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An SH2 Domain-dependent, Phosphotyrosine-independent Interaction between Vav1 and the *Mer* Receptor Tyrosine Kinase

A MECHANISM FOR LOCALIZING GUANINE NUCLEOTIDE-EXCHANGE FACTOR ACTION*

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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 lead to the physiological consequences is not understood. By using yeast two-hybrid assays, we identified the carboxyl-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 independently 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 and subsequent activation of Rac1, Cdc42, and RhoA may explain how *Mer* coordinates precise 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), and hence its physiological function and that of its two family members, Axl and Tyro3, has 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 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.[1] The *Mer* extracellular region, like Axl and Tyro3, comprises 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 and 2.9 nM, respectively) (3–6).

To investigate the physiological function of *Mer*, our group generated a knockout mouse deleting the *Mer* tyrosine kinase domain (Mer^{kd}) (7). Mer^{kd} mice were extremely sensitive to endotoxin (lipopolysaccharide) treatment exhibiting excessive TNF-α production by monocytes resulting in lethal endotoxic shock. The spleens of Mer^{kd} 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^{kd} 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^{kd} mice (7, 11) and evidence of profound autoactivation of the immune system in the triple-knockout mice (10).

The Royal College of Surgeons (RCS)[2] rat exhibits a progressive and postnatal loss of vision because of a failure of retinal pigment epithelial (RPE) cells to ingest 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

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[1] D. K. Graham, T. Dawson, H. R. Snodgrass, and H. S. Earp, unpublished data.

[2] The abbreviations used are: RCS, Royal College of Surgeons; RPE, retinal pigment epithelial; EGF, epidermal growth factor; GEF, guanine nucleotide-exchange factor; aa, amino acid(s); GST, glutathione *S*-transferase; DTT, dithiothreitol; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; EMC, *EGFR-Mer*-chimera; kdEMC, kinase-dead EMC; EGFR, epidermal growth factor receptor; PS, phosphatidylserine.

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 32Dc13 (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 physiological (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 a ligand binding and transmembrane domain of the rat EGF receptor (Fig. 1*A*), using a suitable *Sal*I restriction site at juxtamembrane region of these two receptors. The entire coding region of chimeric receptor of 1142 amino acids was subcloned into pLXSN, a mammalian retroviral expression vector (Clontech). This ***E**GFR-**M**er **C***himeric receptor was named EMC. For construction of the "bait" plasmid pNCMY, 290 amino acids of the carboxyl-terminal intracellular region of *Mer* (amino acids 546–836), 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. 1*A*). 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 (Invitrogen). The PCR products were digested with *Xho*I and *Hin*dIII and subcloned into the corresponding sites of pcDNA4.1/Myc-His vector (Invitrogen) in-frame with a myc epitope-encoding region (Fig. 1*B*). The *Mer* truncation constructs AMer (aa 529–999), BMer (aa 621–999), CMer (aa 690–999), DMer (aa 529–696), EMer (aa 755–999), and FMer (aa 777–999) were generated by PCR amplification using full-length *Mer* as template and *Pfx* polymerase. The PCR products were digested with *Xho*I and *Hin*dIII 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. 5*A*). For expression of GST-Mer fusion proteins, intracellular region (carboxyl-terminal 415 amino acids) of *Mer* was PCR-amplified using *Pfx* polymerase. The PCR product was digested with *Bam*HI and *Sal*I and subcloned into pGEX-4T vector in appropriate reading frame (Fig. 1*A*). 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 69 kDa, detected by Western blotting with an antibody against the GAL4 DNA-binding domain (Upstate Biotechnology Inc.). A human bone marrow cDNA library was screened. 1×10^6 transformants were plated on SD/-Trp/-Leu/-His/-Ade plates. The colonies that grew on these plates were further screened and "true positive" 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 carboxyl-terminal 90 amino acids of *Mer* was expressed as GST fusion protein in DH5α *Escherichia 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 h, which was followed by isopropyl-1-thio-β-D-galactopyranoside addition (0.1 mM final concentration). Culture was grown for 2 more h, and cells were harvested, 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 Na_2VO_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 phenylmethylsulfonyl fluoride). Lysates were incubated with glutathione beads for 2 h, followed by washing with lysis buffer and elution in PBS containing 10 mM glutathione. Eluted protein was dialyzed against 50 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT, to remove all glutathione. The purified polypeptide was then used to generate rabbit polyclonal antibody. Anti-Mer Ab2 (Clone 110) was obtained from FabGennix International. Anti-Vav1 antibody was purchased from UBI. Anti-phosphotyrosine (RC20) antibodies were purchased from BD Transduction Laboratories. Anti-Rac1, anti-RhoA, and anti-Cdc42 antibodies were purchased from Santa Cruz Biotechnology. Goat anti-rabbit immunoglobulin G (IgG)-HRP, and goat anti-mouse IgG-HRP antibodies were purchased from Amersham Biosciences. Anti-myc monoclonal antibody was obtained from Invitrogen. Human SV40-transformed embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 32D cells were maintained in RPMI medium containing 15% fetal calf serum and 5% WEHI cell-spent media (IL-3). 293T cells were transfected using FuGENE (Invitrogen) as per the manufacturer's protocol. Thirty-two hours after transfection, cells were serum-starved for 16 h 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 mM Tris (pH 7.5), 175 mM NaCl, 1% Triton X-100, 1 mM DTT, 15% glycerol, phosphatase inhibitors (10 mM NaF, 1 mM Na_2VO_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 phenylmethylsulfonyl fluoride). Equivalent amounts of protein were incubated with respective primary antibodies for 2 h or overnight at 4 °C, followed by incubation with protein A/G-Sepharose (Santa Cruz Biotechnology) for 30 min. The beads were washed three times in the RLB buffer, resuspended in the appropriate volume of Laemmli gel loading buffer, and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and blocked in 3% bovine serum albumin (for pTyr blots) or 5% milk in TBST buffer (Tris-buffered saline, pH 7.5, 0.1% Tween 20) for 1 h. Blocked filters were probed with primary antibodies in the same buffer, followed by secondary antibody conjugated to HRP in blocking solution and developed using enhanced chemiluminescence (ECL) detection (Amersham Biosciences). The blots were re-probed with a second set of antibodies (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 (Invitrogen-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 *Xba*I and *Sal*I sites of pDR120 vector, named as 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 (6×10^7 cells) were suspended in an extraction buffer containing 10 mM Tris-HCl, pH 8.0, 50 mM sodium phosphate, pH 8.0, 0.5 M NaCl, 10% glycerol, 20 mM β-mercaptoethanol, and protease inhibitor mixture (Roche Applied Science). The extract was sonicated for 1-min pulses and then clarified by centrifugation twice at 15,000 × *g* for 15 min. The supernatant was added to 2 ml (50% slurry) of pre-equilibrated Talon affinity beads (Clontech). Binding was done for 2 h in the cold room. The unbound proteins were removed by centrifugation at 2000 rpm for 2 min. The beads were washed with 10 ml of extraction buffer thrice. After final wash the beads were transferred to 15-ml chromatographic columns (Bio-Rad). The beads were subsequently washed with 10 bed volumes of wash buffer containing 50 mM sodium phosphate (pH 7.0), 500 mM NaCl, 20 mM imidazole, 10% glycerol, 20 mM β-mercaptoethanol. The bound proteins were eluted in buffer containing 20 mM Tris HCl, pH 8.0, 100

mM NaCl, 100 mM imidazole, 10% glycerol, and 20 mM β-mercaptoethanol and collected as 0.5-ml fractions. The fractions were analyzed by SDS-PAGE and Coomassie Blue staining. The fractions containing the purified protein were pooled and dialyzed against 50 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT. The protein concentration was determined by using the 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. Culture, grown overnight, was added to 300 ml of fresh LB containing ampicillin and grown for 2 h, which was followed by isopropyl-1-thio-β-D-galactopyranoside addition (0.1 mM final concentration). Culture was grown for 4 more hours, and cells were harvested, 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 Na_2VO_4), and protease inhibitor mix. Lysates were incubated with glutathione beads for 2 h, followed by washing with lysis buffer and elution in PBS containing 10 mM glutathione. Eluted protein was dialyzed against 50 mM Tris, pH 7.5, 10% glycerol, 100 mM NaCl, and 1 mM DTT, to remove all glutathione.

For the *in vitro* binding assay, 50 nM purified LVav or KVav were added to Talon beads resuspended in modified RLB containing 25 mM Tris (pH 7.5), 175 mM NaCl, 0.4% Triton X-100, 1 mM DTT, 15% glycerol, 1% protease-free bovine serum albumin, and protease inhibitor mix. After 1 h, 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 h, beads were washed thrice with lysis buffer. Bound protein was dissociated from beads by boiling in SDS sample buffer and assessed by gel electrophoresis, transfer, and detection by immunoblotting with anti-*Mer* antibody. In a 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 ligand 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 $MgCl_2$, 1 mM EDTA, 10% glycerol, protease, and phosphatase inhibitor mixture). Samples of 500 μg of protein lysates were mixed with 20 μl of PBD beads and incubated at 4 °C for 45 min. 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 $MgCl_2$, 1 mM EDTA, 10% glycerol, protease, and phosphatase inhibitor mixture). Samples of 500 μg of protein lysates were mixed with 30 μl of RBD beads and incubated at 4 °C for 45 min. 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 phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 10% glycerol, protease, and phosphatase inhibitor mixture) 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 2000 rpm for 30 min the mononuclear cell layer was collected and washed four times with PBS and plated in RPMI media containing 10% fetal bovine serum. After 2 h 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 GEF and 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. 1*A*) as bait. Of nine positive colonies, three encoded the carboxyl-terminal SH3-SH2-SH3 domains of Vav1 ($CVav\Delta_N$ depicted in Fig. 1*B*). This prototypic GEF is a 97-kDa protein with multiple structural motifs (Fig. 1*B*), including, from the carboxyl to the amino termini, 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.

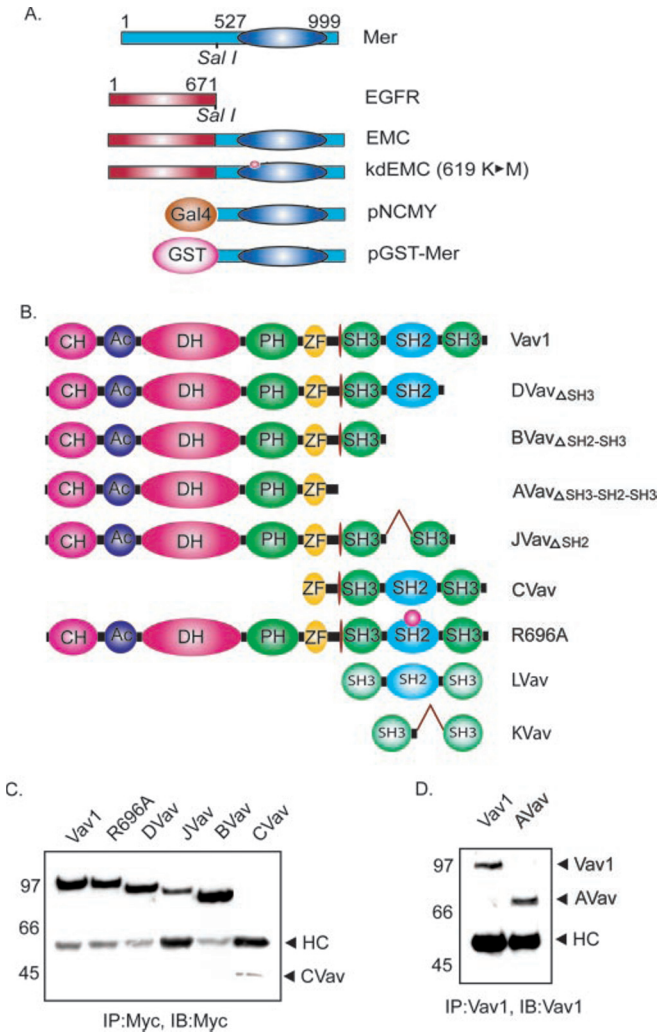

FIG. 1. **Domain structure of *Mer* and Vav1.** *A*, schematic of *Mer*, *Mer* chimera, and the cytoplasmic domain constructs used for yeast two-hybrid and *in vitro* binding assays. *B*, domain structure of Vav1 and schematics of the truncation, deletion, and structural mutants used for co-transfections.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

Because 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. 1*A*). 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 (17). 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 physiological role of the *Mer*/Vav1 complex.

For these studies full-length Vav1 and truncated constructs were generated using vector pcDNA4 (depicted in Fig. 1*B*) and tagged with an myc epitope. Each construct gave generally equivalent expression when transfected into 293T cells (Fig.

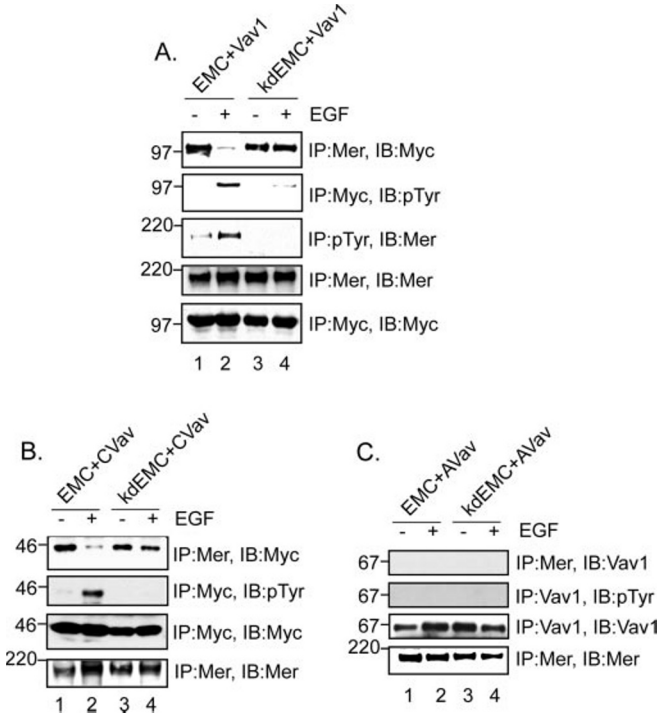

FIG. 2. **Full-length Vav1 is constitutively associated with unphosphorylated *Mer*.** Following co-transfection of EMC or kdEMC with Vav1 (*A*) or Vav1 deletion constructs (*B* and *C*), lysates from unstimulated or EGF-stimulated cells were immunoprecipitated (*IP*) using anti-*Mer*, anti-myc, or anti-pTyr 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 nor phosphorylated by EMC

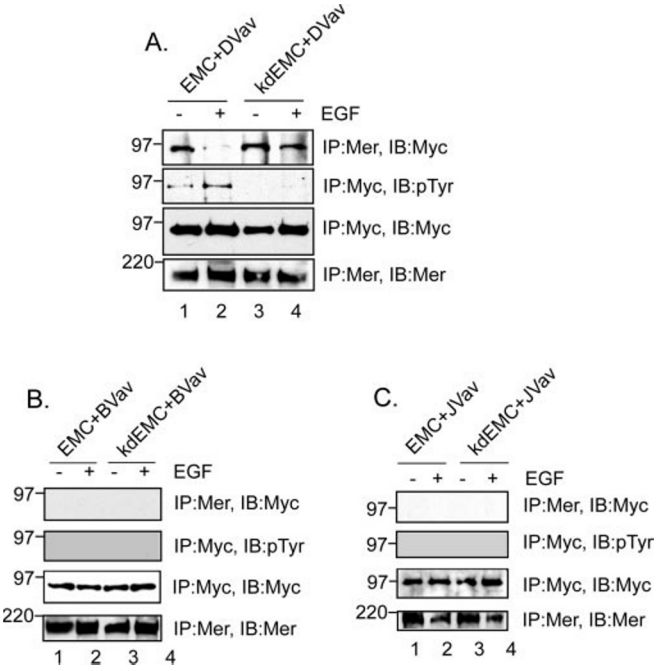

FIG. 3. **The SH2 domain of Vav1 interacts with *Mer* in a phosphotyrosine-independent manner.** EMC or kdEMC were co-transfected with $DVav_{\Delta SH3}$ (*A*), $BVav_{\Delta SH2\text{-}SH3}$ (*B*), and $JVav_{\Delta SH2}$ (*C*). Lysates from unstimulated or EGF-stimulated were immunoprecipitated and analyzed as described in Fig. 2. *A*, the mutant lacking the terminal SH3 domain, $DVav_{\Delta SH3}$, behaved like the Vav1 C terminus. *B* and *C*, deletion of terminal SH2-SH3 domains $BVav_{\Delta SH2\text{-}SH3}$, or the SH2 domain itself JVav, resulted in loss of association with or phosphorylation by *Mer*.

1*C*). The AVav construct, which was not myc-tagged and thus was detected using Vav1 antibodies, also expressed equivalently to Vav1 (Fig. 1*D*). Vav1 interaction with the *Mer* intracellular domain was studied by co-transfection with either EMC or an EMC with a K619M site-directed mutation that destroyed kinase activity (kinase-dead EMC or kdEMC).

Activation of Mer Lead 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.[3] To study this interaction in more detail, myc-tagged Vav1 constructs were co-transfected in 293T cells with EMC or kdEMC. Vav1 was readily detected in *Mer* immunoprecipitates from unstimulated cells, but the level of *Mer*-associated Vav1 decreased considerably with ligand stimulation (Fig. 2*A*, *first panel*, *lanes 1* and *2*). When co-transfected with kdEMC, Vav1 was present in the kdEMC immunoprecipitates from both untreated and EGF-treated lysed cells (Fig. 2*A*, *first panel*, *lanes 3* and *4*). Activation of EMC increased Vav1 tyrosine phosphorylation (Fig. 2*A*, *second panel*, *lanes 1* and *2*). This was not observed in kdEMC-expressing cells (Fig. 2*A*, *second panel*, *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. 2*A*, *first panel*, *lane 2* to *second panel*, *lane 2*).

Upon co-transfection, the carboxyl-terminal SH3-SH2-SH3, CVav, was also readily detected in EMC immunoprecipitates from unstimulated cells and was tyrosine-phosphorylated and released from this association upon *Mer* stimulation (Fig. 2*B*, *second panel*). This indicates that at least one site for *Mer*-dependent Vav1 tyrosine phosphorylation differs from the N-terminal Tyr-174 known to be phosphorylated by LCK (21). When co-transfected with kdEMC, CVav was bound to *Mer* regardless of ligand addition (Fig. 2*B*, *first panel*) 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. 2*C*). 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 carboxyl-terminal SH3, $DVav\Delta_{SH3}$, behaved in a fashion similar to full-length Vav1 (Fig. 3*A*, *first panel*, lanes 1 and 2). However deletion of the SH2 domain abolished *Mer*/Vav1 interaction. For example deletion of the carboxyl-terminal SH2-SH3 domains produced a construct, $BVav\Delta_{SH2\text{-}SH3}$, that neither associated with *Mer* nor was tyrosine-phosphorylated (Fig. 3*B*, *first panel*, *lanes 1* and *2*). $JVav\Delta_{SH2}$, Vav1 with just the SH2 deleted, neither interacted with *Mer* nor was it tyrosine-phosphorylated (Fig. 3*C*, *first* and *second panels*, *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

[3] N. P. Mahajan and H. Shelton Earp, unpublished results.

interact with the phosphate group of the phosphotyrosine (22–25). Mutation of this arginine results in loss of phosphotyrosine binding and impaired Ras activity in Shc (26). To further confirm the unusual phosphotyrosine-independent Vav1 binding to *Mer*, we mutated Vav1 arginine 696 to alanine (R696A, Fig. 1*B*). 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. 4*A*, *first panel*, *lanes 3* and *4*), whereas wild-type Vav1 interacted with EGFR in an EGFR phosphotyrosine-dependent manner (Fig. 4*A*, *first panel*, *lanes 1* and *2*). In contrast, R696A bound to both EMC and kdEMC irrespective of *Mer* activation (Fig. 4*B*, *first panel*). Thus, Vav1 recruitment to *Mer* in unstimulated cells is not the product of a small amount of the 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. 2*A*, *first panel* with Fig. 4*B*, *first panel*) 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 SH2 domain of Vav1 and *Mer* observed in multiple immunoprecipitation experiments is direct, the *Mer* cytoplasmic domain was subcloned into bacterial expression vector PGEX4T (Fig. 1*A*), and the resultant 75-kDa GST fusion protein was expressed and purified using glutathione beads (GST-*Mer* in Fig. 4*C*, *third panel*). 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 a baculovirus expression vector (Fig. 1*B*). Both Vav domain-containing proteins were expressed in insect cells, purified (Fig. 4*C*, *second panel*), and used for *in vitro* binding assay (Fig. 4*C*, *first panel*). Fifty nM purified LVav and KVav was immobilized on Talon beads, and the beads alone or beads with Vav1 domains were incubated with purified GST-*Mer* (50 nM). After tumbling for 1 h, 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. 4*C*, *first panel*). Only the LVav, which possesses the Vav1 SH2 domain, interacted with *Mer* with sufficient affinity to allow detection, whereas KVav, which lacked the SH2 domain, failed to interact with the purified GST-*Mer* intracellular domain (Fig. 4*C*, *first panel*, *lanes 3* and *2*, respectively). Although 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.

Carboxyl-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. 5*A*), were made and were co-expressed with myc-tagged full-length Vav1. Our *Mer* polyclonal antibody was raised against the carboxyl-terminal region and recognized A-CMer, EMer, and FMer but not DMer (Fig. 5*B*, *third panel*), 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. 5*B*, *fourth panel*, *lane 4*). The results show that Vav1 binds to A, B, CMer, but not DMer (Fig. 5*B*, *first panel*, *lane 4*), placing at least one important site of interaction carboxyl-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. 5*B*, *first panel*, *lanes 5* and *6*).

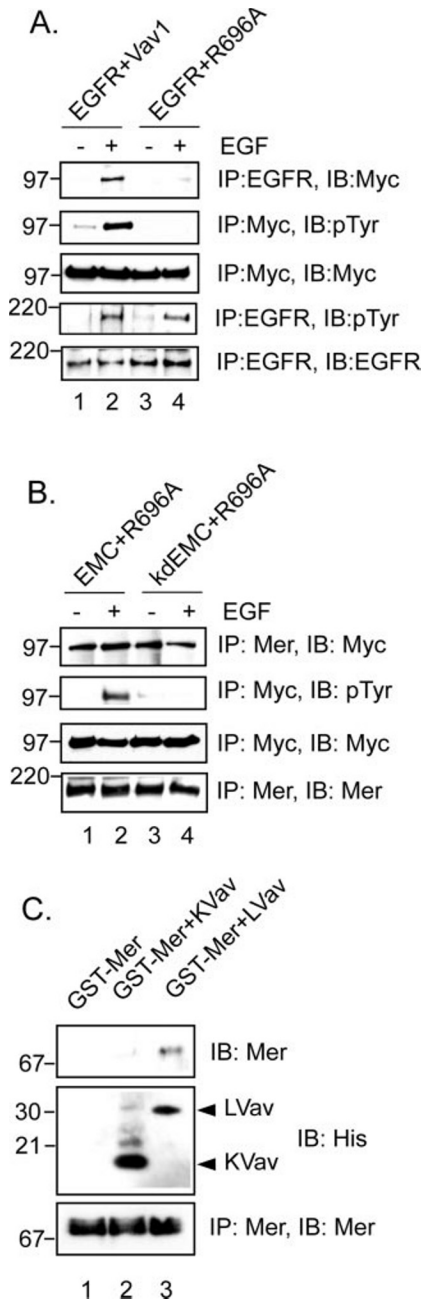

FIG. 4. **Vav1 Arg-696 mutant was constitutively associated with *Mer*.** *A*, the EGFR was co-transfected with Vav1 or the R696A mutant, a mutation designed to abolish SH2 domain-phosphotyrosine interaction. Lysates were immunoprecipitated using anti-EGFR antibodies, and immune complexes were analyzed as in Fig. 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 the baculovirus expression system were bound to Talon beads. Purified GST-*Mer* protein was incubated with the Talon beads, unbound protein was washed, and beads were boiled, separated on SDS-PAGE, then immunoblotted with anti-*Mer* antibodies.

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,

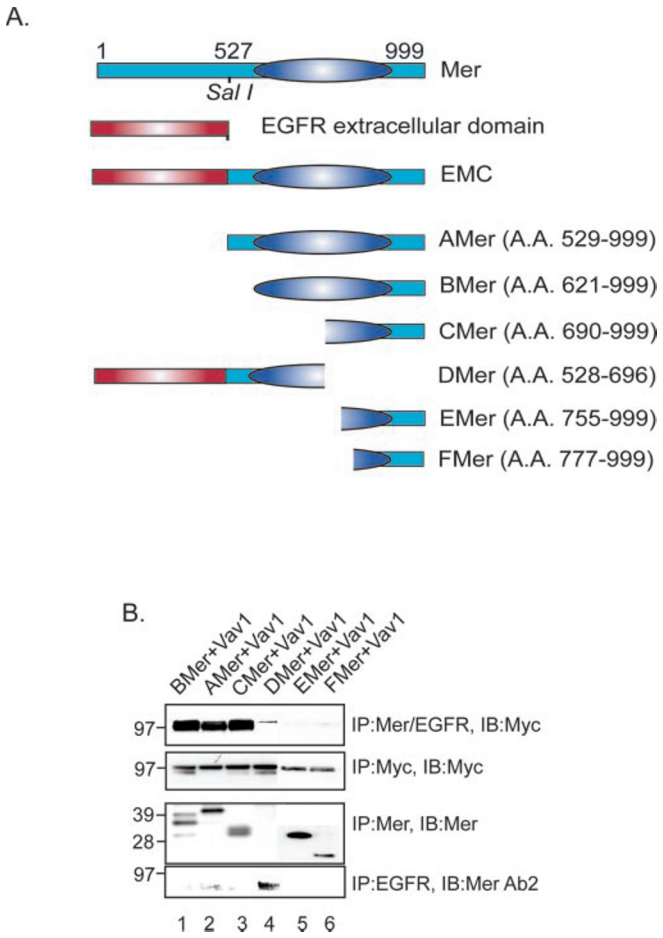

FIG. 5. **Vav1 recognize carboxyl-terminal region of *Mer*.** *A*, schematic of *Mer*, *Mer* chimera, and various intracellular regions representing constructs used for co-immunoprecipitation assay. A-FMer constructs were co-transfected with Vav1 in 293T cells, and lysates were immunoprecipitated using anti-*Mer* (in the cases of A-CMer, EMer, and FMer) or anti-EGFR (in the case of DMer) antibodies. Immune complexes were analyzed using anti-myc, anti-*Mer*, and anti-*Mer* Ab2 antibodies. *B*, Vav1 co-immunoprecipitated with A, B, and CMer but failed to bind DMer, EMer, and FMer.

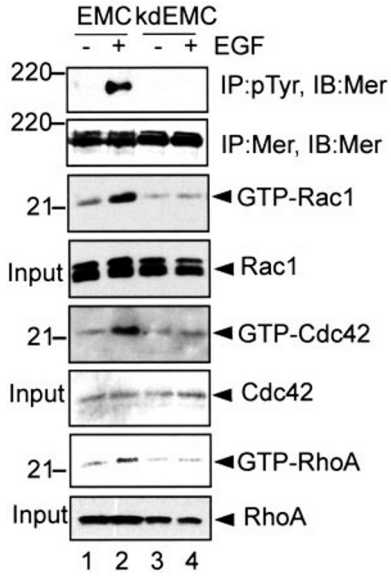

FIG. 6. ***Mer* signaling stimulates guanine nucleotide exchange in 32D cells.** In 32D cells, ligand activation of *Mer* stimulated accumulation of GTP-bound Rac1, RhoA, and Cdc42. kdEMC was without effect. 32D cell lines stably expressing EMC or kdEMC were treated with EGF; ligand-dependent, EMC tyrosine phosphorylation was observed. GTP-bound Rac1, RhoA, and Cdc42 were assessed as described under "Experimental Procedures."

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, whereas 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, *first panel*). Analysis of GTP-bound Rac1, Cdc42, and RhoA was performed 20 or 30 min 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, *third*, *fifth*, and *seventh panels*; *lanes 1* and *2*). The cells expressing kdEMC exhibited no such increase (Fig. 6, *third*, *fifth*, and *seventh panels*; *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. 7*A*, *first* and *third panels*). Gas6 stimulation also produced substantial GDP/GTP exchange in all the three members of RhoA family members, Rac1, Cdc42, and RhoA (Fig. 7*A*, *fifth*, *seventh*, and *ninth panels*). Untreated monocytes/macrophages had low levels of GTP-bound RhoA family members and Vav1 tyrosine phosphorylation.

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

DISCUSSION

A *Mer* splice acceptor site mutation in the RCS rat strain (12), *Mer* mutations in at least three 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

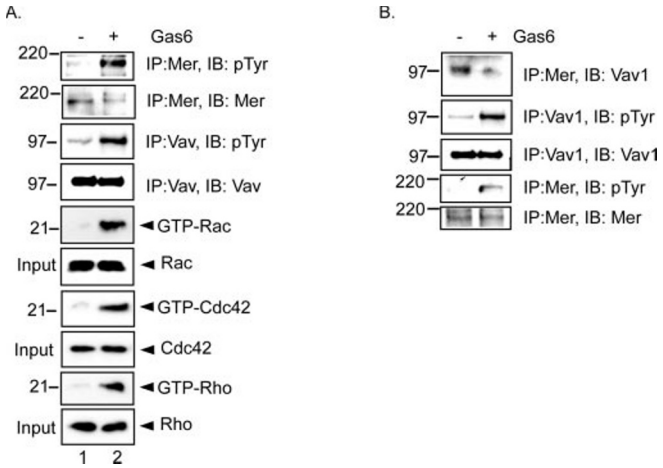

FIG. 7. **Gas6-mediated activation of *Mer* leads to tyrosine phosphorylation and dissociation of bound Vav1 and GDP/GTP exchange in monocytes/macrophages.** *A*, in monocytes/macrophages, activation of endogenous *Mer* in response to 10 min of Gas6 treatment resulted in tyrosine phosphorylation of endogenous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

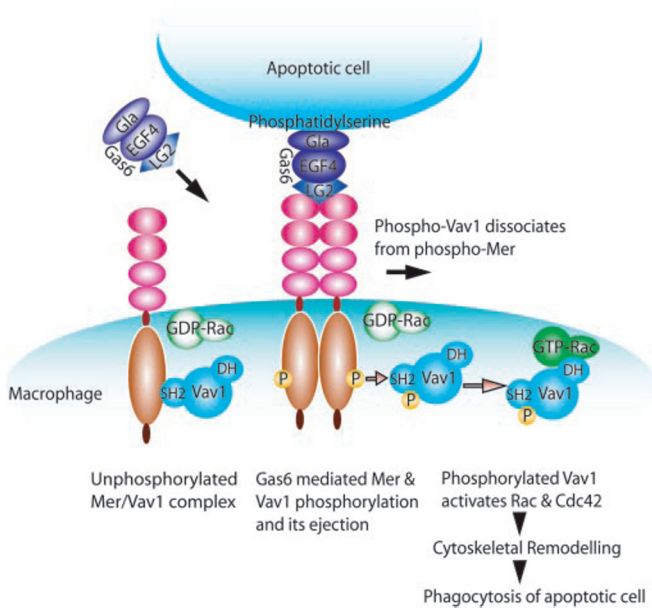

FIG. 8. **Constitutive *Mer*/Vav1 binding provides apoptotic cell recognition and control of GEF activity: a model.**

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). Because 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 type of phagocytic receptor engaged in particle internalization, distinct signaling cascade would be triggered, leading to activation of specific member/s of 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, whereas 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 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 phosphatidylserine (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, Rac1, and Cdc42 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 signaling 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 (Fig. 8) *Mer*, Vav1, and Rho family member process 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 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 β_3 integrin cytoplasmic tail through a phosphotyrosine-independent process involving the tandem SH2 domain region of Syk (37).

Here we find through deletion analysis that the SH2 domain of Vav1 interacts with an unphosphorylated *Mer* receptor tyrosine kinase. A more selective approach in which mutation of the phosphotyrosine coordinating Arg-696 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. 2*A*). 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. 4*B*). Therefore, the Vav1 carboxyl 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. 2*B*) is not sufficient to eject Vav1 from the complex (R696A, Fig. 4*B*). An intact SH2 phosphotyrosine site appears to be necessary. We do not yet know what role the carboxyl-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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