The Actin Regulatory Protein HS1 Interacts with Arp2/3 and Mediates Efficient Neutrophil Chemotaxis*

Peter J. Cavnar‡, Kevin Mogen†, Erwin Berthier§, David J. Beebe⁵, and Anna Huttenlocher†,‡,§

From the ‡Departments of Pediatrics and Medical Microbiology and Immunology, 4205 Microbial Sciences Building, University of Wisconsin-Madison, Madison, Wisconsin 53706 and the §Wisconsin Institutes for Medical Research & Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, Wisconsin 53705

Background: HS1 is a cortactin homolog. However, its role in neutrophil chemotaxis is not known.

Results: HS1 interacts with Arp2/3, regulates Vav1 and Rac signaling, and is necessary for efficient neutrophil chemotaxis.

Conclusion: HS1 tyrosine phosphorylation is critical for its interaction with Arp2/3 and efficient neutrophil chemotaxis.

Significance: This work increases our understanding of how actin regulatory proteins mediate efficient cell migration.

HS1 is an actin regulatory protein and cortactin homolog that is expressed in hematopoietic cells. Antigen receptor stimulation induces HS1 phosphorylation, and HS1 is essential for T cell activation. HS1 is also expressed in neutrophils; however, the function of HS1 in neutrophils is not known. Here we show that HS1 localizes to the neutrophil leading edge, and is phosphorylated in response to the chemoattractant formyl-Met-Leu-Phe (fMLP) in adherent cells. Using live imaging in microchannels, we show that depletion of endogenous HS1 in the neutrophil-like PLB-985 cell line impairs chemotaxis. We also find that HS1 is necessary for chemoattractant-induced activation of Rac GTPase signaling and Vav1 phosphorylation, suggesting that HS1-mediated Rac activation is necessary for efficient neutrophil chemotaxis. We identify specific phosphorylation sites that mediate HS1-dependent neutrophil motility. Expression of HS1 Y378F, Y397F is sufficient to rescue migration of HS1-deficient neutrophils, however, a triple phosphomutant Y222F, Y378F, Y397F did not rescue migration of HS1-deficient neutrophils. Moreover, HS1 phosphorylation on Y222, Y378, and Y397 regulates its interaction with Arp2/3. Collectively, our findings identify a novel role for HS1 and its phosphorylation during neutrophil directed migration.

Neutrophils are key mediators of the innate immune response and are the first responders to sites of infection or acute injury. The rapid recruitment of neutrophils requires a highly specialized form of motility that translates chemoattractant signals from the environment into polarized intracellular signals that mediate directed protrusion and efficient migration (1). Furthermore, defects in signaling pathways required for neutrophil chemotaxis are associated with primary immune deficiencies, such as congenital neutropenia and leukocyte adhesion deficiency (2–4).

Chemotaxtactant-induced cell polarization is characterized by an asymmetric distribution of dynamic F-actin at the leading edge that is regulated by a complex array of actin regulatory proteins and lipid products of PI(3)-kinases, such as phosphatidylinositol (3, 4, 5)-triphosphate (PIP₃) (5, 6). Downstream effectors of PIP₃ include Src and Syk family tyrosine kinases, and Rho GTPases, such as Rac and Cdc42, which regulate the Arp2/3 complex and actin dynamics at the leading edge (7, 7–9). To establish and maintain polarity, these signaling mechanisms become amplified through positive feedback loops. For example, PIP₃ stimulates Rac activation through binding to Rho GTPase guanine exchange factors (GEFs), and active Rac can further activate PI(3)-kinase at the leading edge of chemoattractant-stimulated neutrophils (10). In addition, Hem-1, a scaffold component of the Scar/WAVE complex, can regulate actin polymerization by producing self-organizing waves of actin nucleation that provide a platform for cell signaling to mediate efficient neutrophil motility (11, 12). However, it remains unclear how neutrophils spatiotemporally coordinate these circuits to maximize migration efficiency, and what components are essential to regulate actin dynamics and signaling at the leading edge.

Hematopoietic lineage cell-specific protein 1 (HS1)² (13) is the hematopoietic homolog of the actin binding protein cortactin, that regulates leading edge actin dynamics, invadopodia formation, and cell invasion (14, 15). HS1 is structurally similar to cortactin and contains an N-terminal Arp2/3 binding region, three and one-half 37 amino acid repeating units that bind F-actin, a proline-rich region, and a C-terminal Src homology 3 (SH3) domain important for binding to Vav1 and the WIP and WASp heterodimer (16–18). HS1 is a substrate of Syk and Src family kinases (19, 20), and has been reported to regulate lamellipodial protrusions through stabilization of branched actin filaments (21, 22). Syk phosphorylation of HS1 occurs on tyrosines 378 and 397, and generates high affinity binding sites for SH2 domain-containing proteins. This promotes subsequent association and secondary phosphorylation of HS1 tyro-

* This work was supported, in whole or in part, by National Institutes of Health Grant RO1EB010039 (to A. H. and D. J. B.) and AHA Grant 10POST3230031 (to P. J. C.).
† This article contains supplemental Fig. S1 and Movies S1–S9.
‡ To whom correspondence should be addressed: 4205 Microbial Sciences Building, Department of Medical Microbiology and Immunology, 1550 Linden Drive, University of Wisconsin-Madison, WI 53706. Tel.: 608-265-4642.
§ The abbreviations used are: HS1, hematopoietic lineage cell-specific protein 1; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo-(3,4d)pyrimidine; SH, Src homology.
sine 222 by Src family kinases (23, 24). Tyrosine phosphorylation of HS1 is important for regulating lymphocyte trafficking (25), and for mediating antigen-receptor signal transduction, growth arrest, and apoptosis (26, 27). Furthermore, phosphorylation of HS1 can regulate protein-protein interactions, such as HS1 interaction with Vav1 (16).

HS1 has been implicated in T cell signaling and podosome formation (16; 28); however its role in neutrophil chemotaxis is not known. In this study, we used the neutrophil-like PLB-985 cell line to investigate the function of HS1. We demonstrate that HS1 is highly expressed in PLB-985 cells, and HS1 co-localizes with F-actin at the leading edge in neutrophils. We show that HS1 is tyrosine phosphorylated in response to formyl-Met-Leu-Phe (fMLP) in a process that is dependent on Src family kinases. Moreover, HS1 regulates Vav1 and Rac activation in response to fMLP. HS1 and its phosphorylation on tyrosine 222, 378, and 397 are required for efficient neutrophil chemotaxis and binding to Arp2/3 complex. Our findings identify a role for HS1 phosphorylation in neutrophil-directed migration.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies and reagents used in this report are mouse anti-HS1 (BD Biosciences), rabbit anti-HS1 (D83A8), rabbit anti-phosphoHS1 (Y397), and rabbit anti-Rac1/2/3 (Cell Signaling Technology). Mouse anti-vinculin clone VIN—11–5 and mouse anti-β-actin clone AC-15, mouse anti-HA clone HA-7 (Sigma-Aldrich). Mouse anti-cortactin clone 4F11 and mouse anti-phosphotyrosine clone 4G10 (Millipore). Mouse anti-Rac2 (Proteintech Group), mouse anti-Vav1 clone B-6 (Santa Cruz Biotechnology), rabbit anti-phospho-Vav1(Y174) (Epitomics), goat anti-rabbit Alexa Fluor 680, goat anti-mouse Alexa Fluor 800, mouse anti-GFP (Invitrogen). FITC donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and Rhodamine phalloidin (Invitrogen). Glutathione-agarose (Clontech) and GammaBind G Sepharose (GE Healthcare).

Human fibroigen, f-Met-Leu-Phe (fMLP), PP2, BSA, and phosphatase inhibitor mixture 2 (Sigma-Aldrich). ICAM-1 clone HA-7 (Sigma-Aldrich). Mouse anti-cortactin clone 4F11 and phospho-mutants were subsequently generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GAGCTCCTGAAGATT-3′, and reverse 5′-CCTGCAGGCTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′.

**Cell Culture and Primary Neutrophil Isolation**—Primary human neutrophils were obtained from healthy donors with informed consent and purified using polymorphprep solution (Nycomed, Oslo, Norway) according to the manufacturer’s instructions. PLB-985 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at a concentration of 0.1–1 × 10^6 cells/ml. To differentiate, 1.25% DMSO was added to 2 × 10^5 cells/ml for 6 days. HEK293T cells (American Type Culture Collection) and Platinum-GP cells (Cell Biolabs, Inc.) were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**DNA Constructs, Transfections, and Viral Infections**—HS1 was PCR amplified using the following primers: forward, 5′-ATGTGGAGAGGCTGGAATCTGTGAGTATTGGGAGCTCATGATG-3′ and reverse, 5′-CTCCAGAAGCTTGACATATTTTGCAGGGAAGAGTC-3′ and cloned into pEGFP-N1 (Takara Bio Inc.). HS1-GFPN1 was subcloned into the retroviral vector pMX-IRES-GFP (Cell Biolabs, Inc) from which the IRES-GFP sequence was excised using the following primers: forward, 5′-AGTGGAGAGGCTGGAATCTGTGAGTATTGGGAGCTCATGATG-3′ and reverse, 5′-CTCCAGAAGCTTGACATATTTTGCAGGGAAGAGTC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GATGAGGAAGGTTCCTTGTGAGTGATGATTACCAGCTGATGTTGGAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GTATGGCTTTCCTTGTGAGTGATGATTACCAGCTGATGTTGGAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GAGCTCCTGAAGATT-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GAGCTCCTGAAGATT-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GAGCTCCTGAAGATT-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GAGCTCCTGAAGATT-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′. An shRNA resistant full length HS1-GFP was generated by Quikchange site-directed mutagenesis (Agilent Technologies) of pMX-HS1-GFP using the following primers, sense 5′-GGATATCCCGTACATTCTATGGTGACAGGAGG-3′ and antisense 5′-GCCCTCTGCCACCATCTCTGATATGGACAGGGGC-3′, and reverse 5′-CCTCCAGAAGCTTGACATAATTTGAGGAGGTGCTCTGGAGCATCC-3′.
HS1 Is Required for Efficient Chemotaxis

in the presence of 15 μg/ml polybrene. Cells were sorted for equal GFP expression levels using the cell sorter (FACSaria; BD) at the University of Wisconsin flow cytometry facility.

Transwell Chemotaxis Assay—Transwell filters (Corning) with 3-μm pores were coated with 10 μg/ml fibrinogen for 1 h at 37 °C, blocked in 2% BSA for 30 min at 37 °C, and allowed to dry. 2 × 10^6 differentiated PLB-985 cells were plated in the top chamber and were allowed to migrate for 90 min toward mHBSS (HBSS, 20 mM HEPES, 0.1% HSA) containing 10 nM fMLP at 37 °C, 5% CO_2. Cells in the lower chamber were detached by treatment with 45 mM EDTA for 15 min at 4 °C. Cells were quantified by flow cytometry. Percentage of cells transmigrated relative to loading control was determined and graphed either as percent migrated or percent migrated relative to control shRNA-fMLP from at least three independent experiments.

Rho GTPase Pull-down Assay—Rho GTPase pull-down assays were performed as described (31). In brief, differentiated PLB-985 cells were serum starved for 24 h in RPMI supplemented with 0.1% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin, and plated on 10 μg/ml fibrinogen for 10 min. Cells were stimulated with 1 μM fMLP for 1 min, immediately placed on ice, and lysed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl_2, 1% Nonidet P-40, 10 mM MgCl_2, 1 mM EDTA, 10% glycerol, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 200 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% protease inhibitor mixture). 30 μg of bacterially expressed GST-PAK-Rac/Cdc42 (p21)-binding domain (PBD) was incubated with the lysates for 1 h at 4 °C. The affinity-purified products were run on SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted for Rac. Quantification of total Rac for all samples was normalized to control shRNA-fMLP. The mean ± S.E. ratio of Rac-GTP/RacGDP was graphed relative to control shRNA-fMLP from three independent experiments.

Immunoblot Analysis and Immunoprecipitation—Differen-
tiated PLB-985 or HEK293 cells were placed in lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl_2, 1% Nonidet P-40, 10 mM MgCl_2, 1 mM EDTA, 10% glycerol, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 200 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% protease inhibitor mixture) on ice for 10 min and clarified by centrifugation. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Immunoblotting of cell lysates was performed and blots were imaged with an infrared imaging system (Odyssey; LI-COR Biosciences). For immunoprecipitations, differentiated PLB-985 cells were plated on 10 μg/ml fibrinogen or 2% BSA for 10 min at 37 °C, 5% CO_2. In some cases cells were pre-treated for 30 min with 10 μM PP2 or pervanadate (100 μM sodium orthovanadate, 3 mM H_2O_2). Cells were stimulated with 1 μM fMLP for the indicated time and were lysed by addition of ice-cold lysis buffer and clarified by centrifugation. Protein concentrations were determined using a BCA assay and equal protein amounts were incubated for 1 h at 4 °C with 3 μg of mouse anti-HS1 (BD Biosciences), rabbit anti-HS1 (Cell Signaling), mouse anti-Vav1 (Santa Cruz Biotechnology), or mouse anti-GFP (Invitrogen). Immune complexes were captured on GammaBind G-Sepharose beads (GE Healthcare), washed three times in lysis buffer, and analyzed by immunoblotting. For HS1-GFP and Vav1-HA coimmunoprecipitations, HEK293 cells were transfected with GFP or HS1-GFP and Vav1-HA (32). GFP was immunoprecipitated with mouse anti-GFP (Invitrogen), captured on GammaBind G-Sepharose beads, and analyzed by immunoblotting for HA.

Adhesion Assays—100,000 control shRNA and HS1 shRNA 2 cells were labeled with calcein AM (Invitrogen) and plated in the presence or absence of 100 nM fMLP for 30 min in quadruplicate in a 96-well black microplate (Greiner Bio-one) coated with fibrinogen, fibronectin, or ICAM-1 followed by blocking with 2% BSA at 37 °C and 5% CO_2. Fluorescence was measured using a plate reader (Victor3 V; PerkinElmer). Plates were gently washed with mHBSS (HBSS, 20 mM HEPES, 0.1% HSA) in between readings until the remaining control shRNA-fMLP cells were <10% of the original input.

Microfluidic Chemotaxis Assays and Immunofluorescence—Microfluidic devices were fabricated as previously described (33). In brief, the devices enable the generation of a static gradient of chemotactant between a source chamber containing fMLP and a sink chamber containing the PLB-985 cells, which were connected by a 1-mm-long, 400-μm-wide channel in which the fMLP gradient is generated. Microfluidic chambers were coated with 20 μg/ml fibrinogen for 1 h at 37 °C in PBS. 3 μl of neutrophil suspension (4 × 10^6 cells/ml) was added to the device, and 1 μM fMLP was added to the source port. The gradient was allowed to set up and equilibrate in a 37 °C humidified chamber for 15 min before imaging. Time-lapse imaging was performed using a 10×, NA of 0.45, or 60×, NA of 1.4 objective and motorized stage (Ludl Electronic Products) on an inverted microscope (Eclipse TE300) using a charge-coupled device camera (CoolSNAP ES2) and captured into MetaVue imaging software v6.2. Images were taken every 30 s for 30–45 min with up to eight devices being imaged simultaneously. Tracking and velocity measurements were performed using ImageJ analysis software (National Institutes of Health). For immunofluorescence, cells were allowed to chemotax in the microfluidic devices for 20 min, then fixed in 3.7% paraformaldehyde and stained with antibodies following standard protocols.

Statistical Analysis—For statistical comparisons, unpaired t test or one-way analysis of variance (ANOVA) were used with Tukey post-tests with p < 0.05 considered significant.

RESULTS

HS1 Localizes to the Leading Edge during Neutrophil Chemotaxis—To determine how HS1 regulates amoeboid cell chemotaxis, we used the myeloid leukemia cell line, PLB-985, which can be terminally differentiated into neutrophil-like cells with DMSO (34). Using immunoblotting to detect cortactin and HS1 in PLB-985 cells, we found that HS1, but not cortactin, is expressed in both differentiated and undifferentiated cells (Fig. 1B). HS1 is up-regulated in terminally differentiated PLB-985 cells, similar to actin and vinculin as reported previously (2), suggesting that HS1 expression is important for neutrophil function (Fig. 1B).

To characterize the intracellular localization of HS1 during chemotaxis, we detected endogenous HS1 using immunofluo-
rescent staining in both differentiated PLB-985 cells and human peripheral blood neutrophils (Fig. 1C). In unstimulated neutrophils, HS1 colocalized with F-actin at the cell periphery; upon stimulation with fMLP, HS1 colocalized with F-actin at the leading edge in polarized neutrophils during chemotaxis. To determine if Arp2/3 and F-actin binding mediates this localization, we ectopically expressed the N-terminal Arp2/3 and F-actin binding repeats of HS1 (HS1-ΔPS-GFP) in PLB-985 cells (Fig. 2). We found that the F-actin and Arp2/3 binding region of HS1 was sufficient for leading edge localization. In contrast, a HS1-GFP construct containing only the C-terminal proline-rich region and SH3 domain (HS1-PS-GFP) displayed a more diffuse localization, similar to GFP expression alone, suggesting that the F-actin binding region is critical for HS1 localization (Fig. 2 and supplemental Movies S1 and S2).

**Chemoattractant-induced Phosphorylation of HS1 Is Dependent on Adhesion and Src Family Kinases**—To determine if HS1 is phosphorylated in chemoattractant stimulated neutrophils, we immunoprecipitated HS1 from fMLP-stimulated PLB-985 cells and immunoblotted for total phosphotyrosine (Fig. 3, A and B). We found that fMLP induced HS1 phosphorylation with maximal phosphorylation after 3 min of stimulation. Treatment of cells with the Src inhibitor PP2 abolished fMLP-induced HS1 phosphorylation, indicating that Src family

---

**FIGURE 1. HS1 is expressed in neutrophils and localizes to the leading edge during chemotaxis.** A, schematic diagram of HS1 illustrating the Arp2/3 and F-actin binding regions, the proline-rich and SH3 domain, and tyrosines 222, 378, and 397 that are phosphorylated by Syk and Src family kinases. B, HS1 and cortactin expression analysis by Western blot. Equal total protein loaded from HEK293, undifferentiated, and differentiated PLB-985 cells were immunoblotted for cortactin, HS1, and actin. Cortactin expression is limited to HEK293 cells while HS1 expression is only in PLB-985 cells. Furthermore, HS1 and actin protein expression are up-regulated with terminal differentiation induced by DMSO for 6 days. Western immunoblot is representative of three independent experiments. C, immunofluorescent localization of HS1 (green) and F-actin (red) in differentiated PLB-985 cells (dPLB-985) and primary neutrophils in the presence and absence of a linear gradient of fMLP (1 μM). During chemotaxis in response to fMLP, HS1 colocalizes with F-actin in both cell types. Scale bar, 10 μm.
HS1 Is Required for Efficient Chemotaxis

**FIGURE 2. The N-terminal Arp2/3 and F-actin binding regions mediate HS1 localization.** A, schematic of HS1 and two truncation mutants. HS1-ΔPS-GFP contains amino acids 1 through 280 including the N-terminal acidic domain that binds Arp2/3, and the F-actin binding region. HS1-PS-GFP contains amino acids 280 through 486 including the proline-rich region and SH3 domain. B, representative images from time-lapse movies of HS1-deficient PLB-985 cells stably expressing GFP, HS1-PS-GFP (supplemental Movie S1), or HS1-ΔPS-GFP (supplemental Movie S2) during chemotaxis to fMLP (1 μM) in microfluidic gradient generators. GFP- and HS1-PS-GFP-expressing PLB-985 cells showed diffuse cytoplasmic localization. HS1-ΔPS-GFP localized to the leading edge. Scale bar, 10 μm.

kinases are necessary for HS1 phosphorylation. In addition, tyrosine phosphorylation of HS1 was dependent on adhesion to fibrinogen because fMLP stimulation of cells plated on BSA did not significantly induce phosphorylation of HS1 (Fig. 3). To further characterize HS1 phosphorylation, an antibody to phospho-tyrosine 397 was used in fMLP-treated cells (Fig. 3, C and D). Accordingly, phosphorylation on Y397 induced by fMLP required both Src signaling and adhesion to fibrinogen (Fig. 3, C and D). Collectively, the data show that fMLP induces tyrosine phosphorylation of HS1 in adherent neutrophils.

**HS1 Is Required for Efficient Neutrophil Chemotaxis**—To characterize the function of HS1 during neutrophil motility, HS1-deficient PLB-985 cells were generated using lentiviral delivered shRNA (Fig. 4A). Two unique shRNA targets were found to significantly deplete endogenous HS1 levels. Using transwell assays to investigate chemotaxis to fMLP, we found that HS1-deficient cells had impaired migration compared with control cells (Fig. 4B). The effects on migration were dependent on the efficiency of the knockdown, with more significant effects observed with HS1 shRNA 2. Neutrophil random motility was not affected by HS1 depletion, suggesting that the defect in motility was downstream of chemoattractant-induced signaling. To determine if the defects in migration were due to altered adhesion, adhesion assays to fibrinogen, fibronectin, and ICAM-1 were performed (Fig. 4C). We observed no significant defects in fMLP-induced neutrophil adhesion in HS1-deficient cells.

To further characterize the chemotaxis of HS1-deficient PLB-985 cells, we used microfluidic gradient generation in custom-designed chemotaxis chambers that allow the precise control of chemoattractant gradients over extended durations under flow free conditions (Fig. 4D) (35). Using live imaging, HS1-deficient cells had impaired chemotaxis to fMLP compared with control cells (Fig. 4E and supplemental Movies S3 and S4). Automated cell tracking revealed a significant decrease in cell speed and directionality in HS1-deficient cells compared with control cells in response to fMLP (Fig. 4, F and G). Taken together, our findings suggest that HS1 is essential for efficient neutrophil chemotaxis to fMLP.

**HS1 Is Necessary for Rac Activation and Vav1 Phosphorylation**—HS1 phosphorylation regulates Rac and Cdc42 activation in Natural Killer cells (36). To determine if Rac activation is altered in HS1-deficient neutrophils, we performed GST-PAK-PBD pulldown assays for Rac-GTP. We found a significant reduction in total Rac-GTP levels in HS1-deficient cells compared with control cells treated with fMLP (Fig. 5, A and B). Moreover, there was a reduction of Rac2-GTP, the predominant Rac isoform in neutrophils (37) (Fig. 5, A and C). Vav1, a GEF for Rac, binds to the SH3 domain of HS1 (16). To determine if HS1 is necessary for Vav1 phosphorylation induced by fMLP, we tested the effects of HS1 depletion on fMLP-induced phosphorylation of Vav1 using phospho-Vav1(Y174) antibodies (Fig. 6A). Control cells exhibited maximal phosphorylation of Vav1 after 3 min of fMLP stimulation, however depletion of HS1 significantly impaired chemoattractant induced Vav1 phosphorylation (Fig. 6). Together, these data demonstrate that HS1 is required for activation of Rac2 and Vav1 phosphorylation in response to fMLP.

**Phosphorylation of Tyrosines 222, 378, and 397 Is Necessary for Efficient Neutrophil Chemotaxis**—Syk phosphorylation of HS1 tyrosines 378 and 397 is required for Src family kinase interaction with HS1 and subsequent phosphorylation of tyrosine 222 (24). To investigate the role of HS1 tyrosine phosphorylation during neutrophil chemotaxis, we generated control shRNA cells that stably express GFP, and HS1-deficient cells that stably express either GFP, HS1-GFP, or one of two phospho-mutant HS1 constructs: HS1(Y2F)-GFP with Y378,397F mutations, and HS1(Y3F)-GFP with Y222,378,397F mutations (Fig. 7A). To detect phosphorylation of these constructs, GFP- and HS1-GFP-expressing cells were treated with pervanadate and analyzed for phosphorylation at tyrosine 397 (Fig. 7A). HS1-deficient cells expressing wild-type HS1-GFP showed specific phosphorylation, however, HS1(Y2F)-GFP and HS1(Y3F)-GFP were not phosphorylated at tyrosine 397. Differences in
phosphorylation at tyrosine 397 and total tyrosine phosphorylation were also observed in response to fMLP (supplemental Fig. S1). To determine if phosphorylation of HS1 regulates localization to the leading edge of cells during chemotaxis, live-cell imaging in microfluidic chemotaxis chambers was performed (Fig. 7B). GFP-expressing cells showed diffuse localization throughout the cytoplasm (supplemental Movies S5 and S6), however wild-type HS1-GFP, HS1(Y2F)-GFP, and HS1(Y3F)-GFP localized to the leading edge of cells during chemotaxis, similar to Fig. 1C (supplemental Movies S7–S9). These results demonstrate that phosphorylation of HS1 tyrosines 222, 378, and 397 is not necessary for leading edge localization of HS1 in neutrophils.

Transwell migration assays were used to determine if phosphorylation of HS1 affects neutrophil chemotaxis. Similar to Fig. 4B, HS1-deficient cells expressing GFP had a significant reduction in chemotaxis compared with control shRNA cells (Fig. 7C). This defect could be fully rescued by expression of wild-type HS1-GFP. Surprisingly, expression of HS1(Y2F)-GFP was also able to rescue the chemotaxis defect, suggesting that phosphorylation of tyrosines 378 and 397 is not necessary for fMLP-mediated chemotaxis. However, HS1(Y3F)-GFP-expressing cells displayed a significant reduction in neutrophil chemotaxis compared with control, suggesting that phosphorylation of tyrosines 222, 378, and 397, is required for efficient neutrophil chemotaxis.

To determine if phosphorylation of tyrosine residues 222, 378, or 397 alone is required for neutrophil chemotaxis we generated HS1 shRNA cells that stably express HS1(Y222F)-GFP, HS1(Y378F)-GFP, or HS1(Y397F)-GFP (Fig. 7D). In response to fMLP, cells expressing HS1(Y222F)-GFP showed increased phosphorylation on tyrosine 397 similar to control. However, phosphorylation of tyrosine 397 in both HS1(Y378F)- and HS1(Y397F)-expressing cells was reduced (Fig. 7D), raising the possibility that the commercially available polyclonal antibody against phospho-HS1(Y397) cross-reacts with phospho-HS1(Y378). Using transwell migration assays we found that expression of HS1(Y222F), HS1(Y378F), or HS1(Y397F) were sufficient to rescue migration of HS1-deficient cells (Fig. 7E). Collectively, these findings indicate that phosphorylation of tyrosines 222, 378, and 397 of HS1 is important for efficient neutrophil chemotaxis.
HS1 Phosphorylation Regulates Interactions with Vav1 and Arp2/3—To determine how phosphorylation on tyrosines 222, 378, and 397 regulates neutrophil chemotaxis, we characterized the role of tyrosine phosphorylation on interactions with Vav1 and Arp2/3. HEK293 cells were co-transfected with HA-tagged Vav1, and either GFP, HS1-GFP, or the two phospho-mutant
constructs, HS1(Y2F)-GFP and HS1(Y3F)-GFP, and treated with pervanadate. HS1(Y2F) and HS1(Y3F) phospho-mutants had significantly impaired interactions with Vav1 compared with wild-type HS1 (Fig. 8, A and B). In accordance with previous publications, this suggests that Y378 and Y397 are necessary for Vav1 binding (17, 36), but this does not explain the specific defect in chemotaxis in HS1-deficient cells that express HS1(Y3F).

It has also been reported that HS1 can interact with Arp2/3, a key regulator of cell motility. We therefore investigated how tyrosine phosphorylation of HS1 regulates binding to the Arp2/3 complex. HS1-deficient PLB-985 cells stably expressing wild-type HS1 or HS1(Y2F)-GFP were able to interact with endogenous Arp2/3 complex, subunit 2 (ARPC2). However, cells expressing the triple-mutant HS1(Y3F)-GFP had a significant reduction in ARPC2 interactions (Fig. 8, C and D). Collect-
HS1 Is Required for Efficient Chemotaxis

tively, these data demonstrate that tyrosine phosphorylation of HS1 residues 378 and 397 regulates its interaction with Vav1, and that tyrosine phosphorylation of 222, 378, and 397 is required for interactions with the Arp2/3 complex and for efficient neutrophil chemotaxis.

DISCUSSION

There is substantial interest in understanding how the leading edge is regulated during amoeboid motility, including how directional signals are amplified to spatially coordinate cytoskeletal rearrangements during directed motility. We have now identified HS1 as a central component of the leading edge that provides a key link between chemoattractant stimulation and downstream signaling to Vav1 and Rac. Rac is important for regulating dynamic F-actin at the leading edge and is part of a positive feedback loop to PI(3,4,5)P3, which requires actin polymerization (38). Furthermore, HS1 is important in PI(3,4,5)P3 signaling through its direct interaction with PI(3)K and Vav1 (16, 39). Therefore, it is intriguing to speculate that HS1 is a central adaptor protein that regulates spatiotemporal...
HS1 Is Required for Efficient Chemotaxis

Using indirect immunofluorescence we found that HS1 colocalizes with F-actin at the leading edge of neutrophils during chemotaxis and that chemotactant stimulation induces HS1 phosphorylation through Src kinase signaling. Previous reports have shown that Syk phosphorylation of HS1 tyrosines 378 and 397 promotes Src family kinase binding and subsequent phosphorylation of tyrosine 222 (23, 24). However, activation of Syk by integrin signaling is dependent on Src family kinases (40), therefore inhibition of Src kinases using PP2 may disrupt Syk activation and HS1 tyrosine phosphorylation. In any case, our findings suggest that both chemotactant and integrin-mediated adhesion is critical for phosphorylation of HS1 through a Src-dependent pathway. However, the identity of the key Src kinase family member in neutrophils that mediates HS1 phosphorylation is not known.

FIGURE 8. Phosphorylation of HS1 Y222, Y378, and Y397 mediate interactions with Vav1 and Arp2/3. A, immunoprecipitation of GFP and immunoblot for HA-tagged Vav1 from pervanadate treated HEK293 cells co-expressing Vav1-HA and GFP, HS1-GFP, HS1(Y2F)-GFP, or HS1(Y3F)-GFP. Full-length HS1-GFP was able to interact with Vav1, however HS1(Y2F)-GFP and HS1(Y3F)-GFP had a significant decrease in Vav1 interaction. *, p < 0.05; one-way ANOVA with Tukey post-test from three independent experiments. B, quantification of the average ratio of Vav1-HA to immunoprecipitated HS1-GFP constructs relative to wild-type HS1-GFP-expressing cells. *, p < 0.05; one-way ANOVA with Tukey post-test from three independent experiments. C, immunoprecipitation of GFP and immunoblot for Arp2/3 complex subunit 2 (ARPC2) in control PLB-985 cells expressing GFP, or HS1-deficient cells expressing HS1-GFP, HS1(Y2F)-GFP, or HS1(Y3F)-GFP in response to fMLP (1 μM) stimulation for 3 min. HS1(Y3F)-GFP had impaired interactions with Arp2/3 compared with wild-type HS1-GFP and HS1(Y2F)-GFP. D, quantification of the average ratio of ARPC2 to immunoprecipitated HS1-GFP constructs relative to wild-type HS1-GFP-expressing cells. *, p < 0.05; one-way ANOVA with Tukey post-test from three independent experiments.
HS1 Is Required for Efficient Chemotaxis

Few studies have addressed how HS1, the hematopoietic specific homolog of cortactin, regulates leukocyte motility. It is known that HS1 can regulate the actin cytoskeleton in leukocytes through its ability to bind and activate Arp2/3, and by interacting with Vav1 (16, 22). More specifically, a recent report demonstrated that HS1 mediates dendritic cell podosome formation and motility through its interactions with the WIP and WASp heterodimer (28). We now report for the first time, that HS1 is a key regulator of neutrophil chemotaxis. Using time-lapse microscopy in microchannels we show that HS1-deficient cells have impaired velocity and directional persistence in response to a gradient of fMLP. We had previously reported that the HS1 binding partner Hax1 regulates neutrophil chemotaxis through its effects on integrin-mediated adhesion and Rho GTPase activation (2). However, HS1-deficient neutrophils displayed normal integrin-mediated adhesion. This is in contrast to HS1-deficient natural killer cells, which have impaired adhesion to ICAM-1 (36).

T-cell receptor ligation induces HS1 phosphorylation that mediates Vav1 recruitment and activation at the immune synapse (16). Moreover, phosphorylation of tyrosines 378 and 397 is critical for HS1 function in natural killer cells (36). In contrast, we found that phosphorylation on tyrosines 378 and 397 is not necessary for efficient neutrophil chemotaxis since ectopic expression of HS1-(Y2F)-GFP was sufficient to rescue chemotaxis of HS1-deficient cells. However, tyrosine 222 is critical for efficient neutrophil chemotaxis since the triple mutant (Y222F, Y378F, and Y397F) was not sufficient to rescue migration of HS1-deficient cells. Our findings also show that HS1-deficient neutrophils have impaired activation of Rac and Vav1 signaling. Based on our findings we hypothesize that phosphorylation of HS1 regulates its interaction with Vav1 and the spatiotemporal activation of Rac. Accordingly, we showed that phosphorylation of tyrosines 378 and 397 is required for HS1 interaction with Vav1. However, our findings demonstrate the surprising finding that HS1-(Y3F) but not HS1-(Y2F) had impaired interactions with Arp2/3, suggesting that HS1 interaction with Arp2/3 is critical for its effects on neutrophil directed migration. Our findings are consistent with a previous report that the HS1 coiled-coil region is required for F-actin binding and synergistic Arp2/3 activation (22), however our report is the first to show that phosphorylation of HS1 regulates its interaction with Arp2/3. Taken together these findings support a model in which fMLP induced phosphorylation of HS1 mediates Vav1 phosphorylation and activation of Rac and Arp2/3 that amplify leading edge signaling to mediate efficient neutrophil chemotaxis.

In summary, we identified a novel role for HS1 in neutrophil chemotaxis through chemoattractant-induced phosphorylation of tyrosines 222, 378, and 397. An important remaining question is how phosphorylation of tyrosine 222 of HS1 specifically modulates the interaction with Arp2/3 in the N-terminal acidic domain, and what key effector proteins bind to HS1 through the regulated phosphorylation of tyrosine 222. In addition it will be important to address how HS1 orchestrates cross talk between Syk and Src kinases to mediate efficient chemotaxis. These types of questions are challenges for future investigation. Collectively, our findings suggest that HS1 is an important adaptor molecule that links Rac and Vav1 signaling to actin dynamics and represents a central point of regulation to spatiotemporally coordinate feedback mechanisms during chemotaxis.

Acknowledgments—We thank Andrew Wiemer and Linda Boateng for critical review of the manuscript and the Huttenlocher laboratory for review of figures. We also thank David Bennin for help with primary neutrophil purification.

REFERENCES

1. Weiner, O. D., Servant, G., Welch, M. D., Mitchison, T. J., Sedat, J. W., and Bourne, H. R. (1999) Spatial control of actin polymerization during neutrophil chemotaxis. Nat. Cell Biol. 1, 75–81
2. Cavnar, P. J., Berthier, E., Beebe, D. J., and Huttenlocher, A. (2011) Hax1 regulates neutrophil adhesion and motility through RhoA. J. Cell Biol. 193, 465–473
3. Walters, K. B., Green, J. M., Surhus, J. C., Yoo, S. K., and Huttenlocher, A. (2010) Live imaging of neutrophil motility in a zebrafish model of WHIM syndrome. Blood 116, 2803–2811
4. Deng, Q., Yoo, S. K., Cavnar, P. J., Green, J. M., and Huttenlocher, A. (2011) Dual roles for Rac2 in neutrophil motility and active retention in zebrafish hematopoietic tissue. Dev. Cell 21, 735–745
5. Rickert, P., Weiner, O. D., Wang, F., Bourne, H. R., and Servant, G. (2000) Leukocytes navigate by compass: roles of PI3K and its lipid products. Trends Cell Biol. 10, 466–473
6. Yoo, S. K., Deng, Q., Cavnar, P. J., Wu, Y. I., Hahn, K. M., and Huttenlocher, A. (2010) Differential regulation of protrusion and polarity by PI3K during neutrophil motility in live zebrafish. Dev. Cell 18, 226–236
7. Pollard, T. D., and Borisy, G. G. (2003) Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–465
8. Pereira, S., and Lowell, C. (2003) The Lyn tyrosine kinase negatively regulates neutrophil integrin signaling. J. Immunol. 171, 1319–1327
9. Jakus, Z., Fodor, S., Abram, C. L., Lowell, C. A., and Mócsai, A. (2007) Immunoreceptor-like signaling by beta 2 and beta 3 integrins. Trends Cell Biol. 17, 493–501
10. Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C., and Bourne, H. R. (2002) A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. Nat. Cell Biol. 4, 509–513
11. Weiner, O. D., Rentel, M. C., Ott, A., Brown, G. E., Jedrychowski, M., Yaffe, M. B., Gygi, S. P., Cantley, L. C., Bourne, H. R., and Kirschner, M. W. (2006) Hem-1 complexes are essential for Rac activation, actin polymerization, and myosin regulation during neutrophil chemotaxis. PLoS Biol. 4, e38
12. Weiner, O. D., Marganski, W. A., Wu, L. F., Altschuler, S. J., and Kirschner, M. W. (2007) An actin-based wave generator organizes cell motility. PLoS Biol. 5, e221
13. Takemoto, Y., Furuta, M., Li, X. K., Strong-Sparks, W. J., and Hashimoto, Y. (1995) LckBP-1, a proline-rich protein expressed in haematopoietic lineage cells, directly associates with the SH3 domain of protein tyrosine kinase p56lck. EMBO J. 14, 3403–3414
14. Patel, A. M., Incognito, L. S., Schechter, G. L., Wasilenko, W. J., and Sommers, K. D. (1996) Amplification and expression of EMS-1 (cortactin) in head and neck squamous cell carcinoma cell lines. Oncogene 12, 31–35
15. Oser, M., Yamaguchi, H., Mader, C. C., Bravo-Cordero, J. J., Arias, M., Jakus, Z., Fodor, S., Abram, C. L., Lowell, C. A., and Bourne, H. R. (1999) Spatial control of actin polymerization during neutrophil chemotaxis. Nat. Cell Biol. 1, 75–81
HS1 Is Required for Efficient Chemotaxis

Freedman, B. D., Billadeau, D. D., and Burkhartd, J. K. (2009) Hematopoietic lineage cell-specific protein 1 is recruited to the immunological synapse by IL-2-inducible T cell kinase and regulates phospholipase Cγ1 Microcluster dynamics during T cell spreading. J. Immunol. 183, 7352–7361

Martinez-Quiles, N., Ho, H. Y., Kirschner, M. W., Ramesh, N., and Geha, R. S. (2004) Erk/Src phosphorylation of cortactin acts as a switch on-switch off mechanism that controls its ability to activate N-WASP. Mol. Cell. Biol. 24, 5269–5280

Brunati, A. M., Donella-Deana, A., Ruzzene, M., Marin, O., and Pinna, L. A. (1995) Site specificity of p72syk protein tyrosine kinase: efficient phosphorylation of motifs recognized by Src homology 2 domains of the Src family. FEBS Letters 367, 149–152

Yamanashi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., and Yamamoto, T. (1993) Identification of HS1 protein as a major substrate of protein-tyrosine kinase(s) upon B-cell antigen receptor-mediated signaling. Proc. Natl. Acad. Sci. U.S.A. 90, 3631–3635

Weaver, A. M., Karginov, A. V., Kinley, A. W., Weed, S. A., Li, Y., Parsons, J. T., and Cooper, J. A. (2001) Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation. Curr. Biol. 11, 370–374

Hao, J. J., Zhu, J., Zhou, K., Smith, N., and Zhan, X. (2005) The coiled-coil domain is required for HS1 to bind to F-actin and activate Arp2/3 complex. J. Biol. Chem. 280, 37988–37994

Ruzzene, M., Brunati, A. M., Marin, O., Donella-Deana, A., and Pinna, L. A. (1996) SH2 domains mediate the sequential phosphorylation of HS1 protein by p72syk and Src-related protein tyrosine kinases. Biochemistry 35, 5327–5332

Brunati, A. M., Donella-Deana, A., James, P., Quadroni, M., Contri, A., Marin, O., and Pinna, L. A. (1999) Molecular features underlying the sequential phosphorylation of HS1 protein and its association with c-Fgr protein-tyrosine kinase. J. Biol. Chem. 274, 7557–7564

Sciellzo, C., Bertilaccio, M. T., Simonetti, G., Kirschner, M. W., Ramesh, N., and Geha, R. S. (2004) Erk/Src phosphorylation of cortactin acts as a switch on-switch off mechanism that controls its ability to activate N-WASP. Mol. Cell. Biol. 24, 5269–5280

Taniuchi, I., Kitamura, D., Maekawa, Y., Huang, Y., Gomez, T. S., Williamson, E. K., Hamner, D. A., Billadeau, D. D., Argon, Y., and Burkhartd, J. K. (2011) Hematopoietic lineage cell-specific protein 1 functions in concert with the Wiskott-Aldrich Syndrome Protein to promote podosome array organization and chemotaxis in dendritic cells. J. Immunol. 186, 4805–4818

Mcdowell, A., Leitinger, B., Stanley, P., Bates, P. A., Randi, A. M., and Hogg, N. (1998) The I domain of integrin leukocyte function-associated antigen-1 is involved in a conformational change leading to high affinity binding to ligand intercellular adhesion molecule 1 (ICAM-1). J. Biol. Chem. 273, 27396–27403

Ruoslaiti, E., Hayman, E. G., Pierschbacher, M., and Engvall, E. (1982) Fibronectin: purification, immunonochemical properties, and biological activities. Methods Enzymol. 82, 803–831

Lokuta, M. A., Nuzzi, P. A., and Huttenlocher, A. (2003) Calpain regulates neutrophil chemotaxis. Proc. Natl. Acad. Sci. U.S.A. 100, 4006–4011

Moores, S. L., Selfors, L. M., Frederick, J., Breit, T., Fujikawa, K., Alt, F. W., Brugge, J. S., and Swat, W. (2000) Vav family proteins couple to diverse cell surface receptors. Mol. Cell. Biol. 20, 6364–6373

Berthier, E., Surs, C. J., Verbsky, I., Huttenlocher, A., and Atmos, D. J. (2010) An array high-content chemotaxis assay for patient diagnosis. Integr. Biol. 2, 630–638

Tucker, K. A., Lilly, M. B., Beck, L., Jr., and Rado, T. A. (1987) Characterization of a new human diploid myeloid leukemia cell line (PLB-985) with granulocytic and monocyctic differentiating capacity. Blood 70, 372–378

Abhyankar, V. V., Toepke, M. W., Cortesio, C. L., Lokuta, M. A., Huttenlocher, A., and Atmos, D. J. (2008) A platform for assessing chemotactic migration within a spatiotemporally defined 3D microenvironment. Lab. Chip 8, 1507–1515

Butler, B., Kastendieck, D. H., and Cooper, J. A. (2008) Differently phosphorylated forms of the cortactin homolog HS1 mediate distinct functions in natural killer cells. Nat. Immunol. 9, 887–897

Heyworth, P. G., Bohl, B. P., Bokoch, G. M., and Curnutte, J. T. (1994) Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. J. Biol. Chem. 269, 30749–30752

Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D., and Bourne, H. R. (2003) Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J. Cell Biol. 160, 375–385

Kahner, B. N., Dorsam, R. T., Mada, S. R., Kim, S., Stalker, T. J., Brass, L. F., Daniel, J. L., Kitamura, D., and Kunapuli, S. P. (2007) Hematopoietic lineage cell-specific protein 1 is a functionally important signaling molecule in platelet activation. Blood 110, 2449–2456

Mócsai, A., Zhou, M., Meng, F., Túbybelwicz, V. L., and Lowel, C. A. (2002) Syk is required for integrin signaling in neutrophils. Immunity 16, 547–558