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An SH2 domain-dependent, Phosphotyrosine-independent interaction between Vav1, and the Mer receptor tyrosine kinase: A mechanism for localizing GEF action

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Running Title: Interaction between GEF Vav1, and the Mer receptor tyrosine kinase
*Mer* belongs to the *Mer/Axl/Tyro3* receptor tyrosine kinase family, which regulates immune homeostasis in part by triggering monocyte ingestion of apoptotic cells. Mutations in *Mer* can also cause retinitis pigmentosa, again due to defective phagocytosis of apoptotic material. Although some functional aspects of *Mer* have been deciphered, how receptor activation leads to the physiological consequences is not understood. By using yeast two-hybrid assays, we identified the C-terminal region of the guanine nucleotide-exchange factor (GEF) Vav1 as a *Mer*-binding partner. Unlike similar (related) receptors, *Mer* interacted with Vav1 constitutively and independent of phosphotyrosine, yet the site of binding localized to the Vav1 SH2 domain. *Mer* activation resulted in tyrosine phosphorylation of Vav1 and release from *Mer*, whereas Vav1 was neither phosphorylated nor released from kinase-dead *Mer*. Mutation of the Vav1 SH2 domain phosphotyrosine coordinating Arg 696 did not alter *Mer*/Vav1 constitutive binding or Vav1 tyrosine phosphorylation, but did retard Vav1 release from autophosphorylated *Mer*. Ligand-dependent activation of *Mer* in human monocytes led to Vav1 release and stimulated GDP replacement by GTP on RhoA family members. This unusual constitutive, SH2 domain-dependent, but phosphotyrosine-independent, interaction and its regulated local release may explain how *Mer* coordinates precise activation of Rac1 and Cdc42 and the cytoskeletal changes governing the ingestion of apoptotic material by macrophages and pigmented retinal epithelial cells.
The Mer receptor tyrosine kinase was identified by molecular rather than functional assays (1), hence its physiologic function and that of two of its family members, Axl and Tyro3, have been elucidated slowly. Mer is primarily expressed in monocytes and cells of epithelial and reproductive origin with the highest levels of Mer mRNA detected in testis, ovary, prostate, kidney, lung, and peripheral blood monocytes (1,2). Mer is not expressed in normal B- and T-cells, but Mer mRNA expression was detected in a variety of human tumor cells, including neoplastic T and B cell lines; it is also present in the majority of childhood acute lymphoid leukemia samples tested (Graham et al., unpublished data). The Mer extracellular region, like Axl and Tyro3, is comprised of two immunoglobulin-like and two fibronectin type III repeats, a transmembrane domain and an intracellular kinase domain with an unusual KWIAIES motif (1, 2). Biochemical purification identified Gas-6 as a ligand for Axl and Tyro3; subsequent studies showed binding to Mer, but the affinity of Gas6 for Mer is considerably lower (29 nM) than its affinity for Axl and Tyro-3 (0.4 nM and 2.9 nM, respectively) (3, 4, 5, 6).

To investigate the physiological function of Mer, our group generated a knockout mouse, deleting the Mer tyrosine kinase domain (Mer<sup>kd</sup>) (7). Mer<sup>kd</sup> mice were extremely sensitive to endotoxin (LPS) treatment, exhibiting excessive TNF-α production by monocytes, resulting in lethal endotoxic shock. The spleens of Mer<sup>kd</sup> mice were enlarged in some animals, demonstrating accumulation of apoptotic debris (7), and the spleens were markedly enlarged in Mer, Axl, and Tyro3 triple-knockout mice (8). Subsequently, a crucial role of Mer in phagocytosis of apoptotic cells was demonstrated in Mer<sup>kd</sup> mice.
Monocytes bound but did not ingest apoptotic thymocytes, and the thymuses of dexamethasone-treated mice exhibited a marked diminution of apoptotic cell clearance (9). Phagocytosis of other particles was intact, indicating a selective defect for apoptotic material in these animals (9). Indeed, the triple-mutant mice lacking Mer, Axl, and Tyro3 receptors had very high levels of apoptotic cells in many organs, including liver, kidney, muscle, brain, spinal cord, and eye (10). Failure to ingest apoptotic self material led to evidence of autoimmunity in Mer<sup>kd</sup> mice (7, 11) and evidence of profound autoactivation of the immune system in the triple-knockout mice (10).

The Royal College of Surgeons (RCS) rat exhibits a progressive and postnatal loss of vision because of failure of retinal pigment epithelial (RPE) cells to phagocytize shed outer segments of photoreceptor cells (12). This genetic defect was traced to a deletion of a Mer splice acceptor site next to the second exon, resulting in the loss of functional Mer. Gas6-mediated activation of Mer can result in the ingestion of shed photoreceptor outer segments by the cultured rat RPE cells (13), and the retinal dystrophy phenotype can be corrected by delivery of replication-deficient, adenovirus-encoding rat Mer gene to the eyes of young RCS rats (14, 15). Subsequently, separate mutations in Mer, each predicted to abrogate Mer tyrosine kinase activity, were identified in three families with retinitis pigmentosa (16). Individuals harboring these mutations suffer from progressive loss of vision, presumably due to defective phagocytosis of shed photoreceptor cells by RPE cells (16).

To study Mer function we stably transfected the IL-3-dependent murine hematopoietic cell line 32D<sub>c13</sub> (32D) with an EGF receptor extracellular and transmembrane-domain Mer-cytoplasmic domain chimera. In these cells, EGF-dependent
Mer signaling prevented apoptosis upon IL-3 and serum withdrawal. In contrast to transfected full-length EGF receptor and other receptor tyrosine kinases, Mer prevented 32D cell apoptosis without stimulating cellular proliferation. When combined with IL-3, Mer signaling produced dramatic shape changes, suggesting involvement of Mer tyrosine kinase in cytoskeletal remodeling (17). However, the mechanism by which Mer signaling brings about cytoskeletal changes in experimental (32D) or physiologic (monocytes or macrophages) processes remains obscure. Here we demonstrate that Mer interacts constitutively with the SH2 domain of the guanine nucleotide exchange factor (GEF), Vav1. Surprisingly, this SH2 domain/Mer interaction is phosphotyrosine-independent. Mer activation leads to tyrosine phosphorylation and release of Vav1 and activation of Rho family members. This unusual constitutive, SH2 domain-dependent, but phosphotyrosine-independent, interaction and release may provide the circumscribed local cytoskeletal control necessary to trigger ingestion of apoptotic material bound to the surface of phagocytic monocytes or pigmented epithelial cells.

**EXPERIMENTAL PROCEDURES**

*Plasmids and site-directed mutagenesis*- A chimeric receptor was constructed wherein the extracellular domain of Mer was replaced with ligand binding and transmembrane domain of rat EGF receptor (Fig. 1A), using a suitable SalI restriction site at the juxtamembrane region of these two receptors. The entire coding region of chimeric receptor of 1,142 amino acids was subcloned into pLXSN, a mammalian retroviral expression vector (Clontech). This *EGFR-Mer* chimeric receptor was named EMC. For construction of the ‘bait’ plasmid pNCMY, 290 amino acids of the carboxy terminal
intracellular region of Mer (amino acids 546 to 836), which includes the entire kinase
domain was amplified by PCR and inserted in frame to the Gal4 DNA binding domain of
the pAS2-1 vector (Fig. 1A). Full-length Vav1 and all the Vav1 truncation constructs
were generated by PCR amplification using pJC11 plasmid (kindly provided by Prof. C.
J. Der) as template and Pfx polymerase, which has proofreading ability (Gibco BRL). The
PCR products were digested with XhoI and Hind III and subcloned into the
corresponding sites of pcDNA4.1/Myc-His vector (Invitrogen) in frame with the myc
epitope encoding region (Fig. 1B). The Mer truncation constructs AMer (A.A. 529 to
999), BMer (A.A. 621 to 999), CMer (A.A. 690 to 999), DMer (A.A. 529 to 696), EMer
(A.A. 755 to 999), and FMer (A.A. 777 to 999) were generated by PCR amplification
using full-length Mer as a template and Pfx polymerase. The PCR products were digested
with XhoI and Hind III and subcloned into the corresponding sites of pcDNA4.1/Myc-His
vector. The 3’ primers used in Mer-truncation constructs had a stop codon, thus no tag
was present in AMer, BMer, CMer, DMer, Emer, and FMer (Fig. 5A). For expression of
GST-Mer fusion proteins, intracellular region (carboxy terminal 415 amino acids) of Mer
was PCR-amplified using Pfx polymerase. The PCR product was digested with BamHI
and SalI and subcloned into pGEX-4T vector in appropriate reading frame (Fig. 1A).
Mutagenesis of Mer and Vav1 proteins was performed using GeneEditor in vitro site-
directed mutagenesis system (Promega). All the plasmid constructs were sequenced to
confirm the authenticity.

Two-hybrid screening-Matchmaker two-hybrid system-2 was used for
identification of interacting clones (Clontech). Y190 cells transformed with pNCMY
produced expected fusion proteins of 69kDa, detected by Western blotting with an
antibody against the GAL4 DNA-binding domain (Upstate Biotechnology Inc). A human bone marrow cDNA library was screened. $1 \times 10^6$ transformants were plated on SD/-Trp/-Leu/-His/-Ade plates. The colonies that grew on these plates were further screened, and "true positives" were selected based on the criterion that these colonies could not induce His or β-galactosidase production when plasmid DNA isolated from these ‘positive’ colonies was transformed into yeast cells carrying pAS2-1 vector. To further confirm, the colony-lift β-galactosidase filter assay was performed according to manufacturer’s protocol (Clontech).

Antibodies, cell lines, immunoprecipitations, and immunoblot analysis-A polypeptide containing the carboxy terminal 90 amino acids of Mer was expressed as GST fusion protein in the DH5α E. coli strain and plated onto Ampicillin plates. Colonies were picked and grown overnight in 10 ml of LB-containing Ampicillin. Overnight grown culture was added to 300 ml of fresh LB-containing Ampicillin and grown for 2 hours; this was followed by IPTG addition (0.1 mM final concentration). Culture was grown for 2 more hours, and cells were harvested and lysed in Lysis Buffer containing 25 mm Tris (pH 7.5), 150 mM NaCl, 0.4% Triton X-100, 1 mM DTT, 15% glycerol, phosphatase inhibitors (10 mM NaF, 1 mM Na₂VO₄), and protease inhibitor mix (25 µg/ml leupeptin, 25 µg/ml trypsin inhibitor, 25 µg/ml pepstatin, 25 µg/ml aprotinin, 10 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride). Lysates were incubated with Glutathione beads for 2 hours, followed by washing with lysis buffer and elution in PBS-containing 10 mM of Glutathione. Eluted protein was dialyzed against 50mM Tris pH 7.5, 10% glycerol, 100 mM NaCl, and 1mmDTT to remove all Glutathione. The purified polypeptide was then used to generate a rabbit polyclonal
antisera. Anti-Mer Ab2 (Clone 110) was obtained from FabGennix international. Anti-Vav1 antibody was purchased from UBI. Anti-phosphotyrosine (RC20) antibodies were purchased from Transduction laboratories. Anti-Rac1, anti-Rho, and anti-Cdc42 antibodies were purchased from Santacruz laboratories. Goat anti-rabbit immunoglobulin G (IgG)-HRP, and goat anti-mouse IgG-HRP antibodies were purchased from Amersham. Anti-myc monoclonal antibody was obtained from Invitrogen. Human SV40 transformed embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal calf serum. 32D cells were maintained in RPMI medium containing 15% fetal calf serum and 5% WEHI cells spent media (IL-3). 293T cells were transfected using Fugene (Gibco-BRL) as per manufacturer’s protocol. Thirty-two hours after transfection, cells were serum-starved for 16 hours and treated for 20 min with EGF (100 ng/ml). Cells were harvested, immediately washed in ice-cold phosphate-buffered saline (PBS), and lysed in Receptor Lysis Buffer (RLB), containing 25 nm Tris (pH 7.5), 175 mM Nacl, 1% Triton X-100, 1 mM DTT, 15% glycerol, phosphatase inhibitors (10 mM NaF, 1 mM Na$_2$VO$_4$), and protease inhibitor mix (25 µg/ml leupeptin, 25 µg/ml trypsin inhibitor, 25 µg/ml pepstatin, 25 µg/ml aprotinin, 10 mM benzamidine, 1 mM phynylmethysulphonyl fluoride). Equivalent amounts of protein were incubated with respective primary antibodies for 2 hours or overnight at 4°C, followed by incubation with protein A/G-Sepharose (Santa Cruz Laboratories) for 30 min. The beads were washed three times in the RLB buffer, resuspended in the appropriate volume of Laemmli’s gel loading buffer, and subjected to SDS-polyacrylamide-gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and blocked in 3% BSA (for pTyr blots) or 5% milk in TBST.
buffer (Tris-buffered saline [pH 7.5], 0.1% Tween 20) for 1 hour. Blocked filters were probed with primary antibodies in the same buffer, followed by a secondary antibody conjugated to HRP in blocking solution and developed using enhanced chemiluminescence (ECL) detection (Amersham). The blots were re-probed with a second antibody (anti-Mer or anti-myc) to confirm the presence of respective proteins.

Purification of recombinant proteins LVav and KVav from Sf9 insect cells-
Baculovirus expression constructs were generated using the pFast Bac method (Gibco-Brl). SH3-SH2-SH3 and SH3-SH3 domains of Vav1 were PCR-amplified using CVav and JVav as templates and were subcloned along with a 5’ six-histidine tag into the XbaI and SalI sites of pDR120 vector, named LVav and KVav respectively. Expression of LVav and KVav in recombinant-baculovirus-infected Sf9 insect cell extracts was confirmed by Western analysis with anti-polyHistidine antibodies. 200 ml of Baculovirus-infected Sf9 cells were used for the purification of the protein. Cells were harvested 3 days following infection with the virus. Cell pellets from (6x 10^7 cells) were suspended in an extraction buffer containing 10mM Tris-Hcl pH 8.0, 50mM Sodium phosphate pH 8.0, 0.5M NaCl, 10% Glycerol, 20 mM β-mercaptoethanol, and protease inhibitor cocktail (Roche Biochemicals). The extract was sonicated for one minute pulses and then clarified by centrifugation twice at 15000Xg for 15 mins. The supernatant was added to 2mls (50% slurry) of preequilibrated Talon affinity beads (Clontech). Binding was done for 2 hrs in the cold room. The unbound proteins were removed by centrifugation at 2000 RPM for 2 mins. The beads were washed with 10mls of extraction buffer thrice. After final wash the beads were transferred to 15ml chromatographic columns (BioRad). The beads were subsequently washed with 10 bed volumes of wash
buffer containing 50mM Sodium phosphate (pH 7.0), 500 mM NaCl, 20mM Imidazole, 10% Glycerol, and 20 mM β-mercaptoethanol. The bound proteins were eluted in buffer containing 20mM Tris HCl pH 8.0, 100 mM NaCl, 100mM Imidazole, 10% Glycerol, and 20 mM β-mercaptoethanol and collected as 0.5ml fractions. The fractions were analysed by SDS PAGE and Coomassie staining. The fractions containing the purified protein were pooled and dialysed against 50mM Tris pH 7.5, 10% glycerol, 100 mM NaCl, and 1mmDTT. The protein concentration was determined by Bradford method. Small aliquots of the purified protein were stored at –80 °C.

**Purification of GST-Mer and in vitro binding assay-GST-Mer** construct was transformed into BL21 (DE3) cells and plated onto Ampicillin plates. Colonies were picked and grown overnight in 10 ml of LB-containing Ampicillin. Three hundred ml of fresh LB-containing Ampicillin was added to the culture for an additional 2 hours; this was followed by IPTG addition (0.1 mM final concentration). Culture was grown for 4 more hours, and cells were harvested and lysed in Lysis Buffer containing 25 nm Tris (pH 7.5), 150 mM Nacl, 0.4% Triton X-100, 1 mM DTT, 15% glycerol, phosphatase inhibitors (10 mM NaF, 1 mM Na₂VO₄), and protease inhibitor mix. Lysates were incubated with Glutathione beads for 2 hours, followed by washing with lysis buffer and elution in PBS-containing 10 mM of Glutathione. Eluted protein was dialyzed against 50mM Tris pH 7.5, 10% glycerol, 100 mM NaCl, and 1mmDTT to remove all Glutathione.

For the *in vitro* binding assay, 50nMoles of purified LVav or KVav were added to Talon beads resuspended in modified Receptor Lysis Buffer (MRLB) containing 25 nM Tris (pH 7.5), 175 mM Nacl, 0.4% Triton X-100, 1 mM DTT, 15% glycerol, 1%
protease-free BSA, and protease inhibitor mix. After an hour, Talon beads were washed to remove all unbound proteins. Purified GST-Mer (50 mM) was added to similarly treated Talon beads without Vav1 domain proteins or to Talon beads with bound LVav or KVav. Following incubation on ice for 30 min and then incubation with shaking at 4°C for 1 hour, beads were washed thrice with MRLB. Bound protein was dissociated from beads by boiling in SDS sample buffer and assessed by gel electrophoresis and transfer and detection by immunoblotting with anti-Mer antibody. In control experiment, the use of NaCl concentration above 175 mM prevented detection of GST-Mer/Lvav interaction.

**Assay for detection of activated Rac1, RhoA, and Cdc42**—The glutathione-Sepharose beads conjugated with GST-Pak (PBD) or GST-Rok (RBD) were used as specific probes for in vitro binding assays of activated Rac1, Cdc42, and RhoA respectively. Stable cell lines expressing EMC and kdEMC were grown overnight in serum-free media with 5% WEHI cells spent media (IL-3). Next day, cells were stimulated with EGF for 20 or 30 min. Cells were lysed in MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl₂, 1 mM EDTA, 10% Glycerol, protease, and phosphatase inhibitor cocktail). Samples of 500 µg of protein lysates were mixed with 20 µl PBD beads and incubated at 4°C for 45 minutes. The beads were washed three times in MLB buffer and analyzed by Western blotting to detect the bound Rac1 and Cdc42 GTPases. For RhoA, cells were lysed in RLB buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM MgCl₂, 1 mM EDTA, 10% Glycerol, protease, and phosphatase inhibitor cocktail). Samples of 500 µg of protein lysates were mixed with 30 µl RBD beads and incubated at 4°C for 45 minutes. The beads were washed three times in wash buffer (50 mM Tris, pH
7.2, 150 mM NaCl, 1% Triton X-100, 0.1 mM PMSF, 10 mM MgCl₂, 10% Glycerol, protease, and phosphatase inhibitor cocktail) and analyzed by Western blotting.

Isolation of human macrophages-Human blood (buffy coat) was diluted to 50% with PBS and mixed gently. The mixture was gently layered over 50 ml of Ficoll (Histopaque-1077, Sigma). After centrifugation at 2,000 rpm for 30 min the mononuclear cell layer was collected and washed 4 times with PBS and plated in RPMI media containing 10% FBS. After 2 hours of incubation, adherant monocytes and macrophages were retained and allowed to grow for 7-10 days in RPMI media before Gas6 treatment.

RESULTS

Mer interacts with Guanine Nucleotide Exchange factor (GEF), Vav1- Downstream effectors of Mer signaling were sought by yeast-two-hybrid methods (18). A human bone marrow cDNA library was screened with the Mer cytoplasmic domain (pNCMY vector, Fig. 1A) as bait. Of 9 positive colonies, 3 encoded the carboxy terminal SH3-SH2-SH3 domains of Vav1 (CVavAN depicted in Fig. 1B). This prototypic GEF is a 97kD protein with multiple structural motifs (Fig. 1B), including, from C to N terminus, the following domain/motifs: a calponin homology (CH), an acidic region (Ac), a Dbl-homology (DH), a pleckstrin-homology (PH), a zinc finger domain (ZF), a short proline rich region (PR), and two SH3 domains flanking a single SH2 region (21). Clones for two other Mer interacting proteins, Grb2 and Shc (19, 20), were also isolated.

Since Gas6 had not yet been identified as a ligand for Axl, Tyro3, or Mer when our studies began, we created a ligand-activated EGFR-Mer-Chimera (EMC) using the rat EGF receptor extracellular and transmembrane domains and the Mer intracellular...
domain (Fig. 1A). EMC was stably transfected into mouse 32D cells, and when activated with ligand, it prevented apoptosis upon IL-3 withdrawal from this IL-3-requiring cell line. Unlike most receptor tyrosine kinases, Mer activation did not stimulate proliferation, but it did alter cell adherence and shape in these normally suspension-growing cells. This positive effect on cytoskeletal components coupled with the fact that Mer knockout mice exhibited defects in local cytoskeletal regulation (phagocytosis), and the strong positive yeast-two-hybrid signal, led us to pursue the interaction and potential physiologic role of the Mer/Vav1 complex.

For these studies full-length Vav1 and truncated constructs were generated using vector pcDNA4 (depicted in Fig. 1B) and tagged with a myc epitope. Each construct gave generally equivalent expression when transfected into 293T cells (Fig. 1C). The AVav construct, which was not myc tagged and thus was detected using Vav1 antibodies, also expressed equivalently to Vav1 (Fig. 1D). Vav1 interaction with the Mer intracellular domain was studied by cotransfection with either EMC or an EMC with a K619M site-directed mutation that destroyed kinase activity (kinase dead EMC or kdEMC).

*Activation of Mer led to dissociation of Mer/Vav1 complex and Vav1 tyrosine phosphorylation*-To confirm Vav1/Mer interactions, EMC-expressing 32D cells were stimulated with EGF, and increased tyrosine phosphorylation of Vav1 was detected. Surprisingly, Vav1 co-immunoprecipitated with unactivated EMC (unpublished results). To study this interaction in more detail, myc-tagged Vav1 constructs were cotransfected in 293T cells with EMC or kdEMC. Vav1 was readily detected in Mer immunoprecipitates from unstimulated cells, but the level of Mer-associated Vav1
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decreased considerably with ligand stimulation (Fig. 2A, panel 1, lanes 1 and 2). When
cotransfected with kdEMC, Vav1 was present in the kdEMC immunoprecipitates from
both untreated and EGF-treated lysed cells (Fig. 2A, panel 1, lanes 3 and 4). Activation
of EMC increased Vav1 tyrosine phosphorylation (Fig. 2A, panel 2, lanes 1 and 2). This
was not observed in kdEMC-expressing cells (Fig. 2A, panel 2, lanes 3 and 4). Thus, in
unstimulated cells Vav1 is, at least in part, constitutively bound to Mer. Ligand
activation and Mer autophosphorylation resulted in dissociation of tyrosine
phosphorylated Vav1 (compare Fig. 2A, panel 1, lane 2 to panel 2, lane 2).

Upon co-transfection, the carboxy terminal SH3-SH2-SH3, CVav\textsubscript{AN}, was also
readily detected in EMC immunoprecipitates from unstimulated cells and was tyrosine
phosphorylated and released from this association upon Mer stimulation (Fig. 2B, panel
2). This indicates that at least one site for Mer-dependent Vav1 tyrosine phosphorylation
differs from the N terminal Y174 known to be phosphorylated by LCK (2). When
cotransfected with kdEMC, CVav\textsubscript{AN} was bound to Mer regardless of ligand addition (Fig.
2B, panel 1) and was not tyrosine phosphorylated. The construct Avav, encompassing
the amino terminal half of the molecule, neither interacted with Mer nor was tyrosine
phosphorylated when coexpressed with Mer that had been ligand activated (Fig. 2C).
This suggests that the SH3-SH2-SH3 region of Vav1 is sufficient for (i) interaction with,
(ii) tyrosine phosphorylation by, and (iii) release from Mer.

The Vav1 SH2 domain interacts with Mer in a phosphotyrosine-independent
manner—To further define interacting domains three more Vav1 constructs were co-
expressed with EMC or kdEMC. Vav1 lacking the carboxy terminal SH3, DVav\textsubscript{ASH3},
behaved in a fashion similar to full-length Vav1 (Fig. 3A, panel 1, lanes 1 and 2).
However deletion of the SH2 domain abolished Mer/Vav1 interaction. For example, deletion of the C-terminal SH2-SH3 domains produced a construct, BVaVASH2-SH3, that neither associated with Mer nor was tyrosine phosphorylated (Fig. 3B, panel 1, lane 1 and 2). JVaVASH2, Vav1 with just the SH2 deleted, neither interacted with Mer nor was it tyrosine phosphorylated (Fig. 3C, panel 1 and 2, lanes 1 and 2). These results, paradoxically, implicate the SH2 domain in a phosphotyrosine-independent Mer/Vav1 association.

SH2 domains possess a highly conserved arginine residue, which extends up from an interior location in the domain to interact with the phosphate group of the phosphotyrosine (22-25). Mutation of this arginine results in loss of phosphotyrosine binding and impaired transforming activity in Shc (26). To further confirm the unusual phosphotyrosine-independent Vav1 binding to Mer, we mutated Vav1 arginine 696 to alanine (R696A, Fig. 1B). To test the R696A binding properties, it was co-expressed with the full-length EGF receptor. As expected, R696A did not interact with phospho-EGF receptor (Fig. 4A, panel 1, lanes 3 and 4), whereas wild-type Vav1 interacted with EGFR in an EGFR-phosphotyrosine-dependent manner (Fig. 4A, panel 1, lanes 1 and 2). In contrast, R696A bound to both EMC and kdEMC irrespective of Mer activation (Fig. 4B, panel 1). Thus, Vav1 recruitment to Mer in unstimulated cells is not the product of a small amount of tyrosine-phosphorylated Mer attracting Vav1. Interestingly, the R696A mutant became tyrosine phosphorylated by activated Mer presumably due to its contiguity with the active Mer kinase; however, the mutant does not appear to dissociate from Mer as well as wild-type Vav1 (compare Fig. 2A, panel 1 with Fig. 4B, panel 1).
even though Vav1 became tyrosine phosphorylated. This may indicate that the SH2 pocket region is involved in Vav1 dissociation from tyrosine-phosphorylated Mer.

To ascertain whether the interaction between the SH2 domain of Vav1 and Mer observed in multiple immunoprecipitation experiments is direct, the Mer cytoplasmic domain was subcloned into the bacterial expression vector PGEX4T (Fig. 1A), and the resultant 75kDa GST fusion protein was expressed and purified using Glutathione beads (GST-Mer in Fig. 4C, panel 3). In addition, Vav1 construct LVav, encompassing SH3-SH2-SH3 domains, and KVav deleting the SH2 domain and leaving the two SH3 domains were subcloned with hexahistidine tags into Baculovirus expression vector (Fig. 1B). Both Vav domains containing proteins were expressed in insect cells, purified (Fig. 4C, panel 2) and used for an in vitro binding assay (Fig. 4C, panel 1). Fifty nMoles of purified LVav and KVav were immobilized on Talon beads, and the beads alone or beads with Vav1 domains were incubated with purified GST-Mer (50 nMoles). After tumbling for 1 hour, and washing three times, the bound GST-Mer was detected after boiling the Talon beads in SDS, gel electrophoresis, transfer, and anti-Mer immunoblotting (Fig. 4C, panel 1). Only the LVav, which possesses the Vav1 SH2 domain interacted with Mer with sufficient affinity to allow detection while KVav, which lacked the SH2 domain, failed to interact with the purified GST-Mer intracellular domain (Fig. 4C, panel 1, lane 3 and 2, respectively). While this does not preclude the involvement of other molecules in the intracellular association of Mer and Vav, it does indicate that the interaction is at least partially direct.

Carboxy terminal region of the Mer-kinase domain is involved in Vav1 interaction-To determine the region within Mer that is recognized by Vav1, six deletion
constructs of Mer. A-FMer (Fig. 5A), were made and were co-expressed with myc-tagged full-length Vav1. Our Mer polyclonal antibody was raised against the carboxy terminal region and recognized A-CMer, Emer, and Fmer, but not DMer (Fig. 5B, panel 3), which lacks this region. To perform co-immunoprecipitation assay with DMer, anti-EGFR antibody (clone#1382) was used, and DMer was detected using a commercial anti-Mer antibody (Mer Ab2) raised against polypeptide to the region upstream of kinase domain contained in DMer (Fig. 5B, panel 4, lane 4). The results show that Vav1 binds to A, B, CMer, but poorly to DMer (Fig. 5B, panel 1, lane 4), placing at least one important site of interaction C-terminal to amino acid 696. Two other deletions, EMer and FMer, were 65 and 87 amino acids shorter than CMer; both EMer and FMer failed to interact with Vav1 (Fig. 5B, panel 1, lanes 5 and 6).

Mer activation stimulates GDP to GTP exchange in RhoA family members—Tyrosine-phosphorylated Vav1 can catalyze GDP/GTP exchange in Rho family members, e.g., Rac1, Cdc42, and RhoA (27). These proteins when in their GTP-bound form regulate myriad functions, including actin organization, cell adhesion, cell motility, membrane trafficking, gene expression, etc. Their crucial role in plasma membrane and cytoskeletal remodeling during phagocytosis has been well established (28-30). Rac1 and Cdc42 act at distinct stages to promote actin-filament assembly and organization at the site of particle ingestion, while RhoA, though recruited to the attachment site, may participate indirectly in the particle ingestion process (28). First we determined whether regulation of Vav1 tyrosine phosphorylation by the Mer chimera stimulated GDP/GTP exchange on Rho family members using 32D cell lines stably expressing EMC or kdEMC (32D cells express neither Mer nor the EGF receptor). EGF stimulated EMC
tyrosine phosphorylation, but not that of kdEMC (Fig. 6, panel 1). Analysis of GTP-bound Rac1, Cdc42, and RhoA was performed twenty or thirty minutes after Mer chimera activation, followed by cell lysis and “pull down” of GTP-bound Rac1 and Cdc42 with GST-PBD beads and RhoA using GST-RBD beads. Mer-tyrosine-kinase activation in EMC cells stimulated accumulation of GTP bound Rac1, Cdc42, and RhoA (Fig. 6, panels 3, 5, and 7; lanes 1 and 2). The cells expressing kdEMC exhibited no such increase (Fig. 6, panels 3, 5, and 7; lanes 3 and 4).

Mer activation in isolated primary human monocytes stimulates Vav1 tyrosine phosphorylation, Vav1 dissociation from Mer, and GDP/GTP exchange on Rho family members-Monocytes/macrophages isolated from human blood were used to confirm the regulation of Vav1 binding and Rho family members by full-length Mer in a physiologically relevant system. These cells express both Mer and Vav1. In monocytes/macrophages Gas6 (150 nM) activated endogenous Mer and produced Vav1 tyrosine phosphorylation (Fig. 7A, panels 1 and 3). Gas6 stimulation also produced substantial GDP/GTP exchange in all the three members of RhoA family members, Rac1, Cdc42, and RhoA (Fig. 7A, panels 5, 7, and 9). Untreated monocytes/macrophages had low levels of GTP-bound RhoA family members and Vav1 tyrosine phosphorylation.

Lastly, we demonstrated that inactive Mer binds Vav1 in human monocytes; Gas6-mediated activation of endogenous Mer resulted in Vav1 dissociation. Vav1 bound Mer in untreated monocytes/macrophages (Fig. 7B). Treatment with Gas6 (150 nM) resulted in tyrosine phosphorylation of both endogenous Mer and Vav1 (Fig. 7B, panels 2 and 4). Furthermore, Gas6 treatment of monocyte/macrophages decreased Vav1 binding
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to tyrosine-phosphorylated Mer, confirming the previous observations with EMC in 293T and 32D cells.

**DISCUSSION**

A Mer splice acceptor site mutation in the RSC rat strain (12), Mer mutations in at least 3 human kindreds (16), and gene-targeted mouse Mer deletion (8) all result in progressive loss of vision due to defective phagocytosis of shed photoreceptor material by retinal pigment epithelial (RPE) cells. Studies of the immune system in gene-targeted Mer mice reveal a crucial role for Mer in the recognition and/or initiation of selective phagocytosis of apoptotic cells by macrophages (9). Delayed apoptotic cell clearance and lupus-like autoimmunity was demonstrated in mice lacking Mer (11). An even more severe immune dysregulation is seen in the triple-mutant mice lacking Mer, Axl, and Tyro-3; these mice exhibit high levels of apoptotic cells and debris in organs, as well as a profound immune auto-activation syndrome (10). How Mer leads to the selective, local cytoskeletal mediated uptake of extracellular apoptotic material bound to a small area of the cell membrane is unknown.

Monocyte and macrophages use multiple receptors to engage apoptotic cells and presumably to trigger their phagocytosis (31, 32). Since general particle phagocytosis is normal in Mer monocytes, distinct signaling cascades must be initiated by different particles or materials; these signals need to selectively stimulate local cytoskeletal rearrangement. It is very likely that, based on the type of phagocytic receptor engaged in particle internalization, distinct signaling cascades might be triggered, leading to activation of specific member/s of the Rho GTPase subfamily. Rac1, Cdc42, and RhoA
activation leads to different biological consequences that are associated with phagocytosis, and this separate or coordinate regulation may define distinct types of phagocytosis (28). In 32D cells, expressing the Mer chimera, EGF activation stimulated GDP/GTP exchange on Rac1, Cdc42, and RhoA, while in isolated primary human monocyte/macrophages, increases in GTP-bound RhoA, Rac1, and Cdc42 were all stimulated by Gas6 addition. Whether regulation of all the three Rho family members occurs via a Mer/Vav1 process or whether Mer/Vav1 regulates Rac1 and Cdc42 while some downstream process or other GEFs regulates RhoA remains to be determined.

To speculate further on a specific mechanism for Mer involvement in clearing apoptotic material, it is important to know that apoptotic cells externalize membrane phosphatidyl serine (PS), which after externalization can bind to a specific PS receptor on the macrophage surface (33). Gas6, a Mer ligand, also has an affinity for PS (34), and Gas6 has recently been shown to speed ingestion of apoptotic material in pigmented retinal epithelial cells (13). Together PS and Gas6 could form a complex ligand activating local cytoskeletal changes through the PS receptor and Mer. The fact that unstimulated Mer sequesters Vav1 and can release it upon activation provides an interesting potential mechanism for regulating the intense local control of RhoA family members needed to initiate cytoskeletal engulfment of apoptotic material. Mer may hold Vav1 in readiness, releasing it and activating its GEF activity upon sensing local binding of apoptotic material (see Fig. 8 for model).

Tyrosine kinase signalling pathways responsible for the recognition and ingestion of apoptotic cells by both professional and non-professional phagocytes may be evolutionarily conserved (35, 36). Indeed, engulfment of apoptotic cells in
Caenorhabditis elegans is controlled by the genes ced-2 and ced-10, which encode proteins similar to the human adaptor protein CrkII and the human GTPase Rac respectively (35). Whether other intermediate steps and/or conserved molecules are involved in the proposed Mer, Vav1, and Rho family member process (Fig. 8) that we predict controls monocyte/macrophage apoptotic cell ingestion remains to be determined.

SH2 domains are typically found in multidomain signaling proteins, where they are involved in protein-protein interaction. It has been thought that all large multidomain adaptor or signaling proteins with SH2 domain bind with high affinity to specific phosphorylated tyrosines, creating a ‘ligand’ for the SH2 interaction. Recently a few exceptions to this concept have been reported, suggesting an expanded paradigm for SH2 domain function, a phosphotyrosine-independent, SH2 domain interaction. The X-linked lymphoproliferative-disease (XLP) gene product SAP, a small protein that consists almost entirely of one SH2 domain, interacts with the unphosphorylated SLAM (36). Syk, a non-receptor tyrosine kinase, was observed to associate with the β3 integrin cytoplasmic tail through a phosphotyrosine-independent process involving Syk’s tandem SH2 domain region (37).

Here we find through deletion analysis that the SH2 domain of Vav1 interacts with unphosphorylated Mer receptor tyrosine kinase. A more selective approach, in which mutation of the phosphotyrosine coordinating Arg696 to Ala, confirmed that the Mer/Vav1 interaction was SH2 domain-dependent, but phosphotyrosine-independent. Phosphorylation of Mer appears to eject Vav1 from this constitutive interaction, a process that involves an intact functional SH2 domain and did not occur after ligand stimulation of kinase-dead EMC (Fig. 2A). Interestingly, the Vav1 mutant R696A was tyrosine
phosphorylated by the activated EMC to which it is bound, but failed to dissociate from EMC after ligand treatment (Fig. 4B). Therefore Vav1 carboxy terminus phosphorylation by Mer is only one part of the proposed mechanism. The Vav1 SH2 pocket region must play a significant role in dissociation from phosphorylated Mer. The physical insertion of a Mer phosphotyrosine residue may be needed to alter Vav1 conformation, reversing the affinity for Mer, in essence the opposite of the SH2 domain function in most examples.

As indicated above other proteins may be involved in stabilizing the Mer/Vav1 complex or in regulating Vav1 dissociation. The Mer-dependent tyrosine phosphorylation of Vav1 in the SH3-SH2-SH3 region (see CVav tyrosine phosphorylation, Fig. 2B) is not sufficient to eject Vav1 from the complex (R696A, Fig. 4B). An intact SH2 phosphotyrosine site appears to be necessary. We do not yet know what role the carboxy terminal Vav1 tyrosine phosphorylation plays in activating guanine nucleotide exchange function or relocalizing Vav1 to its site of action. However, the novel interaction described herein, an SH2-dependent, phosphotyrosine-independent constitutive binding of Vav1 to Mer, does provide a mechanism for localizing Vav1 GEF action to the site surrounding the cell surface interface at which Mer has been activated by apoptotic cell binding.
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Figure Legends

FIG. 1. Domain structure of Mer and Vav1. A, Schematic of Mer, Mer chimera, and the cytoplasmic domain constructs used for yeast-two-hybrid assay and in vitro binding assay. B, Domain structure of Vav1 and schematics of the truncation, deletion, and structural mutants used for co-transfections. C, Western analysis for expression of Vav1 and its myc-tagged derivatives. D, Western analysis for expression of Vav1 and its untagged derivative AVav.

FIG. 2. Full-length Vav1 is constitutively associated with unphosphorylated Mer. Following co-transfection of EMC or kdEMC with A, Vav1 or B, and C, Vav1 deletion constructs, lysates from unstimulated or EGF-stimulated cells were immunoprecipitated (IP) using anti-Mer, anti-myc, or anti-pTyr antibodies as indicated. The immune complexes were electrophoresed, transferred, and immunoblotted (IB) with anti-myc, anti-Mer, or anti-pTyr antibodies. Molecular weights are displayed on the left of each panel. A, Vav1 is phosphorylated in an EGF-dependent manner and no longer co-immunoprecipitated with EMC. KdEMC is not phosphorylated and maintains its association with Vav1 even after ligand binding. B, The C terminus of Vav1 behaves like full-length Vav1 upon co-transfection with EMC or kdEMC. C, The amino terminus of Vav1 is neither bound or phosphorylated by EMC.

FIG. 3. The SH2 domain of Vav1 interacts with Mer in a phosphotyrosine-independent manner. EMC or kdEMC were co-transfected with A, DVav\textsubscript{ASH3}, B, BVav\textsubscript{ASH2-SH3}, and C, JVav\textsubscript{ASH2}. Lysates from unstimulated or EGF-stimulated were
immunoprecipitated and analyzed as described in Figure 2. A, The mutant lacking the terminal SH3 domain, \( \text{DVav}_{\text{ASH3}} \), behaved like the Vav1 C terminus. B, and C, Deletion of terminal SH2-SH3 domains \( \text{BVav}_{\text{ASH2-SH3}} \), or the SH2 domain itself \( \text{JVav} \), resulted in loss of association with or phosphorylation by Mer.

**FIG. 4. Vav1 Arg696 mutant was constitutively associated with Mer.** A, The EGFR was co-transfected with Vav1 or the R696A mutant, a mutation designed to abolish SH2 domain-phosphotyrosine interaction. Lysates were immunoprecipitated using anti-EGFR antibodies, and immune complexes were analyzed as in Figure 2 using anti-myc, anti-pTyr, and anti-EGFR antibodies. EGF-dependent, EGFR tyrosine phosphorylation was observed and resulted in Vav1/EGFR interaction in the phosphotyrosine-dependent fashion. As expected, the R696A mutant prevented Vav1/EGFR phosphotyrosine-dependent interaction. B, EMC or kdEMC were co-expressed with Vav1 R696A. Lysates were immunoprecipitated and analyzed as above. In contrast to the EGFR, R696A Vav1 was constitutively associated with EMC. Ligand treatment resulted in Mer and R696A Vav1 tyrosine phosphorylation, but R696A failed to dissociate from EMC. C, *In vitro* binding assay of Mer and Vav1. LVav and KVav purified from baculovirus expression system were bound to Talon beads. Purified GST-Mer protein was incubated with the Talon beads, unbound protein was washed, beads boiled, separated on SDS-PAGE, followed by immunoblotting with anti-Mer antibodies.

**FIG. 5. Vav1 recognize carboxy terminal region of Mer.** A, Schematic of Mer, Mer chimera, and various intracellular region representing constructs used for co-
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immunoprecipitation assay. A-FMer constructs were co-transfected with Vav1 in 293T cells, and lysates were immunoprecipitated using anti-\textit{Mer} (in case of A-CMer, EMer, and FMer) or anti-EGFR (in case of DMer) antibodies. Immune complexes were analyzed using anti-myc, anti-\textit{Mer}, and anti-\textit{Mer} Ab2 antibodies. B, Vav1 co-immunoprecipitated with A, B, and CMer, but failed to bind DMer, EMer, and FMer, indicating that at least one Vav1 SH2 domain binding region localizes C-terminal to amino acid 696 of \textit{Mer} protein.

\textbf{FIG. 6.} \textit{Mer} signaling stimulates guanine nucleotide exchange in 32D cells. In 32D cells, ligand activation of \textit{Mer} stimulated accumulation of GTP bound Rac1, RhoA, and Cdc42. KdEMC was without effect. 32D cell lines stably expressing EMC or kdEMC were treated with EGF; ligand-dependent, EMC tyrosine phosphorylation was observed. GTP bound Rac1, RhoA, and Cdc42 were assessed as described in experimental procedures.

\textbf{FIG. 7.} Gas6-mediated activation of \textit{Mer} leads to tyrosine phosphorylation and dissociation of bound Vav1 and GDP/GTP exchange in monocytes/ macrophages. \textit{A}, In monocytes/macrophages, activation of endogenous \textit{Mer} in response to 10 minutes of Gas6 treatment resulted in tyrosine phosphorylation of endogenous Vav1. The subsequent increase in GTP bound Rac1, Cdc42, and RhoA were assessed as described in experimental procedure. \textit{B}, Monocytes/macrophages derived from human blood were treated with 150 nM of Gas6 for 10 minutes, decreasing Vav1 binding to \textit{Mer}. Lysates were immunoprecipitated using anti-\textit{Mer} or anti-Vav1 antibodies, and the immune
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complexes were electrophoresed, transferred, and immunoblotted with anti-pTyr, anti-
Mer, or anti-Vav1 antibodies.

FIG. 8. Constitutive Mer/Vav1 binding provides apoptotic cell recognition and
control of GEF activity: A model.
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**Figure 1**

A. 

Sal I

1 527 999

Mer

1 671

Sal I

EGFR

EMC

kdEMC (619 K•M)

Gal4

GST

pNCMY

pGST-Mer

B. 

CH Ac DH PH ZF SH3 SH2 SH3

Vav1

DVavΔSH3

BVavΔSH2-SH3

AVavΔSH3-SH2-SH3

JVavΔSH2

CVav

R696A

LVav

KVav

C. 

IP:Vav1, IB:Vav1

97

66

45

Vav1

R696A

DVav

JVav

BVav

CVav

IP:Vav1, IB:Vav1

97

66

45

Vav1

AVav

CVav

HC
Figure 2

A.

|          | EMC+Vav1 | kdEMC+Vav1 |
|----------|----------|------------|
| EGF      | -        | +          |
|          | -        | +          |
|          | EGF      | -          | +        |
|          | EGF      | -          | +        |

IP: Mer, IB: Myc
IP: Myc, IB: pTyr
IP: pTyr, IB: Mer
IP: Mer, IB: Mer
IP: Myc, IB: Myc

B.

|          | EMC+Vav1 | kdEMC+Vav1 |
|----------|----------|------------|
| EGF      | -        | +          |
|          | -        | +          |
|          | EGF      | -          | +        |
|          | EGF      | -          | +        |

IP: Mer, IB: Myc
IP: Myc, IB: pTyr
IP: Myc, IB: Myc
IP: Mer, IB: Mer

C.

|          | EMC+Vav1 | kdEMC+Vav1 |
|----------|----------|------------|
| EGF      | -        | +          |
|          | -        | +          |
|          | EGF      | -          | +        |
|          | EGF      | -          | +        |

IP: Mer, IB: Vav1
IP: Vav1, IB: pTyr
IP: Vav1, IB: Vav1
IP: Mer, IB: Mer
Figure 4

A.

| EGFR+Vav1 | EGFR+R696A |
|-----------|------------|
| - | + | - | + |
| 97 | EGF | IP: EGFR, IB: Myc |
| 97 | IP: Myc, IB: pTyr |
| 97 | IP: Myc, IB: Myc |
| 220 | IP: EGFR, IB: pTyr |
| 220 | IP: EGFR, IB: EGFR |

B.

| EMC+R696A | kdEMC+R696A |
|-----------|------------|
| - | + | - | + |
| 97 | EGF | IP: Mer, IB: Myc |
| 97 | IP: Myc, IB: pTyr |
| 97 | IP: Myc, IB: Myc |
| 220 | IP: Mer, IB: Mer |

C.

| GST-Mer | GST-Mer+LVav | GST-Mer+KVav |
|---------|-------------|-------------|
| 67 | IB: Mer |
| 30 | LVav |
| 21 | IB: His, KVav |
| 67 | IP: Mer, IB: Mer |
Figure 5

A.

1  527  999
Sal I

EGFR extracellular domain

EMC

AMer (A.A. 529-999)

BMer (A.A. 621-999)

CMer (A.A. 690-999)

DMer (A.A. 528-696)

EMer (A.A. 755-999)

FMer (A.A. 777-999)

B.

BMer+Vav1
AMer+Vav1
CMer+Vav1
DMer+Vav1
EMer+Vav1
FMer+Vav1

IP:EMC fragments, IB:Myc

IP:Myc, IB:Myc

IP:Mer, IB:Mer

IP:EGFR, IB:Mer Ab2
**Figure 6**

![Western blot analysis of proteins](image)

- **EMC** and **EMCkd** were treated with **EGF**.
- **IP**: pTyr, **IB**: Mer
- **IP**: Mer, **IB**: Mer
- **GTP-Rac1**
- **Rac1**
- **GTP-Cdc42**
- **Cdc42**
- **GTP-RhoA**
- **RhoA**

The figure shows the interaction and activation of GTPases under different conditions and treatments.
Figure 7

A.

| -  | +  |
|----|----|
| 220|    |
| 220|    |
| 97 |    |
| 97 |    |
| 21 |    |

IP: Mer, IB: pTyr
IP: Mer, IB: Mer
IP: Vav, IB: pTyr
IP: Vav, IB: Vav
GTP-Rac
Rac
GTP-Cdc42
Cdc42
GTP-Rho
Rho

B.

| -  | +  |
|----|----|
| 97 |    |
| 97 |    |
| 220|    |
| 220|    |

IP: Mer, IB: Vav1
IP: Vav1, IB: pTyr
IP: Vav1, IB: Vav1
IP: Mer, IB: pTyr
IP: Mer, IB: Mer
An SH2 domain-dependent, Phosphotyrosine-independent interaction between Vav1, and the Mer receptor tyrosine kinase: A mechanism for localizing GEF action
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