Dock180 and ELMO1 Proteins Cooperate to Promote Evolutionarily Conserved Rac-dependent Cell Migration

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Cell migration is essential throughout embryonic and adult life. In numerous cell systems, the small GTPase Rac is required for lamellipodia formation at the leading edge and movement ability. However, the molecular mechanisms leading to Rac activation during migration are still unclear. Recently, a mammalian superfamily of proteins related to the prototype member Dock180 has been identified with homologues in Drosophila and Caenorhabditis elegans. Here, we addressed the role of Dock180 and ELMO1 proteins, which function as a complex to mediate Rac activation, in mammalian cell migration. Using mutants of Dock180 and ELMO1 in a Transwell assay as well as transgenic rescue of a C. elegans mutant lacking CED-5 (Dock180 homologue), we identified specific regions of Dock180 and ELMO1 required for migration in vitro and in a whole animal model. In both systems, the Dock180-ELMO1 complex formation and the ability to activate Rac were required. We also found that ELMO1 regulated multiple Dock180 superfamily members to promote migration. Interestingly, deletion mutants of ELMO1 missing their first 531 or first 330 amino acids that can still bind and cooperate with Dock180 in Rac activation failed to promote migration, which correlated with the inability to localize to lamellipodia. This finding suggests that Rac activation by the ELMO-Dock180 complex at discrete intracellular locations mediated by the N-terminal 330 amino acids of ELMO1 rather than generalized Rac activation plays a role in cell migration.

Cell migration is essential for many normal and abnormal biological processes including embryonic development, wound healing, the immune response, and tumor cell metastasis. In virtually every cell type examined, cell movement requires the activity of Rac, a member of the Rho family of small GTPases (1–4). Rac is preferentially activated at the leading edge of migrating cells where it induces the formation of actin-rich lamellipodia protrusions thought to be a key driving force for membrane extension and cell movement (5–8). Rac can also mediate the assembly of multi-molecular signaling and adhesion complexes associated with these leading edge protrusions (9, 10). Despite their importance, the upstream signaling mechanisms that facilitate Rac activation during migration are not fully understood (11, 12).

Recent work (13–15) has revealed an evolutionarily conserved protein superfamily with homology to Dock180 comprised of at least 11 mammalian members. Dock180, the prototype member of this superfamily, forms a basal complex with ELMO1 and together this complex functions as an unconventional two-part guanine nucleotide exchange factor (GEF) specific for Rac (16). As with other GTPases, Rac functions as a binary switch by cycling between an inactive GDP-bound form and an active GTP-bound form. GEFs promote the exchange of GDP for GTP by stimulating the dissociation of GDP and stabilizing the nucleotide-free form, thereby facilitating association of GTP (17, 18). However, neither Dock180 nor ELMO1 contains an obvious Dbl homology domain, which is present in most other known mammalian GEFs for Rho family GTPases (19, 20). Instead, Dock180 and its homologues contain a Docker domain that can interact directly with nucleotide-free Rac and mediate Rac GDP/GTP exchange in vitro (14, 16).

In intact cells, however, the Dock8 domain alone is insufficient for efficient Rac GTP-loading and an interaction between Dock180 and ELMO1 is required for GEF activity (16). Moreover, Dock180 and ELMO1 functionally cooperate to promote phagocytosis of apoptotic cells, a Rac-dependent process that involves dynamic reorganization of the actin cytoskeleton (for review see Ref. 21). Neither Dock180 nor ELMO1 alone can promote phagocytosis. Interestingly, these two proteins also colocalize on membrane ruffles (16), which are actin-rich protrusions associated with the leading edge of migrating cells. One other member of this superfamily, termed Dock9 or Zizimin, was found to bind and specifically activate Cdc42 (another Rho family member) rather than Rac (13). Thus, it is likely that the other members of this superfamily also function as GEFs. The biological roles of this new superfamily and the

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; DTC, distal tip cell; ELMO, engulfment and cell motility; GFP, green fluorescent protein; SII, Src homology; CMV, cytomegalovirus; HA, hemagglutinin; GST, glutathione S-transferase; PH, pleckstrin homology; CRIB, Cdc42/Rac interactive binding.
Dock180 and ELMO1 Cooperate in Migration

Genetic studies in Drosophila and Caenorhabditis elegans have suggested that highly conserved homologues of Dock180 and ELMO1 function as critical upstream regulators of Rac. In Drosophila, mutations in the gene encoding Myoblast City (Dock180), which acts upstream of Drac1 (Rac), lead to defects in myoblast fusion, dorsal closure, and border cell migration (22–25). Worms deficient in either CED-5 (Dock180) or CED-12 (ELMO1) display defects in engulfment of apoptotic debris and onal pathfinding and migration of the distal tip cells (DTCs) (20, 26–30). Genetically, CED-5 and CED-12 were shown to function at the same step upstream of CED-10 (Rac) in these processes.

It remains unknown, however, whether the Dock180 and ELMO1 proteins also regulate Rac-dependent mammalian cell migration and which structural features of these proteins are involved. Here, we provide evidence that Dock180 and ELMO1 functionally synergize to promote Rac-dependent cell migration using an in vitro Transwell migration assay. We also confirm these observations at an organismal level by rescue of CED-5 deficient worms with mutants of Dock180. Interestingly, based on studies using ELMO1 mutants, generalized Rac activation in cells alone is not sufficient to enhance migration but rather targeting via the N terminus of ELMO1 appears to be critical for Dock180-ELMO1-mediated migration.

MATERIALS AND METHODS

Plasmids and Antibodies—GFP-tagged ELMO1, ELMO1–Δ330-GFP and the ELMO1–FLAG-CAAX have been described previously (20). ELMO1–Δ511-FLAG-CAAX was generated by replacing the coding region (from full-length ELMO1 in ELMO1–FLAG-CAAX) with a region for residues 532–727. GFP-tagged T707, 6M, and Δ531 mutants of ELMO1 and the ΔSH3 mutant of Dock180 were generated by a PCR-based approach and were sequenced to confirm the appropriate mutations. The mutations in the 6M mutant are as follows: L689A, M691S, E692D, R696K, L697A, and L698A. Full-length Dock180, the DOHR3 mutant plasmid, Dock180-CAAX, and FLAG-tagged Dock2 were provided by Dr. Matsuda (19, 32, 38). The Δ575 and Dock-ISP mutants of Dock180 and the ELMO1-T625 mutant have been described previously (16). The plasmid-encoding Tian1 (C1199) was provided by Dr. John Collard (Netherlands Cancer Institute) (48). The pG3L-CMV-luciferase plasmid was obtained from Dr. Michael Smith (University of Wisconsin). A Δac-neg, expression construct (20) was modified with Nco1-Net1 sites in which FLAG-tagged Dock180 (wild type), Dock180 (ISP– AAA, DOCK180 (358–1865) was cloned for subsequent expression in C. elegans. HA-tagged Dock3 was kindly provided by David Schubert (Salk Institute). The FLAG-tagged Rac1wt, and Rac1T17N plasmids were obtained from Dr. Tom Parsons (University of Virginia). Purified rabbit polyclonal anti-ELMO1 has been described previously (16). Mouse monoclonal anti-GFP (clone B2, mouse monoclonal anti-HA (clone F7), goat polyclonal anti-Dock180 (clones N19 and C19), rabbit polyclonal anti-GST (clone Z5), and horseradish peroxidase-conjugated donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Rac (clone M20) was from Sigma. Mouse monoclonal anti-Rac (clone 23A8) was from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Amersham Biosciences. All of the immunoblots were developed using enhanced chemiluminescence (Pierce).

Cell Culture and Transfection—293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1% penicillin/streptomycin/glutamine. LR73 cells were maintained in Eagle’s modified α-L-glutamine Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin/glutamine. 293T cells were transiently transfected by the calcium phosphate method, and LR73 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. In all of the experiments, carrier DNA was added to keep equal plasmid concentration between different samples.

Migration and Adhesion Assay—LR73 cells at ∼70% confluence were transiently transfected with pGL3-CMV-luciferase as a reporter construct in addition to the indicated plasmids in a 6-well plate. After 20 h, cells were harvested using trypsin-EDTA and resuspended in Opti-MEM medium supplemented with 2% fetal bovine serum and 105 cells were added in duplicate to the upper chamber of an untreated polycarbonate Transwell filter with 8-μm pores (Costar). Opti-MEM medium supplemented with 2% fetal bovine serum was also added to the lower chamber. After a 6-h incubation, Transwell filters were removed and the number of cells migrating completely through the filter to the lower chamber was assessed by quantification of luciferase activity (Promega). 1 × 105 cells were also separately added in parallel to wells without Transwell filters for estimating total luciferase activity, upon which the percent migration was estimated for each transfection condition. The percent migration of control cells transfected with luciferase alone was set at 100%. The percentage of cells remaining attached to the filter was assessed by mechanically removing cells from the top of the filter with a cotton swab and determining the luciferase counts of the remaining cells. An aliquot of cells from each transfection condition was analyzed for expression of transfected proteins by immunoblotting. To examine adhesion under the migration assay conditions, cells were transfected, harvested, and resuspended as described above for the migration assay. 1 × 105 cells were then either plated in duplicate on the same 24-well Transwell filter placed in a 12-well plate (so that the filter lies flush with the bottom of the well, eliminating the bottom chamber) or plated in a separate well without a Transwell filter to estimate the total luciferase counts for each condition. At the indicated time points, the filters were then gently washed with phosphate-buffered saline and the percentage of transfected cells remaining attached to the top of the filter was determined with a luciferase assay.

Scoring of C. elegans DTC Migration—The indicated Dock180 and CED-5-coding sequences were subcloned into the transgenic vector driven by the Pant-3 promoter. To create DOCK180-transgenic lines, worms were injected with test DNA at a concentration of 10 ng/μl along with P or GFP as described previously (52). Injected hermaphrodites were picked and allowed to have progeny. Transgenic progeny (that expressed Pant::GFP) were moved to a plate that was seeded with OP50 bacteria and allowed to propagate one generation. Worms were then scored under a Zeiss M1 Bio-dissecting microscope equipped with epifluorescence. Worms with a gonad that deviated from the standard U-shaped tube was scored as migration defective. Only worms in which both the anterior and posterior arms were clearly visible were scored.

Immunoprecipitation and Immunoblotting—Lysis, immunoprecipitation, and immunoblotting were performed as described previously (16, 20). 293T cells were transiently transfected with 10 μg of Dock180 and Dock180 homologues and 3 μg of ELMO1 plasmids. For FLAG immunoprecipitations, cells were harvested and transfected and immunoprecipitated using anti-FLAG antibody directly coupled to Sepharose. For ELMO1 immunoprecipitations, anti-ELMO1 antibody was incubated with protein A-Sepharose (Santa Cruz Biotechnology, Inc.) for 1 h and washed. Cells were then harvested and lysed 4 h after transfection and incubated with the beads for 1 h. Precipitated proteins were then assessed by SDS-PAGE and immunoblotting (16).

Rac GTP-loading Assay—Bacterially produced GST-CRIB proteins bound to glutathione-Sepharose beads were incubated with lysates of LR73 or 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 beads were analyzed by SDS-PAGE and immunoblotting for Rac.

Phagocytosis Assay—LR73 cells were transiently transfected in duplicate with the indicated plasmids (either with GFP or fused to GFP) in a 24-well plate. 20 h after transfection, the cells were incubated with 2 μm of carboxylate-modified red fluorescent beads in serum-free medium (Sigma). After 2 h, the wells were then washed twice with cold PBS, resuspended in buffered saline, resuspended, resuspended in cold PBS with 0.5% sodium azide, and analyzed by two-color flow cytometry. The transfected cells were recognized by their GFP fluorescence. Further and side scatter parameters were used to distinguish unbound beads from cells. For each point, 30,000 events were collected and the data were analyzed using Cell Quest software. As shown previously, the majority of doubly positive cells were in the fluorescence activated cell sorter assay represents particles engulfed by transfected cells or particles in the process of engulfment and do not represent beads simply bound to the cell surface.

Microscopy—The indicated plasmids were transiently transfected...
into LR73 cells plated on Labtek chamber slides using LipofectAMINE 2000 reagent at the following concentrations: ELMO1-GFP or /
H9004 531-GFP (1.0 g) and Dock180 (1.5 g). At 24 h post-transfection, the cells were fixed in 3.7% paraformaldehyde and permeabilized with phosphate-buffered saline, 0.1% Triton X-100, 0.1% bovine serum albumin. Cells were then stained with Alexa Fluor-568 phalloidin (Molecular Probes, Eugene, OR) for 20 min at room temperature and analyzed by confocal microscopy. The regions of overlay of the green ELMO1-GFP fluorescence with the red fluorescence of Alexa Fluor-568 phalloidin are represented in yellow. The images shown are representative of multiple cells with similar phenotypes from three independent experiments. To quantitate morphology, cells were classified as 1-rounded appearance, 2-spread but not polarized, or 3-polarized with visible leading and tail edges using confocal and epifluorescence analyses. The expression of Dock180 in a duplicate chamber was confirmed by direct immunostaining for Dock180 using a goat polyclonal anti-Dock180 antibody (1:40) for 30 min at 4 °C followed by a Texas Red-labeled donkey anti-goat antibody (1:40) for 30 min at 4 °C to visualize expression of Dock180.

Cells were mounted using Vectashield-mounting medium (Vector Laboratories, Inc., Burlingame, CA 94010). An Olympus Fluoview BX50WI laser-scanning microscope with a H11003 60 LumPlanFI lens with an aperture of 2 (zoom ×1.5) was used to obtain images. The acquisition software was Fluoview FV200, version 3.3, and images were processed as entire pictures using Adobe PhotoShop, version 6.0.

RESULTS

Dock180 and ELMO1 Coexpression Promotes Mammalian Cell Migration—To probe the role of Dock180 and ELMO1 proteins in mammalian cell migration, we developed a Transwell migration assay with LR73 cells. This is a variant of the Chinese hamster ovary cell line commonly used for cell migration studies and has also been used previously to investigate the role of Dock180 and ELMO1 in phagocytosis of apoptotic

FIG. 1. Dock180 and ELMO1 cooperate to promote Rac-dependent cell migration. A, LR73 cells were transiently transfected with the indicated plasmids plus a luciferase reporter construct. After 24 h, 1 × 10^5 cells were plated in duplicate on top of a 24-well Transwell chamber filter and allowed to migrate for 6 h. Equal cells were also plated in a separate chamber without a filter to estimate total luciferase activity for each condition. The percentage migration was calculated by dividing counts in the bottom chamber (migrated cells) by the total cell counts for each condition. Each point represents the mean percentage ± S.E. of two duplicate migration chambers. The luciferase alone control is set at 100%. Aliquots of cells from each transfection condition were lysed and immunoblotted with anti-FLAG to confirm expression of Dock180 and RacT17N and anti-GFP to confirm expression of ELMO1. These data are representative of >20 independent experiments. B, the total luciferase activity (open bars) and the number of transfected cells migrating through the Transwell filter to the bottom chamber (striped bars) was quantitated by a luciferase assay. Cells migrate to the bottom chamber and do not remain attached to the filter underside, and total luciferase activity was not affected by expression of Dock180 or ELMO1 constructs. Where not visible, the bars were too small to appear on the graph. C, expression of Dock180 and ELMO1 does not affect adhesion to the Transwell filter under the conditions of the migration assay. LR73 cells were transiently transfected with the indicated plasmids and plated on a 24-well filter placed flush with the bottom of a 12-well plate (eliminating the bottom chamber). Cells were also plated in a chamber without a filter to estimate the total luciferase activity for each condition (not shown). After 6 h, filters were washed to remove unattached cells and the percentage of cells adhering to the Transwell filter was quantitated by a luciferase assay.
cells (16, 31). During the development of this migration assay, we found that LR73 cells completely migrated to the bottom chamber when placed on an uncoated Transwell filter and did not move to the underside of the filter. To score the movement of transfected cells, we used a cotransfected luciferase reporter gene, which provided a convenient and quantitative readout for the simultaneous marking of transfected cells and scoring of their motility.

In a 6-h migration assay, coexpression of wild type Dock180 and ELMO1 strongly promoted migration compared with control cells expressing luciferase alone (generally 4–6-fold in >20 independent experiments) (Fig. 1A). Under similar conditions, expression of Dock180 alone or ELMO1 alone did not promote migration, indicating a requirement for both proteins for this effect. Immunoblotting an aliquot of cells from the same experiment also confirmed that Dock180 and ELMO1 were comparably expressed under the different transfection conditions.

It is noteworthy that an equal number of cells from each transfection condition were also plated in a separate chamber (without a filter) for estimating the total luciferase activity in each transfected population. By analyzing these luciferase counts, we confirmed that the luciferase expression, driven by a constitutive CMV promoter, was unaffected by cotransfection with either the Dock180 or ELMO1 plasmids (Fig. 1B). Furthermore, no significant difference in luciferase expression was detected in any of the transfection conditions reported in this paper. In addition, we also found that there was no increase in cell attachment to the underside of the Transwell filter in any of the transfection conditions (Fig. 1B).

Because an alternate explanation for the observed enhancement of motility with Dock180-ELMO1 coexpression could be differences in the ability of these cells to attach to the Transwell filter, we examined this possibility more closely under similar conditions. Cells were independently plated from the different transfection conditions directly on an isolated filter (without a bottom chamber), after which their relative adherence was measured. Under these conditions, we found comparable attachment of cells transfected with Dock180 alone, ELMO1 alone, or Dock180-ELMO1 in the 6-h time frame of the migration assay (Fig. 1C). We also detected no significant differences among the various transfected samples in filter adhesion at 30 min and at 1 and 3 h (data not shown). This finding suggests that the enhanced migration due to Dock180-ELMO1 coexpression does not result from overall differences in the ability of the cells to adhere to the filter.

We next examined whether the enhanced migration due to Dock180-ELMO1 coexpression was dependent on Rac activity. Cotransfection of a dominant negative form of Rac (RacTH77K) inhibited the Dock180-ELMO1-dependent increased migration (Fig. 1A), suggesting that the enhancement of migration with Dock180-ELMO1 coexpression depends on Rac activation.

**ELMO1- and Rac-binding Regions of Dock180 Are Required for Migration**—It has previously been determined that ELMO1 binding requires the N-terminal 357 amino acids of Dock180, whereas Rac binding occurs via the Docker domain (amino acids 1111–1657) (Fig. 2A) (16). Therefore, we examined the importance of these regions in migration. Coexpression of ELMO1 with the Δ357 mutant of Dock180 failed to enhance migration in the Transwell assay (Fig. 2B). It is noteworthy that although the Δ357 mutant can interact with nucleotide-free Rac, it also fails to enhance Rac-GTP loading in vivo (16). Under the same conditions, a mutant of Dock180 lacking only the first 83 amino acids (ΔSH3), which retains the ability to bind ELMO1 and to promote Rac GTP loading in vivo (data not shown), still cooperated with ELMO1 in migration (Fig. 2B). We also tested Dock-ISP, a mutant of Dock180 with three amino acid changes within the Docker domain that abrogates Rac binding (yet can still interact with ELMO1). This Dock-ISP mutant also failed to synergize with ELMO1 in promoting migration. Thus, both ELMO1-binding and Rac-binding regions of Dock180 appear to be essential for migration.

We also examined whether Dock180 carrying deletions of the known Crk-binding regions (DOHRS mutant) (32) can cooperate with ELMO1 in migration. Surprisingly, the C-terminal Crk-binding region of Dock180 was not essential for the synergy with ELMO1 in this assay (Fig. 2B). Although CrkII overexpression alone enhanced migration in this system as in other Transwell systems in which CrkII is known to promote migration (33, 34), a version of CrkII with a mutation in the particular SH3 domain linked to Dock180 binding still promoted migration (data not shown). This finding is consistent with our other observations that although Dock180-ELMO1 and CrkII can form a trimolecular complex (20), Dock180-ELMO1 can also function independently of CrkII.

**Structural Features of Dock180 Required for Migration in Vivo**—The *C. elegans* DTCs migrated in a stereotypical U-shaped pattern during development to determine the shape of the adult hermaphrodite gonad (Fig. 3) (35). Mutations in the highly conserved Dock180 homologue *ced-5* are associated with pathfinding defects during this migration, resulting in worms...
with abnormal gonadal morphology. Previous studies have shown that expression of Dock180 in worms deficient in CED-5 can partially rescue DTC migration defects (26). To address which features of Dock180 are required for migration at an organismal level and in a situation where no endogenous Dock180 is expressed, we generated transgenic worms that express wild type or mutant forms of Dock180. We then crossed these forms into the ced-5 mutant background and scored the DTC migration defects by observing the shape of the adult gonad.

In ced-5 mutant animals alone, we observed DTC mismigration in 35% of gonadal arms using a transgenic fluorescent reporter construct that is expressed in the somatic sheath cells surrounding the germ line (Fig. 3). This marker allows for rapid scoring of DTC migration defects under a standard epifluorescence-dissecting microscope. To focus solely on migration defects resulting from the loss of CED-5, we only scored worms whose gonadal arms developed but were abnormally shaped, excluding those gonads that never developed (which may account for any differences in our scores and published results) (26).

Expression of either CED-5 or Dock180 partially rescued the DTC migration defects observed in ced-5 mutants, although the worm CED-5 was more efficient than mammalian Dock180 in its rescue. Under these conditions, the Dock-ISP mutant, which fails to associate with Rac, did not rescue the DTC migration defects (Fig. 3). Similarly, the Δ357 mutant of Dock180, which fails to associate with ELMO1, also did not rescue the migration defect. In fact, both the Dock-ISP and the Δ357 mutants increased the percentage of gonads displaying migration defects in the CED-5-deficient background and in the wild type background. These data pointed to key evolutionarily conserved structural features of Dock180 that are required for migration at the organismal level and supported the findings of our in vitro migration studies.

Multiple Members of the Dock180 Superfamily Promote Migration Together with ELMO1—11 mammalian members of the Dock180 superfamily have been identified and classified into four families, Dock-A, B, C, and D. Although Dock-A family members (Dock180 and Dock2) can specifically activate Rac, the specificity of the other three families (with the exception of Dock9 or zizimin in the Dock-D family, which specifically activates Cdc42) is still unclear. Among the 11 members, Dock180, Dock2, Dock3 (also called MOCA) (36), and Dock4 (37) carry an
Therefore, we examined whether Dock2 (which is most homologous to Dock180 and in the Dock-A family) or Dock3 (a member of the Dock-B family) can interact and cooperate with ELMO1 to promote migration.

As shown in Fig. 4A, both Dock2 and Dock3 associated with ELMO1 when coexpressed. Previously, it has been shown that Dock2 can bind to nucleotide-free Rac and can lead to Rac-GTP loading in 293T cells (38) but whether Dock3 can also regulate Rac-GTP levels has not been determined. In intact cells, we could detect some level of Rac-GTP loading with expression of Dock3 alone, which was enhanced by coexpression with ELMO1 (Fig. 4B). Cell lysates were immunoprecipitated in the presence of EDTA with GST affinity-agarose. The coprecipitation of Dock180 and Dock3 was assessed by immunoblotting with a mixture of anti-FLAG and anti-HA antibodies. Precipitation of the GST-Rac was confirmed using anti-GST antibody. D, Dock2 and Dock3 synergize with ELMO1 to promote Rac-dependent migration. Percentage migration was determined as in Fig. 1. Cell lysates were immunoblotted with anti-FLAG (Dock180 and Dock2), anti-HA (Dock3), and anti-GFP (ELMO1) antibodies to confirm expression of all of the proteins. E represents ELMO1.

Dock180 homologues synergize with ELMO1 to promote Rac-dependent migration. A, ELMO1 associates with Dock2 and Dock3. 293T cells were transiently transfected with the indicated plasmids. After 24 h, the interaction of Dock180, Dock2, and Dock3 with ELMO1 was assessed by immunoprecipitation with anti-ELMO1 antibody and immunoblotting with anti-FLAG antibody (Dock180 and Dock2), or anti-HA antibody (Dock3). Anti-GFP was used to confirm appropriate immunoprecipitation of transfected ELMO1-GFP. B, Dock3 cooperates with ELMO1 to promote Rac-GTP loading in vivo. 293T cells were transiently transfected with the indicated plasmids. After 24 h, levels of Rac-GTP were assessed by incubation of lysates with the bacterially produced CRIB domain of p21-activated kinase followed by anti-Rac immunoblotting. Expression of the transfected proteins was confirmed by immunoblotting total lysates with anti-HA (Dock3), anti-FLAG (Dock180), and anti-GFP (ELMO1). C, Dock3 binds nucleotide-free Rac. GST-Rac was coexpressed in 293T cells with the indicated plasmids. Cell lysates were immunoprecipitated in the presence of EDTA with GST affinity-agarose. The coprecipitation of Dock180 and Dock3 was assessed by immunoblotting with a mixture of anti-FLAG and anti-HA antibodies. Precipitation of the GST-Rac was confirmed using anti-GST antibody. D, Dock2 and Dock3 synergize with ELMO1 to promote Rac-dependent migration. Percentage migration was determined as in Fig. 1. Cell lysates were immunoblotted with anti-FLAG (Dock180 and Dock2), anti-HA (Dock3), and anti-GFP (ELMO1) antibodies to confirm expression of all of the proteins. E represents ELMO1.

Dock180-binding Regions of ELMO1 Are Required for Migration—We next examined the specific regions of ELMO1 required for promoting mammalian cell migration (Fig. 5A). A deletion mutant of ELMO1 (T625), previously shown to no longer interact with Dock180 (16), was unable to promote migration when coexpressed with Dock180 (Fig. 5B). Under these conditions, another truncation mutant of ELMO1 (T707) that retains the ability to bind Dock180 still promoted cell migration. In addition, a version of ELMO1 (denoted as 6M) with point mutations in a conserved stretch of residues required for binding to Dock180 failed to promote migration. This confirms that the interaction between Dock180 and ELMO1 is critical for the enhancement of migration.

Dock180 and ELMO1 Cooperate in Migration—Interestingly, under the same conditions, overexpression of a constitutively active form of Rac (Rac1S17N) failed to promote migration (Fig. 5A). Moreover, a constitutively active form of Tiam1 (which activates endogenous Rac comparably to Dock180-ELMO1, see Fig. 6A) also
failed to promote migration. This finding suggests that generalized Rac activation per se is not sufficient to promote migration under these conditions and that additional regulation of Rac activation during migration achieved through Dock180-ELMO1 might be necessary.

We then examined whether particular regions of ELMO1 not essential for Rac GTP-loading per se by the Dock180-ELMO1 complex might be crucial for regulating migration in our assay. As part of our structure-function studies on ELMO1, we identified that C-terminal regions of ELMO1 (either lacking residues 1–330 or lacking residues 1–531) were necessary and sufficient to interact with Dock180 and increased Rac-GTP within cells when coexpressed with Dock180 as determined by a CRIB pull-down assay (Fig. 6, A and B, and data not shown) (20). To further test the ability of the Δ330 and Δ531 mutants of ELMO1 to promote activation of Rac, we also looked at the role of these mutants in Dock180-ELMO1-dependent phagocytosis. We, along with others, have observed that increasing the levels of Rac-GTP in cells either by expression of a GEF for Rac or by other means is sufficient to promote engulfment (Fig. 6D) (31). Consistent with this notion, the Δ330 and Δ531 mutants were fully capable of cooperating with Dock180 in promoting phagocytosis (Fig. 6D). We tested whether these ELMO mutants could still cooperate with Dock180 to promote migration. Both the Δ330 and Δ531 mutants completely failed to cooperate with Dock180 in migration (Fig. 6A). Together, these data suggest that generalized Rac activation, even when mediated by mutated forms of the Dock180-ELMO1 complex, is not sufficient to promote cell migration and that additional regulation of Rac activation mediated by the first 330 amino acids of ELMO is required.

We additionally tested whether a CAAX-tagged version of Dock180, shown previously to target Dock180 to the membrane (19, 39), could promote migration when expressed alone. This Dock180-CAAX version was not capable of promoting migration by itself, although it could still promote migration when coexpressed with ELMO1 (Fig. 6A). Moreover, a CAAX-tagged version of the Δ531-ELMO1 mutant also failed to cooperate with Dock180 to promote migration (Supplemental Fig. 1). These data indicate that residues 1–531 of ELMO1 perform a crucial role in migration above simple targeting of Dock180 to the membrane. Expression of ELMO1 together with Rac1Q61L also failed to promote migration (Supplemental Fig. 2). This finding further suggests that ELMO1 plays an important role in regulating Rac activation rather than regulating active Rac itself.

One likely explanation for the inability of the Δ330 and Δ531 versions of ELMO1 to promote migration, despite their ability to cooperate with Dock180 in increasing Rac-GTP levels, was that they failed to properly localize to specific sites within cells. This would be expected to disrupt the proper localization of Rac activation, which is likely crucial for proper migration. Therefore, we examined the intracellular localization of full-length ELMO1 and the Δ531 ELMO1 mutant when expressed alone or with Dock180. When expressed alone, both ELMO1 and the Δ531 mutant displayed similar cytoplasmic localization and no membrane ruffling was visible (Fig. 7). However, coexpression of Dock180 together with full-length ELMO1 promoted modest membrane ruffling with readily detectable localization of ELMO1 to the ruffles. Interestingly, the Δ531 mutant when coexpressed with Dock180 could still promote ruffles in the cells. This finding is consistent with the ability of this Δ531 mutant to promote a generalized increase in the levels of Rac-GTP in cells when coexpressed with Dock180. However, this Δ531 mutant failed to localize to these membrane ruffles (Fig. 7). This finding indicates that the first 531 amino acids are indeed critical for determining the proper intracellular localization of ELMO1. Although correlative, it is also noteworthy that cells expressing Dock180 and full-length ELMO1 frequently had a polarized morphology where ruffles were mainly on one end of the cell, resembling “leading” and “tail” edges (48% (n = 62) in transfected cells versus 6% (n = 514) in untransfected cells). However, this polarized morphology was not seen in cells expressing Dock180 and the Δ531 mutant of ELMO1 (11%, n = 99) (Fig. 7). No increase in polarized morphology was present in cells expressing either full-length ELMO1 or the Δ531 mutant alone without coexpression of Dock180 (data not shown). Taken together, these data suggest that proper localization of the Dock180-ELMO1 complex dependent on the N terminus of ELMO1, probably facilitates the polarized Rac activation and polarized morphology important for migration ability.

**DISCUSSION**

Previous studies have shown that the Dock180-ELMO1 complex functions as a GEF for Rac. Activation of Rac via these proteins has also been shown to be important for phagocytosis...
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in mammalian cell systems (16, 40). Here, we present evidence that Dock180 and ELMO1 can also synergize to facilitate movement of mammalian cells. We observed that this motility depends on the ability of these proteins to form a complex and to activate Rac. In addition, the importance of the ELMO1 and Rac binding features of Dock180 has been highly conserved throughout evolution, because these regions of Dock180 were also required for rescuing DTC migration defects in CED-5-deficient worms.

Interestingly, however, the role of Dock180-ELMO1 and Rac activation in phagocytosis and migration appears to be subtly different. Although activation of Rac alone is sufficient to increase phagocytosis in vitro, it is not sufficient to increase migration. Mutants of ELMO1 (Δ531 or Δ330) that were fully capable of cooperating with Dock180 to promote Rac activation and phagocytosis were completely defective in promoting migration and in localizing to membrane ruffles. This observation suggests that the specific intracellular localization of the Dock180-ELMO1-mediated activation of Rac is critical for migration.

Fluorescence resonance energy transfer techniques have shown that Rac is preferentially activated at the leading edge in several migratory cell types including motile Swiss 3T3 fibroblasts (5), primary neutrophils (41), and motile H1080 cells (42). Similarly, levels of active Rac are increased in biochemically purified protruding pseudopodia as compared with the cell body (43). Activated Pak1, a downstream effector of Rac, is also predominantly found in protruding lamellipodia upon growth factor stimulation of fibroblasts (44). This indicates that the activation of Rac and the protrusive activity stimulated by Rac must be tightly regulated spatially for forward movement to occur. This is supported by observations that constitutively active versions of Rac often fail to promote or inhibit migration both in vitro (2, 45, 46) and in whole...
animal studies (22), depending on the cell type, stimulus, and substratum. It is also supported by our observations that expression of constitutively active Rac or expression of a constitutively active version of Tiam1 fails to promote migration. Our in vitro studies in LR73 cells suggest that the Dock180-ELMO1 complex may be one factor that can determine the intracellular location of Rac activation. By confocal analysis, coexpression of Dock180 and ELMO1 promotes what resembles a polarized morphology (48% compared with 6% in untransfected cells). Full-length ELMO1 is also readily detectable in membrane ruffles, which are actin-rich structures associated with Rac activity at the leading edge (7). In contrast, cells coexpressing Dock180 and the Δ531 mutant of ELMO1, which fails to cooperate in migration, no longer display this polarized morphology and this mutant no longer localizes to membrane ruffles. This finding suggests that the Dock180-ELMO1 complex probably leads to the polarized and localized activation of Rac required for migration.

Previous genetic studies in the nematode C. elegans also support the notion that the localization of Rac activation by Dock180-ELMO1 homologues is critical to migration. Overexpression of CED-10/Rac can rescue engulfment defects in

![Image of Figure 7: Role of the ELMO1 N terminus in membrane ruffle localization.](image-url)
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