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Munc13-4 Regulates Granule Secretion in Human Neutrophils

Christophe Pivot-Pajot,* Frederique Varoqueaux,§ Geneviève de Saint Basile,§ and Sylvain G. Bourgoin2*†

The neutrophil plays a central role in the innate host immune defense. Regulated exocytosis of its granules and release of antimicrobial and cytotoxic substances are key events to limit the spread of pathogens. However, the molecular mechanisms that control exocytosis of neutrophil granules are ill-defined. Recently, it was shown that Munc13-4 is essential for the priming of granules in several hematopoietic cells. In this study, we show that Munc13-4 is expressed in human neutrophils, and that its expression is increased during granulocytic differentiation of HL-60 and PLB-985 cells. Cell fractionation analysis reveals that Munc13-4 is mainly cytosolic and is recruited rapidly to membranes following stimulation with FMLF (N-formyl-methionyl-leucyl-phenylalanine). Moreover, a pool of Munc13-4 associated with mobilizable secondary and tertiary granules is relocalized to the plasma membrane after stimulation with FMLF. The FMLF-induced translocation of Munc13-4 is strictly dependent on calcium in neutrophils. C2 domains of Munc13-4 are essential for binding to phospholipid vesicles in a Ca2+-independent manner. Finally, down-regulation of Munc13-4 using small interfering RNA decreases exocytosis of tertiary granules in PLB-985 cells, whereas overexpression of Munc13-4 enhances secretion of MMP-9 (matrix metalloproteinase-9) from tertiary granules. Our findings suggest a role for Munc13-4 as a component of the secretory machinery in neutrophils. The Journal of Immunology, 2008, 180: 6786–6797.

polymeronuclear leukocytes (neutrophils) are the most abundant white cells found in peripheral blood and form the first line of cellular defense against infection. In inflammatory conditions, most neutrophil responses are dependent on the mobilization of cytoplasmic granules. Granule exocytosis is a critical event in converting inactive, circulating neutrophils to fully activated cells capable of chemotaxis, phagocytosis, and destruction of pathogens (1). Human neutrophils contain four distinct types of granules, which, upon stimulation, are transported to and fuse with the phagosome or the plasma membrane, allowing the release of their content and providing plasma membrane with functional proteins and receptors. Primary (azurophil) granules, secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles differ in their respective contents and mobilization hierarchy (2, 3). Regulatory mechanisms underlying mobilization of neutrophil granules and control of exocytosis are complex and ill-defined. Neutrophil activation in vitro by a stimulus like the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF) leads to the exocytosis of secondary and tertiary granules as well as secretory vesicles, whereas release of primary granule contents requires a pretreatment with cytochalasin B, an inhibitor of actin filament formation that acts as a priming reagent in neutrophils (4–7). The FMLF-mediated transient increase in cytosolic calcium is required for the mobilization of neutrophil granules (4, 8–10). However, synergistic signals generated through FMLF receptor activation seem to be required for full exocytosis (8, 11). Calcium may play a central role in promoting neutrophil degranulation. In particular, it may regulate the interactions between soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) located on granules/vesicles (v-SNAREs) and the plasma membrane (t-SNAREs) (1, 2, 12). In a variety of secretory cells, the formation of SNARE complexes is a major event preceding membrane fusion and mediator release (13, 14). A range of SNARE complexes is involved in secretory granule degranulation, and there is much evidence that indicates their implication in the regulation of granule fusion (13, 15–19).

Munc13 proteins constitute a family of four mammalian homologs of Caenorhabditis elegans Unc-13. With the exception of a ubiquitously expressed Munc13-2 splice variant, ubMunc13-2, also called hMunc13, Munc13-1/2/3 proteins are mainly expressed in brain. In several secretory cell types, especially in neuronal cells, chromaffin, and pancreatic β cells and in some hematopoietic cells, Munc13 proteins are key components of the secretory pathway (20–28). These proteins are important regulators of SNARE complex formation, and they are thought to be involved in promoting vesicle priming (29, 30). Munc13-4, the most recently identified Munc13 homolog, is highly expressed in hematopoietic cells (31). Munc13-4 exhibits the typical Munc13 domain structure with two Munc13 homology domains (MHDs) and two C2 domains known to bind lipids (32, 33). In patients suffering from familial hemophagocytic lymphohistiocytosis syndrome subtype 3, Munc13-4 is mutated and this deficiency results in defective...

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3 Abbreviations used in this paper: fMLF, N-formyl-methionyl-leucyl-phenylalanine; ADA, adenosine deaminase; CB, cytochalasin B; C2, C2 domains of Munc13-4 are essential for binding to phospholipid vesicles in a Ca2+-independent manner. Finally, down-regulation of Munc13-4 using small interfering RNA decreases exocytosis of tertiary granules in PLB-985 cells, whereas overexpression of Munc13-4 enhances secretion of MMP-9 (matrix metalloproteinase-9) from tertiary granules. Our findings suggest a role for Munc13-4 as a component of the secretory machinery in neutrophils. The Journal of Immunology, 2008, 180: 6786–6797.

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exocytosis of CTL lytic granules (23, 34). Munc13-4 is absolutely necessary for the regulated secretion of these cytotoxic granules at the priming stage of the exocytic pathway, downstream of the docking of vesicles with the plasma membrane. Several studies have confirmed the positive regulatory role of Munc13-4 in the secretion of dense granules in platelets, of basophilic secretory granules in mast cells, and of lytic granules in CTLs and NK cells (24–27).

Given that degranulation plays a key function in the physiology of the neutrophil, and because of its critical role in regulating the exocytosis of granules in several hematopoietic cells, we investigated whether Munc13-4 could regulate the exocytosis of the different granules of the neutrophil. In the present study, we show for the first time that a member of the Munc13 protein family is expressed in terminally differentiated human neutrophils, and we provide evidence for the involvement of Munc13-4 in neutrophil exocytosis.

Materials and Methods

Reagents

BAPTA/AM was purchased from Calbiochem. fMLF, cytochalasin B (CB), PMSF, ionomycin, dibutyryl cyclic AMP (dbcAMP), TCA solution, 1,3-diolein, t-α-phosphatidylserine, and DMSO were obtained from Sigma-Aldrich. Phosphatidylcholine and phosphatidylethanolamine were obtained from Avanti Polar Lipids. Adenosine deaminase (ADA) was from Roche Diagnostics. Dextran T-500 and Percoll were purchased from GE Healthcare. Aprotinin and leupeptin were purchased from Boehringer Ingelheim. Ficol-Paque and Mg2+-free HBSS were obtained from Wisent, and dextran sulfate (DSP) (DPF) was from Serva. His-tagged recombinant PKCα was purchased from Upstate Biotechnology. Fura 2-AM was obtained from Invitrogen.

Plasmid constructs

pDsRED2-N1-Munc13-4 vector was used as template for subcloning in pcDNA3.1/HisB (Invitrogen) and pAcHLT-C vectors. Deletion mutants were generated by PCR and confirmed by DNA sequencing. Mutant C2A corresponded to amino acids 1–267. The ΔC2A&B mutant was constructed by deleting amino acids 1–239 and 927-1090. The ΔC2A and the ΔC2B mutants were constructed by deleting amino acids 1–239 or 297-1090, respectively.

Antibodies

Anti-actin monoclonal and anti-lactoferrin Abs were purchased from Sigma-Aldrich. Anti-MMP9 Ab was obtained from Abcam, and anti-myeloperoxidase Ab was from Dako Canada. Anti-fibronectin I Ab was purchased from BD Biosciences, and FITC-anti-CD63 Ab was from Beckman Coulter Canada. Anti-His Ab was obtained from Santa Cruz Biotechnology, and anti-lactate dehydrogenase (LDH) Ab was from Fitzgerald. Anti-CD32A (FcεRIIA) is an IgG-purified fraction of a polyclonal rabbit antiserum against the cytoplasmic domain of CD32A (35). Polyclonal anti-Munc13-4 antisera was produced in rabbits by using the His-tagged N-terminal domain of Munc13-4 (amino acids 1–273) as immunogen. HRP-labeled donkey anti-rabbit and sheep anti-mouse IgGs were purchased from Jackson ImmunoResearch Laboratories and from GE Healthcare, respectively.

Isolation of human neutrophils

Venous blood was collected from healthy adult volunteers in isocitrate anticoagulant solution. Neutrophils were separated as described previously (36). Briefly, whole blood was centrifuged at 180 × g for 10 min, and the resulting platelet-rich plasma was discarded. Leukocytes were obtained following erythrocyte sedimentation in 2% dextran T-500. Mononuclear cells were removed by centrifugation on Ficoll-Paque cushions, and contaminating erythrocytes in the neutrophil pellets were removed by a 20 s hypotonic lysis in water. Neutrophils were resuspended in HBSS (pH 7.4) containing 1.6 mM CaCl2 but no MgCl2.

Transfection assays

Neutrophils (4 × 107 cells/ml) were treated with 1 mM DFP for 15 min at room temperature. The cells suspensions were centrifuged and resuspended in HBSS containing 1.6 mM CaCl2 or not at 107 cells/ml. After warming for 5 min at 37°C, neutrophils were treated 5 min with 10 μM CB and 0.1 U/ml ADA to eliminate endogenous adenosine, and stimulated with 10−7 M fMLF at 37°C for indicated times, or incubated with an equal volume of DMSO as control. In experiments with calcium chelator, cell suspensions were treated 30 min at 37°C in the dark with 10 μM BAPTA/AM. CB (10 μM) and ADA (0.1 U/ml) were added 5 min before stimulation with 10−7 M fMLF for 30 s. In experiments with ionomycin or thapsigargin, neutrophils were preincubated 5 min with 10 μM CB and 0.1 U/ml ADA before stimulation with 2 μM ionomycin or with 10−7 M thapsigargin for 10 min at 37°C. Incubations were stopped by diluting the cell suspensions 5-fold with ice-cold HBSS, and total membrane proteins were collected as described previously (36). Samples were assayed for protein content using the Bradford assay. Protein samples (30 μg) were resolved by SDS/PAGE and analyzed by Western blot.

Subcellular fractionation

Neutrophil subcellular organelles were isolated according to a procedure adapted from Kjeldsen et al. with modifications (37). Neutrophils (4 × 107 cells) were treated with 1 mM DFP for 15 min at room temperature. Cells were stimulated for 30 s with 100 nM fMLF at 37°C or with DMSO as control, and incubations were stopped by diluting the cells 5-fold with ice-cold HBSS. The cells were then centrifuged and resuspended in 10 ml ice-cold KCI-HEPES relaxation buffer (100 mM KCl, 50 mM HEPES, 5 mM NaCl, 1 mM MgCl2, 0.5 mM EGTA, 1 mM DFP, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 2.5 mM PMSF (pH 7.2)). Neutrophils were presurized (400 psi, 10 min) in a nitrogen bomb (Pan Instrument). Cavitates were centrifuged at 400 × g for 5 min to pellet the unbroken cells. Supernatants were laid onto 3 × 4.5 ml of a Percoll step gradient (1.050, 1.090, and 1.120 g/ml). After centrifugation (37,000 × g for 30 min), 18 fractions were collected (1 ml each), starting from the bottom of the tube. This procedure allows the distinct separation of primary, secondary, and tertiary granules, a plasma membrane-enriched fraction, and cytosol (37). Each fraction was centrifuged at 100,000 × g for 90 min to pellet Percoll. Fractions (50 μl) were aspirated with a syringe and processed for immunoblot analysis using marker proteins corresponding to individual compartments: myeloperoxidase (α-chain/azurophilic or primary granules), lactoferrin (β1-band/secondary or specific granules), gelatinase (β2-band/gelatinae or tertiary granules), CD32A (γ-band/plasma membrane), and LDH (cytosol).

Electrophoresis and immunoblotting

Proteins were analyzed on 7.5–20% gradient SDS-polyacrylamide gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Immunoblotting was performed using the indicated Abs and revealed with the HRP-conjugated secondary anti-rabbit or anti-mouse Abs (1:20,000) and the Renaissance detection system (NEN/PerkinElmer Life Sciences).

Cell culture and transfection

PLB-985 cells (German Collection of Microorganisms and Cell Culture) and HL60 cells (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% FBS and 2 mM l-glutamine at 37°C in a humidified atmosphere of 5% CO2. To induce a neutrophil-like phenotype, cells were cultured in medium supplemented with 0.3 mM dbcAMP for 3 days. PLB-985 cells were transiently transfected using the Nucleofector system from Amaxa Biosystems. Cells were transfected with DNA after 2 days of differentiation. Cells (105) were suspended in 100 μl of nucleofection buffer containing 8 μg of pcDNA Munc13-4 plasmid or empty vector as control, and transfections were performed with the electrical setting U-02. After nucleofection, the cells were immediately transferred to prewarmed complete medium containing 0.3 mM dbcAMP. Cells were harvested 24 h after transfection and resuspended in HBSS containing 1.6 mM CaCl2. For experiments with small interfering RNA (siRNA), 2 × 106 cells were transfected after 1 day of differentiation with 20 nM Munc13-4-specific siRNA (Qiagen) or nonsilencing siRNA (AllStars negative control siRNA, Qiagen) in 100 μl of nucleofection buffer and using the Nucleofector program U-02. The Munc13-4 siRNA sequence used was 5’-ggcagagcagccggagaa. After nucleofection, cells were resuspended in complete medium in the presence of 0.3 mM dbcAMP. Cell functions were monitored at 48 h posttransfection.

Expression and purification of Munc13-4 proteins

Munc13-4 cDNAs were inserted into the pAcHLT-C baculovirus shuttle vector and cotransfected with linearized BaculoGold viral DNA (BD Biosciences) into S9 insect cells. Culture supernatants were used to infect S9 cells with a multiplicity of infection of >1. Insect cells were collected 48 h
the HEPES buffer described above. Munc13-4 and PKC
antibodies were used to analyze the supernatants (SN) and the pellets (P) were analyzed by immunoblotting with
(MMP-9) by ELISA (RayBiotech).

was analyzed by monitoring the release of matrix metalloproteinase-9
was preincubated in HBSS containing 1.6 mM CaCl2 for 5 min at 37°C in
the presence of 0.1 U/ml ADP and 10 µM CB. Cell suspensions were stimulated with 10⁻⁷ M FMLF for the indicated time or with DMSO as control, and the incubations were stopped as described in Materials and Methods. Membrane fractions (30 µg of protein per lane) were analyzed by Western blot using Abs against Munc13-4 and Flotillin1 (loading control). A. The immunoblots shown are representative of at least three independent experiments. B. The data from densitometric analyses are expressed as fold increase relative to DMSO control, and are the means ± SD from at least three experiments.

Measurement of the intracellular calcium concentration
Changes in intracellular calcium concentrations were monitored by measuring Fura-2 fluorescence using a Fluorolog-3 spectrofluorimeter (HORIBA Jobin Yvon). Fluorescence was recorded at 510 nm with alternating excitation wavelengths of 340 and 380 nm. Intracellular calcium concentrations were calculated using the following formula: 224(y–Fmin)/(Fmax–y), in which y represents the fluorescence of the samples. Fmin was obtained by adding 2.5 µM calcium ionophore to the cells and Fmax was obtained by adding 25 mM MnCl2. Differentiated PLB-985 cells and blood neutrophils (10⁷ cells/ml) were loaded with 1 µM Fura 2-AM, washed twice, and then transferred to the spectrofluorometer. The cell suspension was continuously stirred and maintained at 37°C while Fura-2 fluorescence measurements were made. Cells were stimulated with 10⁻⁷ M FMLF or with 2 µM ionomycin at the time indicated by the arrow.

RT-PCR
Total RNA was extracted from neutrophils, dbcAMP-differentiated PLB-985, human cortex, and cerebellum using TRIzol reagent (Invitrogen). RNA samples were subjected to DNase treatment (Promega) for 30 min at 37°C (1 unit per µg of RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) using random hexamer primers. The following primers were used to amplify human Munc13-1/2/3/4 and GAPDH as control; Munc13-1, 5′ primer TGGTGGACTGAGGCGTTGGGTTGTGAC GTAG, 3′ primer GGCGACACTCCTCTCCCATCCGCAGC; Munc13-2, 5′ primer CGCGGCCATCACGCCACAGTTTCC. PCR reactions were conducted using 5 µl of cDNA, and PCR products were separated on a 2% agarose gel.

Immunofluorescence analysis
dbcAMP-differentiated PLB-985 were treated for 5 min at 37°C with 10 µM CB, and stimulated for 30 s with 10⁻⁷ M FMLF or DMSO as control. Cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.1% Triton X-100 for 15 min. Blocking was performed for 20 min with 10% goat serum and 10% human serum in PBS. After washing with PBS, cells were incubated at room temperature for 1 h with FITC-
anti-CD63 and affinity purified anti-Munc13-4 Abs (1 µg of each Ab diluted in PBS containing 5% goat serum, 5% human serum, and 0.1%
Munc13-4 expression in human neutrophils and during granulocytic differentiation

Munc13-4 is expressed in several hematopoietic cell types but its expression in neutrophils has not yet been reported. To address this issue, we first generated an Ab against the N-terminal region of human Munc13-4. Fig. 1A shows that this Ab recognizes specifically a band at 120 kDa that corresponds to Munc13-4 overexpressed in CHO cells. We found a high expression of Munc13-4 in platelets and lymphocytes/monocytes, and a lower level of expression in neutrophils. Next, we examined Munc13-4 expression during granulocytic differentiation by using the human myeloid cell lines HL60 and PLB-985. Granulocytic differentiation of HL60 and PLB-985 cells to induce a neutrophil-like functional phenotype was achieved by treating cells with dbcAMP for 3 days. Western blot analyses of cell lysates revealed that Munc13-4 expression was up-regulated during differentiation of both cell lines (Fig. 1B), suggesting a role for the protein in the functional responses of mature granulocytes. A similar increase in Munc13-4 expression was observed in DMSO-differentiated cells (data not shown). To analyze the subcellular distribution of Munc13-4, neutrophils were disrupted by sonication or solubilized in buffer containing 1% Triton. As shown in Fig. 1C (left panel), Munc13-4 was predominantly localized in the soluble fraction (SN) of sonicated neutrophils, whereas a second pool of the protein was recovered in the crude membrane fraction (P). When neutrophils were lysed in buffer containing detergent, the totality of Munc13-4 was localized in the soluble fraction (Fig. 1C, right panel). As control, the transmembrane receptor CD32A (FcγRIIA) was found exclusively in membrane fraction of sonicated neutrophils and was efficiently solubilized when neutrophils were lysed with Triton.

fMLF-induced Munc13-4 translocation to the membranes

We next investigated the subcellular distribution of Munc13-4 in neutrophils stimulated with the chemotactic factor fMLF. Fig. 2A shows that Munc13-4 in neutrophils stimulated with fMLF was translocated to the membranes. This was confirmed using membrane fractions prepared as described in Materials and Methods and analyzed by Western blot using Abs against Munc13-4 and Flotillin1. The immunoblots shown are representative of at least three independent experiments. The data from densitometric analyses are expressed as fold increase relative to DMSO control, and are the means ± SD from at least three experiments. Means were compared using a Student’s t test. The differences between indicated conditions were statistically significant with p < 0.05 (⁎).


FIGURE 4. C2 domain-dependent and Ca\(^{2+}\)-independent Munc13-4 binding to phospholipid vesicles. A, Domain organization of Munc13-4 and truncation constructs. The positions of the two C2 domains (C2A and C2B) as indicated by amino acid number were obtained by submitting the Munc13-4 sequence to the SMART server. B, Expression of PKC\(\alpha\) and Munc13-4 constructs purified from Sf9 cells as described in Materials and Methods. Purified fractions for each construct were analyzed by Western blot using anti-His tag Ab. C, Binding of PKC\(\alpha\) and Munc13-4 constructs to phospholipid vesicles in the presence of 0, 1, or 50 \(\mu\)M CaCl\(_2\). Binding was analyzed by Western blot using anti-His tag Ab. The immunoblots shown are representative of at least three independent experiments.

shows that Munc13-4 rapidly translocates to the crude membrane fraction in response to stimulation with fMLF. Maximal recruitment of Munc13-4 to membranes was achieved 30 s after stimulation and returned to near basal membrane levels by 2 min. As shown in Fig. 1C, a small amount of Munc13-4 was associated with the membrane in unstimulated neutrophils (control DMSO). In contrast to fMLF, the phorbol myristate acetate (PMA), which induced Munc13-1 translocation to the membrane, did not stimulate the translocation of Munc13-4 (data not shown).

**Calcium-dependent recruitment of Munc13-4 to membranes**

Thereafter, we attempted to elucidate the mechanism by which Munc13-4 translocates to membranes. Because one of the early neutrophil responses to chemotactic peptides is a transient increase in the levels of cytosolic calcium ([Ca\(^{2+}\)]\(_{i}\)), we examined the calcium dependence of fMLF-induced Munc13-4 translocation. Fig. 3A shows that treatment of neutrophils with the permeable calcium-free chelator BAPTA/AM abolishes the fMLF-induced Munc13-4 translocation. To analyze the role of extracellular Ca\(^{2+}\), cells were resuspended in Ca\(^{2+}\)-free HBSS immediately before stimulation with fMLF. As shown in Fig. 3B, Munc13-4 translocation was significantly impaired by the removal of Ca\(^{2+}\) from the incubation medium. To analyze further the role of calcium and distinguish it from other fMLF receptor-induced transduction pathways, we evaluated the effect of ionomycin, a calcium ionophore that bypasses the receptor activation step, on the subcellular distribution of Munc13-4 in neutrophils. As described previously (38), fMLF induced a rapid and transient increase in [Ca\(^{2+}\)]\(_{i}\), whereas ionomycin induced a sustained and long-lasting increase in [Ca\(^{2+}\)]\(_{i}\) (Fig. 3C). Interestingly, treatment with ionomycin induced a significant translocation of Munc13-4 to the membrane (Fig. 3D). As observed for fMLF, ionomycin-induced Munc13-4 translocation was dependent on the presence of calcium in the extracellular medium. These results indicate that the elevations in [Ca\(^{2+}\)]\(_{i}\) induced by fMLF and ionomycin are sufficient to promote Munc13-4 translocation to the membranes.

**Calcium-independent binding of Munc13-4 C2 domain to lipid**

Thereafter, we investigated the ability of Munc13-4 to bind phospholipids. Munc13-4 has no transmembrane domain and also lacks the Munc13-1 typical N terminus with the phorbol ester-binding C1 domain. However, it contains two C2 domains (31). C2 domains are calcium-dependent phospholipid-binding motifs that can also function in a calcium-independent manner and mediate protein-protein interactions (33). To determine whether the Munc13-4 C2 domains can bind to liposomes and whether the interaction is calcium-dependent, we expressed wild-type Munc13-4 or various mutants deleted of C2 domains in Sf9 cells (Fig. 4A). Proteins were affinity purified and similar amounts were used for the liposome binding assay (Fig. 4B). Despite numerous attempts testing several culture and infection conditions, we were not able to produce the Munc13-4 C2B domain in Sf9 cells. Fig. 4C shows that the binding of the C2A domain to liposomes is similar to that seen for the full-length protein. Deletion of C2A or C2B domains markedly reduced the binding of these Munc13-4 mutants to liposomes. Moreover, deletion of both C2 domains abrogated almost completely the binding of Munc13-4 to liposomes. Interestingly, the in vitro binding of Munc13-4 to these defined liposomes did not require calcium. In contrast and as previously reported (39), PKC\(\alpha\), which contains a C2 domain known to bind lipid in a calcium-dependent manner, bound to liposomes in the presence of calcium. These results suggest that Munc13-4 C2 domains can promote Munc13-4 vesicle binding in a calcium-independent manner, whereas its translocation from cytoplasm to membranes takes place in a calcium-dependent manner in intact neutrophils. It is not excluded that calcium-dependent events would be required for the mobilization and fusion of Munc13-4-positive granules with the plasma membrane.
**Munc13-4 association with human neutrophil granules**

To analyze further the subcellular localization of Munc13-4 and determine the nature of the membranes to which Munc13-4 is associated with and recruited to, we separated the different granule populations of human neutrophils using a three-layer Percoll gradient (37). Gradient fractions were collected and characterized by immunoblotting for granule-specific and plasma membrane markers. Fig. 5A shows a typical separation profile of resting neutrophils on a three-layer Percoll gradient. The α-band contained primary granules and was enriched in myeloperoxidase (MPO), the β1-band contained secondary granules and was enriched in lactoferrin (LF), and the β2-band contained tertiary granules and was enriched in gelatinase (MMP-9). The marker of primary granules, MPO, was also recovered in a small population of granules (fractions 7–9) that colocalized between the secondary and tertiary granule fractions on the three-layer Percoll gradient. FcγRIIA (CD32A) was recovered in the plasma membrane fraction (α-band), and LDH in the cytosol. Immunoblot analyses revealed the presence of Munc13-4 in secondary (fractions 6 and 7) and tertiary granules (fractions 8 and 9), in the plasma membrane fractions (fractions 10 and 11), and in cytoplasmic fractions (fractions 13–18) (Fig. 5B, upper panel). Stimulation with fMLF resulted in the total disappearance of Munc13-4 from the secondary granule fractions, and in a reduction of Munc13-4 associated with tertiary granule fractions and present in the cytosolic fractions. This was concomitant with a relocationalization of the protein to the plasma membrane fractions (Fig. 5B, lower panel). The distribution of the granule markers was not significantly altered following stimulation with fMLF (Fig. 5C). These results suggest that in human neutrophils a pool of Munc13-4 is associated with secondary and with tertiary granules that are mobilized to the plasma membrane upon fMLF stimulation. We cannot exclude the possibility that a cytoplasmic pool of Munc13-4 is also directly recruited to the plasma membrane in response to stimulation with fMLF.

**Munc13-4-dependent exocytosis of tertiary granules in neutrophil-like-differentiated PLB-985 cells**

The localization of Munc13-4 to fMLF-recruitable secondary and tertiary granules led us to investigate whether Munc13-4 has a functional role in neutrophil granule exocytosis. To this aim, we used neutrophil-like-differentiated PLB-985 cells, which were shown to be a suitable cellular model to study fMLF-mediated degranulation (40–42). To induce granulocytic differentiation of PLB-985 cells toward the neutrophil-like phenotype, 0.3 mM...
dbcAMP was added to the culture for 3 days. The differentiation into neutrophil-like cells was demonstrated by showing an increase in CD32A expression (Fig. 6A) and by measuring fMLF-induced calcium mobilization (Fig. 6B). In undifferentiated PLB-985 cells, no calcium mobilization was observed in response to fLMF, whereas in differentiated PLB-985 cells as well in blood neutrophils, a rapid and transient increase in $[Ca^{2+}]_{i}$ was measured. We then evaluated other functional responses of these differentiated PLB-985 by measuring fMLF-induced increase in cell-surface expression of CD63, a specific primary granule marker, and CD11b, a marker located in secondary and tertiary granules as well as in secretory vesicles. As shown in Fig. 6C, stimulation with fMLF enhanced the expression of both CD63 and CD11b at the cell surface relative to cells incubated with DMSO. The fMLF-induced expression of CD63 at the surface of differentiated PLB-985 cells was similar to that obtained with neutrophils, whereas that of CD11b was weaker. No increase in cell surface expression of the specific secondary granule marker CD66b was observed. Similarly, lactoferrin, which is normally present in neutrophil secondary granules, was not secreted by differentiated PLB-985 cells, suggesting the absence of secondary granules in differentiated PLB-985 cells. Altogether, these results reflect the efficient terminal granulocyte maturation of differentiated PLB-985 cells and a normal degranulation capacity of primary and tertiary granules, as well as secretory vesicles.

In a first approach, we used RNA interference to selectively decrease Munc13-4 expression in PLB-985 cells transfected with Munc13-4-specific siRNA. As shown in Fig. 7A, the silencing approach resulted in a dramatic decrease in the level of Munc13-4 expression, whereas control siRNA had no effect. Exocytosis of primary granules was analyzed by measuring up-regulation of the specific primary granule membrane marker CD63 at the cell surface. Knockdown of Munc13-4 with siRNA inhibited fMLF-induced CD63 up-regulation by 15% ($p < 0.05$) (Fig. 7B). We also monitored primary granule exocytosis by analyzing the release of MPO in cell supernatants. As shown in Fig. 7C, the fMLF-induced MPO secretion was reduced by $\sim 15\%$ in Munc13-4 siRNA-transfected cells when compared with control siRNA-transfected cells. Degranulation of tertiary granules was analyzed by measuring the release of gelatinase (MMP-9) in cell supernatant with an ELISA assay. Thereafter, we also found that the fMLF-mediated MMP-9 secretion was significantly decreased by treatment with Munc13-4 siRNA when compared with nonsilencing siRNA control-transfected cells (Fig. 7D). In a complementary approach, we overexpressed Munc13-4 by transfecting differentiated PLB-985 cells with pcDNA3.1 Munc13-4. Protein expression was examined by immunoblotting with an anti-His Ab (Fig. 7E), and fMLF-induced MMP-9 secretion was analyzed. As shown in Fig. 7F, Munc13-4 overexpression resulted in a marked increase in MMP-9 secretion. Taken together, these results suggest an important role for Munc13-4 in the degranulation of neutrophils, especially in the exocytosis tertiary granules.

Subcellular localization of Munc13-4 in dbcAMP-differentiated PLB-985 cells

Despite the lack of association of Munc13-4 with primary granules in resting or stimulated neutrophils, we observed that Munc13-4 silencing led to inhibition of primary granule exocytosis in differentiated PLB-985 cells. The different granule populations of PLB-985 cells could not be separated using the three-layer Percoll gradient technique (data not shown). To circumvent this problem and to understand the potential contribution of Munc13-4 to primary granule exocytosis, we examined the subcellular localization of CD63, a specific primary granule marker, and of Munc13-4 in dbcAMP-differentiated PLB-985 cells by immunofluorescence microscopy. Confocal imaging on resting cells revealed that Munc13-4 and CD63 were diffusely distributed throughout cells.
Fifteen hours after knockdown, weak colocalization was observed in overlaid images (Fig. 8, A and B). Stimulation of PLB-985 cells with fMLF induced a marked relocalization of both Munc13-4 and CD63 to the cell periphery at the level of the plasma membrane (Fig. 8, D and E) and a significant increase of overlap (yellow) in their distribution. Primary granule secretion was also determined by analyzing MPO release. Supernatants from fMLF- and DMSO-treated cells were precipitated with 20% TCA, and protein pellets were analyzed by Western blot using an anti-MPO Ab (C). MMP-9 in the supernatants of fMLF- and DMSO-treated cells was measured by ELISA as described in Materials and Methods (D).

 FIGURE 7. Involvement of Munc13-4 in the secretion of neutrophil granules. A, Munc13-4 knockdown in differentiated PLB-985 cells. Munc13-4 siRNA (Munc siRNA) and negative control siRNA (Ct siRNA) were transfected into PLB-985 cells as described in Materials and Methods. Munc13-4 expression was analyzed 48 h posttransfection by immunoblotting with anti-Munc13-4 and anti-actin (loading control) Abs. B–D, Effect of Munc13-4 knockdown on secretion of primary and tertiary granules. Forty-eight hours after Munc13-4 or control siRNA transfection, PLB-985 cells were resuspended in HBSS containing 1.6 mM CaCl2 and stimulated for 30 min with 10−7 M fMLF or with DMSO. Cell-surface CD63 expression was determined by FACS as described in Materials and Methods (B). Primary granule secretion was also determined by analyzing MPO release. Supernatants from fMLF- and DMSO-treated cells were precipitated with 20% TCA, and protein pellets were analyzed by Western blot using an anti-MPO Ab (C). MMP-9 in the supernatants of fMLF- and DMSO-treated cells was measured by ELISA as described in Materials and Methods (D). E, Munc13-4 overexpression in neutrophil-like differentiated PLB-985 cells. pcDNA-M unc13-4 and control vector were transfected into differentiated PLB-985 cells, and Munc13-4 expression was analyzed 24 h after transfection by Western blot using anti-His Ab. F, Effect of Munc13-4 overexpression on MMP-9 secretion. Twenty-four hours after transfection, PLB-985 cells were resuspended in HBSS. Cells were stimulated as described in B, and MMP-9 was quantified as in C, A, C, and E. Representative autoradiograms of three independent experiments are shown. B, D, and F. Data represent the means ± SD of three independent experiments done in duplicates. Means were compared using a Student’s t test. The differences between indicated conditions were statistically significant with p < 0.05 (*).

fMLF-induced ubMunc13-2 translocation to the plasma membrane

Because the knockdown of Munc13-4 expression had only a modest effect on primary and tertiary granule secretion in differentiated PLB-985 cells, we wondered whether some functional redundancy due to another Munc13 isoform could exist. To address this issue, we looked for Munc13 isoform expression in dbcAMP-differentiated PLB-985 cells and human neutrophils. The expression pattern of the genes encoding Munc13-1, ubMunc13-2, and Munc13-3 was analyzed by RT-PCR in neutrophils, differentiated PLB-985 cells, and brain tissues. Fig. 9A shows that the isoform ub-Munc13-2 is expressed in neutrophils as well as in differentiated PLB-985 cells. No expression of Munc13-1 and Munc13-3 was observed. As reference, Munc13-4 and GAPDH expression was also monitored. The expression of Munc13 isoforms in human brain tissues was similar to those described in rat brain by Augustin et al. (43). The expression of ubMunc13-2 was confirmed by Western blot (Fig. 9B). Higher levels of ubMunc13-2 were detected
in differentiated PLB-985 cells as compared with human neutrophils. Moreover, ubMunc13-2 was predominantly localized in the soluble fraction (SN) of sonicated neutrophils as well as of PLB-985 cells (Fig. 9C). In contrast, CD32A was recovered exclusively in the membrane fraction (P), as already shown in Fig. 1C. We next investigated subcellular distribution of ubMunc13-2 in resting or fMLF-stimulated neutrophils. Immunoblots of fractions obtained following fractionation of neutrophils on Percoll gradients revealed that ubMunc13-2 was not associated with any granule populations (fractions 1–8), but was only recovered in the cytosolic fractions (fractions 12–18) of resting neutrophils (Fig. 9D, upper panel). Following stimulation with fMLF, ubMunc13-2 was recruited to the CD32A positive plasma membrane fractions (Fig. 9D, middle and lower panels). These results suggest that ubMunc13-2 could also act as a component of exocytic signaling machinery following its translocation to the plasma membrane.

Discussion
Neutrophils are the first line of defense against invading microorganisms. Mobilization and exocytosis of cytoplasmic granules play crucial roles in most of the neutrophil functions leading to the destruction of pathogens. Nevertheless, exocytosis of neutrophil granules must be tightly regulated to prevent uncontrolled release of components that may damage tissues. Many studies have increased our understanding of the molecular mechanisms controlling exocytosis of neutrophil granules, but these mechanisms remain incompletely defined. The data reported in the present study

**FIGURE 8.** Subcellular localization of Munc13-4 and CD63 in dbcAMP-differentiated PLB-985 cells. Immunofluorescence images of CD63 (green) and Munc13-4 (red), dbcAMP-differentiated PLB-985 cells were preincubated for 5 min at 37°C in the presence of 10 μM CB and then stimulated with 10^{-7} M fMLF for 30 s or with DMSO as control. Cells were fixed, permeabilized, and stained with FITC-anti-CD63 Ab, anti-Munc13-4 Ab, and Alexa Fluor 594-conjugated anti-rabbit IgG as indicated in Materials and Methods. Scale bars represent 12 μm. Images are representative of three independent experiments.

**FIGURE 9.** fMLF-induced membrane translocation of ubMunc13-2 in neutrophils. A, Analysis of Munc13 isoform expression in neutrophils and dbcAMP-differentiated PLB-985 cells. PCR using specific primers for Munc13-1, ubMunc13-2, Munc13-3, and Munc13-4, as well as GAPDH as control, were performed on reverse-transcription products (+) obtained from total RNA of the indicated cells and brain tissues, in comparison to negative controls (−) without (−) reverse transcriptase (RT). B, ubMunc13-2 expression in dbcAMP-differentiated PLB-985 cells and neutrophils. Protein samples (30 μg per lane) were resolved by SDS-PAGE (8%) and analyzed by Western blot using Abs against ubMunc13-2 and actin (loading control). C, Analysis of ubMunc13-2 subcellular localization. Neutrophils and dbcAMP-differentiated PLB-985 cells were sonicated. Cytosolic and membrane fractions were prepared as indicated in Materials and Methods. Protein samples (30 μg) from the supernatants (SN) and the pellets (P) were analyzed by immunoblotting with anti-ubMunc13-2 and anti-CD32A (FcγRIIA) Abs. D, ubMunc13-2 was detected by immunoblotting of fractions purified from nontreated (control DMSO) or fMLF-stimulated (10^{-7} M for 30 s) neutrophils as shown in Fig. 5A. Localization of plasma membranes in gradient fractions is indicated by CD32A immunoblot. The data shown are representative of three independent experiments with similar results.
show for the first time that Munc13-4, a member of the Munc13 protein family, is expressed in human neutrophils. We have conducted a cellular and subcellular characterization of Munc13-4 in resting and stimulated human neutrophils, and we provide evidence for a role of Munc13-4 in fMLF-induced neutrophil secretion.

Munc13-4 function was first described in CTLs, and its expression is abundant in hematopoietic tissues such as the spleen and thymus (23). Munc13-4 expression is not restricted to CTLs, and it has been reported in other hematopoietic cells, including platelets, mast cells, and NK cells (24, 25, 27). In particular, in platelets, Munc13-4 is equally distributed between cytosolic and membrane fractions, but it fails to associate with dense core granules (24).

Conversely, in mast cells, Munc13-4 localizes exclusively with secretory lysosomes (26). Munc13-4 is found in the cytosol and associates with cytotoxic granules when transiently overexpressed in CTLs (23). In our study, we found lower levels of Munc13-4 in neutrophils, as compared with those in lymphocytes/monocytes and platelets. However, its expression is markedly up-regulated during granulocytic differentiation. We found that in resting neutrophils, Munc13-4 is in part cytoplasmic and associated with secondary and tertiary granules. A small pool of Munc13-4 colocalizes with plasma membrane markers under basal conditions. The fractionation method we used did not allow us to separate the plasma membrane from the secretory vesicles. Therefore, we cannot exclude an association of Munc13-4 with secretory vesicles. Nevertheless, our findings are consistent with a recent proteomic analysis of neutrophil granule proteins that identified the presence of Munc13-4 in tertiary granules (44).

Although Munc13-4 is present in the granules of several hematopoietic cells, there is little information about its recruitment to the plasma membrane during exocytosis. The neutrophils represents an interesting prototype of cell for the study of exocytosis, as it contains four different classes of granules that undergo regulated exocytosis. A major finding of our study is that upon stimulation with fMLF, cytosolic and granule-associated Munc13-4 translocates to plasma membranes. In human neutrophils, fMLF-stimulated exocytosis is completed within 30 s (8). This is consistent with the kinetics of Munc13-4 translocation to the plasma membrane, which peaks at 15–30 s. In neuronal and chromaffin cells, Munc13-1 isoform translocates to the plasma membrane upon stimulation with PMA (21, 45). In contrast to other Munc13 isoforms, Munc13-4 lacks the long N terminus containing the phosphor- ester-binding C1-domain. Interestingly, we observed no membrane translocation of Munc13-4 upon neutrophil stimulation with PMA (data not shown), suggesting that the translocation and membrane association of Munc13-4 involve different mechanisms than those reported for Munc13-1.

Our study suggests that the redistribution of Munc13-4 at the plasma membrane may act as a priming factor for the exocytosis of tertiary granules, at least in neutrophil-like-differentiated PLB-985 cells. A fraction of Munc13-4 associated with secondary, with tertiary granules, and possibly with secretory vesicles is recruited to the plasma membrane upon stimulation of neutrophils with fMLF. The functional relevance of Munc13-4 granule association was examined in dbcAMP-differentiated PLB-985 cells. Using MPO and MMP-9 as specific markers for primary and tertiary granule exocytosis, respectively, we observed that silencing of Munc13-4 by siRNA in these cells decreases fMLF-mediated MPO and MMP-9 release. Secretion of secondary granules could not be investigated because PLB-985 cells do not possess these granules (46). Despite its association with these granules in neutrophils, it was not possible to determine whether Munc13-4 plays a role in secondary granule exocytosis using PLB-985 cells. Although the silencing of Munc13-4 is achieved to almost 90%, it results in a reduction of only 15% of primary and tertiary granule exocytosis in this cell model. We cannot exclude that the residual amounts of Munc13-4 would be sufficient to regulate exocytosis. In contrast, we do not disregard the possibility that Munc13-4 regulates exocytosis of only a certain pool of primary and tertiary granules, or that regulation of exocytosis of these granules could involve additional exocytic pathways or other effectors. We show that another isoform of Munc13 proteins, ubMunc13-2, is also expressed in neutrophils and differentiated PLB-985 cells. ubMunc13-2 is a cytosolic protein that translocates to the plasma membrane in response to fMLF stimulation, where it could act as a component of regulated exocytosis in neutrophils. As suggested by Augustin et al. for other Munc13 isoforms in brain structures, a partial functional redundancy between Munc13-4 and ubMunc13-2 could explain in part the significant but weak inhibitory effect of Munc13-4 silencing (43). Further studies should provide interesting new insight into ubMunc13-2 function in human neutrophils. Despite the fact that subcellular fractionation demonstrated that Munc13-4 is not constitutively associated with primary granules of unstimulated cells, silencing of Munc13-4 results in reduction of exocytosis of these granules. Recently, Munafò et al. have shown that in neutrophils, MPO was distributed into two populations of granules (47). These observations are consistent with a previous study that suggested that primary granules are heterogeneous in terms of density (48). The neutrophil fractionation method we used (three-layer Percoll gradient) allowed us to distinguish these two populations. We detected a major MPO-containing granule population and a minor MPO-containing granule population (Fig. 5). Our observation that only a minor subpopulation of MPO-positive granules is associated with Munc13-4 suggests that Munc13-4 knockdown only affects the exocytosis of this subpopulation of granules. This hypothesis can explain the 15% inhibition of MPO release we have observed. Moreover, using immunofluorescence microscopy, we observed in dbcAMP-differentiated PLB-985 cells that Munc13-4 colocalization with primary granule markers was increased at the level of the plasma membrane after fMLF stimulation. Thus, it is possible that cytoplasmic Munc13-4, which is mobilized to the plasma membrane in response to stimulation with fMLF, is required for primary granules fusion with the plasma membrane. Alternatively, we cannot exclude the possibility that Munc13-4 is recruited first to primary granule membrane and thereby participates in the regulation of exocytosis of these granules. Our experiments cannot distinguish between these two scenarios. In CTLs and NK cells, Munc13-4 is involved in the priming of cytotoxic granules (23, 27). In neutrophil-like PLB-985 cells, overexpression of Munc13-4 clearly indicates a role for Munc13-4 in the release of tertiary granule protein content such as MMP-9.

A common characteristic of exocytosis in many cellular systems is its regulation by [Ca2+]i (49). In neutrophils, elevation of [Ca2+]i is one of the earliest cellular events induced by exposure to fMLF, and it constitutes a crucial event for degranulation. Using calcium chelator, IMLF, or calcium ionophore stimulation, we demonstrated that Munc13-4 translocation to the membranes is strictly dependent on the [Ca2+]i elevation. The importance of this [Ca2+]i elevation is supported by the finding that neutrophil stimulation with PMA, which does not trigger calcium mobilization in cells, is unable to induce Munc13-4 translocation. This suggests that extracellular calcium may play an important role in promoting Munc13-4 recolonization to the membranes.

Translocation of Munc13-1 to the membrane is not induced by calcium ionophores (45). Moreover, Munc13-1, Munc13-2, and Munc13-3 C2 domains bind to phospholipids in a calcium-independent manner (50). Very few C2 domain-containing proteins
have been reported to bind phospholipid in a calcium-independent manner. The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), whose C2 domain lacks the canonical calcium binding motif, is one of them (51). Munc13-4 C2 domains display two putative calcium binding sites, but the insertion of a large α-helix within the C2A domain suggests that this domain may promote membrane or protein interaction independently of calcium binding (23). Neef and coworkers have investigated the colocalization of serotonin and several Munc13-4 deletion constructs by immunofluorescence in mast cells, and found that the MHDs but not the C2 domains were required for Munc13-4 targeting to secretory lysosomes (26). Recently, similar observations were made in CTLs. The MHD1–MHD2 region allows the vesicular localization of Munc13-4, whereas the C2 domains displayed essentially a cytosolic localization (52). However, using a liposome binding assay, we demonstrated that Munc13-4 has the capacity to bind phospholipid in a calcium-independent manner and that both C2 domains were required for Munc13-4 binding to liposomes. The reasons for this discrepancy are not clear, but they could be the result of differences in the assay conditions used. Further studies are needed to evaluate more precisely the contribution of C2 and MHDs of Munc13-4 in membrane binding. In the present study, we found that IMLF-mediated redistribution of cytosolic and granule-associated Munc13-4 to the plasma membrane is a calcium-dependent event in neutrophils. In contrast, we showed that Munc13-4 binds to phospholipids in a calcium-independent manner. It seems that Munc13-4 translocation toward the plasma membrane occurs by a calcium-dependent mechanism; whereas the subsequent binding to membrane phospholipids takes place in a calcium-independent manner. Munc13-4 association with secondary and tertiary granules in resting neutrophils in the absence of elevated intracellular calcium is consistent with the observation that Munc13-4 membrane binding does not require calcium. However, we cannot also exclude an interaction of Munc13-4 with proteins that function as cytosolic calcium sensors.

The final step of vesicle transport is docking to and fusion with the target membrane, which, according to the SNARE hypothesis, is mediated by formation of trans-SNARE complex (53). In neuronal cells, the formation of the SNARE complex is regulated by several factors such as Munc18 homologs and Munc13-1. Munc18-1 binds tightly to syntaxin 1 and prevents SNARE complex assembly. Munc13-1 is generally thought to mediate dissociation of Munc18-1 from syntaxin 1, thereby promoting trans-SNARE complex formation (54, 55). A range of SNARE proteins homologous to those found in neuronal tissue are present in neutrophils, and studies have shown that exocytosis of the different granules is regulated in a differential manner by the formation of combinatorial SNARE complexes (13, 15, 17–19, 56, 57). The conserved C-terminal region of Munc13-4 contains a domain that is homologous to the syntaxin-1-binding site of Munc13-1 (29). Interestingly we detected the presence of Munc18 isoforms in neutrophils (our unpublished data). As previously reported for Munc13-1 in neuronal cells, regulation by Munc13-4 of an interaction between Munc18 and syntaxin in neutrophils may be required for docking and granule fusion with the target membrane.

Munc13-4 is an effector of the small GTPase Rab27a, and Munc13-4 binding to active GTP-bound Rab27a constitutes a critical event in the exocytic mechanism of hematopoietic cells (24–26, 52). Rab27a is expressed in human neutrophils and associates with the three types of granules (Refs. 40, 43 and our unpublished data). As previously described (24), we were also able to pull down Munc13-4 from neutrophil lysates using recombinant GTP-loaded Rab27a. The next step would be to investigate whether Munc13-4 and Rab27a interaction takes place in neutrophils, and what could be the functional relevance of this interaction in granule exocytosis.

The data reported in the present study suggest that Munc13-4 is involved in regulation of exocytosis of tertiary cytoplasmic granules in human neutrophils. Uncontrolled release of soluble mediators is likely to cause toxic effects and exacerbate inflammation. It is therefore important to understand the molecular mechanisms of neutrophil secretion. Moreover, the proteins implicated in the regulation of exocytosis may represent new targets for antiinflammatory treatments.

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Disclosures

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