Monocyte migration requires the dynamic redistribution of integrins through a regulated endo-exocytosis cycle, but the complex molecular mechanisms underlying this process have not been fully elucidated. Glia maturation factor-\(\gamma\) (GMFG), a novel regulator of the Arp2/3 complex, has been shown to regulate directional migration of neutrophils and T-lymphocytes. In this study, we explored the important role of GMFG in monocyte chemotaxis, adhesion, and \(\beta_1\)-integrin turnover. We found that knockdown of GMFG in monocytes resulted in impaired chemotactic migration toward formyl-Met-Leu-Phe (fMLP) and stromal cell-derived factor 1 (SDF-1\(\alpha\)) as well as decreased \(\alpha_5\beta_1\)-integrin-mediated chemoattractant-stimulated adhesion. These GMFG knockdown impaired effects could be reversed by cotransfection of GFP-tagged full-length GMFG. GMFG knockdown reduced the cell surface and total protein levels of \(\alpha_5\beta_1\)-integrin and increased its degradation. Importantly, we demonstrate that GMFG mediates the ubiquitination of \(\beta_1\)-integrin through knockdown or overexpression of GMFG. Moreover, GMFG knockdown retarded the efficient recycling of \(\beta_1\)-integrin back to the plasma membrane following normal endocytosis of \(\alpha_5\beta_1\)-integrin, suggesting that the involvement of GMFG in maintaining \(\alpha_5\beta_1\)-integrin stability may occur in part by preventing ubiquitin-mediated degradation and promoting \(\beta_1\)-integrin recycling. Furthermore, we observed that GMFG interacted with syntaxin 4 (STX4) and syntaxin-binding protein 4 (STXBP4); however, only knockdown of STXBP4, but not STX4, reduced monocyte migration and decreased \(\beta_1\)-integrin cell surface expression. Knockdown of STXBP4 also substantially inhibited \(\beta_1\)-integrin recycling in human monocytes. These results indicate that the effects of GMFG on monocyte migration and adhesion probably occur through preventing ubiquitin-mediated proteasomal degradation of \(\alpha_5\beta_1\)-integrin and facilitating effective \(\beta_1\)-integrin recycling back to the plasma membrane.

The migration of monocytes from the circulation toward the surrounding extravascular space is a crucial event in physiological and pathological immune and inflammatory responses. The extravasation of monocytes is a highly orchestrated multistep adhesion cascade that is mediated by dynamic regulation of adhesion molecules expressed on both monocytes and endothelial cells, including integrins (1, 2). Integrins are a large family of heterodimeric transmembrane proteins consisting of an \(\alpha\)- and \(\beta\)-subunit that bind extracellular matrix components to provide sites of adhesion and signals for the dynamic rearrangement of cytoskeletal elements in various stages of cell migration (3, 4). Monocytes, like other leukocytes, express distinct integrins, but those most relevant to monocyte migration belong to the \(\beta_1\) - and \(\beta_2\)-integrin subfamilies. Although the important role of \(\beta_1/\beta_2\)-integrins in regulating monocyte transmigration across the endothelium to sites of inflammation has been relatively well studied (5–7), the molecular mechanisms underpinning the dynamic regulation of endo-exocytic trafficking of these integrins are only beginning to emerge.

Accumulating evidence indicates that integrin endocytic trafficking plays an important regulatory role in cell migration and disease progression by affecting integrin function and cell surface distribution as well as the turnover of extracellular matrix molecules (8–10). The trafficking of newly synthesized or recycled integrin molecules to the plasma membrane requires the tight regulation of the actin cytoskeleton, actin-modulating proteins, and vesicular trafficking machineries that selectively sort cargo proteins via various adaptor proteins (11). The final step for integrin trafficking to the cell surface is mediated by the fusion of integrin-containing transport vesicles with the plasma membrane, a process that is poorly understood at the molecular level (12).

A growing number of studies have demonstrated that the interaction of SNARE proteins on vesicles (v-SNAREs) and on target membranes (t-SNAREs) drives intracellular vesicle fusion (13–16). Several SNARE proteins have been implicated in integrin trafficking. For example, the interaction of t-SNARE protein STX6 (syntaxin 6) with the v-SNARE VAMP3 (vesicle-associated membrane protein 3) appears to play a role in cell migration by mediating recycling of heterodimers containing \(\beta_1\)-integrin in endothelial cells and HeLa cells (17, 18). In macrophages, the plasma membrane t-SNARE proteins STX4 and SNAP23 (soluble N-ethylmaleimide-sensitive fusion protein 23) interact with VAMP3 to mediate endocytic recycling of \(\alpha_5\beta_1\)-integrin (19). STX3 and STX4 mediate cell migration by regulation of \(\alpha_5\beta_1\)- and \(\alpha_3\beta_1\)-integrin trafficking to the cell surface in HeLa cells (12). In addition, STX4 and STXBP4 (syntaxin-binding protein 4) are specifically critical in the vesicular transport of GLUT4-containing vesicles to the plasma mem-

**Glia Maturation Factor-\(\gamma\) Regulates Monocyte Migration through Modulation of \(\beta_1\)-Integrin**

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Wulin Aerbajinai\(^{1}\), Lunhua Liu\(^{5}\), Jianqiong Zhu\(^{4}\), Chutima Kumkhaek\(^{3}\), Kyung Chin\(^{4}\), and Griffin P. Rodgers\(^{1}\)

From the \(^{4}\)Molecular and Clinical Hematology Branch, NHLBI, National Institutes of Health, Bethesda, Maryland 20892 and the \(^{5}\)Laboratory of Cellular and Molecular Biology, NCI, National Institutes of Health, Bethesda, Maryland 20892

supplemental Movies S1–S8

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[1] This article contains supplemental Movies S1–S8.

[2] To whom correspondence should be addressed: Molecular and Clinical Hematology Branch, Bldg. 10, Rm. 9N-113A, National Institutes of Health, Bethesda, MD 20892-2560. Tel.: 301-496-5741; Fax: 301-402-2125; E-mail: gr5n@nih.gov.
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brane in skeletal muscle, adipocytes, and cardiomyocytes (20–22). These findings indicate that t-SNARE proteins play an important role in cell migration by mediating β1-integrin trafficking.

Glia maturation factor-γ (GMFG), a newly recognized actin depolymerization factor/cofilin superfamily protein, is a highly conserved protein throughout eukaryotes from yeast to mammals. GMFG is predominantly expressed in inflammatory cells and microendothelial cells (23, 24). We and others have recently shown that GMFG mediates neutrophil and T-lymphocyte migration via regulation of actin cytoskeletal reorganization (25, 26). Functional studies of GMFG in systems ranging from yeast to mammalian cells have shown that GMFG promotes debranching of actin filaments and inhibits new actin assembly through binding of the Arp2/3 (actin-related protein-2/3) complex (27–29). The Arp2/3 complex plays a fundamental role in both cell migration and intracellular trafficking by mediating membrane remodeling through the endocytic internalization and recycling, degradation, and exocytic transport of membrane components (30, 31). Despite these advances, it remains unclear whether GMFG mediates monocyte migration through modulation of intracellular trafficking of β1-integrin.

Here, we show that GMFG is important for maintaining the stability of β1-integrin protein and the efficient recycling of β1-integrin in monocytes and is required for efficient monocyte chemotaxis, probably through association with STXBP4. These results further support the important role of GMFG in monocyte migration.

Experimental Procedures

Cells and Cell Cultures—Peripheral blood mononuclear cells were isolated from buffy coats of healthy adult donors by Ficoll-Paque (GE Healthcare) density gradient centrifugation according to a protocol approved by the institutional review board of NHLBI, National Institutes of Health, and consistent with federal regulations. Monocytes were purified using a negative selection human monocyte enrichment kit (STEMCELL Technologies) according to the manufacturer’s instructions. Monocytes (2 × 10⁶/ml) were cultured in complete RPMI 1640 medium (Invitrogen) containing 10% human AB serum (Sigma-Aldrich). The human monocytic cell line THP-1 and HEK-293T cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 or DMEM containing 10% heat-inactivated FBS.

RNA Interference and Transfection—Monocytes (1 × 10⁶ cells) or THP-1 cells (2 × 10⁶ cells) were transiently transfected with a GMFG siRNA, STX4 siRNA, STXBP4 siRNA, control negative siRNA, GFP vector, and/or GMFG-GFP vector using the Nucleofector Kit V and Nucleofector I Program U-01 (Lonza), according to the manufacturer’s protocol. The GMFG siRNA (Silencer Select siRNA s18302), STXBP4 siRNA (Silencer Select siRNA s48386), STX4 siRNA (Silencer Select siRNA s13595), and control negative siRNA (Neg-siRNA-2) were purchased from Applied Biosystems. GFP vector and GMFG-GFP plasmid (human cDNA clone, ORF with C-terminal GFP tag) were obtained from ORIGENE. Functional experiments with siRNA-transfected cells were performed 48 h after transfection, when GMFG, STX4, or STXBP4 expression levels were reduced 75% in relevant siRNA-transfected cells compared with control negative siRNA-transfected cells. For GMFG rescue experiments, cells were first transiently transfected with a control negative siRNA or GMFG siRNA for 48 h, followed by cotransfection of GFP vector or GMFG-GFP expression plasmid and continuously cultured in complete growth medium for 24 h prior to use.

Monocyte Chemotaxis Assays—Primary human monocytes (5 × 10⁶ cells) or THP-1 cells (5 × 10⁶ cells) in 0.5% BSA-RPMI medium were placed in the upper chamber of a Transwell culture insert (5-µm pore size; Corning Inc.) coated with or without 10 µg/ml fibronectin (FN) (Sigma-Aldrich) for 1 h. Formyl-Met-Leu-Phe (fMLP) (1–100 nM; Sigma-Aldrich) or SDF-1α (stromal cell-derived factor 1α) (1–100 ng/ml; R&D Systems) was added to the lower well, and the cells were allowed to migrate for 3 h at 37 °C under a 5% CO₂ atmosphere. Migrated cells in the lower wells were quantified by placing 10-µl aliquots of the medium from the lower wells on a hemacytometer and counting four fields in duplicate.

EZ-TAXIScan Chemotaxis Assays—The EZ-TAXIScan chamber (Effector Cell Institute, Tokyo, Japan) was assembled as described by the manufacturer. Coverslips and chips used in the chamber were coated with 1 µg/ml FN or 1% BSA at room temperature for 1 h, and then monocytes or THP-1 cells were added to the chamber and allowed to migrate. Cell migration toward fMLP (100 nM) or SDF-1α (100 ng/ml) was recorded every 15 s for 30 min at 37 °C in a humidified environmental chamber. Cell migration analysis was conducted using MATLAB software as described previously (32). Cell tracks were subsequently used to compute chemotactic index (directionality) and mean migration speed for each tracked cell at each time point in each experiment. Each image shows a representative section of the final frame in the experiment plus the locations of all cells identified and tracked in the section in all frames.

Adhesion Assays—Ninety-six-well flat bottom plates (BD Biosciences) were coated with 10 µg/ml FN for 1 h. Wells were washed and blocked with 1% BSA in PBS for 1 h. Monocytes or THP-1 cells (50 µl; 1 × 10⁶ cells/ml) were seeded onto precoated plates and incubated at 37 °C for 15 min. Cells were then stimulated with fMLP (100 nM) or SDF-1α (100 ng/ml) at 37 °C for 15 min, and unattached cells were subsequently removed by gentle washing with washing buffer (0.1% BSA/PBS) twice. Attached cells were then fixed and stained with crystal violet. The bound crystal violet was dissolved with 1% SDS and analyzed at 590 nm. Values were calculated as -fold increase over levels in unstimulated cells treated with control negative siRNA.

Coimmunoprecipitation Assays—For endogenous coimmunoprecipitation analysis, human THP-1 cells were lysed in IP lysis buffer (Thermo Scientific) and immunoprecipitated overnight with monoclonal GMFG antibody (LifeSpan BioSciences), monoclonal STXBP4 antibody (Thermo Scientific), or monoclonal STX4 antibody (BD Biosciences) and then subjected to Western blotting. For immunoprecipitation analysis using transient transfection, HEK-293T cells were transiently
cotransfected with FLAG-tagged GMFG and GFP-tagged STXB4, with FLAG-tagged GMFG and Myc-tagged STX4 (ORIGENE), or with expression vectors for all three proteins together (Myc-tagged STX4, GFP-tagged STXB4, and FLAG-tagged GMFG). Forty-eight h after transfection, cells were lysed and immunoprecipitated overnight with anti-FLAG, anti-GFP, or anti-Myc antibodies (ORIGENE). Dynabeads protein G (Life Technologies) was added and incubated for 3 h at 4 °C. The immunoprecipitated complexes-beads were washed three times in the washing buffer, and the proteins were eluted with 2× sample buffer (46.7 mM Tris-HCl, pH 6.8, 5% glycerol, 1.67% SDS, 1.55% dithiothreitol, and 0.003% bromphenol blue). Proteins were then subjected to Western blotting.

**Western Blotting**—Primary monocytes or THP-1 cells lysed in radioimmune precipitation assay buffer (Thermo Scientific), or immunoprecipitated proteins were electrophoresed on 4–20% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies to GMFG (Abgent), β1-integrin (MAB2000; Millipore), fMLP receptor and CXCR4 (Bioss), β-actin, ubiquitin (P4D1), or α-tubulin (Santa Cruz Biotechnology); α5-integrin, STXB4, biotin, or STX6 (Cell Signaling Technology); or STX3, STX4, VAMP2, VAMP3, or VAMP4 (Proteintech Group). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and the Amersham Biosciences ECL Western blotting system (GE Healthcare) was used for detection.

**Flow Cytometry Analysis**—For the detection of monocyte cell surface proteins, cells were fixed with 1% paraformaldehyde in PBS for 15 min and then blocked in 10% FBS in PBS for 15 min. The cells were incubated with 20 μg/ml control mouse IgG (Sigma-Aldrich) or monoclonal antibodies to total β1-integrin (both the active and inactive forms, MEM101A) (Novus Biologicals) or α5-integrin (clone NKK-SAM1) (BioLegend) or polyclonal antibodies to fMLP receptor or CXCR4 (Bioss) for 1 h at 4 °C. After washing, the cells were labeled with Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit secondary antibody (Invitrogen), diluted 1:500, for 1 h at 4 °C. Samples were analyzed by EPICS ELITE ESP flow cytometry (Beckman Coulter), and the data were analyzed using CellQuest software (BD Biosciences).

**Flow Cytometry-based Integrin Internalization and Recycling Assays**—The internalization assay was performed as described previously (33). Human monocytes were transfected with control negative siRNA, GMFG siRNA, or STXB4 siRNA for 48 h. The cells (5 × 10⁶ cells/ml) were incubated with anti-β1-integrin antibody (MAB2000; Millipore) at a concentration of 10 μg/ml in HBSS plus 0.5% BSA for 1 h at 4 °C. After washing with ice-cold HBSS, labeled cells were incubated in prewarmed 10% FBS-RPMI medium for the indicated times at 37 °C to allow for integrin internalization. Samples were then quenched with twofold washes of 50 mM glycine in HBSS. The cells were incubated in 10% FBS-RPMI medium for 30 min at 37 °C to allow internalization. Samples were then returned to ice and washed with ice-cold HBSS three times, and the surface biotin was reduced by incubating the cells with reducing solution (50 mM reducing glutathione, 75 mM NaCl, 10 mM EDTA, and 75 mM NaOH) for two treatments of 15 min each at 4 °C. The cells were then washed with ice-cold HBSS for a second round of surface reduction with reducing solution to remove the biotin from recycled integrins, followed by a second quenching with iodoacetamide. Cells were then washed and lysed in 300 μl of immunoprecipitation lysis buffer containing a protease inhibitor.
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**FIGURE 1. GMFG knockdown reduces chemotaxis in human monocytes.** A, knockdown efficiency of GMFG siRNA in monocytes or THP-1 cells. Cells were transfected with a control negative siRNA (Ctrl) or GMFG siRNA, and lysates were examined by Western blotting. β-Actin was used as a loading control. B and C, Transwell migration assays were performed on 5-μm pore filters coated with 10 μg/ml FN in primary human monocytes (B) or THP-1 cells (C) transfected with control negative siRNA (Ctrl siRNA) or GMFG siRNA in response to vehicle alone (0.1% BSA) or increasing concentrations of fMLP (1–100 nM) or SDF-1α (1–100 ng/ml). The number of migrated cells was quantitated after 3 h, as described under “Experimental Procedures.” D and E, Transwell migration assays were performed in human monocytes or THP-1 cells cotransfected with control negative siRNA (Ctrl siRNA) or GMFG siRNA and GFP vector or GFP-GMFG vector in response to vehicle alone (0.1% BSA) or fMLP (100 nM) or SDF-1α (100 ng/ml). The number of migrated cells was quantitated after 3 h, as described under “Experimental Procedures.” F–I, EZ-TAXIScan chemotaxis toward fMLP (100 nM) or SDF-1α (100 ng/ml) in human monocytes or THP-1 cells transfected with control negative siRNA or GMFG siRNA. F and G, representative images of migrating cells in a gradient of fMLP or SDF-1α after 3 h. Scale bars, 20 μm. Data are representative of three experiments. H and I, migration speed and chemotactic index were quantified from the captured images during the course of the EZ-TAXIScan chemotaxis assay, as described under “Experimental Procedures.” J, cell surface expression of fMLP receptor (fMLP-R) or chemokine receptor CXCR4 in control negative siRNA- or GMFG siRNA-transfected human monocytes or THP-1 cells. Cells were stained with polyclonal anti-fMLP receptor or anti-CXCR4 antibodies for 1 h at 4 °C. After washing, the cells were labeled with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody and subjected to flow cytometry analysis. K, Western blotting analysis of GMFG, fMLP receptor, and chemokine receptor CXCR4 expression in whole-cell lysates from monocytes or THP-1 cells transfected with control negative siRNA or GMFG siRNA. β-Actin was used as a loading control. Data represent the mean ± S.D. (error bars) from three independent experiments. *p < 0.05 compared with control siRNA-transfected cells. **p < 0.01 compared with control siRNA-transfected cells.

Results

GMFG Is Required for Efficient Chemotaxis in Human Monocytes—To test our hypothesis that GMFG mediates monocyte migration as well as the mechanisms underlying this process, we first set out to evaluate the effect of GMFG knockdown on the chemotactic ability of human monocytes in response to two well known chemoattractants, fMLP and SDF-1α, using Transwell migration assays. The successful knockdown of GMFG protein expression in human monocytes or THP-1 cells was confirmed by Western blotting analysis (Fig. 1A) (75% reduction in GMFG protein expression in GMFG siRNA-transfected cells). Consistent with previous studies in other types of cells (25, 26), we observed that GMFG siRNA-transfected primary human monocytes or THP-1 cells displayed significantly reduced chemotactic migration toward the chemoattractants fMLP (1–100 nM) or SDF-1α (1–100 ng/ml) at all concentrations tested, when compared with control siRNA-transfected cells (Fig. 1, B and C). We further determined whether an ectopically expressed GMFG was capable of

mixture (Roche Applied Science). Biotinylated cell surface β1-integrin remaining inside the cells was immunoprecipitated from lysates using anti-β1-integrin antibody (MAB2000) (Mili- lipore) and analyzed by Western blotting using anti-biotin antibody or streptavidin-HRP.

Isolation of Plasma Membrane Protein Fraction—The plasma membrane fraction of human monocytes or THP-1 cells was isolated using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher) according to the manufacturer’s protocol.

Integrin Degradation Assays—Monocytes were incubated with cycloheximide (100 μM) for the indicated time period in the presence or absence of the lysosomal inhibitor leupeptin (500 μg/ml; Sigma-Aldrich) or chloroquine (10 μM; Sigma-Aldrich) or the proteasomal inhibitor MG132 (10 μM; Calbiochem). After treatment, cells were subjected to Western blotting analysis using an anti-β1-integrin or α5-integrin antibody.

Statistical Analyses—Statistical significance for all experiments was determined by Student’s paired t test analyses. A p value of <0.05 was considered statistically significant.
rescuing the impaired chemotactic cell migration in GMFG knockdown cells. Human monocytes and THP-1 cells were first transiently transfected with a control negative siRNA or GMFG siRNA for 48 h, followed by cotransfection of GFP vector or GMFG-GFP expression plasmid for another 24 h. Western blotting analysis confirmed that successful rescue of the GMFG knockdown by ectopic expression of GMFG-GFP plasmid in human monocytes and THP-1 cells (Fig. 1, D and E, bottom). Importantly, the reduced chemotactic cell migration in GMFG siRNA-transfected monocytes or THP-1 cells could be restored to the levels observed in control siRNA-transfected cells by cotransfection of GMFG-GFP plasmid but not by cotransfection with GFP vector only, indicating that the effects of GMFG siRNA on chemotactic migration are specific rather than a potential off-target effect (Fig. 1, D and E).

To gain further insight into this observation, we examined the dynamics of directional migration in cells transfected with control negative siRNA or GMFG siRNA using an EZ-TAXIScan system, which allows real-time visualization of cell movements and makes time-lapse recording of the speed and directionality of individual cells toward linear gradients of chemoattractants over time. We observed that, in contrast to control siRNA-transfected cells, GMFG knockdown monocytes or THP-1 cells revealed a marked impairment in their chemotactic behavior, because GMFG knockdown cells migrated over a much shorter distance with slower speed toward the fMLP or SDF-1α gradient when compared with control siRNA-transfected monocytes (Fig. 1, F and G, and supplemental Movies S1–S8). However, GMFG knockdown cells were able to sense the chemoattractant gradient, because these cells were seen to quickly form protrusions and polarize toward chemoattractants after the initiation of chemotaxis (supplemental Movies S1–S8). To perform a detailed analysis of time-dependent chemotactic ability, we measured mean migration speed and chemotactic index (directionality) in GMFG knockdown and control negative siRNA-transfected cells. Quantification by cell tracking revealed that GMFG knockdown monocytes or THP-1 cells displayed significantly decreased migration speed and directionality during the chemotaxis, indicating that GMFG is important for monocyte chemotaxis (Fig. 1, H and I). Furthermore, these defects were not due to alteration in total or cell surface levels of fMLP receptor or chemokine receptor CXCR4, because knockdown of GMFG did not alter total or cell surface levels of these relevant receptors in human monocytes or THP-1 cells compared with control siRNA-transfected cells (Fig. 1, J and K).

**GMFG Regulates α5β1-integrin-mediated Chemoattractant-stimulated Adhesion in Human Monocytes**—Integrin-mediated cell adhesion to the endothelium or the extracellular matrix is a prominent mechanism for monocyte recruitment to sites of inflammation. Therefore, we next sought to determine whether the effects of GMFG on chemotaxis might occur through regulation of monocyte adhesion. For this purpose, the effect of knockdown of GMFG on monocyte adhesion was determined with adhesion assays that measure the fraction of cells that attach to an FN-coated surface in steady-state conditions or after stimulation by fMLP or SDF-1α. We found that knockdown of GMFG had no significant effect on adhesion to FN in the absence of stimulation. However, when GMFG knockdown monocytes or THP-1 cells were stimulated with fMLP or SDF-1α, their adhesion to FN was significantly reduced compared with control siRNA-transfected cells (Fig. 2A). Importantly, reduced adhesion in GMFG knockdown monocytes or THP-1 cells could be rescued to the levels observed in control siRNA-transfected cells by cotransfection with GMFG-GFP vector but not by cotransfection with GFP vector only (Fig. 2B), demonstrating that impaired adhesion is indeed due to suppression of endogenous GMFG expression. These results clearly indicated that GMFG regulates α5β1-integrin-mediated adhesion and that the inhibition of monocyte migration observed upon knockdown of GMFG may be a consequence of reduced adhesion.

Cell migration and adhesion are affected by surface integrin levels (34). Therefore, we investigated whether the reduced cell adhesion induced by knockdown of GMFG was associated with alteration of specific integrins. We restricted our analysis to the integrins expressed in human monocytes that are most critical for monocyte adhesion to FN (α5- and β1-integrins). First, flow cytometry analysis indicated that GMFG knockdown in human monocytes or THP-1 cells resulted in a decrease in the surface levels of α5- and β1-integrin (both the active and inactive forms, MEM101A) compared with control negative siRNA-transfected cells (Fig. 2, C and D). We further determined the surface levels of α5- and β1-integrin in GMFG knockdown monocytes or THP-1 cells using cell surface biotinylation. Biotinylated surface proteins were pulled down with avidin beads and then examined by Western blotting using α5- and β1-integrin antibodies. Consistent with flow cytometry analysis results, cell surface biotinylation showed that GMFG knockdown resulted in markedly decreased levels of surface α5- and β1-integrin in monocytes or THP-1 cells (Fig. 2E). Moreover, Western blotting analysis of plasma membrane protein extracts confirmed reduced membrane levels of α5- and β1-integrin in GMFG knockdown monocytes or THP-1 cells (Fig. 2F). These results strongly suggest that knockdown of GMFG expression reduces the abundance of α5- and β1-integrin at the cell surface in human monocytes and THP-1 cells. Finally, Western blotting analysis revealed that knockdown of GMFG in monocytes or THP-1 cells led to markedly decreased total protein levels of mature (M, 125,000) β1-integrin and α5-integrin compared with control siRNA-transfected cells (Fig. 2G). These reduced α5- and β1-integrin protein levels were not due to insufficient messenger RNA transcription, because quantitative Q-PCR analysis showed that the levels of α5- and β1-integrin transcripts were unchanged in GMFG knockdown cells compared with control siRNA-transfected cells (Fig. 2H). Together, these results indicate that modification of GMFG expression levels can serve to regulate the protein levels of α5β1-integrin by posttranscriptional events, such as altered α5β1-integrin degradation.

**GMFG Overexpression Enhances Chemoattractant-stimulated Migration and Adhesion to FN in Human Monocytes**—To confirm the role of GMFG in α5β1-integrin-mediated monocyte migration and adhesion, we next examined the effect of GMFG overexpression in monocytes or THP-1 cells. Western blotting analysis showed that GFP-tagged GMFG was
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FIGURE 2. GMFG knockdown decreases chemoattractant-stimulated cell adhesion to FN and cell surface expression of α5β1-integrin in human monocytes. Human monocytes or THP-1 cells were transfected with control negative siRNA (Ctrl siRNA) or GMFG siRNA for 48 h. A, siRNA-transfected human monocytes or THP-1 cells were subjected to adhesion assays on 10 μg/ml FN-coated wells (in triplicate samples) in the absence (Ctrl) or presence of fMLP (100 nM) or SDF-1α (100 ng/ml). Values were calculated as fold-increase over levels in unstimulated cells treated with control negative siRNA. Each assay was performed in triplicate. Data represent the mean ± S.D. (error bars). *, p < 0.05 compared with control siRNA-transfected cells. B, human monocytes or THP-1 cells were cotransfected with control negative siRNA or GMFG siRNA and GFP vector or GFP-GMFG vector and then subjected to an in vitro adhesion assay on 10 μg/ml FN-coated wells (in triplicate samples) in the absence or presence of 100 nM fMLP or 100 ng/ml SDF-1α. Values were calculated as fold-increase over levels in unstimulated cells treated with control negative siRNA. Data represent three independent experiments and are expressed as the mean ± S.D. (error bars). *, p < 0.05 compared with control negative siRNA-transfected cells. G, representative flow cytometry results for cell surface expression of β1-integrin (top panels) or α5-integrin (bottom panels) in siRNA-transfected human monocytes or THP-1 cells. Cells were labeled with antibodies to β1-integrin (both the active and inactive forms, MEM101A) or α5-integrin (NKI-SAM1) for 1 h at 4°C, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody, and then subjected to flow cytometry analysis. D, quantification of integrin cell surface expression flow cytometry results as in C. Data represent the mean fluorescence intensity ± S.D. of three independent experiments. *, p < 0.05 compared with control siRNA-transfected cells. E, surface-biotinylated β1-integrin or α5-integrin in siRNA-transfected human monocytes or THP-1 cells. Surface-biotinylated proteins were precipitated using avidin beads, followed by Western blotting analysis with β1-integrin (MAB2000) or α5-integrin (4705P, Cell Signaling) antibodies (top panels). 20% input total cell lysates were analyzed by Western blotting analysis of α-tubulin or GMFG (bottom panels). α-Tubulin was used as a loading control. The densitometric analysis of the relative quantification of β1- and α5-integrin signal intensities (normalized to control siRNA-transfected integrin signal set at 1) is shown below each blot. F, plasma membrane expression of β1- or α5-integrin in siRNA-transfected monocytes or THP-1 cells. The plasma membrane fraction of cells was isolated and then subjected to Western blotting analysis with β1-integrin (MAB2000) or α5-integrin (4705P, Cell Signaling) antibodies (top panels). 20% input total cell lysates were analyzed by Western blotting analysis of α-tubulin or GMFG (bottom panels). α-Tubulin was used as a loading control. The densitometric analysis of the relative quantification of β1- and α5-integrin signal intensities (normalized to control siRNA-transfected integrin signal set at 1) is shown below each blot. G, Western blotting analysis of β1-integrin, α5-integrin, and GMFG expression in whole-cell lysates from siRNA-transfected monocytes or THP-1 cells. α-Tubulin was used as a loading control. H, quantitative PCR analysis of the expression of α5-integrin and β1-integrin transcripts in control siRNA- or GMFG siRNA-transfected human monocytes or THP-1 cells. Data represent the mean ± S.D. of three independent experiments. IP, immunoprecipitation.

successfully transfected into monocytes or THP-1 cells (Fig. 3A). Consistent with the results of GMFG knockdown experiments, GMFG overexpression led to a significant increase in chemotactic cell migration toward fMLP or SDF-1α compared with the GFP control-transfected cells (Fig. 3B). Moreover, GMFG overexpression significantly enhanced cell adhesion to FN after stimulation with fMLP or SDF-1α (Fig. 3C). Flow cytometry analysis did not demonstrate any significant differences in cell surface expression of α5- or β1-integrin between GFP-transfected and GMFG-GFP-transfected cells (Fig. 3D). However, overexpression of GMFG appeared to slightly increase total cellular protein levels of α5- and β1-integrin compared with GFP vector only-transfected cells (Fig. 3E). Together, these results indicate that
overexpression of GMFG enhances chemotactic cell migration and cell adhesion in monocytes.

**GMFG Is Required for the Maintenance of α5β1-Integrin Stability**—To investigate whether GMFG might influence the stability of α5- and β1-integrin proteins, we performed integrin degradation assays. For these experiments, we inhibited protein synthesis by the addition of cycloheximide and then assessed degradation by Western blotting analysis of expression of GMFG or GMFG-GFP in monocytes or THP-1 cells. α-Tubulin was used as a loading control. Western blotting analysis of expression of GMFG or GMFG-GFP in monocytes or THP-1 cells. α-Tubulin was used as a loading control.

Ubiquitin-dependent proteasome degradation is an important process involved in protein down-regulation. To investigate whether the decreased levels of β1- and α5-integrin protein expression observed in GMFG knockdown monocytes may result from ubiquitin-mediated degradation, we immunoprecipitated endogenous β1- or α5-integrin in THP-1 cells transfected with GMFG siRNA or GMFG-GFP vector and subjected the immunoprecipitates to Western blotting analysis with ubiquitin antibody. We observed that ubiquitinated β1-integrin levels were significantly increased in GMFG knockdown cells, whereas the ubiquitinated levels were dramatically reduced in GMFG-overexpressing cells compared with the control siRNA- or GFP vector-transfected cells (Fig. 4C). These results were verified by assessing the effect of proteasomal inhibitor MG132 on ubiquitination of β1-integrin steady-state levels in GMFG siRNA- or GMFG-GFP vector-transfected cells (Fig. 4D). MG132 treatment caused further accumulation of ubiquitinated β1-integrin levels in GMFG-overexpressing cells to the levels observed in control GFP-transfected cells (Fig. 4D), indicating that GMFG indeed mediated β1-integrin ubiquitination. However, Western blotting analysis of α5-integrin immunoprecipitates using anti-ubiquitin antibody in GMFG siRNA-, GMFG-GFP vector-, control

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**FIGURE 3.** GMFG overexpression enhances chemoattractant-stimulated cell migration and adhesion to FN. Human monocytes or THP-1 cells were transfected with GFP vector or GFP-tagged GMFG plasmid for 48 h. A, Western blotting analysis of expression of GMFG or GMFG-GFP in monocytes or THP-1 cells. α-Tubulin was used as a loading control. B, Transwell migration assays were performed on 5-μm pore filters coated with 10 μg/ml FN in transfected primary human monocytes or THP-1 cells in the absence (Ctrl) or presence of fMLP (100 nM) or SDF-1α (100 ng/ml). The number of migrated cells was quantitated after 3 h, as described under “Experimental Procedures.” Data represent the mean ± S.D. (error bars). *, p < 0.05 compared with control GFP-transfected cells. C, transfected human monocytes or THP-1 cells were subjected to adhesion assays on 10 μg/ml FN-coated wells (in triplicate samples) in the absence or presence of fMLP (100 nM) or SDF-1α (100 ng/ml). The number of attached cells was quantitated after 15 min, as described under “Experimental Procedures.” Data represent the mean ± S.D. *, p < 0.05 compared with control GFP-transfected cells. D, representative flow cytometry results for cell surface expression of α5- or β1-integrins in GFP- or GMFG-GFP-transfected human monocytes. Monocytes were incubated with antibodies to α5-integrin or β1-integrin (both the active and inactive forms, MEM101A) for 1 h at 4 °C. After washing, the cells were labeled with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and subjected to flow cytometry analysis. E, Western blotting analysis of α5-integrin or β1-integrin in whole-cell lysates from GFP- or GMFG-GFP-transfected monocytes or THP-1 cells. α-Tubulin was used as a loading control.
FIGURE 4. GMFG knockdown enhances degradation of α5- and β1-integrin in human monocytes. A and B, α5-integrin and β1-integrin degradation in GMFG knockdown cells. Human monocytes were transfected with control negative siRNA (Ctrl siRNA) or GMFG siRNA for 48 h. siRNA-transfected cells were treated with 100 µM cycloheximide (CHX) for the indicated time periods (A) or with cycloheximide (100 µM) for 6 h in the presence of DMSO, the lysosomal inhibitors leupeptin (Leu; 500 µg/ml) or chloroquine (Chlo; 10 µM), or the proteasomal inhibitor MG132 (10 µM), as indicated. Relative quantification of degradation of β1- and α5-integrin is shown in the bottom panels. The signal intensities of β1- and α5-integrin were standardized to the signal intensity of α-tubulin and normalized to 100% at time 0. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05 compared with control siRNA-transfected cells. **, p < 0.01 compared with control siRNA-transfected cells. B, total cell lysates were subjected to Western blotting analysis using anti-α5- and β1-integrin antibody. α-Tubulin was used as a loading control. Relative quantification of degradation of β1- and α5-integrin is shown in the lower panels. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05 compared with control siRNA-transfected cells. **, p < 0.01 compared with control siRNA-transfected cells. C and D, THP-1 cells were transfected with control negative siRNA or GMFG siRNA and GFP vector or GFP-GMFG vector for 48 h and then treated with DMSO (C) or 10 µM MG132 for an additional 2 h (D). The cell lysates were immunoprecipitated with anti-β1-integrin antibody, and ubiquitinated β1-integrin was detected by Western blotting using anti-ubiquitin antibody. Relative quantification of ubiquitinated β1-integrin levels is shown in the bottom panels. Data represent the mean ± S.D. of three independent experiments. *, p < 0.05 compared with control siRNA-transfected cells. NS, no statistical significance. E and F, stability of α5- and β1-integrin after fMLP (E) or SDF-1α (F) stimulation of GMFG knockdown cells. siRNA-transfected human monocytes were treated with fMLP (100 nM) or SDF-1α (100 ng/ml) for the indicated time periods. Total cell lysates were subjected to Western blotting analysis using anti-α5- and β1-integrin antibody. GMFG was used as a loading control.
siRNA- or GFP vector-transfected cells failed to detect a ubiquitin signal (data not shown), indicating that GMFG mediates the ubiquitination of β1-integrin, but not α5-integrin, in human monocytes. Finally, to investigate whether GMFG knockdown decreases β1- and α5-integrin stability during cell migration, we determined the degradation kinetics of β1- and α5-integrin proteins in response to fMLP or SDF-1α stimulation. Western blotting analysis showed that both β1- and α5-integrin levels were dramatically decreased at late time point treatments with fMLP or SDF-1α in GMFG knockdown monocytes compared with control siRNA-transfected monocytes (Fig. 4, E and F). Together, these results suggest that GMFG regulates monocyte migration and adhesion by preventing β1-integrin degradation through ubiquitin-mediated proteasomal and lysosomal pathways.

**GMFG Is Not Involved in β1-Integrin Endocytosis**—Having implicated GMFG as a mediator of β1-integrin turnover, and knowing that β1-integrin turnover is also affected by the dynamic regulation of integrin internalization and recycling (which plays a pivotal role in cell adhesion and motility) (35, 36), we hypothesized that GMFG knockdown might affect β1-integrin internalization or recycling. To functionally address this point, we first performed antibody-based internalization assays in GMFG knockdown monocytes compared with control siRNA-transfected monocytes (Fig. 4). After surface-labeling with β1-integrin antibody at 4 °C, internalization was initiated by transferring cells to serum-free medium at 37 °C for the indicated time period. To quantify this process, we measured the surface-remaining β1-integrin using flow cytometry. We observed no significant differences in the amount of labeled surface-remaining β1-integrin following endocytosis in GMFG knockdown monocytes or THP-1 cells compared with control siRNA-transfected monocytes (Fig. 5A). Immunofluorescence analyses of β1-integrin following endocytosis at 37 °C over time demonstrated similar patterns of endocytosis in GMFG knockdown monocytes or THP-1 cells compared with control siRNA-transfected cells (Fig. 5, B and C). These results indicated that GMFG has no effect on the internalization of β1-integrin in monocytes. Therefore, GMFG may instead regulate β1-integrin turnover by altering its exocytosis.

**GMFG Is Required for β1-Integrin Recycling**—Given that increased β1-integrin degradation in GMFG knockdown monocytes appears not to result from altered β1-integrin internalization, we next examined the role of GMFG in exocytic trafficking of β1-integrin to the plasma membrane using antibody-based, immunofluorescence-based, and biotinylation-based recycling assays. We first performed an antibody-based recycling assay in control negative siRNA- or GMFG siRNA-transfected monocytes. In this assay, monocytes with internalized, antibody-labeled β1-integrin were surface-stripped of antibody and then transferred to serum-containing medium at 37 °C for the indicated time period to allow recycling, followed by incubation with a fluorescently labeled second antibody to detect those integrin molecules that had been initially internalized and subsequently recycled to the cell surface. Flow cytometry analysis showed that GMFG knockdown monocytes had reduced levels of internalized β1-integrin rapidly returning to the cell surface and increased levels of internalized β1-integrin remaining inside the cell, compared with control siRNA-transfected monocytes, indicating a role for GMFG in β1-integrin recycling (Fig. 6A). These data were further confirmed by immunofluorescence analyses, which demonstrated reduced β1-integrin trafficking back to the cell surface following chase with serum at 37 °C in GMFG knockdown cells, compared with control siRNA-transfected cells (Fig. 6B). Finally, an integrin recycling assay employing cell surface biotinylation analysis revealed that the fraction of internalized biotinylated β1-integrin remaining inside the cells was remarkably increased at the 5-min time point in GMFG knockdown cells compared with control siRNA-transfected cells, indicating a retardation of the efficient recycling of β1-integrin back to the plasma membrane in GMFG knockdown cells (Fig. 6C). Taken together, these results indicate that GMFG regulates β1-integrin recycling without affecting β1-integrin endocytosis.

**GMFG Interacts with STXBP4 and STX4**—Although endocytic recycling of β1-integrin is dependent upon a number of diverse protein families and cytoskeletal adaptor proteins, including small GTPases of the Arf and Rab families, the most studied of the Rab GTPases are Rab4 and Rab11, which play key roles in β1-integrin rapid and slow recycling, respectively. Therefore, we examined whether GMFG interacts with Rab4 and Rab11 and mediates β1-integrin recycling in human monocytes. Our immunofluorescence assays showed that GMFG did not colocalize with Rab4, Rab11, or β1-integrin under steady-state conditions (Fig. 7A). However, the intracellular vesicular-like distribution of GMFG suggests that GMFG might be associated with other proteins that mediate constitutive exocytic trafficking of β1-integrin (Fig. 7B).

To gain further insights into the role of GMFG in β1-integrin recycling, we attempted to identify its potential binding partners that relate to GMFG function. In recent studies, SNARE proteins have emerged as regulators in exocytic trafficking of integrins by mediating membrane fusion (37). The SNARE protein STX3, STX4, and STX6 have been reported to mediate β1-integrin recycling in human monocytes (Fig. 7A). Therefore, we screened the possible interaction of GMFG with these SNARE proteins and VAMP proteins in monocytes by performing endogenous coimmunoprecipitation assays using a specific GMFG antibody. We observed that the STX4-binding protein STXBP4 was detected strongly by anti-GMFG immunoprecipitation, and STX4 was also weakly detected, whereas all other syntaxins (STX3 and STX6) or VAMP isoforms (VAMP2, VAMP3, and VAMP4) were not detectable in GMFG coimmunoprecipitates in human monocytes (Fig. 8A, top). Conversely, interaction with GMFG was barely detectable in coimmunoprecipitates using anti-STX4 or anti-STXBP4 antibodies (Fig. 8A, bottom), indicating that these antibodies may not be suitable for immunoprecipitation assays. Therefore, to further test this interaction, we exogenously coexpressed GFP-tagged STXBP4 or Myc-tagged STX4 together with FLAG-tagged GMFG in
HEK-293T cells. Immunoprecipitation with anti-GFP or anti-Myc antibody efficiently precipitated GMFG protein (Fig. 8, B and C). Conversely, immunoprecipitation using anti-FLAG antibody precipitated STXBP4 or STX4 (Fig. 8, B and C, respectively). Furthermore, coimmunoprecipitation analysis following coexpression of FLAG-tagged GMFG together with Myc-tagged STX4 and GFP-tagged STXBP4 in HEK-293T cells demonstrated that GMFG was detected in a complex with STXBP4 and STX4 (Fig. 8D). These data indicate that GMFG is specifically associated with both endogenous SNARE proteins STX4 and STXBP4 in human monocytes. We were unable to detect a clear colocalization between GMFG and STX4 or STXBP4 in THP-1 cells using immunofluorescence confocal microscopy (data not shown), suggesting that these STX4 or STXBP4 antibodies might also not be suitable for immunofluorescence assays. However,
our coimmunoprecipitation results suggest that GMFG-mediated β1-integrin trafficking may be related to its association with STX4 and STXBP4.

STXBP4 Facilitates Cell Directional Migration and β1-Integrin Recycling—Given that GMFG interacts with STX4 and STXBP4, we next investigated whether the GMFG associated with STX4 or STXBP4 is involved in directional migration of monocytes by mediating β1-integrin endocytic trafficking to the cell surface. To test this possibility, we knocked down endogenous STX4 or STXBP4 to compare the role of each of these two molecules in human monocyte migration and β1-integrin recycling. STX4 or STXBP4 siRNA knockdown efficiency was confirmed by Western blotting (Fig. 9A). In Transwell migration assays, we observed that STXBP4 knockdown significantly reduced chemotactic migration of THP-1 cells toward fMLP or SDF-1α, when compared with control siRNA-transfected cells, whereas knockdown of STX4 had no effect on monocyte migration (Fig. 9, B and C). We next evaluated whether knockdown of STX4 or STXBP4 expression in THP-1 cells affected cell surface β1-integrin protein expression using cell surface biotinylation. Knockdown of STXBP4, but not STX4, markedly reduced the surface expression level of β1-integrin (Fig. 9D). Given that down-regulation of STXBP4, but not STX4, decreased the surface levels of β1-integrin and reduced monocyte migration, we further examined the role of STXBP4 in β1-integrin recycling using a biotinylation-based recycling assay. Knockdown of STXBP4 substantially increased the fraction of
internalized biotinylated β1-integrin remaining inside the cells at the 5-min time point in STXBP4 knockdown cells compared with control siRNA-transfected cells, demonstrating that recycling of β1-integrin back to the plasma membrane was impaired in STXBP4 knockdown cells (Fig. 9, E and F). Taken together, our results support the conclusion that GMFG interacts with STXBP4 to regulate cell migration and β1-integrin recycling and that the loss of either GMFG or STXBP4 can impair β1-integrin trafficking back to the cell surface, disrupting directional monocyte migration.

Discussion

Because the endocytic trafficking of integrins toward the leading edge has been established to be a critical step during
leukocyte migration (38), finding the molecular mechanisms that coordinate regulation of integrin recycling and degradation has been a matter of considerable research interest. Here we provide evidence demonstrating the important role of GMFG in directional monocyte migration through protecting β1-integrin from ubiquitin-mediated proteasome degradation and maintaining β1-integrin stability during cell migration as well as promoting β1-integrin recycling back to the plasma membrane. These effects of GMFG may be coordinated through association with STXBP4 to modulate fusion of β1-integrin-containing vesicles with the leading plasma membrane during chemotactic migration of monocytes.

Directional migration requires an asymmetric distribution of actin cytoskeletal and adhesive molecules, such as integrins (39–42), but the underlying regulatory mechanisms are not clearly understood. Recently, a growing amount of evidence has begun to emerge suggesting that actin filaments (cytoskeleton) play essential roles in endocytic vesicle trafficking and that actin-regulatory proteins are required for endocytic integrin trafficking and regulating integrin-mediated cell motility. These include the Arp2/3 regulator proteins N-WASP (43), cortactin (44), annexin A2 (45), and Arp2/3 (46, 47). GMFG, another Arp2/3 regulator protein, has been reported to be required for efficient directional migration of neutrophils and T-lymphocytes through regulation of actin cytoskeletal and integrin-mediated adhesion (25, 26). Consistent with this, our study confirmed that GMFG is also required for human monocyte chemotactic migration. We found that GMFG knockdown was correlated with a significant reduction in chemoattractant-stimulated adhesion to FN, the extracellular matrix substrate of β1-integrin. Although a previous study reported that GMFG silencing enhanced cell surface β1-integrin and led to increased

GMFG Modulates β1-Integrin-dependent Monocyte Migration

FIGURE 9. Knockdown of STXBP4 inhibits human monocyte migration and is correlated with inefficient β1-integrin recycling to the cell surface. A, knockdown efficiency of STXB4 and STX4 siRNA in THP-1 cells. Cells were transfected with a control negative siRNA (Ctrl), STX4 siRNA, or STXB4 siRNA, and lysates were examined by Western blotting. β-Actin was used as a loading control. B and C, Transwell migration assays were performed on 5-μm pore filters coated with 10 μg/ml FN in THP-1 cells transfected with control negative siRNA (Ctrl siRNA), STXB4 siRNA, or STX4 siRNA in response to vehicle alone (0.1% BSA) or increasing concentrations of fMLP (1–100 nM) or SDF-1α (1–100 ng/ml). The number of migrated cells was quantitated after 3 h, as described under “Experimental Procedures.” Data represent the mean ± S.D. (error bars) of at least three independent experiments. *, p < 0.05 compared with control siRNA-transfected cells. D, THP-1 cells transfected with the indicated siRNAs were surface-labeled with 0.5 mg/ml sulfo-NHS-SS-biotin at 4 °C for 1 h. Surface-biotinylated proteins were then isolated using streptavidin beads, followed by Western blotting analysis of β1-integrin. α-Tubulin was used as a loading control. E, biotinylation-based assay of β1-integrin recycling. THP-1 cells transfected with the indicated siRNAs were biotinylated and allowed to internalize surface proteins for 30 min at 37 °C. Surface biotin was removed by reduction (glutathione solution), and internalized proteins were chased at 37 °C for the indicated time periods to allow recycling back to the surface. Surface biotin was reduced again, and cells were lysed. Top, biotinylated cell surface proteins remaining inside the cells were immunoprecipitated using an anti-β1-integrin antibody and subsequently detected by Western blotting analysis using streptavidin-HRP or β1-integrin antibody. Bottom, samples of the total lysates; each sample corresponds to 5% of the cell lysate used in each immunoprecipitation. Shown is a representative Western blot of one experiment (n = 3). α-Tubulin was used as a loading control. F, quantification of β1-integrin recycling determined by densitometry signal as in E. The fraction of internalized β1-integrin remaining was quantified from the signal intensity of internalized protein at each time point relative to control siRNA-transfected cells that had not been placed at 37 °C after the first surface reduction.
cell adhesion in T-lymphocytes (26), our findings from flow cytometry, cell surface biotinylation, and plasma membrane protein extract analyses revealed decreased cell surface levels of β1- and α5-integrin in human monocytes and THP-1 cells following GMFG knockdown. This discrepancy regarding the effect of GMFG on cell adhesion is probably due to the use of different experimental conditions and cell types. Lippert and Wilkins (26) examined the role of GMFG on cell adhesion under steady-state conditions in T-lymphocytes, whereas we performed studies of chemoattractant-stimulated adhesion in monocytes to FN. Importantly, our findings indicate that reduced protein levels of β1- and α5-integrin in GMFG knockdown cells appear to be due to accelerated integrin degradation during monocyte directional migration. Moreover, the ectopic expression of GMFG markedly enhanced chemotactic cell migration and cell adhesion in monocytes and THP-1 cells although it showed no effects on cell surface expression levels of α5-or β1-integrin, further supporting the results from GMFG knockdown studies.

The fate of internalized integrin trafficking for degradation or recycling back to the plasma membrane is determined in early sorting endosomes (48) and requires the concerted action of diverse protein families, including kinases, actin-associated proteins, sorting nexins, and small GTPases of the Arf and Rab families (49, 50). Our findings revealed that GMFG knockdown reduced total and cell surface levels of β1-integrin and accelerated ubiquitin-mediated β1-integrin degradation, indicating that GMFG is probably involved in β1-integrin sorting rather than in influencing β1-integrin transcript. Indeed, the ubiquitination of α5β1-integrin has recently arisen as a crucial regulator of cell adhesion and migration via sorting integrin to the lysosome (51, 52). We have shown that knockdown of GMFG markedly increases ubiquitination of β1-integrin, whereas overexpression of GMFG decreases its ubiquitination. These results suggest that one of the important functions of GMFG is to maintain α5β1-integrin stability through regulation of β1-integrin ubiquitination. These findings might explain the observed impairments in chemoattractant-stimulated cell adhesion as well as in directional cell migration upon GMFG knockdown in monocytes, indicating that β1-integrin functions are severely impaired when their degradation and recycling are not well controlled by GMFG. Moreover, overexpression of GMFG induced remarkable decreases in ubiquitination of β1-integrin, which was reversed by proteasomal inhibitor, indicating that GMFG prevents β1-integrin ubiquitination and proteasomal degradation. However, further studies are needed to identify the underlying mechanism of GMFG-regulated α5β1-integrin degradation and ubiquitination.

Effective β1-integrin trafficking from the rear of the cell, with timely delivery to the membrane at the leading edge, is critical in a migrating cell. Impairment of the exocytic trafficking of integrins dramatically affects cell adhesion and polarity and leads to attenuation of the directionality of cell migration (10, 34, 53). Considering these studies together with our finding that GMFG is required to maintain β1-integrin stability during monocyte chemotactic migration, we speculate that GMFG may play a role in β1-integrin endocytic trafficking. In support of this hypothesis, our recycling assays of GMFG knockdown monocytes revealed impairment in β1-integrin recycling following internalization and a marked increase in β1-integrin degradation, whereas β1-integrin endocytic internalization events were not affected. Similar to our observations, others have demonstrated that the sorting nexin protein SNX17 regulates the β1-integrin recycling-trafficking step that sequesters β1-integrins away from traffic destined for lysosomal degradation and facilitates integrin recycling back to the membrane (54, 55). Hence, the ability to sequester β1-integrin away from degradation in lysosomes and toward entry into recycling endosomes represents an important regulatory process during monocyte migration.

Endocytic recycling of β1-integrin requires a number of steps, including docking, sorting, and delivery of β1-integrin-containing vesicles recycling to the plasma membrane by trafficking machineries (34, 39, 53, 56). However, the final steps that control fusion of β1-integrin recycling endosomes with the plasma membrane remain poorly understood. It has recently emerged that the SNARE family of proteins is involved in all membrane-trafficking pathways by regulating membrane fusion events (37). In this study, we found that GMFG directly interacted with the SNARE protein STX4 and its binding protein STXBP4 in monocytes, indicating that GMFG-mediated β1-integrin recycling might occur in cooperation with these SNARE proteins. Indeed, it has been reported that STXBP4 is involved in the insulin-stimulated trafficking of the glucose transporter GLUT4 vesicle to the cell surface by regulation of membrane fusion in muscle cells and adipocytes (21, 57). However, other reports suggest that STXBP4 plays an inhibitory role in SNARE-dependent membrane fusion with the plasma membrane (58, 59). Recently, further studies have shown that phosphorylation of STXBP4 is required for GLUT4 translocation to the plasma membrane in response to insulin stimulation in both adipocytes and podocytes, indicating that the phosphorylation site of STXBP4 might be important for membrane fusion (22, 60). These results, combined with our finding that knockdown of STXBP4, but not STX4, inhibits the chemotactic migration and recycling of β1-integrin in monocytes, suggest that GMFG-facilitated recycling of β1-integrin might coordinate with STXBP4 to modulate fusion of β1-integrin-containing vesicles to the leading edge membrane during monocyte migration. However, it remains to be determined whether GMFG-mediated recycling of β1-integrin occurs in cooperation with other components of the trafficking machinery (e.g. SNX17, Ras small GTPase, or Arf6) or promotes recycling of other β-integrins to the cell surface membrane (61, 62). Therefore, further detailed investigation is needed to determine how GMFG functions in β1-integrin recycling.

In summary, our findings suggest that GMFG interacts with the SNARE protein STXBP4 and that together they are key regulators in β1-integrin recycling and degradation that direct the intracellular trafficking of internalized β1-integrin to recycle back to the plasma membrane rather than to the lysosomes for degradation. Hence, GMFG may play an important role in maintaining appropriate levels of β1-integrin and efficient monocyte migration and adhesion.
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