knockdown experiments, we propose that ezrin anchorage to the plasma membrane is likely due to the concerted activity of many binding partners.

**DISCUSSION**

ERMs function as regulated linking proteins, so to learn more about them, we took a global proteomics view, not only to uncover new ligands and regulators but also to understand the role of conformational change in ERM function.

Our use of cross-linking immunoprecipitation rather than a direct interaction assay, as reported previously (28), biased the analysis toward proteins bound to ezrin in vivo, including transient interactors, rather than those with tenacious in vitro affinity. This revealed many new interactors. We provide three validations of our approach as follows: 1) greater than one-quarter of the interactors were expected based on previous studies, and
a large fraction (almost half) of the interactors contained transmembrane helices or membrane-binding domains; 2) where tested, we were able to biochemically validate the mass spectrometry results; 3) many of the interactors, including three novel interactors, accumulate not just on the plasma membrane, but also strongly in microvilli.

Our analysis defined three conformational states of ezrin (closed, active, and fully open) as perceived by the 38 high confidence interactors. One group, designated as active ezrin interactors, bound more to wild-type than unphosphorylatable T567A ezrin, more to phosphomimetic T567E than wild-type ezrin, and more to truncated 1–583 than T567E ezrin. A second group, closed ezrin interactors, bound roughly equally to wild-type and T567A ezrin, less to T567E ezrin, and still less to 1–583 ezrin. Most ERM-binding proteins had a modest (1.2–4.2-fold) preference for truncated 1–583 ezrin over phosphomimetic T567E ezrin. However, EBP50, which binds to the FERM domain more intimately than other known FERM interaction partners (21), was unique in having an extreme (45-fold) preference for 1–583 ezrin over T567E ezrin. This result supports *in vitro* data that, even when phosphorylated, the tail of ezrin still serves as a potent competitive inhibitor of FERM

**FIGURE 7. DAG1, TACSTD2, or CLIC4 overexpression in fibroblastic cells promotes dorsal ERM-containing surface structures.** A, COS7 fibroblastic cells were transfected with indicated protein and stained for endogenous moesin (an ERM protein present in these cells). *Panels*, maximum intensity projections through a field of cells; *insets*, maximum intensity projections through a small dorsal region of one transfected cell. *Bar*, 10 μm. B, cells transfected as in A were scored for the presence of MLSs. GFP-Nuc is nuclear-localized GFP, intended as a negative-control. IRES2EGFP allows expression of free GFP as a reporter of untagged ezrin construct transfection. *Error bars* are standard deviation. *p* values were computed using a two-tailed *t* test. *, *p* < 0.05; ***, *p* < 0.001; ns, not significant. C, cells expressing the transfected proteins as listed in B were subjected to Western blotting (*WB*) with indicated antibodies.
domain accessibility (16). Moreover, it argues that the influence of the tail on FERM domain binding differs in degree for different interaction partners and highlights the uniqueness of EBP50’s interaction with ezrin.

Surprisingly, we identified multiple partners of the inactive state of ezrin. As expected, these are not enriched in microvilli but are found in the cytoplasm. To date, the only known interactor of inactive ezrin is the lipid P(4,5)P₂, which plays a role in the transition to an activated state. Thus, we are looking into the function of this large class of ezrin interactors in ezrin regulation.

The conformational activation model (45) stipulates that interactors binding ezrin on the plasma membrane should bind preferentially to more open forms of ezrin. Consistently, most interactors containing transmembrane helices were active ezrin interactors. But is this merely an effect of the localization of open versus closed ezrin? We can determine this by comparing the binding partners of the two constitutively open forms of ezrin, truncated ezrin(1–583)-iFLAG versus phosphomimetic ezrin-T567E-iFLAG, because both localize identically in a depolarized manner on the plasma membrane (7). Strikingly, all but one transmembrane interactor (8/9) bound truncated ezrin(1–583)-iFLAG to a greater extent than phosphomimetic ezrin-T567E-iFLAG (for instance, see TACSTD2, Fig. 5B). Therefore, we argue that the extent to which an interactor binds to ezrin is not only a function of the localization of the proteins but also ezrin conformation.

This work adds to several recent reports showing that protein complexes involving ERMs are unusually dynamic. Ezrin turns over from microvilli faster than microvilli themselves turnover (14, 15, 46), and EBP50 turns over even faster than ezrin (15). The turnover rate of EBP50 is regulated by PDZ interactions, and impairing these interactions decreases EBP50’s turnover rate from microvilli and increases the recovery of co-immunoprecipitated ezrin (15). Moreover, cross-linking was also used to preserve the interactions between the Droso phila orthologs of ERMs, EBP50, and the LOK/SLK kinase (47). Thus, rapid turnover seems to be a general feature of ERM interactions.

Some known interactors were notably absent from our analysis, possibly reflecting cell type-specific expression differences. None of the peptides in our analysis corresponded to CD44, ICAM-2, ICAM-3, syndecan-2, CLIC5, and S100P (Table 1; data not shown). Thus, these are not major ezrin-associated proteins in Jeg-3 cells and may not be expressed. ICAM-1 is present in Jeg-3 cells but appeared in our immunoprecipitates in amounts too low to accurately quantify.

RNA interference knockdown of two of the top candidate ERM-binding transmembrane proteins in Jeg-3 epithelial cells failed to yield a striking phenotype, although TACSTD2 suppression partially reduced the number of microvilli-bearing cells in Jeg-3 cells (Fig. 6F), and dominant-negative β-dystroglycan (DAG1ΔE) inhibited the formation of active-Cdc42-induced filopodia in a myoblast cell line (41). Conversely, overexpression of TACSTD2, DAG1, or CLIC4 caused the enumeration of MLSs in outexpressing fibroblastic cells, which normally produce dorsal ERM-containing protrusions only rarely (Fig. 7). The lack of overt phenotype in the epithelial cell line can be interpreted in two ways as follows: 1) transmembrane ERM-binding proteins are not required as ERM tethering or clustering agents as has been proposed (20); 2) ERM binding to numerous transmembrane proteins coordinately regulates their tethering or clustering. In support of the second possibility, ERM-binding motifs are minimalistic and present in many transmembrane proteins, and the proteins containing them have evolved entirely separate functions (e.g. β-dystroglycan functions as a receptor for the extracellular matrix component laminin), making it possible that ERMs evolved promiscuously to a wide range of available ligands. Also in support of this model, our calculations suggest that ezrin is stoichiometrically present in a similar abundance to actin (data not shown), suggesting that no one membrane protein is in sufficient abundance to bind all the ezrin molecules, such that many transmembrane proteins would be needed to serve such a function. Thus, our current efforts are to ask whether simultaneous perturbation of multiple transmembrane ezrin interactors described here affects ezrin function.

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