Identification of Tyrosine Residues on ELMO1 That Are Phosphorylated by the Src-Family Kinase Hck†

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ABSTRACT: The SH3 and SH2 domains of hematopoietic cell kinase (Hck) play important roles in substrate targeting. To identify new components of Hck signaling pathways, we identified proteins that bind to the SH3 domain of Hck (Scott et al. (2002) *J. Biol. Chem. 277*, 28238). One such protein was ELMO1, the mammalian orthologue of the *Caenorhabditis elegans* gene, *ced-12*. ELMO1 is an ≈80-kD protein containing a PH domain and a C-terminal Pro-rich sequence. In *C. elegans*, *ced-12* is required for the engulfment of dying cells and for cell migration. In mammalian fibroblasts, ELMO1 binds to Dock180, and functions upstream of Rac during phagocytosis and cell migration. We previously showed that ELMO1 binds directly to the Hck SH3 domain and is phosphorylated by Hck. In this study, we used mass spectrometry to identify the following sites of ELMO1 phosphorylation: Tyr 18, Tyr 216, Tyr 511, Tyr 395, and Tyr 720. Mutant forms of ELMO1 lacking these sites were defective in their ability to promote phagocytosis and migration in fibroblasts. Single tyrosine mutations showed that Tyr 511 is particularly important in mediating these biological effects. These mutants displayed comparable binding to Dock180 and Crk as wild-type ELMO1, but gave a lowered activation of Rac. The data suggest that Src family kinase mediated tyrosine phosphorylation of ELMO1 might represent an important regulatory mechanism that controls signaling through the ELMO1/Crk/Dock180 pathway.

The SH2 and SH3[1] domains of Src-family tyrosine kinases play important roles in substrate recognition (*1*−*4*). In Src-family kinases, two intramolecular interactions regulate enzymatic activity: (i) an interaction between the SH2 domain and the C-terminal tail, and (ii) an interaction between the SH3 domain and a polyproline type II helix in the SH2-kinase linker region (*5*−*7*). Ligands for the SH2 and SH3 domains can disrupt the autoinhibitory interactions and stimulate Src kinase activity (*8*−*10*). Many Src kinase substrates possess SH3 or SH2 ligands; in this way, enzyme activation is coupled to substrate phosphorylation (*4, 11*−*13*). Numerous substrates that bind to the SH3 domain of Src-family kinases have been reported. Well-studied examples of this class of Src-kinase substrates include $p130^{Cas}$ (*14*), FAK (*15*), and AFAP-110 (*16*).

Hematopoietic cell kinase (Hck) is a Src kinase that has been implicated in a variety of signaling pathways in granulocytic and monocytic cells. Hck plays an important role in phagocytosis and lysosome−phagosome fusion; macrophages from Hck-deficient mice show impaired phagocytosis (*17*). To identify novel components of Hck signaling pathways, we previously carried out a mass spectrometry based search for proteins from U937 monocytic cells that bind to the SH3 domain of Hck. We identified approximately 25 potential binding partners for Hck (*18*). One of them, ELMO1, is the subject of the present study.

ELMO1 is the mammalian orthologue of the *Caenorhabditis elegans* gene, *ced-12*. ELMO1/*ced-12* was identified as an upstream regulator of Rac that functions genetically at the same step as Dock180/*ced-5* in the engulfment of apoptotic cells and in cell migration (*19*). Overexpression of mammalian ELMO1 rescues the distal tip cell migration defect of the *ced-12* mutant *C. elegans* (*19*). In mammalian fibroblasts, the ELMO1/Crk/Dock180 complex functions upstream of Rac during phagocytosis and cell migration. Thus, coexpression of ELMO1 with CrkII and Dock180 in LR73 fibroblasts promotes phagocytosis, and causes localization of ELMO1 to the membrane ruffles that are formed. Expression of ELMO1 augments the interaction between Dock180 and Rac. The Dock180−ELMO1 complex functions as an unconventional two-component exchange factor for Rac, and ELMO1 directly participates in the stimulation of Rac GEF activity and Rac GTP loading (*20, 21*). ELMO1

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[1] Abbreviations: GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; SH3, Src homology 3; CRIB, Cdc42/Rac interactive binding; MS, mass spectrometry; PAK, p21-activated kinase; GFP, green fluorescent protein; GEF, guanine nucleotide exchange factor, PH, pleckstrin homology; CMV, cytomegalovirus, LC−ESMS, liquid chromatography−electrospray mass spectrometry.

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has no discernible catalytic domain, but at its C-terminus it possesses a pleckstrin homology (PH) domain, a putative leucine zipper motif, and a proline-rich motif (*19*, *22*). The C-terminal 100 amino acids of ELMO1 are essential for the interaction with Dock180 (*20*), while the N-terminus of ELMO1 is important for mediating cell migration via the ELMO1−Dock180 complex (*23*).

We previously showed that ELMO1 binds directly to the Hck SH3 domain via a polyproline-rich motif. We also demonstrated that coexpression of Hck and ELMO1 in mammalian cells resulted in tyrosine phosphorylation of ELMO1 (*18*). In this report, we have mapped the sites of tyrosine phosphorylation, and we report that phosphorylation has the potential to modulate the ELMO/Dock/CrkII signaling pathway that leads to Rac activation.

MATERIALS AND METHODS

Materials. Monoclonal antibodies against phosphotyrosine (4G10) and Hck were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Dock180, Rac, CrkII, and Hck polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). M45 antibody was a gift from Dr. Pat Hearing (SUNY at Stony Brook). Protein A-agarose, alkaline phosphatase conjugated gel, and anti-Flag antibody were from Sigma. Glutathione-agarose and glutathione S-transferase (GST) antibody were purchased from Molecular Probes (Eugene, OR). Ni-NTA affinity resin was from QIAGEN. The pCM45 ELMO1 and Flag-Dock180 mammalian expression plasmids have been described previously (*18*, *24*). The anti-ELMO antibody was described previously (*20*).

Purification of ELMO1 for Phosphopeptide Mapping. ELMO1 was purified by immunoprecipitation from COS-7 cells transfected with M45-ELMO1 alone, or Hck plus M45-ELMO1, as previously described (*18*). Samples of V5-ELMO1 were applied to SDS−PAGE and analyzed as described below. Alternatively, His-tagged ELMO1 was expressed and purified from *Spodoptera frugiperda* (Sf9) cells as described previously (*18*). After purification, peak ELMO1 fractions were pooled, concentrated, and treated with immobilized alkaline-phosphatase for 5 h at room temperature. ELMO1 (15 μg) was then phosphorylated by Hck (0.48 μg) in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 1 mM DTT for 2 h at 30 °C. Both dephosphorylated and tyrosine-phosphorylated ELMO1 were applied to SDS−PAGE and used for mass spectrometry experiments.

Phosphopeptide Mapping. Coomassie stained SDS−PAGE bands corresponding to ELMO, dephosphorylated ELMO, and dephosphorylated ELMO treated with Hck in vitro were excised, reduced, alkylated, and digested with trypsin as reported (*25*).

Samples were analyzed for phosphopeptide content using phosphopeptide specific LC−ESMS experiments (*26*; M.J.H. and R.S.A., unpublished results). In one set of experiments, the phosphoprotein tryptic digest was separated by reverse phase HPLC and analyzed by negative-ion electrospray mass spectrometry, monitoring for the presence of the phosphopeptide specific marker ions PO_3^- and PO_2^- (*m*/*z* 63 and 79 respectively; ref *27*). HPLC separations were performed using acetonitrile/water gradients at 4 μL/min on an LC-Packings PepMap C18 capillary column (300 μm × 15 cm, 3 μm particles). HPLC mobile phases contained 0.1% formic acid and 0.02% trifluroacetic acid. The column flow was split prior to the MS with 0.4−0.6 μL/min going to the micro-electrospray source and the remainder being manually collected into PCR tubes. MS spectra were recorded on a modified PE-Sciex API III+ (Concord, Ontario, Canada) triple quadrupole mass spectrometer. Phosphopeptides were identified using a precursor ion scan for the phosphopeptide marker ion, PO_3^- (*m*/*z* 79). Precursor scan spectra were recorded on a Sciex API 3000 triple quadrupole mass spectrometer equipped with a nanoES spray source. An aliquot (1 μL) from each phosphopeptide containing ELMO fraction was mixed with 1 μL of 50:40:10 methanol:water:ammonium hydroxide prior to loading into the nanoES sample needle.

Alternatively, the phosphoprotein tryptic digest was separated by reverse phase HPLC and analyzed by positive-ion electrospray mass spectrometry on a Micromass Q-ToF MS for the presence of the phosphotyrosine specific marker ion *m*/*z* 216.043 (*28*). HPLC separations were performed using acetonitrile/water gradients at 300 nL/min on an LC-Packings PepMap C18 capillary column (75 μm × 15 cm, 3 μm particles). HPLC mobile phases were as described above. The collision cell on the mass spectrometer alternated between high energy to produce the marker ion and low energy to produce peptide molecular weight ions. Phosphotyrosine-containing precursor ions were identified by exact chromatographic alignment of the marker ion and the putative precursor (M.J.H. and R.S.A., unpublished results).

Peptide sequencing was performed by targeted LC−MS/MS on a Micromass Q-ToF MS equipped with a nanoflow ion source. Peptide mixtures were separated on a PepMap C18 capillary column (75 um × 15 cm, 15 μm particles) at 0.3 μL/min using a 5−50% gradient in 30 min. HPLC mobile phases contained 0.1% formic acid and 0.02% trifluroacetic acid. Phosphorylation stoichiometry was determined using the positive ion LC−ESMS peak intensity of the phosphorylated and the nonphosphorylated pair for any peptide sequence.

Cell Culture and Transfection. Cos-7 and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% antibiotic antimycotic (penicillin/streptomycin/amphotericin, Invitrogen) at 37 °C. Cells were cultured to 70% confluence in 100 mm dishes. Transfections were performed in OPTI medium (Invitrogen) using 10 μg plasmid DNA and TransIT polyamine transfection reagent (Mirus) according to the manufacturer's instructions. Cells were harvested 40 h after transfection. The Chinese hamster ovary fibroblast line LR73 cells (*29*) were maintained in alpha modified Eagle's medium supplemented with 10% FCS, 1% penicillin/streptomycin, and 292 μg/mL glutamine. Cells were transiently transfected using Lipofectamine 2000 (GIBCO-BRL) according to the manufacturer's instructions (*19*). In all experiments, carrier DNA was added to keep equal plasmid concentration between various samples.

Immunoprecipitation and Western Blotting. Cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed in buffer containing 50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 2 mM Na_3VO_4, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL

aprotinin. The postnuclear lysates were precleared by addition of protein A-agarose for 1 h. Immunoprecipitation reactions were initiated by addition of the appropriate antibody (or a control) to the lysates with 10 μL of protein A-agarose. Incubations were continued 5 h or overnight at 4 °C. The resin was collected and washed 4 times with phosphate-buffered saline plus 0.5% NP-40. The precipitated proteins were analyzed on 8% SDS−polyacrylamide gels and transferred to Immobilon membrane (Millipore, Bedford, MA) in the presence of 0.1% SDS. The membranes were blocked using 5% milk in Tris-buffered saline plus 0.1% Tween 20, then probed with the appropriate antibodies. Blots were visualized using horseradish peroxidase conjugated second antibody with ECL (enhanced chemiluminescence, Amersham).

Site-directed mutagenesis of ELMO1 (Y18F, Y216F, Y511F, Y395F, Y720F, 3YF, 4YF, and 5YF) was carried out on plasmid pCM45-ELMO1 using the Quikchange Mutagenesis System (Stratagene). Mutations were confirmed by automated DNA sequencing prior to transfection.

Transwell Migration Assay. The Transwell migration assay was performed as described previously (*23*). Briefly, LR73 cells were transiently transfected in 6-well plates with the indicated plasmids and a pGL3-CMV-luciferase reporter construct. Twenty hours post-transfection, cells were trypsinized and resuspended in Opti-MEM medium containing 2% FBS. Cells (1×10^5) were added to the upper chamber of the 8 μm pore Transwells. Equal amounts of cells were added into the lower chamber of a separate Transwell to measure total input. The cells were incubated in Opti-MEM supplemented with 2% serum for another 6 h, and the cells in the lower chamber were then collected and quantitated by luciferase detection (Promega). Migration percentage was calculated by dividing the luciferase activity from the trans-migrated cells by that from total input.

Phagocytosis Assay. LR73 cells were transiently transfected in triplicate with GFP and the indicated plasmids in a 24-well plate. Twenty hours post-transfection, the cells were incubated with 2 μm carboxylate-modified red fluorescent beads, which mimic the negative charge on apoptotic cells and serve as a simplified target, in serum-free medium (Sigma Chemical Co., St Louis, MO). After 2 h, the cells were trypsinized, resuspended in cold medium (with 1% sodium azide), and analyzed by two-color flow cytometry. Transfected cells were distinguished by their GFP fluorescence. For each condition (in triplicate), 30 000 events were collected and the data was analyzed using Cell Quest software. As shown previously (*20*), the majority of double positive cells scored in the FACS assay represent transfected cells containing engulfed particles or particles in the process of engulfment, and do not represent beads simply bound to the cell surface. For the Src kinase inhibition experiments, PP2 was added to the medium containing the beads at the indicated concentration. The cells were then incubated with the slurry for 2 h before the assay was performed.

Rac Binding Assay. The PAK-CRIB domain was expressed as a GST fusion protein and purified with glutathione-agarose. This immobilized CRIB domain was used in a pull down assay to measure Rac binding. Cells were lysed in buffer containing Tris-HCl (pH 7.2), 0.5 M NaCl, 10 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitors. Cell lysates (2 mg of protein) were incubated with immobilized GST fusion proteins for 2 h at 4 °C. After gels were washed with a buffer containing Tris-HCl (pH 7.2), 0.15 M NaCl, 10 mM $MgCl_2$, and 1% Triton X-100, bound proteins were eluted with SDS−PAGE sample buffer. The samples were subjected to 12% SDS−PAGE and analyzed by Western blotting with anti-Rac antibody.

RESULTS

Identification of Hck-Specific Phosphorylation Sites on ELMO1. We previously demonstrated that coexpression of ELMO1 with Hck resulted in tyrosine phosphorylation of ELMO1 (*18*). To map Hck-dependent ELMO phosphorylation sites, we used a combination of electrospray mass spectrometry based strategies (*26*; M.J.H. and R.S.A., unpublished results). We first examined the phosphorylation profile of ELMO by monitoring a tryptic digest of the protein during on-line LC−ESMS for the phosphopeptide specific marker ions PO_3^- and PO_2^- (*m*/*z* 79 and 63, respectively). Because this analysis utilizes an LC separation coupled to phosphopeptide detection, it generates a semiquantitative phosphopeptide profile that can be used to compare the phosphorylation state of a protein under two or more sets of conditions. We compared the phosphorylation profile of ELMO1 from ELMO1-transfected COS-7 cells that either coexpressed (+) or did not coexpress (−) Hck. The phosphorylation profile of ELMO1 from the Hck (−) cells was very complex (Figure 1, panel A, gray shaded trace), suggesting a high level of basal non-Hck-dependent phosphorylation. The phosphorylation profiles of ELMO1 from the Hck (+) cells was equally complex (Figure 1, panel A, black trace), and only the peak at ca. 12 min showed a significant increase in phosphorylation over the Hck (−) cells. We were not, however, able to identify any potential tyrosine phosphorylated peptides in this fraction. These results suggested that the stoichiometry for Hck-dependent phosphorylation was low compared to the background levels of serine and threonine phosphorylation. To simplify the identification of Hck-specific sites, we treated ELMO1 purified from Sf9 cells with alkaline phosphatase to remove endogenous phosphorylation (Figure 1, panel B) and then rephosphorylated this sample with purified Hck. Figure 1, panel C, shows the Hck-dependent phosphorylation profile of this sample. The peaks labeled 2−9 were collected for further analysis.

Fractions 2−9 were analyzed by precursor ion scanning for the phosphopeptide specific marker ion PO_3^- (*m*/*z* 79) to determine the molecular weight of each phosphopeptide present in the eight fractions. Based solely on the molecular weights determined in these experiments, we were able to tentatively identify three phosphorylated ELMO sequences (see Table 1) with each mole of peptide containing one mole of phosphate. There were a number of other phosphopeptides which could not be assigned to ELMO sequences based on their determined molecular weights, leading us to suspect that one or more other phosphoproteins had copurified with ELMO. To localize the specific site of phosphorylation on the tentatively assigned ELMO sequences and to identify the unassigned phosphopeptides, we performed direct peptide sequencing using targeted LC−ES-MSMS on all the phosphopeptides detected in each fraction. In this way we confirmed the three assigned ELMO phosphopeptide se-

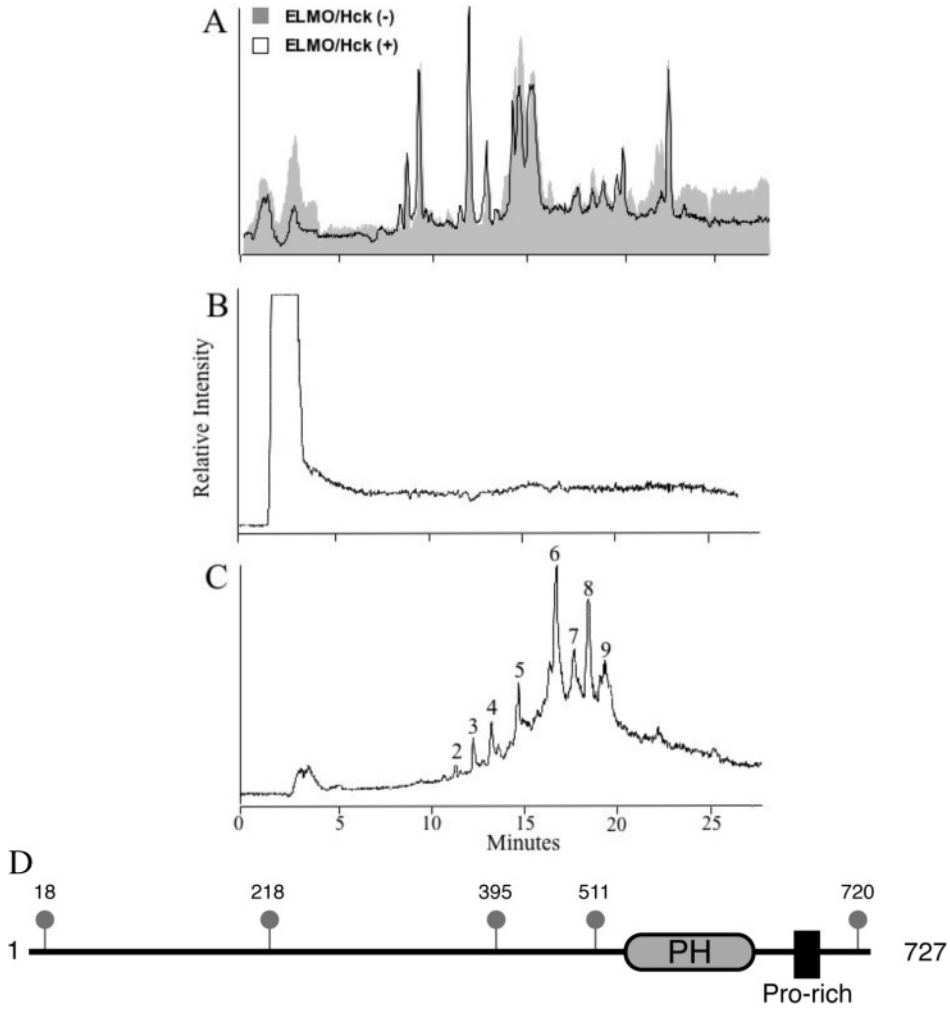

FIGURE 1: Phosphorylation profile of ELMO1. After purification by SDS−PAGE, the band corresponding to ELMO1 was excised, digested with trypsin, and analyzed by LC−ESMS for the presence of a marker ion (PO_3^-) which is specific for phosphopeptides (see text for details). (A) ELMO1 from COS cells. (B) ELMO1 purified from Sf9 cells was treated with alkaline phosphatase. (C) The alkaline phosphatase treated sample from Sf9 cells was phosphorylated in vitro by Hck. Peaks 2−9 were collected for further analysis. (D) Summary of ELMO1 phosphorylation sites.

Table 1: Summary of ELMO Phosphorylation Sites Dependent on Hck[a]

peptide	sequence	M_r obs	M_r calc	% phos
ELMO 10−20	VAIEWPGAYPK	1309.6	1309.6	11
ELMO 201−218	SLAILESMVLNSHDLYQK	2156.0	2157.4	27
ELMO 390−397	HHQDAYIR	1118.4	1118.4	7−10
ELMO 506−516	LQNLSYTEILK	1400.8	1400.7	10−14
ELMO 716−727	APSNYDFVYDCN	1543.6	1543.5	70−77

[a] Peptides from trypsin-digested phosphorylated ELMO were detected and identified by mass spectrometry. All sequences were verified by direct MS-based sequencing. Phosphorylated tyrosines are underlined. % phos = approximate stoichiometry of phosphorylation in an in vitro reaction with Hck. The amino acid numbering is based on the ELMO sequence described previously (*18*), which was cloned from a human testis cDNA library. The testis ELMO contains Ala at position 716, while most other human ELMO sequences have Glu at position 716.

quences and determined the specific phosphorylation sites to be Tyr^{18}, Tyr^{216}, and Tyr^{511}. The approximate phosphorylation stoichiometry for these three sites was determined to be 10% at Tyr^{18}, 14% at Tyr^{216}, and 14% at Tyr^{511}. Additional targeted LC−ES-MSMS peptide sequencing allowed us to identify most of the remaining unassigned phosphopeptides as tyrosine phosphorylated Hck sequences (data not shown).

To identify any additional Hck-dependent phosphorylation sites on ELMO, we analyzed tryptic digests of Hck treated ELMO using an on-line LC−ESMS method that detects the phosphotyrosine-specific marker ion *m*/*z* 216.043 (*28*). Using this approach, we identified three potential phosphotyrosine-containing ELMO peptides (data not shown). Direct sequencing of these peptides by LC−ES-MSMS confirmed that one of the peptides was phosphorylated on the previously identified Tyr^{511} and that the other two peptides were phosphorylated on novel sites, $Tyr^{395}$and Tyr^{720}. The stoichiometry on these two new sites was found to be approximately 10% at Tyr^{395} and 75% at Tyr^{720}. In all we conclusively identified five Hck-dependent tyrosine phosphorylation sites on ELMO1 at residues 18, 216, 511, 395, and 720 (summarized in Table 1 and Figure 1D).

Tyrosine Phosphorylation of ELMO1 Mutants. To elucidate the role of tyrosine phosphorylation in ELMO1 function, we produced mutant forms of ELMO1 in which the sites identified by mass spectrometry were mutated to phenylalanine. The mutants were tagged with the M45 epitope and were coexpressed with Hck in Cos-7 cells. After immunoprecipitation, we measured tyrosine phosphorylation by

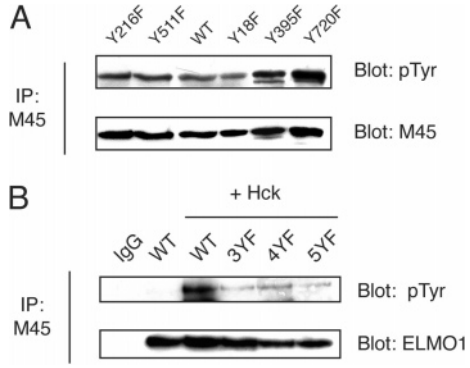

FIGURE 2: Phosphorylation of ELMO 1 mutants. (A) M45-tagged wild-type ELMO1 (WT) or the individual phosphorylation site mutants were coexpressed with Hck in Cos-7 cells. The cells were lysed and ELMO1 was immunoprecipitated using M45 antibody. Precipitated proteins were subjected to SDS−PAGE and analyzed with anti-phosphotyrosine and anti-M45 antibodies. The results presented are representative of three independent experiments. (B) Similar experiments were carried out with mutants containing multiple substitutions (3YF, 4YF, and 5YF). Wild-type ELMO1 and the mutants were coexpressed with Hck in Cos-7 cells. Wild-type ELMO1 was also expressed in the absence of Hck. The cells were lysed and ELMO1 was immunoprecipitated using M45 antibody or IgG as a control. Precipitated proteins were subjected to SDS−PAGE and analyzed with anti-phosphotyrosine and anti-ELMO1 antibodies. The results presented are representative of four independent experiments.

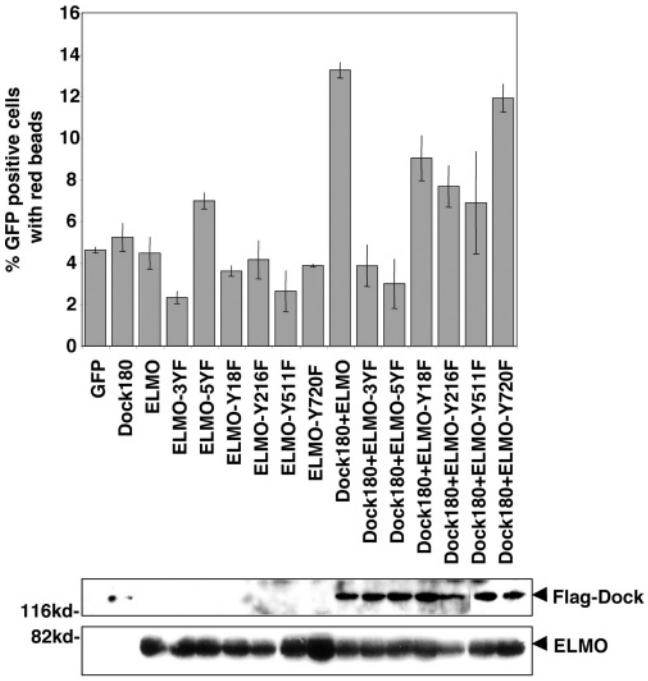

FIGURE 3: ELMO1 mutants lacking tyrosine phosphorylation sites fail to promote phagocytosis. LR73 fibroblasts were transiently transfected with plasmids encoding GFP (0.2 μg/well) with Flag-Dock180 (1.2 μg/well) and M45-tagged ELMO1 (1.0 μg/well) plasmids as indicated. Phagocytosis was measured (see Materials and Methods), and the fraction of GFP positive cells with engulfed particles is depicted. GFP mean fluorescence intensity (MFI) was matched between the different constructs and cells with comparable MFI were analyzed throughout the experiment. If necessary, empty Flag-vector was added to ensure that equal plasmid amounts were transfected in each condition. These data are representative of at least 5 independent experiments.

Western blotting with anti-phosphotyrosine antibody. Mutations at the individual phosphorylation sites Y18, Y216, Y395, Y511, or Y720 showed no reduction in phosphorylation relative to wild-type (Figure 2A), suggesting that multiple sites are phosphorylated under these conditions. We mutated three tyrosine residues together (18, 216, and 511), four tyrosine residues (18, 216, 511, and 395), or all five tyrosine residues (18, 216, 511, 395, and 720); these mutants were designated 3YF, 4YF, and 5YF, respectively. The 3YF, 4YF, and 5YF ELMO1 mutants all showed reduced tyrosine phosphorylation relative to wild-type ELMO1 (Figure 2B). However, even after all five tyrosine sites were mutated to phenylalanine, there was some residual tyrosine phosphorylation of ELMO1 (Figure 2B). This could be due to additional minor phosphorylation sites that were not detected by our mass spectrometry approaches.

Functional Assays of the ELMO1 Mutants. We tested the importance of these sites in a well-established model system for phagocytosis (*19*−*21*, *23*). In LR73 fibroblasts, ELMO1 functions upstream of Rac in signaling pathways that lead to phagocytosis and cell migration. To test the functional importance of the tyrosine phosphorylation sites, ELMO1 mutants lacking sites of tyrosine phosphorylation were transfected into LR73 fibroblasts in the presence or absence of Dock180. We did not overexpress Hck in these experiments, but instead relied on the endogenous expression of Src and other Src-family kinases in LR73 cells (C.D.D. and K.S.R., unpublished observations). We analyzed the 3YF and 5YF mutants, as well as forms of ELMO1 with individual Tyr to Phe substitutions at Y18, Y216, Y511, and Y720, for their role in phagocytosis. (For reasons that are not clear, we were unable to express the Y395F ELMO1 mutant in these cells under a variety of conditions.) A two-color flow cytometry assay was carried out to determine the fraction of GFP-positive cells that can engulf red fluorescent latex beads (*19*). The expression of Dock180 or ELMO1 alone did not promote phagocytosis of the beads (Figure 3). As reported previously (*19*−*21*), coexpression of Dock180 with ELMO1 stimulated phagocytosis about 3-fold (compared to expression of either Dock180 or ELMO1 alone) (Figure 3). In contrast, the 3YF and 5YF mutants of ELMO1 failed to synergize with Dock180 to promote phagocytosis (Figure 3). For the single-site mutants, the total levels of tyrosine phosphorylation were similar to wild-type ELMO1, as judged by anti-phosphotyrosine Western blotting (data not shown). The Y720F ELMO mutant cooperated with Dock180 about as effectively as wild-type ELMO1. The Y18F, Y216F, and Y511F mutants showed reductions in their ability to synergize with Dock180 to promote phagocytosis (Figure 3).

To confirm that endogenous Src-family tyrosine kinases play a role in the ability of ELMO1 to promote phagocytosis under these conditions, we repeated the experiment in the presence of the broad-spectrum Src kinase inhibitor PP2. As shown in Figure 4, PP2 interfered with the ability of ELMO1 to synergize with Dock180, providing additional evidence for the role of Src kinases in this system.

We also carried out cell migration assays with wild-type and mutant forms of ELMO1. As reported previously, LR73 cells coexpressing Dock180 and wild-type ELMO1 showed enhanced migration compared to cells expressing Dock180 alone (Figure 5). Cells coexpressing Dock180 and the 3YF or 5YF mutant forms of ELMO1, however, showed only baseline levels of migration (Figure 5). Coexpression of the

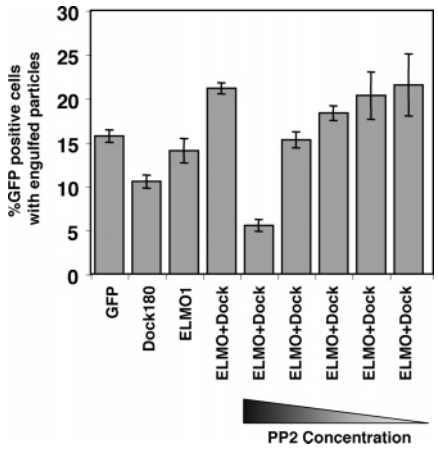

FIGURE 4: PP2 inhibits Dock/ELMO1-mediated phagocytosis. LR73 cells were transiently transfected with plasmids encoding GFP (0.2 μg/well) with Flag-Dock180 (1.2 μg/well) and M45-tagged ELMO1 (1.0 μg/well) plasmids. The Src kinase inhibitor PP2 was added at the following concentrations for the duration of the engulfment period: 10 nM, 100 nM, 1 μM, 10 μM, 100 μM. Phagocytosis was measured as described above in the caption to Figure 3.

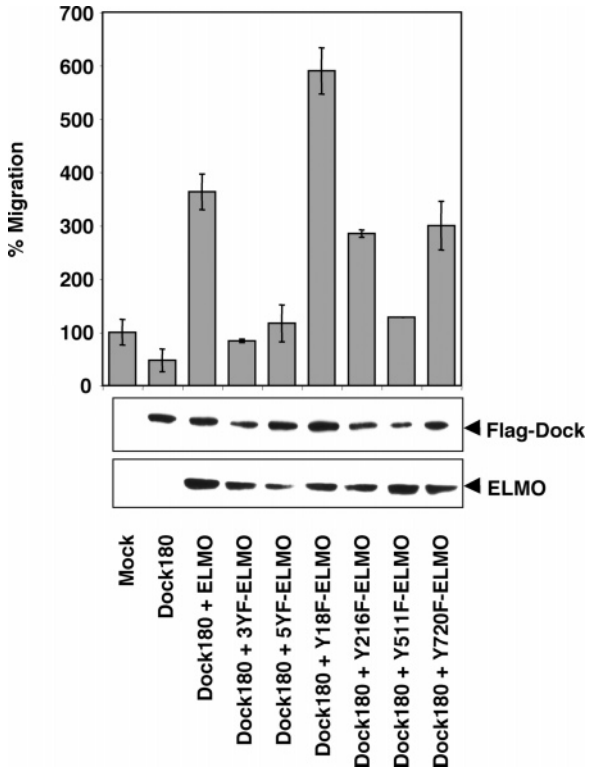

FIGURE 5: ELMO1 mutants fail to cooperate with Dock180 to promote cell migration. LR73 cells were transiently transfected with a luciferase reporter construct (see Materials and Methods), Flag-Dock180 (1.2 μg) and wild-type or mutant and M45-ELMO1 (1.0 μg) plasmids as indicated. The luciferase alone control was set at 100% ("Mock"). Cells from each transfection condition were saved separately, lysed, and immunoblotted to confirm expression of Dock180 and ELMO mutants.

Y18F or Y720F mutants with Dock180 led to levels of migration that were comparable to that of wild-type ELMO1. (In some experiments, such as the example presented in Figure 5, the migration was higher than that of wild-type.) The Y216F ELMO1 mutant displayed migration activity that was slightly reduced from that of wild-type. The Y511F mutant showed a complete inability to promote migration,

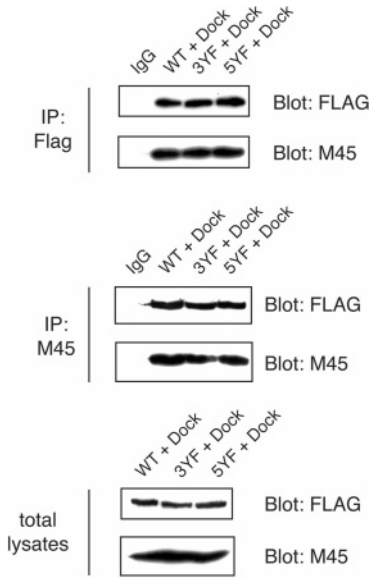

FIGURE 6: Tyrosine mutations of ELMO1 do not affect the association between Dock180 and ELMO1. M45-tagged ELMO1 (wild-type or mutants) were cotransfected with Flag-tagged Dock180 in 293T cells. The cells were lysed and Dock180 was immunoprecipitated with Flag antibody or IgG as control (top panel). Alternatively, ELMO1 was immunoprecipitated with M45 antibody or IgG as control (middle panel). Proteins were separated by SDS−PAGE and analyzed by Western blotting with anti-Flag (Dock180) and anti-M45 (ELMO1) antibodies. The bottom panel shows expression of Dock180 and ELMO1 in 293T cell lysates.

as was the case for the 3YF and 5YF mutants (Figure 5). Lysates were immunoblotted with anti-ELMO1 antibody to confirm similar protein expression of ELMO1 mutants (Figure 5, bottom). Thus, the effects on phagocytosis and migration were similar: the Y18F and Y720F forms of ELMO1 cooperate with Dock180 approximately as effectively as wild-type, Y216F displays a partial cooperation, and Y511F fails to cooperate. These results point to a particularly important role for Y511.

Dock180 and CrkII Association with Tyrosine Mutants of ELMO1. Based on the failure of the ELMO1 tyrosine mutants to synergize with Dock180 in phagocytosis and migration assays, we tested the ability of the mutants to bind to Dock180. Dock180 and ELMO1 have previously been shown to associate directly (*19, 22*). We coexpressed M45-tagged ELMO1 and FLAG-Dock180 together with Hck in 293T cells. We isolated ELMO1 by anti-M45 antibody immunoprecipitation, and examined Dock180 co-immunoprecipitation by anti-FLAG Western blotting. Wild-type ELMO1 associated strongly with Dock180 in these cells (Figure 6). The 3YF (Y18F, Y216F, and Y511F) and 5YF (Y18F, Y216F, Y511F, Y395F, and Y720F) ELMO1 mutants showed essentially no difference in their ability to bind Dock180 (Figure 6). We obtained similar results by doing the reciprocal immunoprecipitation (immunoprecipitation with anti-FLAG, and Western blotting with anti-M45) (Figure 6). We also obtained similar data using endogenous levels of Dock180, although the signal for co-immunoprecipitation was much weaker (data not shown). Thus, mutation of tyrosine phosphorylation sites on ELMO1 did not affect the association between ELMO1 and Dock180.

ELMO1, Dock180, and CrkII form a trimeric complex in which Dock180 acts as a bridge between ELMO1 and CrkII

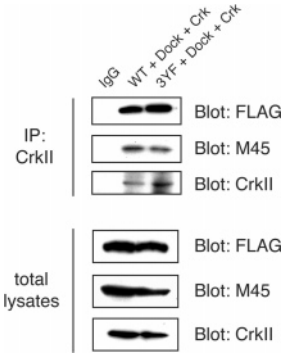

FIGURE 7: Mutations of ELMO1 do not affect CrkII association. 293T cells were transiently transfected with the indicated plasmids. CrkII was immunoprecipitated from cell lysates. The precipitated proteins were analyzed by Western blotting with anti-Flag (Dock180), anti-M45 (ELMO1), and anti-CrkII antibodies. The bottom panel shows the expression of each protein in 293T cell lysates.

(*19*, *23*). Mutants of Dock180 and CrkII that were unable to interact with each other did not synergize in the LR73 engulfment assay (*19*). We tested whether mutation of the Hck phosphorylation sites in ELMO1 affected formation of the ELMO1−CrkII complex. M45-tagged ELMO1 (wild-type or 3YF mutant) was transfected into 293T cells together with FLAG-tagged Dock180 and CrkII. After anti-CrkII immunoprecipitation, the samples were analyzed by SDS−PAGE and Western blotting with anti-FLAG and anti-M45 antibodies (Figure 7). We observed no difference between wild-type ELMO1 and the phosphorylation site mutant with regard to CrkII−Dock180 complex formation or CrkII−ELMO association (which presumably occurs via Dock180) (Figure 7).

Effects of ELMO1 Tyrosine Mutations on Rac Activation. Rac lies downstream of the ELMO1/Dock180/CrkII complex in signaling pathways leading to cell migration and phagocytosis (*19*, *30*). Coexpression of Dock180 and ELMO1 strongly promoted Rac GTP loading (*20*). We reproduced these results by coexpressing ELMO1 and Dock180 in 293T cells, then incubating lysates with an immobilized fusion protein containing the Cdc42/Rac interactive binding (CRIB) domain from PAK. The CRIB domain only interacts with Rac in the GTP-bound state (*20*). Bound proteins were analyzed by SDS−PAGE and Western blotting with anti-Rac antibody (Figure 8A). Coexpression of Hck in 293T cells did not affect the amount of GTP-bound Rac, suggesting that endogenous Src kinases are sufficient for Rac activation in this system (Figure 8A). Next, we compared the abilities of wild-type ELMO1 and the 3YF and 5YF mutants to promote GTP loading of Rac. The immobilized CRIB domain of PAK was incubated with lysates from cells overexpressing Dock180, ELMO1 (wild-type and mutants), and Hck. We observed a significant reduction in the GTP-bound form of Rac from cells expressing the 3YF and 5YF mutant forms of ELMO1 (Figure 8B). There was no significant reduction in the ability of the 3YF and 5YF mutants to promote the association between Rac and Dock180 (Figure 8C). We also analyzed the three individual mutations represented in the 3YF mutant (Y18F, Y216F, and Y511F). In each case, we observed some reduction in Rac activation, suggesting that all three sites are involved in coupling ELMO1 to Rac activation (Figure 8D).

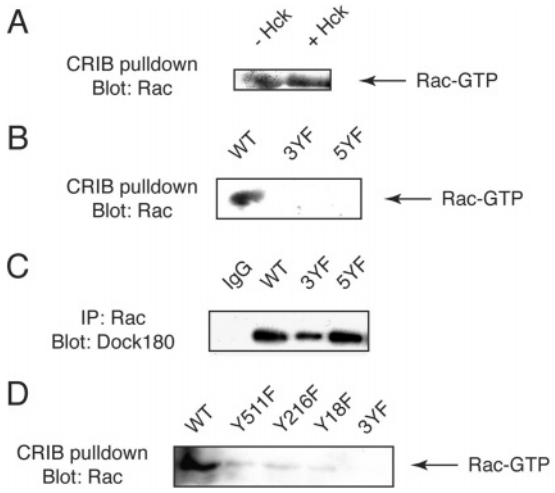

FIGURE 8: ELMO1 mutants fail to promote GTP loading of Rac. (A) 293T cells were cotransfected with ELMO1 and Dock180 in the presence or absence of Hck. Lysates were incubated with immobilized PAK-CRIB domain for 2 h. Bound proteins were separated by SDS−PAGE and probed with Rac antibody. (B) Wild-type, 3YF, or 5YF ELMO1 were cotransfected with Dock180 together with Hck in 293T cells, and PAK-CRIB binding was analyzed as described above. (C) 293T cells were cotransfected with Dock180 and wild-type, 3YF, or 5YF ELMO1. Endogenous Rac was immunoprecipitated from cell lysates, and precipitated proteins were analyzed by anti-Dock180 Western blotting. (D) Wild-type, 3YF, Y18F, Y216F, or Y511F ELMO1 were cotransfected with Dock180 together with Hck in 293T cells, and PAK-CRIB binding was analyzed as described above.

DISCUSSION

Phagocytosis, the process by which cells recognize and engulf large particles, is important in host defense mechanisms as well as in tissue repair and morphogenetic remodeling. Phagocytosis is driven ultimately by the reorganization of filamentous actin (F-actin). The Rho/Rac family of small GTPases regulates the actin cytoskeleton, and signaling through these regulators is critical for dynamic processes such as phagocytosis and cell migration (*31*). Recent work in mammalian and other systems has focused on the upstream components of pathways leading to phagocytosis.

ELMO1 is the mammalian orthologue of the *C. elegans* gene, *ced-12*. Based on genetic studies in the worm and on biochemical studies in cultured mammalian cells, ELMO1 has been shown to functionally cooperate with CrkII and Dock180 during phagocytosis. The ELMO1/Dock180/CrkII complex functions upstream of Rac in this signaling pathway. In *C. elegans*, ELMO1/*ced-12* is also necessary for the proper migration of the gonadal distal tip cells during larval development. The biochemical basis for the stimulation of Rac-GEF activity in these events is that ELMO1 augments complex formation between Dock180 and Rac. The Dock180−ELMO1 complex appears to operate as an unusual, bipartite guanine nucleotide exchange factor for Rac (*20*).

The potential role of phosphorylation in ELMO1 function has not been elucidated. We reported previously that overexpression of Hck leads to tyrosine phosphorylation of ELMO1. We focused here on a cell model system for phagocytosis of apoptotic cells in which ELMO1 has been implicated. We identified five sites on ELMO1 (tyrosines 18, 216, 511, 395, and 720) that are phosphorylated by Hck.

Additional sites of tyrosine phosphorylation may be present, since the 5YF mutant still showed reactivity with anti-pTyr antibody (Figure 2), and the 5YF mutant could still be phosphorylated by Hck in vitro (data not shown). Mutation of three of these sites (Y18, Y216, and Y511) or all five sites caused a decrease in the ability of ELMO1 to cooperate with Dock180 to promote the uptake of fluorescent beads, a simple model of phagocytosis (Figure 3). ELMO1 was unable to cooperate with Dock180 in the presence of the Src-kinase inhibitor PP2, suggesting that endogenous Src-family kinases play a role in the process (Figure 4). Mutation of the tyrosine phosphorylation sites also decreased the ability of ELMO1 to promote cell migration (Figure 5). By testing single-site mutants of ELMO1, we found that tyrosine 511 plays an especially important role in the cooperation with Dock180 to promote phagocytosis and migration. The Y18F and Y720F mutations had minor effects in these assays, while the Y216F mutation gave partial effects. In the migration assay, the Y511F and 5YF mutant forms of ELMO1 behaved similarly, suggesting that Y511 is primarily responsible for this effect. In the phagocytosis assays, Y511F ELMO1 did not show as dramatic a defect as the 5YF mutant, suggesting that more than one site may be involved in this process. Our data suggest that Src kinase-mediated phosphorylation at some of these sites acts to regulate signaling via the ELMO1/Dock180 complex.

The mechanism by which Hck influences the ELMO1/Dock180 complex is not clear. Tyrosine phosphorylation of ELMO1 is not required for the interaction with Dock180, since mutation of three of the tyrosine phosphorylation sites (Y18, Y216, and Y511) or all five sites had no effect on the ability of ELMO1 to bind Dock180 (Figure 6). Previous experiments point to the C-terminal region of ELMO1 as an essential determinant for interaction with Dock180 (*20*). This region presumably interacts with the N-terminal portion of Dock180, as deletion of the Dock180 N-terminus abolishes the interaction with ELMO1. The N-terminal region of ELMO1 (residues 1−330) is required for migration and for proper localization of the ELMO1−Dock180 complex (*23*). Interestingly, ELMO1 mutants lacking the N-terminus were able to cooperate with Dock180 to promote Rac activation and phagocytosis (*23*). The SH3 domain of Hck interacts with the C-terminal polyproline region of ELMO1 (*18*). Tyrosine phosphorylation of ELMO1 by Hck could stabilize the complex, recruit additional complex components, or modulate downstream Rac signaling by an unknown mechanism. Of the five sequences identified here, three (Y18, Y216, and Y395) do not fall in any obvious SH2-binding consensus sequence. The two sites in the C-terminal half of ELMO1 (Y511, with the sequence Tyr-Thr-Glu-Ile, and Y720, with the sequence Tyr-Asp-Phe-Val) are in sequences that, when phosphorylated, could bind to Src-family SH2 domains. One possibility is that phosphorylation of these sites could promote additional phosphorylation of ELMO1 by a processive mechanism (*4, 11*).

One model for activation of the ELMO1/Dock180 complex is that this bimolecular complex exists constitutively in the cytoplasm. CrkII, which is not present in the complex with Dock180 under basal conditions (*24, 32*), might be recruited to the complex by phagocytosis/migration signals (*19, 24, 32*). Integrin stimulation leads to increased Dock180−CrkII binding (*24*). The SH3 domain of CrkII binds to a polyproline sequence at the C-terminus of Dock180 (*32*). Tyrosine phosphorylation of ELMO1 did not appear to be critical for the recruitment of CrkII, however, since the 3YF mutant form of ELMO1 was equally efficient in promoting Dock180−CrkII binding and ELMO1−CrkII binding (via Dock180) as wild-type ELMO1 (Figure 7). This is consistent with the idea that ELMO1−Dock180 can function independently of CrkII (*23*).

Rac is the most downstream component in the CrkII/Dock180/ELMO1 signaling pathways leading to phagocytosis and cell migration. The interaction between ELMO1 and Dock180 is required for Rac activation (*20, 23*). Both ELMO1 and Dock180 do not have an obvious Dbl homology domain, which is present in most other known mammalian GEFs for Rho family GTPases (*19, 32*). However, Dock180 contains a Docker domain that can interact directly with nucleotide-free Rac and catalyze Rac GDP/GTP exchange in vitro (*20, 33*). Both the ELMO1 and Rac binding regions of Dock180 appear to be essential for migration (*21, 23*). In our experiments, wild-type ELMO1 promoted Rac GTP loading when coexpressed with Hck and Dock180, but the 3YF and 5YF mutants did not (Figure 8B). At present it is unclear precisely how the 3YF and 5YF mutants may affect Rac activation. It is notable that both mutants of ELMO1 bind Dock180 similarly to the wild-type ELMO1. Recent studies have suggested that ELMO regulates Dock180 mediated Rac activation by at least three distinct mechanisms: (i) binding of ELMO to activated RhoG and localization of the Dock180/ELMO complex to the membrane; (ii) relief of an intramolecular inhibition within Dock180 by ELMO binding; and (iii) ELMO-PH domain dependent enhancement of Dock180:Rac complex formation. Because Dock180:Rac complex formation was not affected by the 3YF or 5YF mutations (Figure 8C), mechanisms i and ii are more likely explanations. Future detailed experiments may help identify which of these mechanisms is affected by the 3YF or 5YF mutations. In any case, although the upstream mechanism has not been defined, the ultimate output of this signaling pathway is affected by mutation of the tyrosine phosphorylation sites on ELMO1.

Three Rac isoforms (Rac1, Rac2, and Rac 3) have been identified in mammals. Rac1 is ubiquitously expressed, while Rac2 is highly expressed in hematopoietic cells. Dock2, a Dock180 family member expressed in hematopoietic cells, also synergizes with ELMO1 in cell migration (*23*). It will be of interest to investigate ELMO1/Dock2 /Rac2 complexes in hematopoietic cells, because of the high expression of Hck in these cells and the possible role of Hck in multiple types of phagocytosis (*34−36*).

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