A role for Mer tyrosine kinase in αvβ5 integrin-mediated phagocytosis of apoptotic cells

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Summary

Efficient phagocytosis of apoptotic cells is crucial for many cellular processes. One of earliest signals to the phagocyte is the expression of phosphatidylserine (PS) on the outer surface of the apoptotic cell that provides a potent 'eat-me' signal. Recognition of PS occurs either directly, via PS receptor (PS-R), or indirectly via αvβ5(3) integrin or Mer-family tyrosine kinases through the opsonizing proteins milk fat globule-EGF factor 8 protein (MFG-E8), or growth arrest specific factor-6 (Gas6), respectively. Because Mer and αvβ5 integrin share PS-dependent recognition signals, we investigated their post-receptor signaling cascades following receptor activation. Using a constitutively active form for Mer (CDMer) or Gas6 as a ligand to stimulate Mer, we found that Mer activation induced a post-receptor signaling cascade involving Src-mediated tyrosine phosphorylation of FAK on Tyr^{861}, the recruitment of FAK^{Tyr861} to the αvβ5 integrin, and increased formation of p130^{CAS}/CrkII/Dock180 complex to activate Rac1. Coexpression of Mer with αvβ5 integrin had a synergistic effect on Rac1 activation, lamellipodial formation and the phagocytosis of apoptotic cells. Interestingly, Gas6 or CDMer failed to stimulate p130^{CAS} tyrosine phosphorylation or phagocytosis in β5-deficient CS-1 cells or in mutant β5ΔC-expressing cells, suggesting that Mer is directionally and functionally linked to the integrin pathway. The present data indicate that receptors that recognize apoptotic cells in the context of PS functionally crosstalk to amplify intracellular signals to internalize apoptotic cells. Moreover, our data link another PS-dependent signal to the CrkII/Dock180/Rac1 module.

Key words: αvβ5 integrin, Mer tyrosine kinase, Rac1, Phagocytosis, Apoptotic cells

Introduction

Cells undergoing apoptosis are efficiently eliminated from tissues by phagocytosis, and this process is fundamentally important for the homeostasis of the organism (Fadok and Chimini, 2001; Ravichandran, 2003). Both professional and non-professional phagocytes bind apoptotic cells by recognizing 'eat-me' signals on the surface of the apoptotic cells using a wide-variety of cell surface receptors, including membrane-exposed phosphatidylserine (PS) as well as oxidized and modified proteins and lipids (Ravichandran, 2003; Savill et al., 2002). Typically, PS is asymmetrically distributed in the plasma membrane bilayer in healthy cells, with PS restricted to the inner surface. During apoptosis, related to signals from elevated calcium and/or activation of Caspase 3 and 7, plasma membrane asymmetry is inactivated, resulting in PS exposure on the outer surface (Fadok et al., 1998; Korfali et al., 2004). External PS serves as an apoptotic cell recognition signal for multiple phagocytic receptors (Fadok et al., 2000; Ravichandran, 2003; Savill, 1997).

Recent evidence has shown that PS-dependent recognition occurs either by direct interaction with the phagocyte via a PS-Receptor (PS-R), or indirectly, via soluble bridging molecules including milk fat globule-EGF factor 8 protein (MFG-E8), Thrombospondin, growth arrest specific factor-6 (Gas6), Protein S and β2-microglobulin (Fadok et al., 2001; Ravichandran, 2003; Savill et al., 2002). Although the utilization of multiple PS-dependent pathways can increase the diversity of signal transduction, in general, a molecular explanation for the integration of multiple PS-dependent 'inputs' is not well understood. We have recently characterized a basic paradigm for αvβ5 integrin-dependent phagocytosis (Albert et al., 2000), in which MFG-E8, a multi-functional opsonizing protein secreted by phagocytes, activates a signal cascade involving CrkII/Dock180/Rac1 to promote early phagosome formation (Akakura et al., 2004; Gumienny et al., 2001; Wu et al., 2001). Genetic and biochemical studies also suggest that PS-R mediates phagocytosis through the aforementioned module in *Caenorhabditis elegans*, and PS-R may bind directly to ELMO (ced-12) and Dock180 (ced-5) (Wang et al., 2003). The convergence of PS-dependent signals on this evolutionarily conserved signaling module suggests that PS-dependent receptors crosstalk at the molecular level for efficient engulfment.

The Mer-family of receptor tyrosine kinases (RTK) include Mer (also known as Mertk), Axl and Tyro3, and are critical for many aspects of immune function by triggering monocyte-mediated engulfment of apoptotic cells (Anderson et al., 2003; Scott et al., 2001). Targeted disruption of Mer using homologous recombination or in a naturally occurring splice

variation in the Royal College of Surgeons (RCS) rat model result in progressive loss of vision and retinal dystrophy due to impaired clearance of rod outer segments (ROS) by associated retinal pigmented epithelial (RPE) cells (D'Cruz et al., 2000; Peng et al., 2003). Triple null mutants of Mer, Axl and Tyro3 develop a more penetrant phenotype, resulting in failure of apoptotic cell clearance in many tissues, and develop a severe autoimmune systemic lupus erythematosus (SLE)-like disease (Cohen et al., 2002; Lu et al., 1999). This latter phenotype is recapitulated in Mer-kinase-dead (Mer^{KD})-expressing transgenic mice, suggesting that intracellular signals from Mer are required to initiate engulfment signals (Scott et al., 2001). Structurally, Mer is a type I RTK, containing an extracellular domain with two iterative Ig-like motifs, two fibronectin type III repeats, a trans-membrane domain and an intracellular tyrosine kinase domain (Graham et al., 1994). The ligand for Mer is Gas6, a multidomain protein containing a vitamin K-activated laminin G-like domain that binds Mer, and a Gla-like domain that binds PS on the apoptotic cell (Ishimoto et al., 2000). Similarly, the ligand for Tyro 3 is protein S, which binds PS in an analogous fashion, suggesting that this theme is conserved in Mer family members (Anderson et al., 2003).

Binding of apoptotic cells or Gas6 to Mer elicits a number of post-receptor signals, which are proposed to culminate in engulfment signals. Challenge of RPE cells with ROS or with Gas6 results in tyrosine phosphorylation followed by ligand-inducible Rac1 activation (Guttridge et al., 2002; Mahajan and Earp, 2003). Recently, it has been shown that challenge of murine peritoneal macrophages or a J774A.1 macrophage cell line with apoptotic cells results in Mer tyrosine phosphorylation, followed by phospholipase C-γ2 (PLC-γ2) recruitment to Mer and activation resulting in the generation of second messengers DAG and IP3 (Todt et al., 2004). Mer has also been shown to constitutively interact with the Vav1 guanine-nucleotide exchange factor (GNEF) in 32D cells (Mahajan and Earp, 2003). Activation of Mer results in the release of Vav1 from Mer and the activation of Rho-GTPases. Studies by Finnemann also suggest that Mer can be activated by αvβ5 integrin in a FAK-dependent mechanism for ROS engulfment (Finnemann, 2003).

To better understand the role of Mer in the phagocytosis of apoptotic cells, we investigated Mer post-receptor signals, as well as the role of Mer in αvβ5 integrin-mediated phagocytosis. We present evidence that Mer activation induces FAK^{Tyr861} phosphorylation and activation, followed by $p130^{cas}$ tyrosine phosphorylation and the recruitment of a $p130^{cas}$/CrkII/Dock180 complex to activate Rac1. Mer-inducible $p130^{cas}$ tyrosine phosphorylation is abrogated in Src/Fyn/Yes null fibroblasts or by expression of kinase-dead FAK. Moreover, Mer-inducible tyrosine phosphorylation of FAK on Tyr^{861} resulted in recruitment of FAK to the αvβ5 integrin cytoplasmic tail, and αvβ5 integrin and Mer had a synergistic effect on Rac1 activation, lamellipodial formation, and the phagocytosis of apoptotic cells. Interestingly, Mer failed to stimulate phagocytosis when overexpressed in β5 and β3 integrin deficient CS-1 melanoma cells, or when coexpressed with a mutant β5 integrin containing a deleted cytoplasmic tail. These data indicate that αvβ5 and Mer exhibit molecular crosstalk for phagocytosis and signaling, but directionally, the signal initiates from Mer. Our data also suggest that PS-dependent signals, via MFG-E8 and Gas6 and their receptors, converge on a common downstream pathway and 'synapse' to amplify internalization signals for the phagocytosis of apoptotic cells.

Materials and Methods

Antibodies and plasmids

Monoclonal antibodies (mAb) against various proteins and epitopes were purchased from respective vendors that include anti-$p130^{CAS}$ and FAK from BD Bioscience; anti-αvβ5 integrin (P1F6) from Chemicon; mAb against Rac1 from Upstate; mAbs against anti-phosphotyrosine (py99) and anti-HA, as well as polyclonal antibodies (pAb) against CrkII, Dock180, integrin β5 (clone H-96) were all from Santa Cruz Biotechnology. pAb against Mer (anti-Mertk) were from FabGennix (MKT-101AP). Horse Radish Peroxidase (HRP)-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were obtained from Jackson Laboratories. The phospho-FAK antibodies directed to Tyr 861 or 397 were from Biosource.

The full-length mouse Mer (994 amino acids) was cloned from a 16-day-old mouse embryo cDNA library, and subcloned into $pMex^{neo}$ at the *Bam*HI and *Kpn*I sites. Alternatively, Mer was subcloned into pIRES2-EGFP at the sites of *Eco*RI and *Bam*HI. The kinase-negative mutant of Mer, Mer/KD, which was obtained by the mutation of the ATP binding site lysine (K) 614 to methionine (M), was generated by mutagenesis using PCR method with 10 pmol following forward 5′-AAGGTGGCAGTGATGACCATGAAGTTG-3′ and reverse 5′-CAACTTCATGGTCATCACTGCCACCTT-3′ primers. Plasmid $pMex^{neo}$-Mer was used as template, and PCR amplifications were performed for 30 cycles with the proof-reading pfu DNA polymerase (Stratagene). The nucleotide sequence of mutagenesis construct was determined to ensure that the expected mutation was present and that no additional mutations were introduced by PCR. The retroviral expression vectors pLXSN expressing CDMer, and CDMer/KD (kinase-negative mutant, K614M) were generated as previously described (Georgescu et al., 1999). The generation of pCx-β5-IRES-GFP, pCx-β5ΔC-IRES-GFP, pEBB-CrkII, pcDNA-FAK, pcDNA-FAK/KD (kinase negative mutant, K454M), pEBG-$p130^{CAS}$, and pGEX-2T-PAK CRIB were described previously (Albert et al., 2000; Zvara et al., 2001). PAX142-HA-Rac1 was kindly provided by I. Whitehead (UMDNJ).

Reagents

Recombinant Gas6 was provided by Brain Varnum (Amgen); Geneticin, Lipofectamine and Lipofectamine 2000 were purchased from Invitrogen; vitronectin was a product of Chemicon. All other reagents were obtained from Sigma (St Louis, MO) unless otherwise specified.

Cell culture, stable cell lines expressing αvβ5 complex and retrovirus infection

Human embryonic kidney (HEK)-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. CS-1 cells were cultured in RPMI 1640 containing 5% FBS, 2 mM L-Glutamine. Murine fibroblast SYF cells (deficient in Src, Yes and Fyn expression) were obtained from the American Type Culture Collection (ATCC). Stock cultures of these cells were maintained in DMEM, supplemented with 10% FBS. For experimental purposes, SYF and control NIH 3T3 cells were plated in 60-mm dishes at 3×10^5 cells/dish. The murine immature dendritic cell line, DC2.4, was kindly provided by K. Rock (University of Massachusetts) and maintained in RPMI 1640 supplemented with 10% FBS as above. To obtain stable

cell lines expressing αvβ5 integrin, CS-1 cells were co-transfected with pCx-IRES-EGFP-β5 and pMex^{neo} using lipofectamine 2000. Stable transformed cells were selected in 1 mg/ml geneticin-containing medium and maintained in 0.4 mg/ml of geneticin-containing medium. The expression of αvβ5 complex was determined with P1F6 staining by flow cytometry.

Murine NIH3T3 and SYF cells were also used for generating cell lines expressing integrin β5. Briefly, pCx-IRES-GFP or pCx-IRES-GFP-β5 plasmid DNA were co-transfected with pCL-Eco plasmid (Imgenex) at a ratio of 1:1 into a 50% confluent 10-cm tissue culture dish of BOSC23 cells by the lipofectamine (Invitrogen) method. After 5 hours, 10 ml RPMI containing 10% FBS was added, the cells were incubated for 48 hours. The virus containing supernatant was collected and added to NIH3T3 or SYF cells in the presence of polybrene (5 μg/ml, Sigma).

RT-PCR analysis

Detection of Mer mRNA from immature DC2.4 cells was performed by RT-PCR using sequence-specific primers. Briefly, total RNA was prepared using Trizol® reagent, followed by DNase I (1 μg/ml) treatment to remove potential contaminating genomic DNA. RNA (a total of 200 ng) was used in a one-step RT-PCR reaction using 10 pmol primers. The forward primer 5′-ATCATTGCCTCTTGCAC-ACCAGG-3′ and the reverse primer 5′-CGCGGATCCGCTTCAC-ATCAGAACTTCAGAGTCTTC-3′ for mouse Mer yielded a product of 296 bp. The band was sequenced to confirm its identity.

Transfection, immunoprecipitation and western blotting

Transient overexpression was achieved using the lipofectamine method. Briefly, HEK-293T cells were transfected with expression plasmids using lipofectamine as indicated by the manufacturer, and the transfection of CS-1 cells and fibroblasts were performed using lipofectamine 2000. After 48 hours, cells were lysed in 1% HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 1 mM sodium vanadate and 20 μg/ml aprotinin, and kept on ice for 30 minutes. All the subsequent steps were performed at 4°C. The lysates were centrifuged at 15,000 ***g*** for 10 minutes. Total protein was measured by the Bradford Lowry method. For immunoprecipitation, supernatants with equal amount of proteins were incubated with primary antibody for 2 hours at 4°C, followed by incubation with protein A-Sepharose for 1 hour at 4°C. The beads were washed three times in low detergent (0.1% Triton X-100) HNTG lysis buffer, before the addition of Laemmli sample buffer. The samples were separated by SDS-PAGE, and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% milk for 1 hour, after which the blots were incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C, and washed in Tris-buffered saline (TBS) containing 0.05% Tween-20. Antibody binding was detected by using horseradish peroxidase (HRP)-conjugated secondary antibodies diluted at 1:5000, and was visualized with enhanced chemiluminescence reaction reagent (Western Lightening; Perkin Elmer Life Sciences). For reprobing with other antibodies, the antibody bound to PVDF membranes was removed with stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 100 μM 2-mercaptoethanol) at 50°C for 20 minutes. After washing, the membranes were blocked with 5% milk and reprobed with the indicated antibodies.

Immunoprecipitation kinase assay

The cells were lysed using 1% HNTG as described above containing 0.1% SDS and 1% sodium deoxycholate. The immunoprecipitates were washed three times with 0.1% HNTG, followed by further processing for an in vitro kinase assay. The reaction was initiated by the addition of 35 μl kinase reaction buffer (0.1% HNTG, 10 mM $MnCl_2$, and 1 μM cold ATP) containing 5 μCi [γ-^{32}P]ATP. After incubation by vigorous vortexing at room temperature for 20 minutes, reactions were stopped by the addition of Laemmli buffer and then boiled for 5 minutes. After SDS-PAGE, the gel was treated with 1 N KOH at 55°C for 2 hours to discriminate serine/threonine phosphorylation from tyrosine phosphorylation (Cooper et al., 1984) and visualized by autoradiography (Escalante et al., 2000; Zvara et al., 2001).

Flow cytometry and phagocytosis assay

For flow cytometry, 2-5×10^5 cells were collected and washed with FACS Buffer [1× phosphate-buffered saline (PBS) containing 1% pooled human serum, 1% FBS and 0.02% sodium azide]. Cells were resuspended in 100 μl FACS buffer containing 1 μg primary antibody and incubated for 30 minutes at 4°C. After washing twice, the cells were further incubated with 100 μl FACS buffer containing 0.5 μg fluorescence-conjugated secondary antibody. After incubation for 30 minutes, cells were washed and resuspended in 400 μl FACS buffer and analyzed by FACScan using CellQuest software (BD Bioscience) (Akakura et al., 2004).

Phagocytosis assay was performed as previously described (Akakura et al., 2004; Albert et al., 2000). Briefly, human lymphocytes were labeled with a red fluorescent cell linker PKH26-GL (Sigma) and induced to undergo apoptosis by UV-B irradiation at 25 mJ/cm^2. PS-positive cells were verified by staining with AnnexinV. HEK-293T cells or CS-1 cells were transfected with bicistronic expression plasmids that contained EGFP expression genes. The transfected cells were allowed to recover for at least 48 hours before co-culturing with apoptotic cells, and CS-1 cells were cultured on poly-L-lysine-coated dishes. After 48 hours, HEK-293T cells or CS-1 cells were co-cultured with apoptotic cells for 2 hours at a ratio of 1:10, after which the cells were washed with PBS containing 5 mM EDTA to remove surface-bound apoptotic cells. Two-color FACscan analysis was used to determine the percentages of green fluorescent phagocytes that had phagocytosed red fluorescent target cells.

Assay for Rac1 activation

The cells were solubilized with magnesium-containing lysis buffer by the addition of 5 mM $MgCl_2$ and 0.25% sodium deoxycholate to 1% HNTG buffer (Lambert et al., 2002). GST-PAK CRIB fusion protein was used to ‘pull-down’ Rac-GTP and prepared as previously described (Akakura et al., 2004). In brief, the lysates were immediately incubated with glutathione-agarose beads coated with bacterially expressed GST-PAK (human PAK1 amino acid residues 56-272) for 45 minutes. The levels of Rac1 in the GST pull-down assay were analyzed by immunoblotting with anti-HA or anti-Rac1 monoclonal antibody and secondary goat anti-mouse antibody conjugated with HRP.

Cell spreading assay and Rhodamine-Phalloidin staining

96-well culture plates were coated with vitronectin (10 μg/ml in PBS) for 4 hours at 37°C. These wells were subsequently blocked with 5% BSA for 45 minutes prior to use. After starvation for 18 hours, dendritic cells (DCs) were harvested with a 0.25% trypsin/EDTA solution, and incubated in serum-free medium at 37°C for 30 minutes to minimize effects of exogenous matrix proteins that are found in high concentrations in serum (e.g. fibronectin, vitronectin). After pretreatment with 150 nM Gas6 at room temperature for 10 minutes, cells were added at 2×10^4 cells/well and allowed to adhere at 37°C for 30 minutes. To remove non-adherent cells, cells were washed twice with PBS and the remaining adherent cells were fixed with 3.7% paraformaldehyde. Cell spreading on vitronectin was photographed using a phase-contrast microscope (TMS-TE300 Nikon, Japan), and

the percentage of spreading cells was determined by counting cells with a typical polygonal or spindle morphology within four random fields (Filardo et al., 1996; Sipes et al., 1999). To view actin cytoskeletal remodeling, CS-1 cells were plated on a coverslip and transfected with the indicated vectors using lipofectamine 2000. After 48 hours, the transfected cells were fixed with 4% paraformaldehyde in PBS, and the cell membranes were permeabilized with 0.2% Triton X-100 in PBS. The actin cytoskeleton was visualized by staining with Rhodamine-Phalloidin diluted with PBS in a ratio of 1:8000. After several washes, the coverslips were dried and mounted with mounting medium (ProLong® Antifade Kit; Molecular Probes). Stained cells were photographed by fluorescence microscopy. Pictures were captured using Spot Advanced Software (Diagnostic Instruments) and the acquired pictures were further processed and assembled using Adobe Photoshop®5.5.

Presentation of data

Unless stated otherwise, the results shown are from a single experiment representative of at least three separate experiments. Results are shown as mean±s.e.m. of these experiments.

Results

Mer activation stimulates tyrosine phosphorylation of p130^{CAS} and recruits the CrkII-Dock180-Rac1 module

To investigate post-receptor signaling pathways of Mer relevant to phagocytosis of apoptotic cells, we evaluated Gas6-stimulated cells expressing Mer, or a constitutively activated chimeric Mer, fused with the extracellular and transmembrane domains of CD8α and intracellular domain of the mouse Mer receptor, CDMer (Georgescu et al., 1999). The extracellular domain of CD8α induces ligand-independent dimerization and hence constitutive activation of the dimeric receptor by forming intermolecular disulfide bonds (Zong et al., 1996). The kinase-negative receptor CDMer/KD or full-length Mer/KD was obtained through a $K_{614}\rightarrow M$ mutation in the kinase ATP-binding site (Georgescu et al., 1999). Overexpression of CDMer in HEK-293T cells induced robust tyrosine phosphorylation of approximate molecular weight 200, 130, 120, 110, 105, 93, 85, 60 and 55 kDa (Fig. 1A, lane 4), and these effects were abrogated by expression of CDMer/KD (Fig. 1A, lane 5). A similar pattern of phosphorylated proteins was observed by overexpression of saturating amounts of wild-type Mer, although at this level of receptor expression, Mer was constitutively active and not dependent on Gas6. To examine the earliest and most prominent Gas6-inducible events, cells were transfected with 20-40 ng of Mer DNA to achieve low receptor expression, and after further starvation with serum-depleted media, the cells were stimulated with 150 nM Gas6 for 10 minutes (Fig. 1B). Stimulation of parental HEK cells with 150 nM Gas6 triggered tyrosine phosphorylation of p125-p130 and 60 kDa proteins, which was enhanced by coexpression of low levels of Mer, but not Mer/KD. Co-immunoprecipitation of detergent lysates with anti-p130^{CAS} antisera confirmed that p130^{CAS} is the predominant Mer-induced phosphoprotein under conditions of limited receptor expression (Fig. 1B). Unless otherwise stated, all transfection strategies in this paper utilized low Mer expression.

Because Gas6 is a common ligand for additional Mer family members Tyro3 and Axl, and stimulates the kinase activities of all three receptors (Chen et al., 1997; Nagata et al., 1996), we utilized CDMer to specifically explore Mer post-receptor signals without competition from other family members (Georgescu et al., 1999). We have previously shown that tyrosine-phosphorylated p130^{CAS} binds to the CrkII adaptor protein (Birge et al., 1992), a molecular interaction that induces actin reorganization and participates in αvβ5 integrin-mediated engulfment of apoptotic cells (Albert et al., 2000). In the lysates of CDMer-overexpressing cells, several tyrosine phosphorylated proteins of approximate relative molecular weight of 130, 110, 85 and 60 kDa were identified in the anti-CrkII immunoprecipitates (Fig. 1C, lane 2). Among them, the associated protein of 130 kDa was confirmed as p130^{CAS} by immunoblotting, and the level of tyrosine phosphorylated p130^{CAS} bound to CrkII was significantly increased in the presence of CDMer (Fig. 1C, right panel). Reprobing the immunoblot with anti-Dock180 antibody demonstrated further that Dock180 was also associated with CrkII in CDMer-transfected cells (Fig. 1C, right panel), indicating that in an analogous fashion to αvβ5 integrin (Albert et al., 2000), activation of Mer is capable of assembling a complex of p130^{CAS}, CrkII and Dock180. In addition, the complex formation of p130^{CAS}, CrkII and Dock180 were also found to be a Gas6-Mer interaction-dependent event in the HEK-293T cells (data not shown).

To investigate whether CDMer or Gas6/Mer regulates Rac1-GTP loading, we co-transfected HEK-293T cells with plasmid DNA encoding Mer and HA-Rac1. As shown in Fig. 1D, panel i, in the lysates prepared from HEK-293T cells coexpressing HA-tagged Rac1 and CDMer, we observed a notable increase in steady-state Rac1-GTP level, relative to those with empty vector or CDMer/KD-expressing cells. Similarly, stimulation of wild-type Mer-expressing HEK-293T cells by Gas6 also resulted in ligand-inducible Rac1-GTP loading (Fig. 1D, panel ii), demonstrating that Rac1 activation is an early post receptor event. Gas6 also stimulated Mer-dependent phosphorylation of the p130^{CAS}-like molecule, Sin1, in professional phagocytic DC2.4 cells, a rat immature dendritic cell line competent for αvβ5 integrin mediated phagocytosis (data not shown). Prior studies in *C. elegans* have provided genetic evidence that the Ced-2, Ced-5 and Ced-10, which are homologues of mammalian CrkII, Dock180 and Rac1, respectively, are critical for controlling removal of cell corpses (Franc, 2002; Wang et al., 2003). Our studies extend these observations and provide evidence that Mer, as a PS-dependent phagocytic receptor, also converges on this conventional pathway.

Mer receptor-mediated downstream tyrosine phosphorylation is dependent on FAK

p130^{CAS} is a scaffolding protein that is recruited to the focal adhesion upon integrin ligation (O'Neill et al., 2000) and growth factor receptor tyrosine kinase stimulation (Munshi et al., 2000). The SH3 domain p130^{CAS} regulates localization to focal contacts through interaction with FAK, the latter results in tyrosine phosphorylation of p130^{CAS}, whereas non-phosphorylated p130^{CAS} remains diffusely distributed in the cytoplasm (Sakai et al., 1994). To investigate the role of FAK in Mer-induced p130^{CAS} tyrosine phosphorylation, detergent lysates from HEK-293T cells expressing CDMer or Mer were immunoprecipitated using anti-FAK mAb. As shown in Fig. 2A, overexpression of CDMer stimulated tyrosine

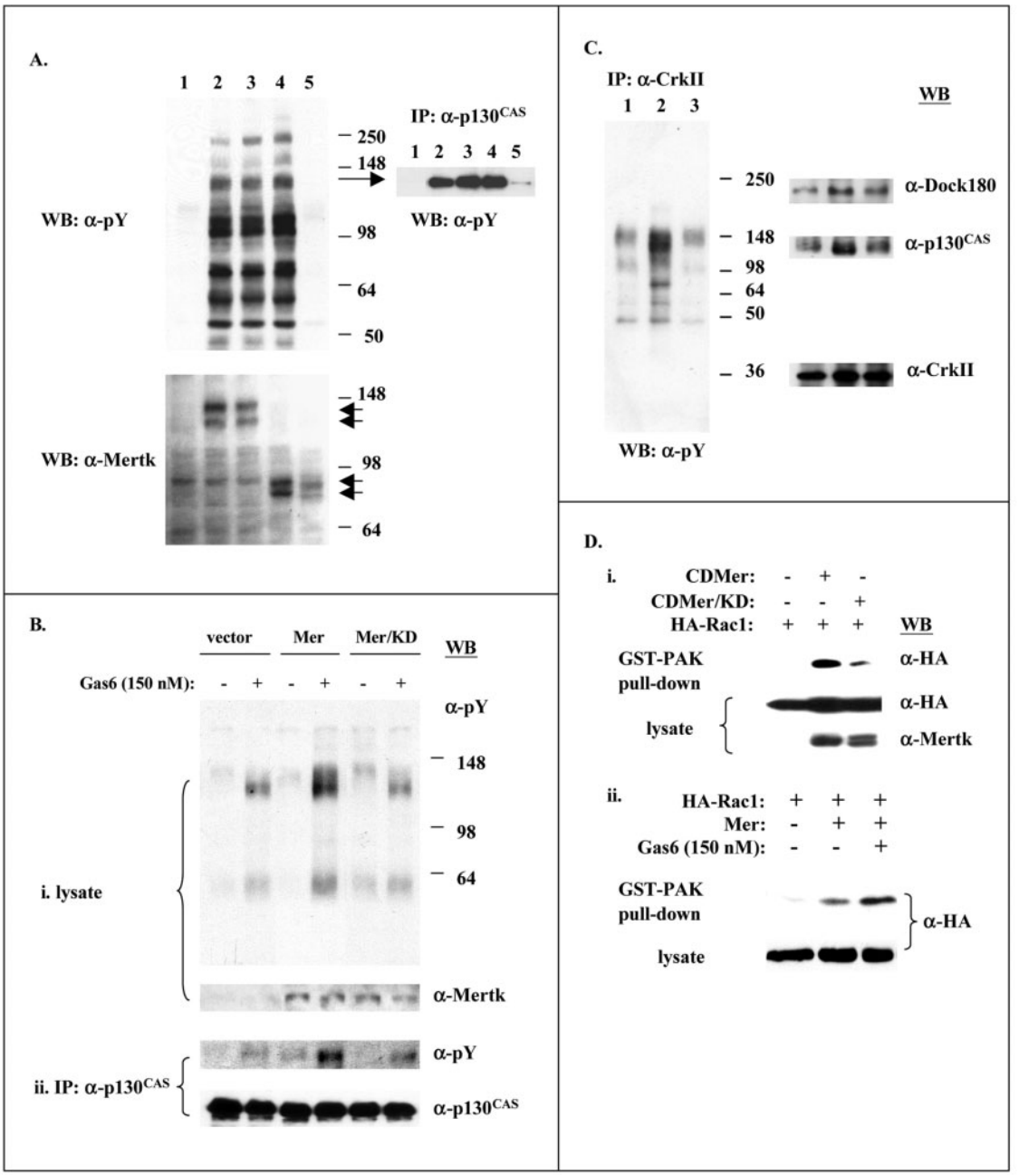

Fig. 1. Mer stimulates tyrosine phosphorylation of p130^{CAS}, complex formation of p130^{CAS}-CrkII-Dock180 and Rac1 activation. (A) (upper panel) HEK-293T cells were transfected with 1.0 μg plasmid encoding empty vector (lane 1), wild-type Mer (lanes 2 and 3), CDMer (lane 4) and CDMer/KD (lane 5). Mer-expressing cells were stimulated with Gas6 (150 nM) for 10 minutes (lane 3), although at this level of receptor expression, Mer activation was saturated. The cell lysates were separated by SDS-PAGE, and analyzed by immunoblotting with anti-phosphotyrosine mAb. (Lower panel) The blot was stripped and reprobed with anti-Mertk Ab, the arrows indicate Mer, CDMer or CDMer/KD. The lysates were immunoprecipitated with anti-p130^{CAS} mAb and immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine Ab (inset). (B) (upper panel) Gas6 stimulates p130^{CAS} phosphorylation in Mer-expressing HEK-293 T cells. HEK-293 T cells were transfected with 40 ng plasmid DNA encoding empty vector, wild-type Mer or Mer/KD as in A. The cells were starved for 18 hours, and stimulated with or without 150 nM Gas6 for 10 minutes as indicated. The cell lysates were immunoprecipitated with anti-p130^{CAS} mAb, and the immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb. The blot was reprobed with anti-p130^{CAS} mAb to show equal recovery of the proteins (lower panel). (C) Mer induces CrkII binding to p130^{CAS} and Dock180. HEK-293 T cells were transfected with empty vector (lane 1), CDMer (lane 2) and CDMer/KD (lane 3). After 48 hours, the cell lysates were immunoprecipitated with anti-CrkII Ab, and the immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine mAb. The blots were then cut and appropriate sections were reprobed with antibodies against p130^{CAS}, Dock180 and CrkII (shown on right). (D) Effects of CDMer and Mer-Gas6 on Rac1 activation. Empty vector, CDMer and CDMer/KD were coexpressed with HA-Rac1 in HEK-293T cells. The detergent lysates were precipitated with 5 μg/ml GST-PAK CRIB Sepharose beads. The levels of Rac1 GTP loading were determined by immunoblotting with anti-HA antisera (i). A similar experiment was performed in Mer-expressing cells stimulated with Gas6 (ii).

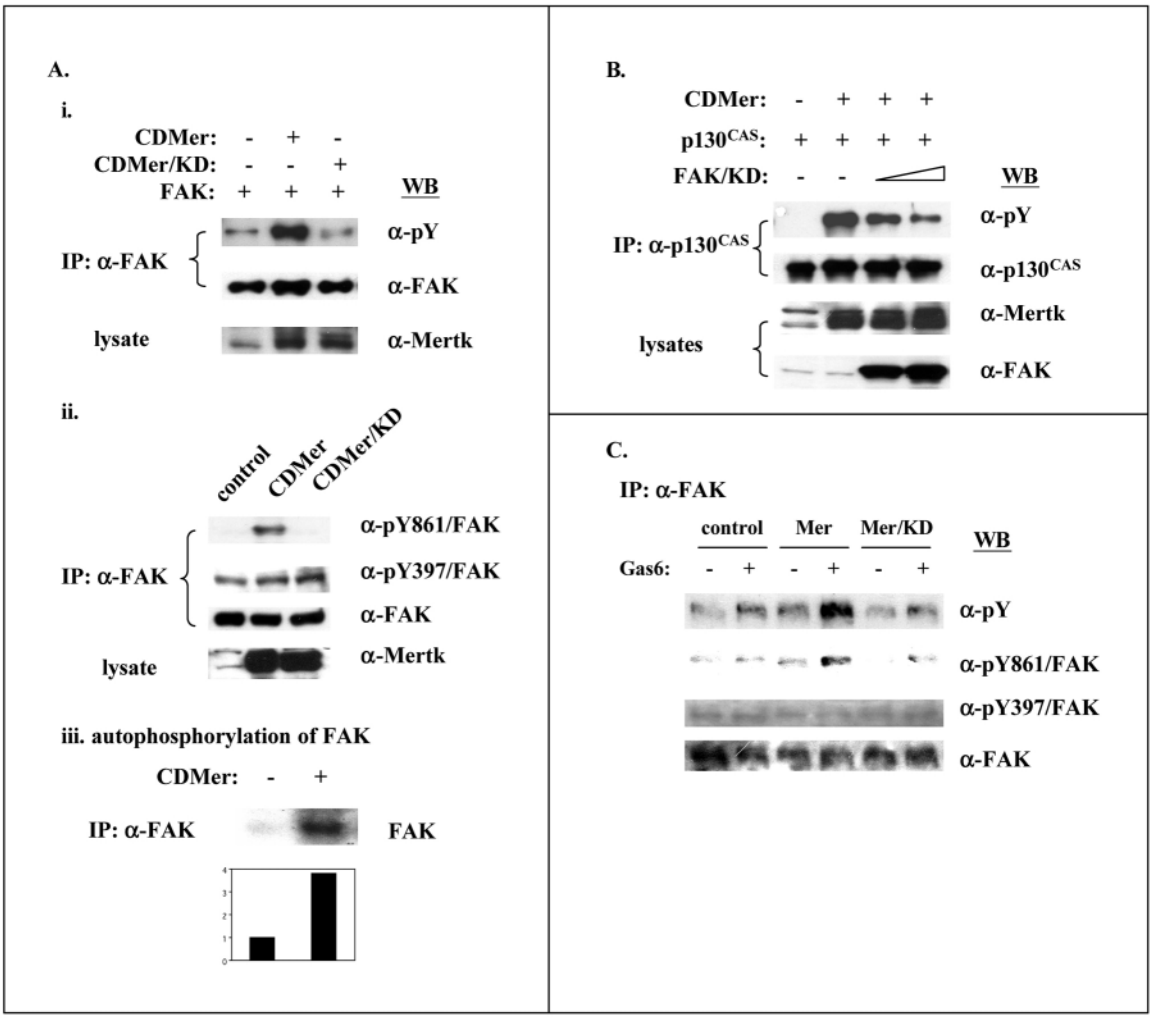

Fig. 2. Mer-mediated tyrosine phosphorylation of $p130^{CAS}$ is dependent on FAK activation. (A) HEK-293T cells were transfected with FAK-expressing plasmid plus empty vector, CDMer, or CDMer/KD as indicated. After 48 hours, cell lysates were immunoprecipitated with anti-FAK mAb, and the immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine Ab (i) or antibodies specific for Tyr 397/FAK or Tyr861/FAK (ii). FAK immunoprecipitates were subjected to an in vitro kinase assay to measure FAK activity and the autophosphorylation level of FAK was analyzed by densitometry (iii). (B) Effect of mutant FAK on $p130^{CAS}$ phosphorylation. HEK-293T cells were co-transfected with vector encoding $p130^{CAS}$ (lanes 1-4), CDMer (lanes 2-4), in combination with 0 (lane 1), 0 (lane 2), 1 (lane 3) and 3 (lane 4) μg of plasmid encoding FAK/KD (kinase negative mutant). $p130^{CAS}$ was immunoprecipitated from extracts containing equal amounts of total proteins and subjected to immunoblotting with mAb against phosphotyrosine. (C) Tyrosine phosphorylation of FAK^{Tyr861} is Gas6-inducible. Mer- or Mer/KD-expressing HEK-293T cells were prepared as in Fig. 1B, and stimulated with or without Gas6, and the cell lysates were immunoprecipitated with anti-FAK mAb, and the immunoprecipitates were analyzed as in A.

phosphorylation of FAK that was abrogated by the kinase-dead mutant. Using phospho-specific FAK antibodies, CDMer-inducible FAK phosphorylation occurred predominantly on Tyr^{861}, and little phosphorylation occurred on Tyr^{397} (Fig. 2A, panel ii). CDMer-modified FAK^{Tyr861} had a three- to fourfold increase in intrinsic activity, as evident from the results of an in vitro kinase assay following the immunoprecipitation of FAK (Fig. 2A, panel iii). Consistent with a pivotal role for FAK in Mer-mediated $p130^{CAS}$ phosphorylation, overexpression of FAK/KD suppressed CDMer-induced tyrosine phosphorylation of $p130^{CAS}$ in a dose-dependent manner (Fig. 2B). Finally, to show that phosphorylation of FAK at Tyr^{861} represents an early post Mer receptor signal, FAK activation was monitored in Mer-expressing cells stimulated by Gas6 (Fig. 2C). Analogous to the case for CDMer, Gas6-inducible phosphorylation of FAK occurred predominantly at Tyr^{861}, and was abrogated by Mer/KD (compare lanes 4 and 6).

Mer recruits FAK^{Tyr861} to the β5 tail to initiate αvβ5 integrin signaling and phagocytosis

Prior studies have provided several examples in which αvβ5 integrin requires collaboration with receptor tyrosine kinases. For example, αvβ5 integrin cooperates with insulin-like growth factor (IGF1) receptor to promote cell motility (Brooks et al., 1997). αvβ5 integrin-mediated cell migration also depends on activation of epidermal growth factor receptor (EGFR) (Klemke et al., 1994), and vascular permeability

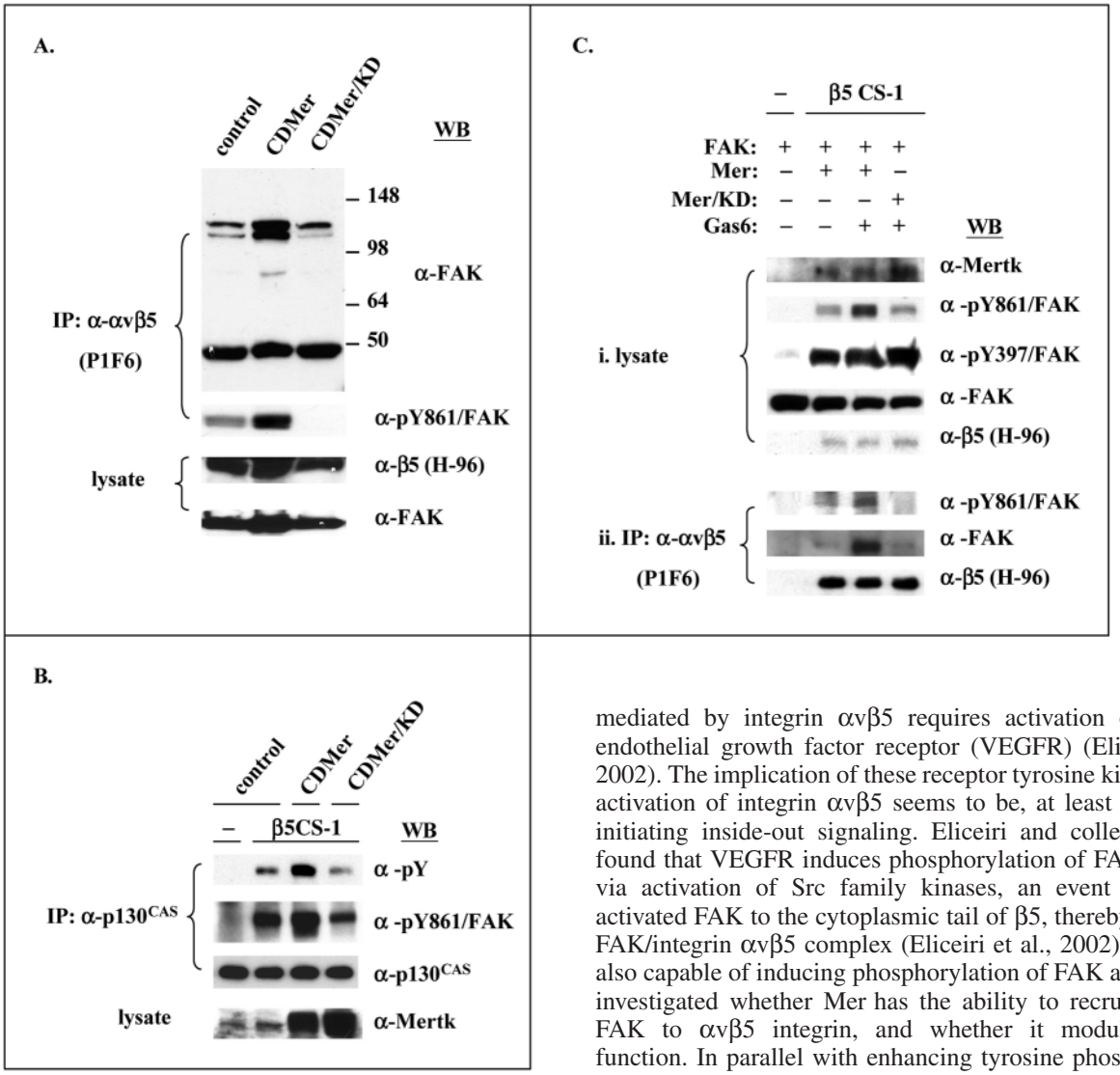

Fig. 3. FAK^{Tyr861} associates with αvβ5 integrin upon Mer activation. (A) Cells were transfected with plasmid DNA encoding FAK and β5 integrin as well as empty vector, CDMer or CDMer/KD as indicated. Subsequently, cell lysates were subjected to immunoprecipitation with anti-αvβ5 mAb (P1F6), and immunoblotting with anti-FAK Ab to detect FAK/αvβ5 integrin complex. The blot was stripped and reprobed with anti-phospho-FAK at Tyr^{861}. (B) FAK^{Tyr861} binding to αvβ5 integrin is linked to the p130^{CAS}/CrkII/Dock180 complex. β5CS-1 cells were transfected with empty vector, CDMer or CDMer/KD. CS-1 cells without transfection serve as control (–). The cell lysates were immunoprecipitated with anti-p130^{CAS} and analyzed by immunoblotting with anti-phosphotyrosine mAb. The blot was stripped and reprobed with anti-phospho-FAK at Tyr^{861} to detect active FAK-p130^{CAS} complex. (C) Gas6 induces a complex between FAK^{Tyr861} and αvβ5 integrin. Empty vector (lane 1), wild-type Mer (lanes 2 and 3), or Mer/KD (lane 4) was expressed with FAK in control CS-1 (lane 1) or β5CS-1 cells (lanes 2-4) for 48 hours. After starvation for 18 hours, the cells were stimulated with 150 nM Gas6 for 10 minutes (lanes 3 and 4). The cell lysates were analyzed by immunoblotting with antibodies specific for Tyr397/FAK and Tyr861/FAK (i). The lysates were also immunoprecipitated with anti-αvβ5 (P1F6) mAb, and immunoblotted with anti-FAK mAb or anti-phospho-FAK at Tyr^{861} to detect the phosphorylated and total FAK recovered with αvβ5 integrin immunoprecipitates (ii).

mediated by integrin αvβ5 requires activation of vascular endothelial growth factor receptor (VEGFR) (Eliceiri et al., 2002). The implication of these receptor tyrosine kinases in the activation of integrin αvβ5 seems to be, at least in part, via initiating inside-out signaling. Eliceiri and colleagues have found that VEGFR induces phosphorylation of FAK at Tyr^{861} via activation of Src family kinases, an event that drives activated FAK to the cytoplasmic tail of β5, thereby forming a FAK/integrin αvβ5 complex (Eliceiri et al., 2002). As Mer is also capable of inducing phosphorylation of FAK at Tyr^{861}, we investigated whether Mer has the ability to recruit activated FAK to αvβ5 integrin, and whether it modulates αvβ5 function. In parallel with enhancing tyrosine phosphorylation of FAK (Fig. 2), CDMer expression in HEK-293T cells mediated association of FAK with αvβ5 integrin, as evident by co-immunoprecipitation (Fig. 3A). Moreover, αvβ5 integrin-associated FAK was predominantly the phosphorylated form, as it was recognized by the antibody against phosphorylated Tyr^{861} within FAK (Fig. 3A, second panel). By contrast, FAK^{Tyr861}-αvβ5 integrin association was completely blocked in the presence of CDMer/KD (Fig. 3A, lane 3), implying that CDMer/KD expression suppresses this complex formation stimulated by activation of endogenous Mer. Interestingly, CDMer expression also increased the association of activated FAK^{Tyr861} with p130^{CAS} (Fig. 3B), suggesting that the αvβ5 integrin-associated FAK^{Tyr861} may directly transduce signals through the p130^{CAS}-CrkII-Dock180 complex.

To address the molecular crosstalk between Mer and αvβ5 integrin in more detail, we examined Mer induced activation signals in non-adherent CS-1 melanoma cells that lack αvβ5 and αvβ3 heterodimers because of their failure to synthesize endogenous β5 or β3 subunit protein, they do not adhere to vitronectin (Filardo et al., 1996; Lewis et al., 1996; Thomas et al., 1993). To verify that FAK^{Tyr861} tyrosine phosphorylation and its subsequent recruitment to the cytoplasmic tail of the αvβ5 integrin was indeed ligand inducible, we first generated

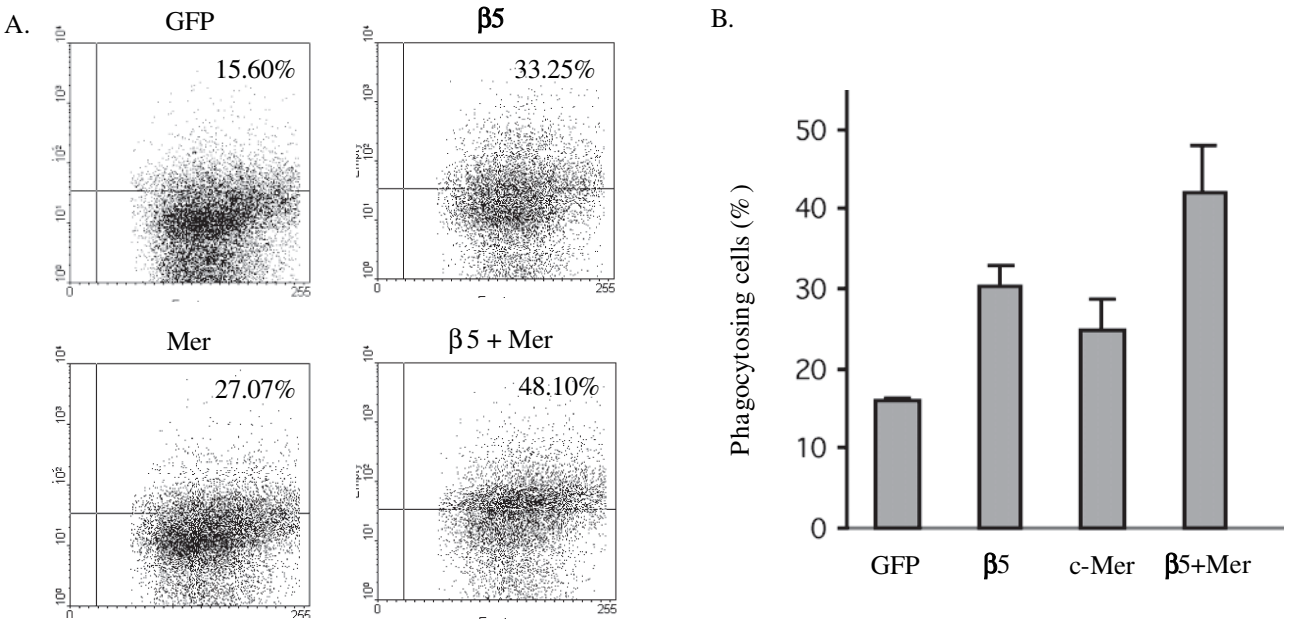

Fig. 4. Collaboration of Mer and αvβ5 integrin in phagocytosis of apoptotic cells. (A) HEK-293T cells were transfected with bicistronic pIRES-EGFP (GFP), pIRES-EGFP-β5 (β5), pIRES2-EGFP-Mer (Mer) or pIRES-EGFP-β5 plus pIRES2-EGFP-Mer (β5+Mer) plasmids. After 48 hours, transfected HEK-293T cells were co-cultured with red-labeled apoptotic T cells. (B) Phagocytosis was assayed 2 hours post co-culture, whereby double positive cells were scored from GFP-expressing cells. Data represent the mean±s.e.m. of triplicate measurements.

stable β5-expressing CS-1 cell lines for stimulation by Gas6. Following selection in G418, αvβ5 integrin-expressing CS-1 cells were detected by flow cytometry with P1F6 mAb (Fig. 6A). In the absence of Gas6, transfection of Mer in β5CS-1 cells resulted in weak FAK^{Tyr861} phosphorylation, and minimal recruitment of FAK^{Tyr861} to the β5 integrin (Fig. 3C). However, when β5CS-1 cells were stimulated with 150 nM Gas6, there was an increase in FAK861 tyrosine phosphorylation, and FAKTyr861 was robustly recruited into a complex with αvβ5 integrin (Fig. 3C, lane 3). Gas6 failed to induce FAK/integrin association in Mer/KD-expressing cells (Fig. 3C, lane 4). Consistent with the results in Fig. 2, Gas6 did not result in inducible FAK^{Tyr397} tyrosine phosphorylation, although this residue was constitutively phosphorylated upon αvβ5 expression. Taken together, these data are consistent with the idea that αvβ5 integrin and Mer impinge on FAK distinctly, and full FAK activation requires the combination of integrin and receptor tyrosine kinase activation.

The fact that Mer mediated the tyrosine phosphorylation of $p130^{cas}$ and FAK suggests its involvement in αvβ5 integrin-mediated uptake of apoptotic cells. To test this hypothesis, we analyzed Mer-mediated phagocytosis. HEK-293T cells were transfected with bicistronic vectors containing wild-type Mer or integrin β5 and the gene for green fluorescent protein (GFP), initiated from an internal ribosomal entry site (IRES). Phagocytosis assay was performed by co-incubating red dye-labeled apoptotic cells with Mer- or integrin β5-expressing HEK-293T cells, and assessing double positive cells by FACScan. As shown in Fig. 4A and B, HEK-293T cells expressing Mer stimulated internalization of apoptotic cells (15.88±0.31% compared to 25.57±3.04%), that was dependent on the intact kinase domain (not shown). Consistent with our previous results, overexpression of β5 (that increases surface αvβ5 integrin) also increased phagocytosis relative to empty vector (15.88±0.31% compared to 31.22±4.57%). However, coexpression of Mer with β5 integrin had a synergistic effect on apoptotic cell engulfment (42.87±6.52%) (Fig. 4B), suggesting molecular cooperation of αvβ5 integrin and Mer in phagocytosis of apoptotic cells.

Mer-mediated FAK^{Tyr861} phosphorylation and engulfment requires a Src-family kinase

Although the aforementioned studies implicate a role for FAK^{Tyr861} in terms of its recruitment to αvβ5 integrin, these studies did not address how Mer induces phosphorylation of FAK. Previous studies have shown that that Src family kinases (SFKs) act upstream of FAK and $p130^{CAS}$ in signaling pathway mediated by integrins and growth factor receptors, and Tyr^{861} residue of FAK is a specific phosphorylation site by SFKs (Avraham et al., 2003; Hanks et al., 2003; Sundberg et al., 2003). As Mer, but not Axl, has been shown capable of activating Src kinases, we tested whether FAK and $p130^{CAS}$ phosphorylation observed in this study might rely on Src activity. Indeed, we observed a 60-kDa phosphoprotein associated with Mer from in vitro kinase assays (Fig. 5A), although we have not been able to detect Src stably associated with Mer. Because this may reflect a transient interaction with rapid dissociation, or might reflect the association of a specific Src member, we used triple-deficient mouse fibroblasts lacking Src, Yes and Fyn, the only Src family kinases expressed in fibroblasts (Klinghoffer, 1999). CDMer-expression increased protein tyrosine phosphorylation in the lysates of control NIH3T3 cells (Fig. 5B, panel i). By contrast, the SYF cells showed weak but notable CDMer-induced

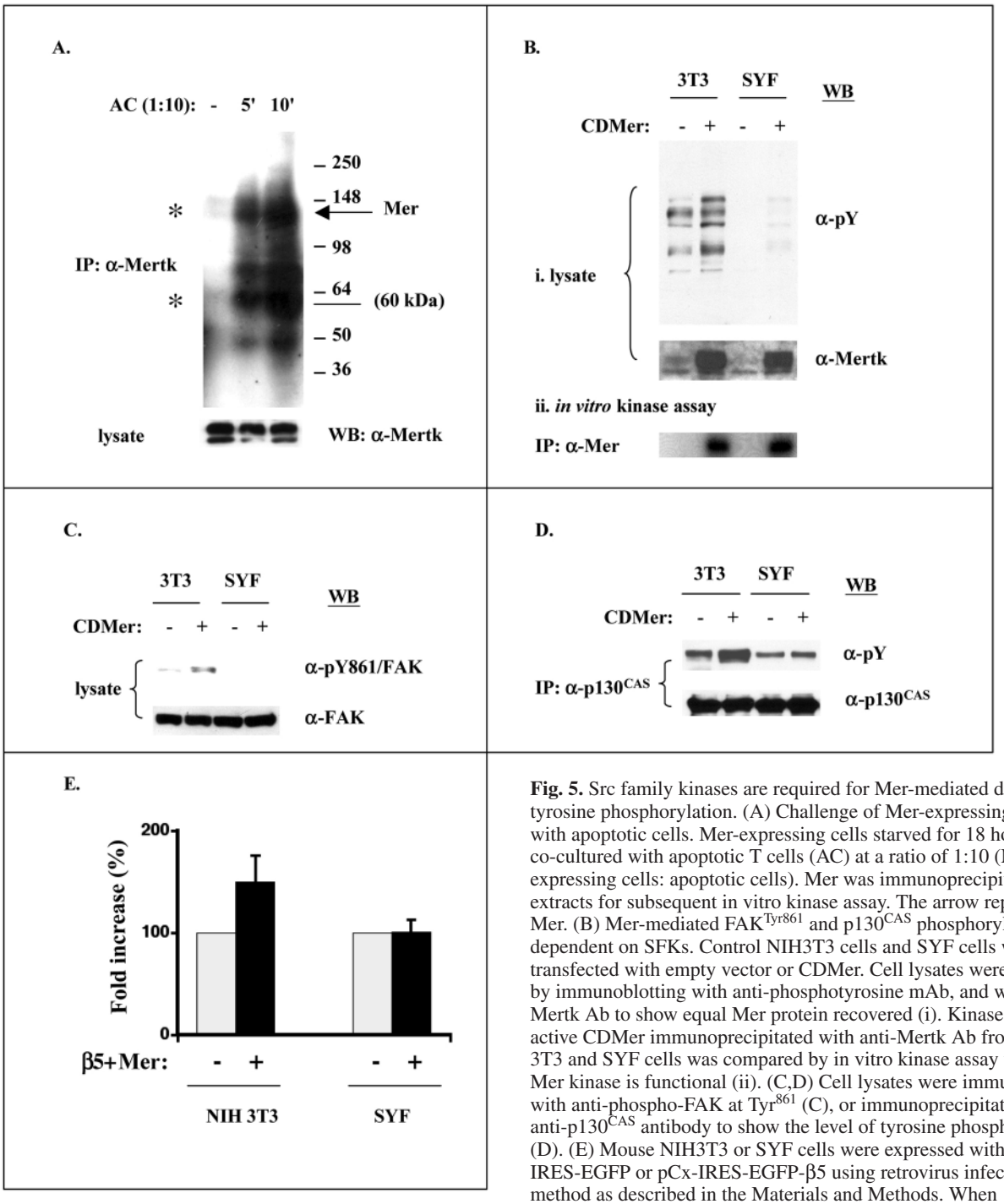

Fig. 5. Src family kinases are required for Mer-mediated downstream tyrosine phosphorylation. (A) Challenge of Mer-expressing cells with apoptotic cells. Mer-expressing cells starved for 18 hours, were co-cultured with apoptotic T cells (AC) at a ratio of 1:10 (Mer-expressing cells: apoptotic cells). Mer was immunoprecipitated from extracts for subsequent in vitro kinase assay. The arrow represents Mer. (B) Mer-mediated FAK^{Tyr861} and $p130^{CAS}$ phosphorylation is dependent on SFKs. Control NIH3T3 cells and SYF cells were transfected with empty vector or CDMer. Cell lysates were analyzed by immunoblotting with anti-phosphotyrosine mAb, and with anti-Mertk Ab to show equal Mer protein recovered (i). Kinase activity of active CDMer immunoprecipitated with anti-Mertk Ab from NIH 3T3 and SYF cells was compared by in vitro kinase assay to show Mer kinase is functional (ii). (C,D) Cell lysates were immunoblotted with anti-phospho-FAK at Tyr^{861} (C), or immunoprecipitated with anti-$p130^{CAS}$ antibody to show the level of tyrosine phosphorylation (D). (E) Mouse NIH3T3 or SYF cells were expressed with pCx-IRES-EGFP or pCx-IRES-EGFP-β5 using retrovirus infection method as described in the Materials and Methods. When coexpressed with or without Mer, the NIH3T3 and SYF cells were analyzed for phagocytosis assay as described in the legend for Fig. 4. As indicated, phagocytotic ability of NIH3T3 or SYF cells expressing control vector pCx-IRES-EGFP was arbitrarily set as 100% (grey) with cells coexpressing β5 and Mer shown in black.

tyrosine phosphorylation, whereas protein expression and kinase activity of CDMer in SYF cells were at comparable level with those in NIH3T3 cells (Fig. 5B, panels i and ii). Interestingly, CDMer-mediated tyrosine phosphorylation of FAK on Tyr^{861} (Fig. 5C) and $p130^{CAS}$ (Fig. 5D) was suppressed in SYF cells. The basal phosphorylation of $p130^{CAS}$ observed in Fig. 5D probably resulted from the substitution by other tyrosine kinases, such as Pyk2 (Astier et al., 1997). Moreover, unlike normal mouse embryonic fibroblasts in which αvβ5 integrin and Mer stimulated engulfment of apoptotic cells, expression of αvβ5 integrin and Mer in SYF cells failed to stimulate engulfment over background (Fig. 5E). Taken together, these data suggest that SFKs localize proximal to Mer receptor and play an important role in protein tyrosine phosphorylation downstream of Mer activation.

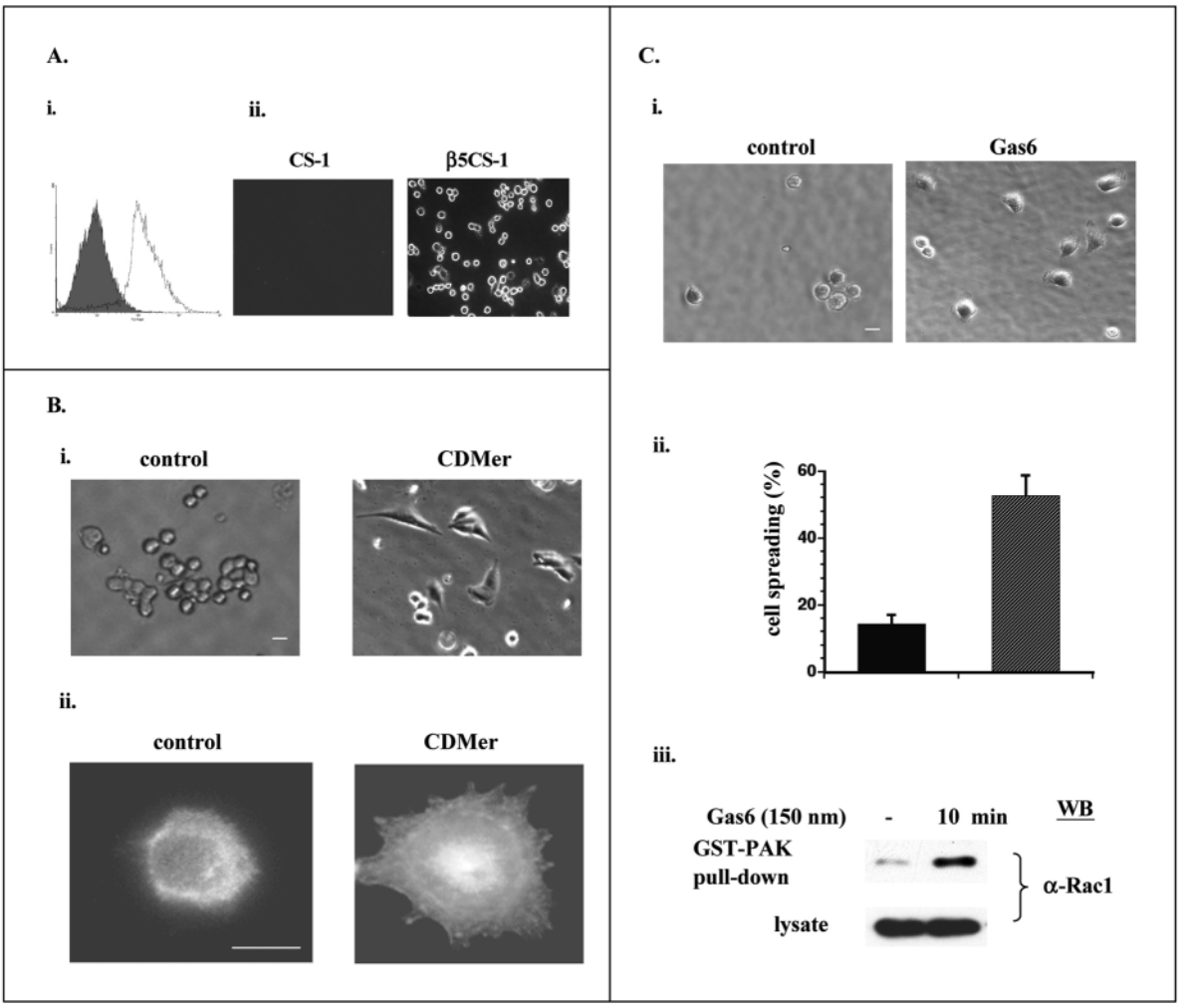

Fig. 6. Activation of Mer enhances αvβ5 integrin-directed actin cytoskeletal remodeling. (A) The surface expression of integrin αvβ5 complex on control CS-1 cells (shaded) and β5CS-1 (unshaded) was analyzed using anti-αvβ5 integrin mAb (P1F6) by flow cytometry (i). Control CS-1 or β5CS-1 cells were allowed to attach to vitronectin-coated culture dishes at 37°C for 60 minutes and were washed with PBS twice to remove non-adherent cells. The adherent cells were visualized by microscopy (ii). (B) β5CS-1 cells were transfected with empty vector or CDMer for 48 hours, cells were then fixed (i). Subsequently, cells were permeabilized by 0.2% Triton X-100 in PBS and stained with Rhodamine-Phalloidin (ii). (C) Gas6 induces Rac1 activation and morphological changes in cells plated on vitronectin. After starvation for 18 hours, DC2.4 cells were pretreated with or without 150 nM Gas6 at 37°C for 10 minutes, and 20,000 cells were seeded onto vitronectin-coated 96-well culture plate. After incubation at 37°C for 30 minutes, the cells were washed twice with PBS, fixed and visualized (i). Cell spreading was assessed by enumerating cells that displayed membrane filopodia and/or lamellipodia. Cells in four fields were counted on each plate (ii). After starvation for 18 hours, the DC 2.4 cells were stimulated with 150 nM Gas6 at 37°C for 10 minutes, and the lysates were precipitated with GST-PAK CRIB Sepharose beads. The levels of Rac1 GTP loading were determined by immunoblotting with anti-Rac1 mAb (iii). Bar, 20 μm.

Mer does not stimulate phagocytosis of apoptotic cells in the absence of the αvβ5 integrin

Stable expression of β5 integrin in CS-1 cells resulted in stable surface expression of αvβ5 heterodimers (Fig. 6A). Consistent with prior reports of Cheresh and colleagues (Filardo et al., 1996), αvβ5-expressing CS-1 cells become adherent to vitronectin, however, these cells remain round and do not exhibit notable lamellipodia or filopodia in the absence of additional stimulation (Fig. 6A). Interestingly, when β5CS-1 cells were transfected with CDMer and cultured on vitronectin, these cells demonstrated increased spreading and notable filopodial structures, as evident by Rhodamine-Phalloidin staining (Fig. 6B), suggesting that αvβ5 integrin and Mer functionally cooperate to reorganize the actin cytoskeleton. Finally, to demonstrate that Gas6 also cooperates with αvβ5 integrin in professional phagocytes, rat DC2.4 cells were cultured on vitronectin-coated surfaces and stimulated with Gas6. By using RT-PCR and immunoblotting methods, we confirmed expression of mRNA and protein of Mer in DC2.4 cells (data not shown). As indicated in Fig. 6C, Gas6 significantly increased spreading and lamellipodial formation, which is in agreement with the finding of Gas6-stimulated membrane ruffling and lamellipodial formation in neuronal cells (Allen et al., 2002). These results are also consistent with previous reports that integrin αvβ5-dependent cell mobility, but not attachment to vitronectin, required prior activation with either cytokine growth factors (Klemke et al., 1994; Yebra et al., 1995) or phorbol myristate acetate (Lewis et al., 1996), and

suggest that Mer possibly initiates inside-out regulation of αvβ5 integrin. Moreover, Gas6-inducible Rac1 activation was also observed in DC2.4 cells (Fig. 6C,iii). Taken together, these results indicate that Mer activation signaling cooperates in αvβ5 integrin-directed actin cytoskeletal reorganization and signaling.

The aforementioned results propose that Mer and αvβ5 integrin cooperate to stimulate phagocytosis, and argue that αvβ5 integrin may be downstream of Mer for proper engulfment signals. Studies by Finnemann also suggest the αvβ5 crosslinking triggers intracellular responses including tyrosine phosphorylation of FAK to activate Mer (Finnemann, 2003), and that αvβ5 and Mer manifest independent signals for engulfment, the former tethering, the latter internalization (Finnemann et al., 1997). Thus, it is important to determine whether Mer-mediated intracellular signaling and phagocytosis require the function of integrin αvβ5. To address this, we co-transfected empty vector, β5 or β5ΔC with or without CDMer. In the absence of αvβ5 integrin, CDMer expression failed to induce $p130^{CAS}$ tyrosine phosphorylation in CS-1 cells (Fig. 7A). However, in β5-coexpressing CS-1 cells, CDMer-induced tyrosine phosphorylation of $p130^{CAS}$ was rescued. By contrast, the rescued phosphorylation of $p130^{CAS}$ was significantly diminished in the cells expressing mutant β5ΔC (Fig. 7A, panel i). Consistent with $p130^{CAS}$ phosphorylation as an upstream regulator of Rac1, a similar pattern of dependence of Rac1 activation on the presence of β5 was also observed (Fig. 7A, panel ii). The defective $p130^{CAS}$ phosphorylation as well as Rac1 activation in β5-null cells or β5ΔC-expressing cells suggests that the αvβ5 integrin, through its cytoplasmic tail, is essential for transduction of Mer-mediated downstream signaling. Although CDMer expression enhances the recovery of β5 integrin distribution in HNTG-extractable buffer, total αvβ5 integrin expression, measured in RIPA-extractable lysates, or by surface staining with anti-P1F6 mAb, revealed the total levels of integrin were unchanged (Fig. 7A,B).

Finally, to assert whether αvβ5 integrin is required for Mer-inducible phagocytosis, we compared Mer and integrin αvβ5 cooperation in wild-type integrin versus β5ΔC-expressing CS-1 cells (Fig. 7C). Whereas Mer and wild-type αvβ5 integrin had a synergistic effect on phagocytosis, importantly, Mer failed to stimulate engulfment in mutant αvβ5ΔC-expressing cells (Fig. 7C). We also found that overexpression of Mer kinase negative mutant (Mer/KD) partially (27%) inhibited αvβ5-dependent phagocytic ability (Fig. 7D). Taken together, these data demonstrate shared PS-initiated extracellular signals between αvβ5 integrin and Mer functionally cooperate in their intracellular signaling to amplify internalization signals.

Discussion

An important conclusion in this study is the demonstration that multiple PS-dependent phagocytic receptors converge on an evolutionarily conserved signaling module for Rac1 activation and the phagocytosis of apoptotic cells. Evidence to support this come from our observations that (1) Mer induces Src kinase-dependent FAK^{Tyr861} tyrosine phosphorylation and FAK recruitment to the cytoplasmic tail of the β5 integrin; (2) Mer recapitulates αvβ5 integrin in promoting $p130^{cas}$ phosphorylation and the recruitment of $p130^{CAS}$/CrkII/Dock180 for Rac1 activation; (3) Mer fails to stimulate Rac1 activation and phagocytosis in β5 and β3-deficient CS-1 cells; and (4) reconstitution of CS-1 cells with wild-type β5 integrin, but not mutant β5ΔC integrin, restores Mer/αvβ5 synergism for the phagocytosis of apoptotic cells. Taken together, our data suggest phagocytic receptors that share PS-dependent extracellular signals, i.e. MFG-E8/αvβ5 integrin and Gas6/Mer, also share common signals to bi-directionally amplify internalization signals. These data offer a possible explanation for the existence of multiple PS-dependent mechanisms for engulfment (Fig. 8).

In addition to the soluble molecules MFG-E8 and Gas6 that engage αvβ5 integrin and Mer and subsequently recruit the CrkII, Dock180, and Rac1 module, recent studies also suggest that PS-R acts in the aforementioned pathway in *C. elegans* (Wang et al., 2003). In PS-R (psr-1-tm469) mutant worms, overexpression of *ced-2* (CrkII), *ced-5* (Dock180), or *ced-10* (Rac1) rescued engulfment defects. Curiously, biochemical analysis and yeast two-hybrid experiments suggest that the intracellular domain of PS-R physically interacts with both ELMO and Dock180, but not CrkII (Wang et al., 2003). Although the nature of the interaction was not revealed, these studies point to yet another PS-dependent convergence of signaling. However, in addition to a receptor-mediated recruitment of signaling proteins mechanism of action, PS-R biology may be more complex than anticipated, as recent studies indicated that PS-R has multiple nuclear localization signals and localizes predominantly in the nucleus (Cui et al., 2004). As PS-R has sequence homology to jumonji family of transcription factors (Clissold and Ponting, 2001), this raises the exciting possibility that PS-R may impinge on CrkII/Dock180 and Rac1 by regulating their expression, or by regulating the expression of receptors that act on this pathway. For example, we have recently shown that Dock180 and MFG-E8 are transcriptionally regulated in immature DCs (Akakura et al., 2004) and are currently testing whether these molecules are down-modulated in $PS^{-/-}$ cells.

The present observations that Mer cooperates with αvβ5 integrin is consistent with a more general biology in which the role of αvβ5 integrin in angiogenesis, cell migration and invasion also requires the participation of RTKs. For example, αvβ5-mediated cell migration is dependent on coactivation of the EGF-R, and αvβ5 integrin can also cooperate with insulin-like growth factor (IGF-1) receptor to promote cell motility (Brooks et al., 1997). Likewise, αvβ5 integrin-mediated angiogenesis and vascular permeability requires VEGF-R and fibroblast growth factor 2 (FGF2) receptor activation (Eliceiri et al., 2002; Klemke et al., 1994). Although the nature of the crosstalk between RTK and αvβ5 integrin is likely complex, one mechanism clearly depends on inside to outside integrin signaling. For example, protein kinase C (PKC) activation via PMA or by various post-RTK signals can increase the number of high affinity αvβ5 receptors on RPEs (Finnemann and Rodriguez-Boulan, 1999). PKC has been shown to be important for αvβ5-integrin mediated endocytosis of vitronectin (Panetti et al., 1995), as well as for FAK tyrosine phosphorylation when αvβ5-expressing CS-1 cells are plated on vitronectin (Lewis et al., 1996). Moreover, unlike the αvβ3 integrin that constitutively binds FAK, αvβ5-association with FAK is dependent on PKC (Lewis et al., 1996), suggesting that PKC may alter the binding surface of the β5 tail. The cytoplasmic domain of β5 is structurally unique among the β

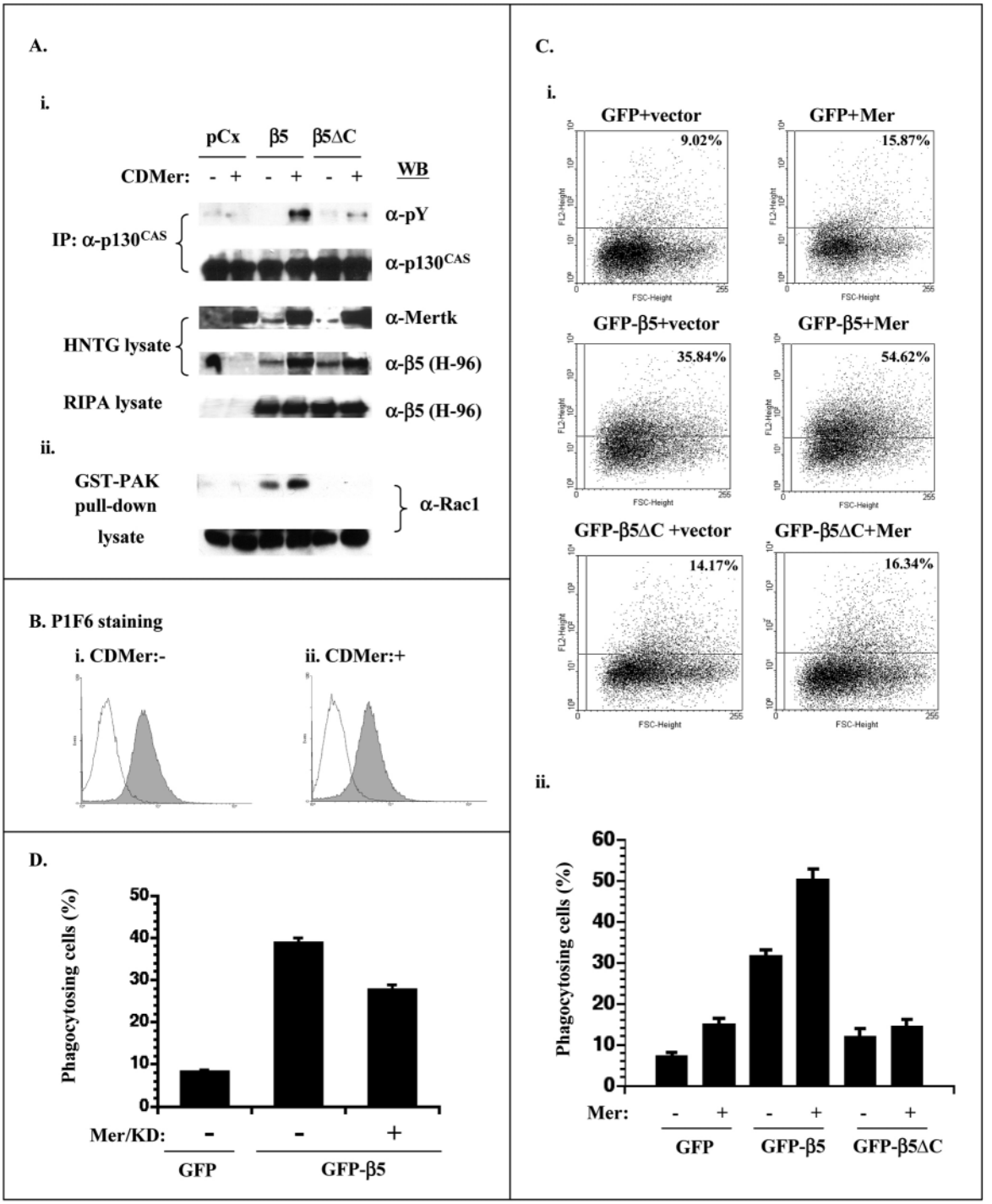

Fig. 7. Integrin αvβ5 is essential for Mer-mediated p130^{CAS} phosphorylation, Rac1 activation, and phagocytosis. (A) β5 null CS-1 cells expressing empty vector, β5 or β5ΔC were expressed with or without CDMer cells. The lysates were immunoprecipitated with anti-p130^{CAS} Ab, and analyzed by immunoblotting with anti-phosphotyrosine Ab (i). Alternatively, the lysates were precipitated with GST-PAK CRIB Sepharose beads, and the levels of Rac1 GTP loading were determined by immunoblotting with anti-Rac1 mAb (ii). β5/β5ΔC expression was comparable between cells co-overexpressed with and without CDMer, as analyzed with anti-β5 immunoblotting in RIPA buffer lysates (A, panel i) and P1F6 staining by FACScan (B). Unshaded, control CS-1 cells; shaded, CS-1 cells expressing β5. (C) Phagocytosis assay. CS-1 cells were transfected with bicistronic pIRES-EGFP, pIRES-EGFP-β5, or pIRES-EGFP-β5ΔC, with empty vectors or Mer. After 48 hours, transfected cells were co-cultured with red-labeled apoptotic T cells (1:10), and phagocytosis assay was performed as described in the legend for Fig. 4. Data represent the mean±s.e.m. percentage of phagocytosing cells from four separate experiments (i and ii). (D) CS-1 cells were transfected with bicistronic pIRES-EGFP or pIRES-EGFP-β5, with control vector (–) or mutant Mer/KD and phagocytosis assay was performed as described above. Data represent the mean±s.e.m. percentage of phagocytosing cells from three separate experiments.

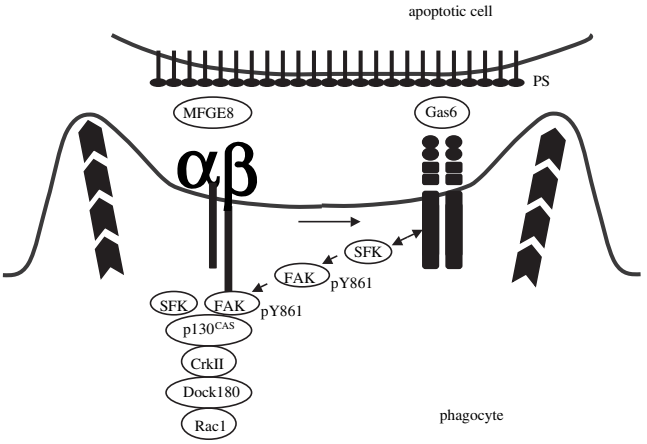

Fig. 8. Schematic model for convergence of PS-dependent receptors αvβ5 integrin and Mer signaling. We propose that Mer activates a Src family kinase, resulting in FAK^{Tyr861} phosphorylation and subsequent recruitment of FAK to the cytoplasmic tail of β5, thereby αvβ5 integrin and Mer functionally cross-talk to amplify internalization signals.

subunits β1, β2, β3, β5, β6 and β7 (Sastry and Horwitz, 1993; Wayner et al., 1991), which are otherwise quite similar. The spacing between two 'NPXY' sequence motifs is conserved as eight amino acid residues in β3 and other integrins, whereas β5 has a longer insertion of 16 amino acid residues. This difference in primary structure accounts for the weaker interaction of αvβ5 with cytoskeletal components, such as actin, α-actinin, talin and tensin, compared with that of αvβ3 (Lewis et al., 1996). However, activation of PKC by PMA somehow increases αvβ5 interaction with α-actinin (Lewis et al., 1996), suggesting that PKC may be capable of inducing conformational change of the cytoplasmic domain of αvβ5, thereby making it accessible for binding activated FAK^{Tyr861}. Recently, in a study relevant to the present findings with Mer, Eliceiri and colleagues reported that VEGFR induces FAK^{Tyr861} tyrosine phosphorylation and the recruitment to the αvβ5 integrin cytoplasmic region as an angiogenesis signal (Eliceiri et al., 2002). Our present results suggest that RTK-inducible formation of a FAK/αvβ5 integrin complex may be a more general mechanism, used by a variety of cellular processes, including migration, invasion and phagocytosis. These ideas also warrant investigation of whether other RTKs on phagocytes, such as Flt1 and FGFR, play a role in the phagocytosis of apoptotic cells.

Our observations that αvβ5 integrin and Mer synergize to activate and phosphorylate FAK, Src and $p130^{CAS}$ suggest parallels between focal adhesion biology and phagocytosis of apoptotic cells and particles. The focal adhesion is a complex dynamic assembly of cytoskeletal and signaling proteins that performs multiple functions. One role involves mechanical stability in which integrins anchor the actin cytoskeleton to the extracellular matrix. However, several focal adhesions colocalize with the formation of the phagocytic cup suggesting that the cellular processes of substratum attachment and phagocytosis are similar. For example, talin, vinculin and paxillin, clearly focal adhesion proteins, are concentrated at FcγR phagosomes and loss of talin in *Dictyostelium* causes defects in phagocytosis indicating a functional interaction (Allen and Aderem, 1995b; Greenberg et al., 1990; Niewohner et al., 1997). During complement (CR3 and CR4)-mediated phagocytosis, which recognize both microbial pathogens and apoptotic cells in the context of partially activated complement factors, Aderem and colleagues noted the appearance of 'punctate-like foci' of focal adhesion and cytoskeletal proteins at CR-mediated phagosomes (Allen and Aderem, 1995a). In addition to its role in phagocytosis of apoptotic cells, αvβ5 integrins anchor cells to the extracellular matrix molecule vitronectin, serve as a co-receptor for adenovirus, and promote endosome to cytosolic transfer during adenovirus internalization (Chiu et al., 1999). These observations suggest that αvβ5, and other integrins such as α5β1, can serve both a tethering function, as well as a receptor-mediated endocytosis/phagocytosis and recycle back to the plasma membrane. Perhaps in the context of inside to outside signals (ex RTK activation) and/or the availability of alternative ligands (MFG-E8 bound to apoptotic cells), that αvβ5 integrin switches from an anchoring integrin to a phagocytic integrin. It is also possible that activation of focal adhesion signals, including FAK and $p130^{cas}$ phosphorylation, function as tethering signals, and require additional internalization signals, for example by PS-R activation. Moreover, very recent studies by Schwartz and colleagues demonstrate that integrin signals regulate lipid raft formation and Rac1 targeting to newly synthesized rafts (del Pozo et al., 2004), although the nature of the integrin activating signal is not explored. As Mer appears to activate αvβ5 integrin, it should also be interesting to determine whether MFG-E8 or Gas6 stabilizes raft formation on the cell surface.

Consistent with the results of this manuscript, studies investigating phagocytosis of rod outer segments (ROS) in RPE demonstrated an important role for FAK in ROS internalization (Finnemann, 2003). Stimulation of RPE with outer segments results in redistribution αvβ5 integrin on the apical surface of the RPE and the recruitment of FAK to the β5 tail. ROS internalization, but not particle binding, was effectively blocked in FAK null cells or by expression of dominant negative FAK (FRNK), suggesting a critical role of FAK in αvβ5 integrin-mediated phagocytosis. Interestingly, OS-dependent FAK activation also resulted in tyrosine phosphorylation of Mer (Finnemann, 2003), suggesting that in addition to the Mer →αvβ5 directionality we report here, crosstalk between αvβ5 and Mer can also occur. Although the lack of Mer-dependent signaling in β5-deficient cells suggests that Mer initiates the tyrosine phosphorylation required for αvβ5 signaling, the fact that signals can be returned from the integrin to Mer suggests the existence of a 'molecular synapse' that may amplify downstream signaling. Indeed, we found that expression of kinase negative Mer (Mer/K614M) also partially blocked αvβ5 integrin-mediated phagocytosis. These data strongly suggest that signals originating from multiple PS-dependent recognition receptors play a synergistic role in apoptotic cell recognition and internalization. Understanding the role of CrkII/Dock180/Rac1-dependent signaling from the PS-R may further elucidate how multiple PS recognition receptors are integrated at the molecular level for physiological clearance.

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