Characterization of a Novel Interaction between ELMO1 and ERM Proteins*\[5\]

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ERMs are closely related proteins involved in cell migration, cell adhesion, maintenance of cell shape, and formation of microvilli through their ability to cross-link the plasma membrane with the actin cytoskeleton. ELMO proteins are also known to regulate actin cytoskeleton reorganization through activation of the small GTP-binding protein Rac via the ELMO-Dock180 complex. We showed that ERM proteins associate directly with ELMO1 as purified recombinant proteins in vitro and at endogenous levels in intact cells. We mapped ERM binding on ELMO1 to the N-terminal 280 amino acids, which overlaps with the region required for binding to the GTPase RhoG, but is distinct from the C-terminal Dock180 binding region. Consistent with this, ELMO1 could simultaneously bind both radixin and Dock180, although radixin did not alter Rac activation via the Dock180-ELMO complex. Most interestingly, radixin binding did not affect ELMO binding to active RhoG and a trimeric complex of active RhoG-ELMO-radixin could be detected. Moreover, the three proteins colocalized at the plasma membrane. Finally, in contrast to most other ERM-binding proteins, ELMO1 binding occurred independently of the state of radixin C-terminal phosphorylation, suggesting an ELMO1 interaction with both the active and inactive forms of ERM proteins and implying a possible role of ELMO in localizing or retaining ERM proteins in certain cellular sites. Together these data suggest that ELMO1-mediated cytoskeletal changes may be coordinated with ERM protein cross-linking activity during dynamic cellular functions.

In eukaryotic cells, rearrangements of the cortical actin cytoskeleton provide the molecular basis for changes in cell shape, motility, adhesion, and division. Organized signaling complexes at the cell membrane are thought to coordinate actin assembly with membrane association during these processes. However, the exact molecular nature of these signaling complexes is not well understood.

The highly conserved ERM (ezrin/radixin/moesin) protein family has been implicated in embryonic development, formation of microvilli, cell motility, formation of membrane ruffles, and formation of cell-cell/cell-matrix adhesion sites through their ability to cross-link the actin cytoskeleton to the plasma membrane (for reviews see Refs. 1–3). Each ERM protein contains an ~300 residue FERM (band 4.1 and ERM) domain at its N terminus followed by an extended α-helical structure (~160 amino acids) and a highly conserved C-terminal region (~90 amino acids). An actin filament-binding domain site is located within the last 34 amino acids of this C-terminal region (4, 5). The FERM domain promotes targeting to the plasma membrane via several transmembrane receptors and membrane-associated proteins. Transmembrane binding partners for ERM proteins identified so far include CD44, CD43, NEP, syndecan-2, and I-CAM-1, -2, and -3 (6–12). Indirect binding of ERM proteins to the membrane occurs through the two closely related scaffolding proteins EBPs/NHE-RF and NHE3 kinase A regulatory protein (E3KARP) (13, 14). ERM proteins also interact with PtdIns(4,5)P₂ at the plasma membrane as well as certain signaling proteins, including RhoGDI, DBL, PALS1, N-WASP, the p85 subunit of phosphatidylinositol 3-kinase, and hamartin via the N-terminal FERM domain (15–20). The functional significance of ERM binding to these various targets is not well understood. In cultured cells, ERM proteins are predominantly coexpressed and enriched at sites just beneath the plasma membrane where actin filaments are densely associated (21, 22).

Biochemical and structural studies suggest that native full-length ERM proteins exist predominantly in a dormant state, by virtue of an intramolecular and/or intermolecular interaction between the N-terminal FERM domain and the C-terminal tail (23–26). In this auto-inhibited conformation, the C-terminal F-actin-binding site and N-terminal protein interaction sites are masked. The dormant forms of ERM proteins have no reported binding partners except for the regulatory subunit of protein kinase A (27). Therefore, to allow N-terminal and C-terminal binding to the plasma membrane and F-actin, respectively, an activation mechanism that opens the molecular structure is required.

Two types of distinct signals have been proposed to generate and/or maintain the active state of ERM proteins. The first involves the phosphorylation of a C-terminal threonine residue that is conserved in all three ERM proteins (T564 in radixin, T567 in ezrin, and T558 in moesin). Phosphorylated ERM proteins are concentrated in actin-rich membrane structures in a variety of cells and tissues, whereas total ERM proteins are distributed in both the cytoplasm and plasma membranes (28–30). Phosphorylation of this threonine residue, or engineered phosphomimetic mutation of this residue (e.g. T564E radixin), reduces the affinity of the C-terminal tail for the FERM domain in all ERM proteins (31, 32), induces cytoskeletal changes (29, 32), and promotes stronger binding to actin filaments in vitro (31, 33). Conversely, non-phosphorylatable Thr → Ala mutants associate poorly with the actin cytoskeleton and act as dominant negative inhibitors of wild type ERM proteins (29, 32, 34). The second ERM activation mechanism involves the binding of PtdIns(4,5)P₂ to the N-terminal FERM domain. PtdIns(4,5)P₂ binding enhances ERM binding to membrane proteins.

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and actin filaments (9, 31, 33, 35). The ability of ERM proteins to function in such a highly regulated fashion, and their association with certain signaling molecules, strongly suggests that ERM proteins might also help organize signaling complexes that serve to regulate cytoskeletal assembly.

Members of the evolutionarily conserved family of ELMO (engulfment and cell motility) proteins have been shown to regulate actin cytoskeleton reorganization and formation of membrane protrusions through an interaction with the protein Dock180 (36–38). The Dock180-ELMO complex functions as a guanine nucleotide exchange factor (GEF) specific for Rac, which mediates actin cytoskeletal reorganization and lamellipodia formation through several downstream effectors (39). ELMO and Dock180 have also been found to localize to actin-rich polarized membrane ruffles in cells (37, 40). One mode of targeting of ELMO to the membrane occurs via ELMO binding to the activated form of another small GTPase, RhoG. The simultaneous interaction of ELMO with active RhoG and Dock180 (via the N- and C-terminal regions of ELMO, respectively) serves as an evolutionarily conserved mechanism for RhoG-dependent Rac activation leading to cell migration and neuronal outgrowth (38).

Here we report that ELMO1 is a direct physiological binding partner for ERM proteins. An interaction of ELMO1 with radixin was observed both in vitro with recombinant proteins and at endogenous protein levels. Most interestingly, the interaction of ELMO1 with radixin appears to be distinct from other ERM-binding proteins, in that ELMO1 associated strongly with the closed, dormant form of the molecule as well as the open, active form of the molecule. Moreover, ELMO1 displayed only weak association with the isolated FERM domain of radixin, in contrast to other known ERM-binding proteins. Therefore, ELMO1 appears to be a novel ERM-binding protein, and the ELMO1-ERM interaction could have important implications for coordinated regulation of actin cytoskeleton reorganization during dynamic cellular functions.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Purified rabbit polyclonal anti-ELMO1 was generated in our laboratory and has been described previously (37). Mouse monoclonal anti-GFP (clone B2), mouse monoclonal anti-HA (clone F7), goat polyclonal anti-Dock180 (C19), rabbit polyclonal anti-GST (clone ZS), mouse monoclonal anti-actin (clone C-2), and horse-radish peroxidase-conjugated donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FLAG peptide and mouse monoclonal anti-FLAG (clone M2) were from Sigma. Mouse monoclonal anti-Myc (9E10) and mouse monoclonal anti-Rac (clone 23A8) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal anti-radixin/moesin antibody was from BD Transduction Laboratories. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences. All immunoblots were developed using enhanced chemiluminescence (Pierce). G-actin purified from muscle was a generous gift from Dorothy Schafer (University of Virginia).

**Plasmids**—All mutant constructs generated were sequenced to confirm the fidelity and presence of the appropriate mutations. PEBlu-O118-GFP and pEBB-ELMO1-FLAG have been described previously (37). The T280, T558, and Δ531 mutants of ELMO1 were generated by a PCR-based approach and were sequenced to confirm the appropriate mutations. The GST-ELMO1, GST-ARM1, GST-ARM2, GST-T115, and GST-T558 mutants of ELMO1 were described previously (41). Full-length pCXN2-FLAG-Dock180 was a generous gift from Dr. Michiyuki Matsuda (42). pEF-radixin-HA, pEF-radixin-T564A-HA, and pEF-radixin-T564E-HA were the generous gifts of Dr. Sachilo Tsukita (Kyoto University). PGEX-2T-radixin was kindly provided by Dr. Christian Roy (Université Montpellier II). We generated the pGEX-2T-radixin-T564A and pGEX-2T-radixin-T564E mutants by replacing wild type pGEX-2T-radixin with the corresponding fragments of pEF-radixin-T564A-HA and pEF-radixin-T564E-HA. pEF-radixin-ERM (residues 1–310) was generated by a PCR-based approach using pEF-radixin as a template. PGEX-2T-erm was a gift of Dr. Monique Arpin (UMR144 CNRS/Institut Curie). PRK-moesin-HA was a gift of Dr. David Braughtan (University of Virginia). pEF-erm-HA and pEF-moesin-HA were generated by a PCR-based approach using pGEX-2T-erm and pRK-moesin-YFP as a template. The plasmid encoding HA-Tiam1 (C1199) was provided by Dr. John Collard (Netherlands Cancer Institute). The FLAG-tagged Rac1Q61L and GFP-FAK plasmids were kindly provided from Dr. Tom Parsons (University of Virginia). pEGFP-C3-RhoGN17 and pEGFP-C3-RhoGL61 for mammalian expression were kindly provided from Dr. Ann Blangy (Centre de Recherches en Biochimie Macromoléculaire; Montpellier, France) (43).

**Yeast Two-hybrid Screen**—The mouse embryonic cDNA library was kindly provided by Dr. Ian Macara (University of Virginia). The cDNAs were cloned into the NotI site of the pVP16 vector. The yeast strain used in the two-hybrid screen was H7FC, with His, Trp, and Leu as selection markers. Yeast cells were transformed using the LiAc-based transformation protocol. Before the library screen, the full-length ELMO1 was tested on a –Trp, –His SCM plate, containing 5 mM 3-amino-1,2,4-triazole, and showed no transcriptional activation. When the ELMO1-Dock180 interaction was tested as a positive control, yeast cells transformed with pGBT10-ELMO1 and pVP16-Dock1–161 grew well on a –Trp, –Leu, –His SCM plate containing 10 mM 3-amino-1,2,4-triazole. The pGBT10-ELMO1-transformed yeast cells were then transformed with library plasmids and spread on –Trp, –Leu SCM plates, and ~3 million colonies were screened. The yeasts grown out of the double dropout plates were replicated onto selection SCM plates (~Trp, ~Leu, ~His, containing 10 mM 3-amino-1,2,4-triazole). The yeast cells were allowed to grow into single colonies. After further re-streaking on selective plates, the plasmid mixtures were then isolated from each clone after culture of the yeast in 10 ml of selective SCM medium. The library plasmids were rescued by transforming the individual plasmids mixture into KC8 Escherichia coli. Plasmids isolated from transformed KC8 were re-transformed into H7FC yeast cells with either the pGBT10-ELMO1 plasmid or the pGBT10 vector to confirm the specific interaction. Once this interaction was confirmed, the pVP16 library vectors derived from the two-hybrid screen were sequenced to identify the inserts.

**Transfection, Immunoprecipitations, and Immunoblotting**—293T and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1% penicillin/streptomycin/glutamine. The J774 macrophage cell line was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, pH 7.4, 0.05 μM β-mercaptoethanol, 4.5 g/liter glucose, and antibiotics. 293T cells were transiently transfected by the calcium phosphate method, and HeLa cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In all experiments, carrier DNA was added to keep equal plasmid concentration between different samples. Lysis, immunoprecipitation, and immunoblotting were performed as described previously (36, 37). Briefly, 293T cells were transiently transfected in 10-cm dishes with ELMO1 (3 μg), radixin (3 μg), Dock180 (10 μg), or other (3 μg) plasmids as indicated. For immunoprecipitation using the FLAG tag, 20 μl of anti-FLAG M2 antibody (Sigma) directly coupled to agarose was used per transfection
condition. For ELMO1 immunoprecipitation, anti-ELMO1 antibody was incubated with protein A-Sepharose (Santa Cruz Biotechnology) for 1 h followed by three washes with lysis buffer. For HA immunoprecipitation, 15 μl of anti-HA 12CA5 antibody (Santa Cruz Biotechnology) directly coupled to agarose was used per transfection condition. For GST precipitation, 20 μl of glutathione-Sepharose beads was used per condition. Cells were then harvested, lysed, and incubated with the beads for 1–2 h. Beads were then washed four times, and precipitation of proteins was assessed by SDS-PAGE and immunoblotting.

Purification of Recombinant Proteins from Bacteria—BL21 transformants were inoculated into 3 ml of LB medium containing 100 μg/ml ampicillin and incubated overnight at 37 °C. The culture was then diluted 1:100 into 250 ml of LB medium containing 100 μg/ml ampicillin and 2% (v/v) of ethanol and incubated for 1 h at 37 °C. One ml of isopropyl 1-thio-β-D-galactopyranoside was then added, and the culture was incubated for 5 h. Bacteria were then collected by centrifugation, and the pellet was stored overnight at −80 °C. The pellet was then resuspended in 8 ml of lysis buffer (100 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme, 1 mM dithiothreitol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 20 min. The suspension was then sonicated three times for 15 s each on ice. Eight mg of sodium deoxycholate in lysis buffer was then added and incubated for 20 min at room temperature. Then 25 μl of 10 mg/ml DNase was added and incubated for 20 min at room temperature. Proteins were then collected by centrifugation and incubated with 300 μl of glutathione-Sepharose beads for 2 h at 4 °C on Nutator. The beads were then washed eight times in washing buffer (50 mM Tris, pH 7.6, 1% (v/v) Nonidet P-40, 150 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The beads were then resuspended in an equal volume of washing buffer containing 20% (v/v) glycerol and stored at −80 °C. Proteins were quantitated by SDS-PAGE followed by Coomassie staining.

Cleavage of GST from ELMO1 and ERM Proteins—GST was cleaved from fusion proteins, where indicated, using a thrombin cleavage kit (Novagen). GST fusion proteins bound to glutathione-Sepharose beads were incubated with 1 μl of thrombin and 100 μl of cleavage buffer in a 1-ml total volume overnight at 4 °C on Nutator. Cleaved proteins were then collected by brief centrifugation and stored at −80 °C. The cleavage appeared virtually complete as determined by SDS-PAGE and staining with Coomassie Blue.

Purification and Elution of FLAG-ELMO1 Proteins—293T cells were transfected in 10-cm dishes, one dish per condition of purified FLAG-ELMO1. Twenty four to 48 h later, cells were lysed, and FLAG-ELMO1 was immunoprecipitated and washed. FLAG-ELMO1 was then eluted using FLAG peptide (Sigma) according to the manufacturer’s instructions.

In Vivo Rac GTP-loading Assay—Bacterially produced GST-CRIB proteins bound to glutathione-Sepharose beads were incubated with lysates from 293T cells transfected with the indicated plasmids for 1 h at 4 °C. The beads were then washed, and the levels of Rac-GTP present in the lysates were analyzed by SDS-PAGE and immunoblotting for Rac (40).

In Vitro GEF Assay—The radioactivity-based in vitro GEF assay was performed as described previously (36). 293T cells in 10-cm dishes were transfected with the indicated plasmids, and the cell lysates were immunoprecipitated with anti-FLAG antibody. Precipitated proteins were then eluted with FLAG peptide following the manufacturer’s instructions into 120 μl. The eluted proteins were quantitated via Western blotting. Levels of Dock180 were kept constant, and conditions were analyzed for Rac GEF activity as follows. 5 μg of bacterially expressed and purified Rac was loaded with 50 μCi of [α-32P]GTP (3000 Ci/mmol) in 40 mM MOPS, pH 7.1, 1 mM EDTA, 1 mg/ml BSA, and 0.3 mM unlabeled GTP for 20 min on ice. 10 mM MgCl2 was then added and incubated on ice for an additional 10 min. 250 ng of [32P]GTP-loaded Rac was added with 2 μl of eluted proteins (for wild type Dock180, volume for other samples was adjusted based on the concentration of eluted Dock180) in reaction buffer (25 mM MOPS, pH 7.1, 6.25 mM MgCl2, 0.6 mM NaH2PO4, 0.5 mg/ml BSA, 1.25 mM unlabeled GDP) in a final volume of 100 ml. After 15 min at 30 °C, 50 ml of the exchange reaction was subjected to nitrocellulose filter binding followed by scintillation counting. The presence of GEF activity was revealed by loss of radioactivity bound to Rac (due to the exchange reaction). [32P]GTP binding to Rac, in the control conditions with precipitates from untransfected 293T cells, was set at 100%. Results are representative of at least three independent experiments.

Microscopy—The indicated plasmids were transiently transfected into LR73 cells or HeLa cells plated on LabtekII chamber slides (4 wells). The transfections were done using Lipofectamine 2000 reagent and 1.0 μg of plasmid DNA. 24 h post-transfection, the cells were fixed in 3.7% paraformaldehyde and permeabilized with phosphate-buffered saline, 0.1% Triton, 0.1% BSA. The permeabilized cells were then blocked with phosphate-buffered saline containing 0.1% BSA and 10% mouse IgG for 20 min at room temperature. Cells were then stained with Alexa-647-phalloidin (Molecular Probes, Eugene, OR) for 1 h at room temperature. This was followed by incubation with anti-HA-Alexa-594 (Molecular Probes) for 1 h on ice. Cells were mounted using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and then analyzed by confocal microscopy. Images were obtained using a laser scanning confocal microscope (model Meta LSM510; Carl Zeiss MicroImaging) with a ×100 objective lens. Images were processed as entire pictures using Adobe Photoshop version 6.0. The images shown are representative of multiple cells with similar phenotypes from at least three independent experiments.

RESULTS

Identification of a Direct Interaction between Radixin and the N-terminal Region of ELMO1—ELMO1 regulates Rac-dependent processes involving dynamic reorganization of the actin cytoskeleton, including phagocytosis, cell migration, and membrane ruffling. However, relatively few molecules that interact with ELMO1 during these processes have been characterized. To identify ELMO1-binding proteins that may contribute to these processes, we performed a yeast two-hybrid screen using full-length ELMO1 as bait against a random-primed murine 10-day embryo library. Initial results of this screen identified radixin as a potential ELMO1-binding protein.

To confirm the ability of radixin to interact with ELMO1 in vivo, we then analyzed the ability of endogenous ELMO1 to interact with endogenous radixin in J774 cells. In these cells, endogenous radixin coimmunoprecipitated with endogenous ELMO1 in the presence of a specific anti-ELMO1 antibody, but not in the presence of an isotype control antibody. This suggests that ELMO1 can form a complex with radixin at endogenous levels in mammalian cells (Fig. 1A).

To test whether the interaction observed between ELMO1 and radixin is direct, we examined the ability of purified ELMO1 and purified radixin to interact in vitro. As shown in Fig. 1B, bacterially produced and purified GST-tagged ELMO1 was able to precipitate recombinant purified radixin. Such coprecipitation was not seen with GST alone (or with mutants of ELMO1, see below). This suggested that the interaction between radixin and ELMO1 is direct.
We next attempted to define the region of ELMO1 responsible for radixin binding by testing the ability of different mutants of ELMO1 to interact with radixin. The ELMO1 mutants tested included H9004Δ531, T558, and T625 mutants. The Δ531 mutant lacks the N-terminal 531 amino acids, whereas the T558 and T625 represent truncations at residues 558 and 625, respectively. After coexpression of these FLAG-tagged ELMO1 mutants with full-length HA-tagged radixin, the interactions were assessed by precipitating ELMO and looking for the coprecipitation of radixin. As shown in Fig. 1C, radixin coprecipitated with full-length ELMO1 and the T625 and T558 mutants of ELMO1 but not with the Δ531 mutant of ELMO1. Radixin did not coprecipitate with other FLAG-tagged proteins such as FLAG-Dock180 or a FLAG-tagged version of the unrelated protein CIN85. In reciprocal experiments, immunoprecipitation of radixin coprecipitated full-length ELMO1 and the T558 mutant of ELMO1 but not the Δ531 mutant of ELMO1 (Fig. 1D). In addition, bacterially produced GST-ELMO1 mutants and recombinant radixin gave essentially similar results (Fig. 1B, 6th to 8th lanes). Thus, the N-terminal 558 amino acids of ELMO1 are necessary and sufficient to mediate radixin binding. It is notable that the removal of the N-terminal 531 amino acids of ELMO1 correlates with a failure of ELMO1 to promote migration and to properly localize to areas of membrane ruffling in LR73 cells (40).

**FIGURE 1. Interaction of ELMO with radixin.** A, endogenous ERM proteins coprecipitate with endogenous ELMO1. Lysates of J774 cells were immunoprecipitated (IP) using antibody to ELMO1 (right lane) or a control antibody, and the coprecipitation of endogenous ERM proteins was analyzed using an antibody to radixin/moesin. Bacterially produced and purified radixin was included as a positive control for immunoblotting (left lane). B, radixin interacts directly with ELMO1. Radixin and ELMO1 were bacterially produced and purified. Untagged radixin was incubated with the indicated GST-tagged ELMO1 proteins, and the coprecipitation of radixin was assessed by immunoblotting with anti-radixin antibody. Precipitation (Ppt) of the GST fusion proteins was confirmed using anti-GST immunoblotting. C, radixin interacts with the N-terminal region of ELMO1. 293T cells were transiently transfected with the indicated plasmids. After 24 h, the interaction of HA-radixin with ELMO1-FLAG or other control FLAG-tagged proteins was assessed by anti-FLAG immunoprecipitation and immunoblotting with anti-HA antibody. Appropriate expression of HA-radixin and ELMO1-FLAG proteins was assessed using anti-GST immunoblotting. D, N-terminal 558 amino acids of ELMO1 are required and sufficient for radixin binding. 293T cells were transiently transfected with the indicated plasmids. After 24 h, the interaction of FLAG-tagged ELMO1 mutants with HA-radixin was assessed by anti-HA immunoprecipitation and immunoblotting with anti-FLAG antibody. Appropriate expression of the FLAG-ELMO1 proteins and HA-radixin was confirmed by immunoblotting total lysates with anti-FLAG or anti-HA antibody.

**Novel Interaction between ELMO1 and ERM Proteins**

Specificity of ELMO1 Binding for ERM Proteins—Radixin shares 75 and 80% sequence identity with ezrin and moesin, respectively (21, 44). Moreover, all three ERM proteins colocalize in most cell types (1–3), suggesting at least partially overlapping functions for the three ERM proteins. As shown in Fig. 2A, ELMO1 associated with ezrin and moesin similarly to radixin, indicating that ELMO1 can interact with all three of the ERM family proteins. We then addressed whether the interaction of ELMO1 with FERM domain-containing proteins was specific to the ERM family of proteins. To this end, we tested ELMO1 binding to FAK, which also contains a FERM-like domain. ELMO1-FLAG was coexpressed with either YFP-moesin or GFP-FAK, and the coprecipitation was then assessed. No association of FAK with ELMO1 was detected, although moesin binding to ELMO1 was readily detected (Fig. 2B). This suggests that the interaction of ELMO1 with FERM domain-containing proteins is relatively specific for the ERM family of proteins.
Novel Interaction between ELMO1 and ERM Proteins

ELMO1 Forms a Trimeric Complex with Radixin and Dock180—The C-terminal 532–727 amino acids of ELMO1 mediate Dock180 binding (40). Because these amino acids were dispensable for radixin binding to ELMO1, we wished to determine whether ELMO1 could bind both radixin and Dock180 simultaneously. We transiently transfected 293T cells with FLAG-Dock180 and HA-radixin in the presence or absence of ELMO1-GFP. As shown in Fig. 3A, radixin was coprecipitated with Dock180 only when ELMO1 was coexpressed, suggesting that ELMO1 bridges Dock180 and radixin. Consistent with this notion, the trimeric complex formation was not detected in the presence of the Δ531 mutant of ELMO1, which can bind Dock180 but is unable to bind radixin. Conversely, trimeric complex formation was also lost with the T558 mutant of ELMO1, which can bind radixin but not Dock180. Taken together, these data suggest that radixin and Dock180 do not interact directly but that a trimeric complex of radixin-ELMO1-Dock180 can be formed, with ELMO1 bridging radixin and Dock180.

We then tested whether radixin might modulate Rac GTP loading induced by Dock180 and ELMO1. We transfected 293T cells with plasmids encoding Dock180 and ELMO1, either with or without a plasmid encoding radixin, and we assessed the levels of Rac-GTP by a GST-CRIB pull-down assay. Similar to our previous observations (36), expression of Dock180 and ELMO1 induced a significant increase in the levels of Rac-GTP in these cells. However, coexpression of radixin with Dock180 and ELMO1 had no apparent effect on this increased Rac GTP loading (Fig. 3B, lanes 2 and 7). This suggests that overexpression of radixin does not detectably alter the ability of the Dock180-ELMO1 complex to promote Rac GTP loading under these conditions.

We also tested whether potential dominant negative or constitutively active versions of radixin may affect Rac GTP loading either with or without Dock180/ELMO expression. We used a point mutant of radixin with the highly conserved T564 mutated to alanine (T564A), as well as a truncation mutant of radixin that is composed of only the FERM domain. These mutants lack the ability to be conformationally activated by phosphorylation or to bind F-actin, respectively. These mutants are thought to interfere with the functions of all three ERM proteins because of the high degree of conservation of the FERM domain between the ERM proteins (1, 29, 34, 45, 46). We also tested a point mutant designed to mimic radixin phosphorylated on T564 (T564E), as this mutant has been reported previously to act as a constitutively active form of radixin (31, 33, 47). Under these conditions, coexpression of either the T564A, FERM, or T564E mutants of radixin had no detectable effect on Rac GTP loading (Fig. 3B).

We also tested whether radixin might affect Rac-GEF activity via the Dock180-ELMO complex in an in vitro Rac-GEF assay. Purified complexes of FLAG-Dock180 and ELMO1-GFP from 293T cells, either with or without HA radixin, were examined for nucleotide exchange on bacte-riorly produced and purified Rac1 in vitro (36). In this assay, GEF activity is detected by loss of radioactivity bound to Rac as a result of the exchange reaction. Although Dock180 does display some Rac-GEF activity on its own in this assay, which is increased by ELMO1 (Fig. 3C), the presence of radixin had no detectable effect on the Rac-GEF activity. Taken together, these results imply that Dock180/ELMO1-mediated Rac GTP loading can be dissociated from endogenous ERM cross-linking activity under these conditions.

ELMO1-binding Sites for RhoG and Radixin Are Distinct—It has been demonstrated previously that the active, GTP-bound form of RhoG can interact with the N terminus of both ELMO2 (38) and ELMO1 (41). Therefore, we tested whether a version of ELMO1-FLAG containing residues 1–280 (T280) was sufficient to mediate the interaction with both radixin and active RhoG. Expression of all YFP- or GFP-tagged proteins, as well as comparable precipitation of ELMO1-FLAG, was confirmed by immunoblotting for GFP or FLAG.

ELMO1 interacts with ezrin and moesin. 293T cells were transiently transfected with plasmids encoding ELFOM1 and HA-ezrin, HA-ezrin, HA-moesin, or control HA-Tiam1-C1199 as indicated. After precipitation with anti-FLAG antibody, association of HA-tagged proteins with ELMO1 was determined. Anti-FLAG immunoblotting was used to confirm immunoprecipitation (IP) of transfected ELMO1-FLAG. B, ELMO1 fails to interact with other FERM domain-containing proteins. 293T cells were transiently transfected with plasmids encoding FLAG-ELMO1 or its mutants and either YFP-moesin or GFP-FAK as indicated. After precipitation with anti-FLAG antibody, association of FERM domain-containing proteins with ELMO1 was determined by immunoblotting for GFP. Expression of all YFP- or GFP-tagged proteins, as well as comparable precipitation of ELMO1-FLAG, was confirmed by immunoblotting for GFP or FLAG.
to bind RhoG, this was unable to bind radixin, suggesting a requirement for residues between 115 and 280. Second, radixin bound well to versions of full-length ELMO1 carrying mutations within either the ARM1 or ARM2 repeats, both of which affect binding to RhoGQ61L (Fig. 4B).

These results imply that ELMO1 likely associates with radixin through a region distinct from the RhoGQ61L binding region.

We next examined whether ELMO1 could form a trimeric complex with both RhoGQ61L and radixin. As shown in Fig. 4C, RhoGQ61L formed...
a complex with radixin but only when ELMO1 was coexpressed. This suggests that a trimeric complex of RhoGQ61L-ELMO1-radixin can be formed, with ELMO1 bridging RhoGQ61L and radixin. This raises the possibility that RhoG-mediated recruitment of ELMO1 to the plasma membrane may concurrently facilitate translocation of ERM proteins to the plasma membrane, where the ERM proteins exhibit cross-linking activity.

**Colocalization of Radixin with Constitutively Activated RhoG on the Plasma Membrane**—To assess the possible physiological relevance of the interaction between ELMO1 and radixin, we analyzed the localization of radixin in HeLa cells, in the presence or absence of ELMO1 and active RhoG. We wanted to explore the possibility that RhoG-mediated recruitment of ELMO1 to the plasma membrane may concurrently facilitate translocation of ERM proteins to the plasma membrane, where the ERM proteins exhibit cross-linking activity.

**Interaction of ELMO1 with Radixin Is Independent of Radixin T564 Phosphorylation**—ERM protein function is regulated by conformational changes. Binding of the N and C terminus of ERM proteins to the cell membrane and actin, respectively, depends upon disruption of an intra- or intermolecular interaction. A body of evidence suggests that phosphorylation of ERM proteins on a conserved C-terminal threonine residue (T564 in radixin) activates ERM proteins by disrupting this interaction. In a parallel condition, radixin also colocalized with ELMO1 in these membrane ruffles (Fig. 5D). ELMO1, but not radixin, was also present in a perinuclear pattern under these conditions. The localization of radixin or ELMO1 in membrane ruffles was not present in cells that were coexpressing a constitutively active Rac1Q61L, even though extensive membrane ruffles were also present (Fig. 5E and supplemental Fig. 1). In addition, this membrane ruffling was not observed in cells coexpressing dominant negative RhoGT17N, radixin, and ELMO1 (data not shown). These data suggest that activation of RhoG may lead to colocalization of RhoG/ELMO1/radixin at the plasma membrane.

**Figure 4.** ELMO1 has distinct binding sites for radixin and active RhoG and can form a trimeric complex. A, 1–280 mutant of ELMO1 interacts with radixin (rad) and constitutively active RhoG. 293T cells were transiently transfected with the indicated plasmids. After 24 h, the interaction of HA-radixin or RhoGQ61L-GFP with the indicated ELMO1-FLAG proteins was assessed by anti-FLAG immunoprecipitation (IP) and immunoblotting with either anti-HA (radixin) or anti-GFP (RhoGQ61L) antibodies. Appropriate expression of HA-radixin and RhoGQ61L was assessed by immunoblotting total lysates. Anti-FLAG was used to confirm appropriate immunoprecipitation of transfected ELMO1 proteins and ELMO1 fails to interact with radixin. Radixin and ELMO1 mutants were bacterially produced and purified as in Fig. 1A. The coperiprecipitation of radixin by the indicated GST-ELMO1 proteins was assessed by immunoblotting with anti-radixin antibody. Equal precipitation (Ppt) of the GST fusion proteins was confirmed by immunoblotting with anti-GST antibody. C, ELMO1 forms a trimeric complex with radixin and active RhoG. 293T cells were transiently transfected with plasmids encoding HA-radixin, ELMO1-FLAG, and RhoGQ61L-GFP as indicated. After precipitation with anti-HA antibody, coperiprecipitation of RhoGQ61L with radixin in the presence or absence of ELMO1 was determined by immunoblotting for GFP. Anti-FLAG was used to confirm immunoprecipitation of ELMO1 with radixin and anti-HA was used to confirm appropriate immunoprecipitation of transfected HA-radixin. Total cell lysates were immunoblotted with anti-GFP to confirm appropriate expression of transfected RhoGQ61L.
We tested the effects of radixin C-terminal phosphorylation on the radixin interaction with ELM01 by comparing wild type radixin, the nonphosphorylatable radixin variant T564A, and the phosphomimetic T564E mutant for ELM01 binding. ELM01-FLAG purified from cells was incubated with immobilized GST-tagged versions of the bacterially produced radixin proteins, and interactions were assessed by anti-FLAG immunoblotting. Most interestingly, ELM01 bound both the T564E and the T564A mutants of radixin, essentially similar to wild type radixin (Fig. 6, lanes 8, 11, and 12). Thus, phosphorylation of radixin on T564 has little, if any, influence on the radixin-ELM01 interaction. This implies that ELM01 can likely associate with both the open and closed forms of radixin and that the binding of ELM01 does not require the unmasking of N- or C-terminal binding regions. This contrasts with most other ERM-binding proteins, which display much higher affinity for the open, activated conformation of the ERM protein.

Because most other ERM-binding proteins have been reported to interact with the isolated FERM domain (presumably after activation/opening up of the FERM domain), usually stronger than the full-length ERM protein, we examined ELM01 binding to the isolated radixin FERM domain or a radixin mutant lacking its FERM domain (ΔFERM). Somewhat surprisingly, the ability of ELM01 to interact with the FERM and the ΔFERM mutants of radixin was substantially diminished compared with full-length radixin (Fig. 6, lanes 8–10). This result suggests that the isolated regions of radixin were not sufficient for ELM01 binding under these conditions and that the binding between ELM01 and radixin likely represents a unique interaction.
Novel Interaction between ELMO1 and ERM Proteins

FIGURE 6. ELMO1 binds to both the open and closed forms of radixin. Mutant forms of GST-radixin were produced and purified from E. coli. The indicated GST proteins were incubated with ELMO1-FLAG eluted/purified from 293T cells. Interactions of ELMO1-FLAG with radixin mutants were assessed by immunoblotting with anti-FLAG antibody. Equal precipitation (Ppt) of the GST fusion proteins was confirmed using anti-GST antibody.

DISCUSSION

This work has identified an important, direct link between ELMO1 and ERM proteins, two molecules that have been known to influence the actin cytoskeleton. We detected endogenous radixin in a complex with endogenous ELMO1. Moreover, this interaction appears direct, because purified, recombinant ELMO1 associated with purified, recombinant radixin in vitro in a cell-free assay.

It was additionally observed that ELMO1 could interact with moesin and ezrin, similar to radixin. This result is perhaps not surprising given the high degree of homology between the three ERM proteins. In fact, many binding properties of one ERM protein have later been found to also extend to the other two. The notion of functional redundancy between the three ERM proteins is also supported by the phenotypic analysis of moesin- and radixin-deficient mice. No severe defects were observed in these mice, although the radixin-deficient mice were not completely normal (49–51). In addition, all three ERM proteins are ubiquitously expressed in most cultured cells (21, 22, 52). However, some tissue-specific requirements for individual ERM proteins probably do exist as one or more of the ERM proteins can be absent or predominate in certain tissues (53–55).

Since the ELMO1-Dock180 complex functions to activate Rac and promote actin polymerization/lamellipodia formation and the ERM proteins mediate cross-linking of filamentous actin to the plasma membrane, an interesting possibility is that the functions of these proteins temporally and spatially coordinate. We made an important observation that the association of ELMO1 with ERM proteins does not appear to affect the exchange activity of the Dock180-ELMO1 complex. Rac GEF activity by the Dock180-ELMO1 complex in vitro was similar in the presence or absence of radixin. In addition, overexpression of radixin had no detectable effect on Dock180/ELMO1-mediated GEF activity in vivo. It is interesting to note that ERM proteins have also been reported to interact with the Rho family GEF Dbl, with no apparent effect on the exchange activity of Dbl (19). Because radixin was readily detected in a trimeric complex with ELMO1 and Dock180, these results support the notion that radixin may be present at sites where Dock180 and ELMO1 promote Rac activation, although it may not have a significant impact on the Rac-GEF activity per se. In this context, we have also analyzed a strain of Caenorhabditis elegans deficient in expression of the single ERM family member erm-1 for defects in gonadal distal tip cell migration (56). This mutant strain (erm-1(tm777)) showed significant defects in gonadal distal tip cell migration (48%, n = 80), compared with it's heterozygous control (0%, n = 70). Distal tip cell migration is similarly defective in worms carrying mutations in genes coding for ELMO1 and Dock180 homologues, ced-12 and ced-5, respectively; these defects are rescued by the mammalian genes expressed as transgenes in the respective mutant worms. This further supports a link between ERM family members and ELMO/Dock180 proteins.

Although a number of proteins linked to cytoskeletal reorganization have been shown to associate with ERM proteins, almost all of them have been shown to bind the “active” form of the ERM proteins. However, a considerable body of evidence suggests that basally the ERM proteins exist in a nonphosphorylated and closed/inactive state. Thus, it is unclear how the inactive versions of ERM proteins get recruited to sites of actin rearrangement and get activated to participate in cellular cytoskeletal reorganization. In addition, recent studies suggest that there may be distinct zones of actin organization at the leading edge of migrating cells. How specific signaling proteins, such as ERM proteins (which remain in a “closed conformation” basally), get shuttled to these sites and remain there (prior to activation or after activation) for given periods of time is poorly understood. Our observations that the active (T564E) and the inactive (T564A) versions of radixin bind equally well to ELMO1 raise some interesting possibilities. Because ELMO1, Dock180, and RhoG proteins can critically regulate cell migration and localize to the leading edge, one possibility is that recruitment of the ELMO1-Dock180 complex from the cytoplasm to the plasma membrane by active RhoG may concurrently target inactive, cytoplasmic ERM proteins to specific sites in the plasma membrane. The observations that ELMO binding to active RhoG is not affected by radixin binding and that the three proteins can colocalize at the plasma membrane suggest that RhoG-mediated recruitment of ELMO1 to the plasma membrane could occur when radixin is bound to ELMO1. In this regard, the ELMO1 binding might help control the targeting of radixin to specific complexes in the plasma membrane. Ultimately, this would facilitate the interaction of radixin with its binding partners. Considering that regulated cross-linking of F-actin to membrane proteins is essential for many fundamental processes, the novel interaction between ELMO1 and ERM proteins described here could play a role in events such as phagocytosis, cell motility, and morphogenesis.

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