Role of Vesicle-Associated Membrane Protein-2, Through Q-Soluble *N*-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor/R-Soluble *N*-Ethylmaleimide-Sensitive Factor Attachment Protein Receptor Interaction, in the Exocytosis of Specific and Tertiary Granules of Human Neutrophils[1]

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We have examined the role of the R-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) synaptobrevin-2/vesicle-associated membrane protein (VAMP)-2 in neutrophil exocytosis. VAMP-2, localized in the membranes of specific and gelatinase-containing tertiary granules in resting human neutrophils, resulted translocated to the cell surface following neutrophil activation under experimental conditions that induced exocytosis of specific and tertiary granules. VAMP-2 was also found on the external membrane region of granules docking to the plasma membrane in activated neutrophils. Specific Abs against VAMP-2 inhibited Ca^{2+} and GTP-γ-S-induced exocytosis of CD66b-enriched specific and tertiary granules, but did not affect exocytosis of CD63-enriched azurophilic granules, in electropermeabilized neutrophils. Tetanus toxin disrupted VAMP-2 and inhibited exocytosis of tertiary and specific granules. Activation of neutrophils led to the interaction of VAMP-2 with the plasma membrane Q-SNARE syntaxin 4, and anti-syntaxin 4 Abs inhibited exocytosis of specific and tertiary granules in electropermeabilized neutrophils. Immunoelectron microscopy showed syntaxin 4 on the plasma membrane contacting with docked granules in activated neutrophils. These data indicate that VAMP-2 mediates exocytosis of specific and tertiary granules, and that Q-SNARE/R-SNARE complexes containing VAMP-2 and syntaxin 4 are involved in neutrophil exocytosis. ***The Journal of Immunology,*** **2003, 170: 1034–1042.**

Exocytosis plays an important role in neutrophil physiology, and secretion of readily mobilizable granules seems to regulate early neutrophil responses, including adhesion, extravasation, and the onset of respiratory burst (1, 2). Human neutrophils contain four major different types of granules, namely: azurophilic or primary granules, specific or secondary granules, gelatinase-rich or tertiary granules, and alkaline phosphatase-rich granules, also named secretory vesicles (2–7). Azurophilic granules are sluggishly mobilized upon neutrophil activation, whereas specific, tertiary, and alkaline phosphatase-rich granules are readily exocytosed following neutrophil activation (1, 7–10). Azurophilic granules contain a large number of lytic enzymes that can be detrimental to the surrounding tissue if secreted in a deregulated way. Specific and tertiary granules constitute a reservoir of plasma membrane proteins that are translocated to the cell surface upon neutrophil activation, and contain a wide array of proteins involved in the adhesion and extravasation of human neutrophils as well as enzymes implicated in the generation of soluble mediators of inflammation (1, 2, 5, 9–16). Thus, secretion of neutrophil granules must be tightly controlled to prevent deregulated release of harmful components or unwanted inflammatory events.

The membrane fusion process requires a molecular mechanism to juxtapose and fuse the two membranes involved, and a regulatory mechanism that confers specificity to a particular type of fusion. In neuronal tissue and other systems, fusion of a vesicle with its target membrane is mainly mediated by a set of proteins collectively referred to as soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[3] (17). The SNARE hypothesis, initially postulated for synaptic vesicle release in the neuronal system, assumes that docking and fusion of vesicles with the plasma membrane is mediated by the specific interaction of vesicle proteins, v-SNAREs, including synaptobrevins/vesicle-associated membrane proteins (VAMPs), with target plasma membrane-located proteins, t-SNAREs, including syntaxins, synaptosome-associated protein (SNAP)-25, and SNAP-23 (25- and 23-kDa, respectively) (18–20).

The neuronal SNAREs VAMP-2, syntaxin 1A, and SNAP-25 have been shown to assemble into a very stable ternary complex

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[3] Abbreviations used in this paper: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP, synaptosome-associated protein; VAMP, vesicle-associated membrane protein; TeTx, tetanus toxin; PMN, polymorphonuclear neutrophil.

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with a 1:1:1 stoichiometry, referred to as the core complex, that consists of a twisted four-helical bundle (VAMP-2 and syntaxin 1A contributing with one α-helix each and SNAP-25 contributing with two α-helices) with all the chains aligned in parallel (21). Sequence comparison analyses have showed that all SNAREs share a homologous domain of ~60 aa, referred to as the SNARE motif (22–24), that mediates the association of SNAREs into core complexes forming the above four-helical bundle. All known SNARE motifs fall into two major subfamilies that contain either a conserved glutamine (Q-SNARE) or a conserved arginine (R-SNARE) at a central position, leading to a reclassification of SNARE proteins into Q- and R-SNAREs (22). In the neuronal SNARE complex, three glutamine residues from the Q-SNARE motifs (one contributed by syntaxin 1A and two by SNAP-25) and one arginine residue from the R-SNARE motif (contributed by VAMP-2) form an ionic layer in the four-helical bundle (21). Neuronal SNARE complex seems to be paradigmatic for all SNARE complexes, and thereby most of the SNARE complexes involved in membrane fusion consist of four-helix bundles, formed from three Q-SNAREs and one R-SNARE (3 (Q-SNARE)/1 (R-SNARE) type).

Recently, we and others (25–30) have demonstrated the presence of a number of SNAREs in human neutrophils by genetic and immunological approaches. We have further shown evidence for the functional involvement of the Q-SNAREs SNAP-23 and syntaxin 6 in neutrophil exocytosis (29), leading to postulate that the selective presence of certain SNAREs in distinct granule membranes might explain the different mobilization of neutrophil granule populations during cell activation (28, 29). The R-SNARE VAMP-2 has been previously found to be expressed and present in cytoplasmic granules in human neutrophils (25, 30). Because neutrophils contain a high number of granules that upon mobilization after cell activation play a major role in the distinct neutrophil functions, we have investigated the functional role of the human synaptobrevin-2/VAMP-2 in human neutrophil exocytosis. In this study, we show the involvement of VAMP-2 through its interaction with the Q-SNARE syntaxin 4 in the exocytosis of tertiary and specific granules in human neutrophils.

Materials and Methods

Antibodies

Anti-synaptobrevin-2/VAMP-2 IgG1 mAb (clone Cl 69.1) was purchased from Synaptic Systems (Göttingen, Germany). This Ab, raised against the N-terminal domain of rat VAMP-2, is able to recognize VAMP-2 from different species including human, is very specific for VAMP-2, and shows no cross-reactivity with synaptobrevin-1/VAMP-1, cellubrevin/VAMP-3, or any other protein in brain extracts (31). Anti-human syntaxin 4 IgG1 mouse mAb (clone Cl 49) was purchased from Transduction Laboratories (Lexington, KY). No cross-reaction between anti-VAMP-2 and anti-syntaxin 4 Abs was assessed by pull-down experiments with rat brain extracts. Anti-CD20 IgG1 mouse mAb was kindly provided by Dr. M. Romero (Hospital Rio Hortega, Valladolid, Spain). Rabbit anti-human lactoferrin polyclonal Ab was from Cappel Laboratories (Cochranville, PA). Rabbit anti-gelatinase polyclonal Ab (32) was generously provided by Dr. N. Borregaard (Department of Hematology, National University Hospital, Copenhagen, Denmark). Specific IgG1 mAbs against human CD63 (clone CLB-gran/12,435) and CD66b (clone CLB-B13.9) were from Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). CD66b was previously clustered as CD67 in the Fourth Human Leukocyte Differentiation Ag Workshop and renamed CD66b in the Fifth Human Leukocyte Differentiation Ag Workshop. Biotinylated goat anti-mouse IgG was from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). FITC-conjugated anti-mouse Ig was from Dakopatts (Glostrup, Denmark). P3×63 myeloma culture supernatant (a kind gift from Dr. F. Sánchez-Madrid, Hospital de la Princesa, Madrid, Spain) was used as a negative control.

Neutrophil isolation and activation

Neutrophils were isolated from fresh heparinized human peripheral blood as previously described (28, 33), resuspended at 3–5 × 10^6 cells/ml in HEPES/glucose buffer (150 mM NaCl, 10 mM HEPES, 5 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose, pH 7.5), and incubated at 37°C for 10 min with 1 μg/ml PMA. Release of granule markers following PMA activation was determined as previously described (1, 6, 9).

Subcellular fractionation

Resting neutrophils were resuspended in 50 mM Tris-HCl, pH 7.5 containing 2 mM PMSF, and then disrupted by repeated freeze-thaw. Homogenates were centrifuged at 1,200 rpm in a Sorvall T 6000D centrifuge for 10 min, and the supernatant, representing the postnuclear extract, was saved. After centrifugation of the postnuclear extract at 45,000 rpm in a TLA rotor for 90 min at 4°C using an Optima TL Ultracentrifuge (Beckman Instruments, Palo Alto, CA), supernatant (soluble fraction) and pellet (membrane fraction), resuspended in 50 mM Tris-HCl (pH 7.5), containing 2 mM PMSF, were saved.

To prepare the distinct subcellular fractions, freshly prepared neutrophils (~3–5 × 10^8) were gently disrupted, and the postnuclear fractions were fractionated in 15–40% (w/w) continuous sucrose gradients as described previously (5, 34). Subcellular fractions were analyzed for marker proteins for each organelle, namely lactate dehydrogenase (cytosol), HLA (plasma membrane), latent alkaline phosphatase (secretory vesicles), gelatinase (tertiary granules), lactoferrin (specific granules), and peroxidase (azurophilic granules) (34–37). Secretory vesicles were not resolved from the plasma membrane under the fractionation conditions used (34). Membranes from each fraction were obtained by diluting the fractions with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and centrifugation at 45,000 rpm for 90 min at 4°C using a 70 Ti type rotor (Beckman Instruments). Then, the pellets were resuspended in 50 mM Tris-HCl (pH 7.5) containing 2 mM PMSF and stored at −20°C until use.

RT-PCR, cDNA cloning, and sequencing

Total RNA (10 μg) was primed with oligo(dT) and reverse-transcribed into cDNA with 30 U of Moloney murine leukemia virus reverse transcriptase from Promega (Madison, WI). The generated cDNA was amplified by using primers for human neuronal VAMP-2 (5′-ATGTCTGCTACCGCT GCCAC-3′ and 5′-TTAAGAGCTGAAGTAAACTA-3′). A 25-μl PCR mixture contained 1 μl of the RT reaction, 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 5 U of EcoTaq DNA polymerase derived from *Thermus aquaticus* (ECOGEN, Barcelona, Spain). The PCR profile was as follows: an initial denaturation step at 95°C for 5 min, then denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 90 s (30 cycles), followed by further incubation for 15 min at 72°C. The PCR products were cloned into the pCR 2.1 vector using the TA cloning kit (Invitrogen, San Diego, CA), and DNA sequencing was performed on both strands from 10 independent cDNA clones using a PE Applied Biosystems 377 DNA sequencer (PerkinElmer, Wellesley, MA).

Western blotting

Proteins were separated by SDS-15% PAGE and immunoblotted as described previously (27, 35). After blocking with 5% powdered defatted milk in TBST buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20), blots were incubated overnight with anti-VAMP-2 mAb (dilution 1/1000 in TBST), or anti-syntaxin 4 mAb (dilution 1/2000 in TBST). Ab reactivity was monitored with biotinylated anti-mouse IgG (diluted at 1/1000 in TBST), followed by streptavidin-HRP conjugate (diluted 1/1000 in TBST), using an ECL detection system (Amersham Pharmacia Biotech).

Treatment with tetanus toxin (TeTx)

TeTx, a kind gift from Dr. J. Blasi (Universidad de Barcelona, L'Hospitalet de Llobregat, Spain), was preactivated by incubation with 10 mM DTT at 37°C for 1 h. Neutrophil membranes (30 μg protein) were incubated in the presence of 0.5% Triton X-100 for 1 h at 37°C with or without 400 nM preactivated TeTx.

Electropermeabilization and immunofluorescence flow cytometry

Neutrophils were permeabilized immediately before use as reported previously (29, 38, 39). In brief, 5 × 10^6 cells were washed with PBS, resuspended in 0.5 ml of ice-cold electropermeabilization buffer (120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES, pH 7.0), and subjected to two discharges of 5 kV/cm, 25 μF, and 72 Ω using a BTX electroporator (Biotechnologies & Experimental Research, San Diego, CA). Permeabilized cells were immediately transferred to plastic tubes containing buffer or

different concentrations of the Abs used in the study, and incubated for 5 min at room temperature to allow incorporation of Abs into electroporated neutrophils (29). In TeTx experiments, cells were transferred after electropermeabilization to plastic tubes containing preactivated TeTx (400 nM), and incubated for 15 min at 37°C. To activate electropermeabilized neutrophils, cells were incubated for 5 min with 5 μg/ml cytochalasin B at 37°C, and then were stimulated with 1 μM Ca^{2+} (0.1 mM $CaCl_2$, 5.37 mM $MgCl_2$, 5 mM HEDTA, 10 mM glucose) and 50 μM GTP-γ-S for 10 min at 37°C. Free Ca^{2+} concentration was checked by fura-2 measurement. After incubation, cells were placed on ice, fixed with 1% paraformaldehyde, and processed for immunofluorescence flow cytometry. Control untreated electropermeabilized cells were run in parallel. Cell surface expression of CD63 and CD66b was measured in paraformaldehyde-fixed neutrophils as described previously (29) using a FACScan flow cytometer at a log scale.

Immunoelectron microscopy

Resting and PMA-activated neutrophils were fixed for 24 h in 4% paraformaldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described (40). Forty-five-nanometer cryosections were cut at −125°C using diamond knives (Drukker International B.V., Cuijk, The Netherlands) in an ultracryomicrotome (Leica, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids (41). The grids were placed on 35-mm petri dishes containing 2% gelatin. For double immunolabeling, the procedure described by Slot et al. (42) was followed with 10- and 15-nm protein-A conjugated colloidal gold probes (Electron Microscopy Lab, Utrecht University, Utrecht, The Netherlands). After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Philips, Eindhoven, The Netherlands). Negative controls were prepared by replacing the primary Ab by a nonrelevant rabbit or mouse Ab, showing no staining of the samples.

Coimmunoprecipitation

Resting and PMA-activated cells (5×10^6) were lysed with 200 μl of lysis buffer (20 mM Tris-HCl, 100 mM KCl, 0.9% Triton X-100, 10% glycerol, 2 mM orthovanadate, 2 mM PMSF), and lysates were precleared with protein A-Sepharose as previously described (29). Abs to VAMP-2 or to syntaxin 4 were precoupled to protein A-Sepharose by incubation for 2 h at 4°C in lysis buffer. The precoupled beads were pelleted, washed twice with lysis buffer, and added to the supernatant from the preclearing step. Lysate and Ab coupled to Sepharose beads were incubated for 2 h at 4°C with constant rotation. Sepharose beads were then pelleted and washed five times with lysis buffer. Then, 30 μl SDS sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were added, samples were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose and immunoblotted as described above.

Results

Synaptobrevin-2/VAMP-2 in human neutrophils

Because VAMPs constitute the major SNARE proteins located in vesicles in different systems (17–20, 43–45), neutrophils contain a high number of granules, and VAMP-2 has been previously reported to be expressed in neutrophils (25, 30), we studied the role of VAMP-2 in human neutrophils.

The presence of VAMP-2 in human neutrophils was confirmed at both mRNA and protein levels by cloning through RT-PCR and subsequent sequencing, and by the use of the Cl 69.1 anti-VAMP-2 mAb that has been previously reported to be highly specific for the VAMP-2 N terminus region (residues 2–17) (31). The amino acid sequence of neutrophil VAMP-2 (GenBank/European Molecular Biology Laboratory database accession no. AJ225044) was identical with the sequence of human neuronal VAMP-2 (46), corroborating recent data (30). This VAMP-2 amino acid sequence contains a SNARE motif (residues 31–85) with 8 heptad repeats and the R-56 residue that confers R-SNARE character on VAMP-2. The Cl 69.1 anti-VAMP-2 mAb recognized a band of ~18 kDa in the postnuclear extract and in the membrane fraction of human neutrophils, but not in the soluble fraction containing the cytosol (Fig. 1*A*), indicating that VAMP-2 was membrane-bound.

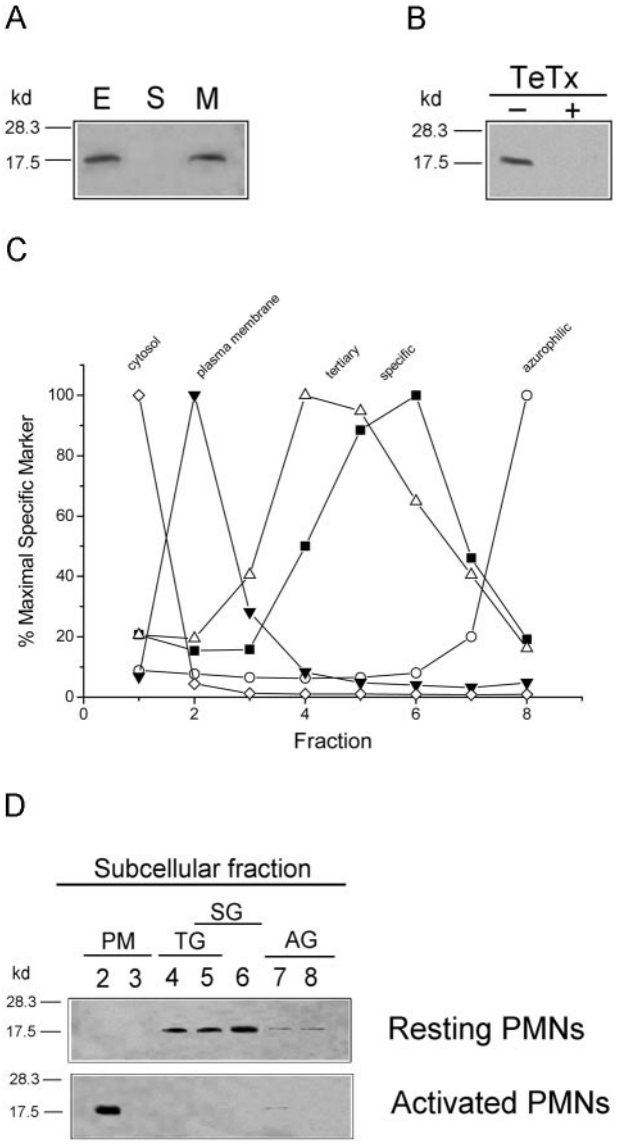

FIGURE 1. Subcellular distribution and TeTx cleavage of VAMP-2 in human neutrophils (polymorphonuclear neutrophils; PMNs). *A*, Equal amounts of postnuclear extract (E), soluble (S), and membrane (M) proteins (50 μg) from resting human neutrophils were run on SDS-PAGE and analyzed by immunoblotting using anti-VAMP-2 mAb. *B*, Membrane proteins (30 μg) were incubated in the absence (−) or in the presence (+) of 400 nM TeTx, and then subjected to SDS-PAGE and immunoblotted for VAMP-2. *C*, Resting human neutrophils were gently disrupted and subjected to subcellular fractionation as described in *Materials and Methods*. Fractions (4 ml each, save fraction 1-cytosol-, 6-ml) were collected and analyzed for the activity of specific organelle markers, which are plotted normalized to the fraction with maximal activity. The following markers were assayed. Cytosol:lactate dehydrogenase (◇); plasma membrane:HLA (▼); tertiary granules:gelatinase (△); specific granules:lactoferrin (■); azurophilic granules:peroxidase (○). *D*, Membrane proteins (30 μg) from the subcellular fractions 2–8 of resting and activated (1 μg/ml PMA, 10 min) human neutrophils were assayed for VAMP-2 by immunoblotting. Membranes from fractions enriched in plasma membrane (PM), tertiary granules (TG), specific granules (SG), and azurophilic granules (AG) were analyzed. The molecular masses (kilodaltons) of protein markers are indicated on the *left*. All data shown are representative of three separate experiments.

The immunoreactive 18-kDa band was extensively degraded by treatment with TeTx (Fig. 1*B*) that cleaves VAMP family proteins specifically (47), further confirming its identity as VAMP-2.

Subcellular fractionation assays under conditions that resolved cytosol, plasma membrane, gelatinase-rich tertiary granules, specific granules, and azurophilic granules (Fig. 1*C*) showed that VAMP-2 was mainly localized in the membranes prepared from subcellular fractions 4–6 (Fig. 1*D*) enriched in tertiary and specific granules in resting neutrophils (Fig. 1*C*). When human neutrophils were activated with 1 μg/ml PMA for 10 min, we found that VAMP-2 was translocated from the above cytoplasmic granules toward the plasma membrane (Fig. 1*D*). This subcellular translocation was correlated with a high release of gelatinase (90% secretion) and lactoferrin (78% secretion), markers for gelatinase-rich tertiary and specific granules, respectively. However, the azurophilic granule markers peroxidase and β-glucuronidase were hardly released (<6% secretion) under PMA treatment, indicating that azurophilic granules were not mobilized. This was corroborated by subcellular fractionation analysis of PMA-activated neutrophils that showed that plasma membrane remained in fraction 2 and azurophilic granules in fraction 8, whereas the amount of specific and tertiary granules was highly decreased (data not shown). These data suggest that VAMP-2 is mainly located in the membranes of both specific and gelatinase-containing tertiary granules in resting neutrophils and is translocated to the cell surface following cell activation under experimental conditions that induced the secretion of specific and tertiary granules.

Ultrastructural localization of VAMP-2 in resting and activated human neutrophils

To further identify the subcellular localization of VAMP-2, resting neutrophils were immunolabeled for VAMP-2 and analyzed by immunogold electron microscopy. Gold label was detected on the membrane of some granules. When neutrophils showed a partly dissolved content of the cytosol, the number of VAMP-2-positive granules was higher (Fig. 2*A*), probably because the VAMP-2 epitope recognized by this Ab became more accessible. To confirm that VAMP-2 is present in both tertiary and specific granules, cryosections of neutrophils were double labeled with anti-VAMP-2 Ab and anti-gelatinase or anti-lactoferrin Ab, respectively. Colocalization of VAMP-2 was observed with both granule markers, gelatinase (Fig. 2*B*) and lactoferrin (Fig. 2*C*). After double labeling with gelatinase and VAMP-2, we have scanned 200 positive granules: 42 were positive for both, gelatinase, and VAMP-2; 131 were positive for only gelatinase; and 27 were positive for only VAMP-2. After double labeling with lactoferrin and VAMP-2, we have scanned 212 positive granules: 27 were positive for both; 172 were positive for lactoferrin only; and 13 were positive for VAMP-2 only. In the immunoelectron microscopy studies, the signal detected with anti-VAMP-2 Ab was much weaker than the corresponding ones to gelatinase and lactoferrin. This could be due to either a lower presence of VAMP-2 and/or to a poor accessibility of the Ab for its epitope. This can explain the low percentage of colocalization of VAMP-2 with gelatinase or lactoferrin when only the gelatinase-positive and lactoferrin-positive granules are counted. However, the actual percentages of colocalization may be higher because only one section was examined for each granule. Thus, it cannot be ruled out that those granules only positive for the corresponding granule marker can be positive for both granule marker and VAMP-2 in another section of the same granule. Due to this lower labeling for VAMP-2, it is more reliable to analyze colocalization in VAMP-2-positive granules. In this regard, we obtained a high percentage of colocalization between gelatinase and VAMP-2 (61%) and between lactoferrin and VAMP-2 (67.5%) when only VAMP-2-positive granules were analyzed. Although electron microscopy data are semiquantitative, these data clearly indicate that VAMP-2 is located mainly in both specific and gelatinase-containing tertiary granules, and are in good agreement with the fractionation experiments (Fig. 1*D*). In Fig. 2*B*, we show a gelatinase-positive tertiary granule docked to the plasma membrane and labeled for VAMP-2 on the granule membrane side facing the plasma membrane.

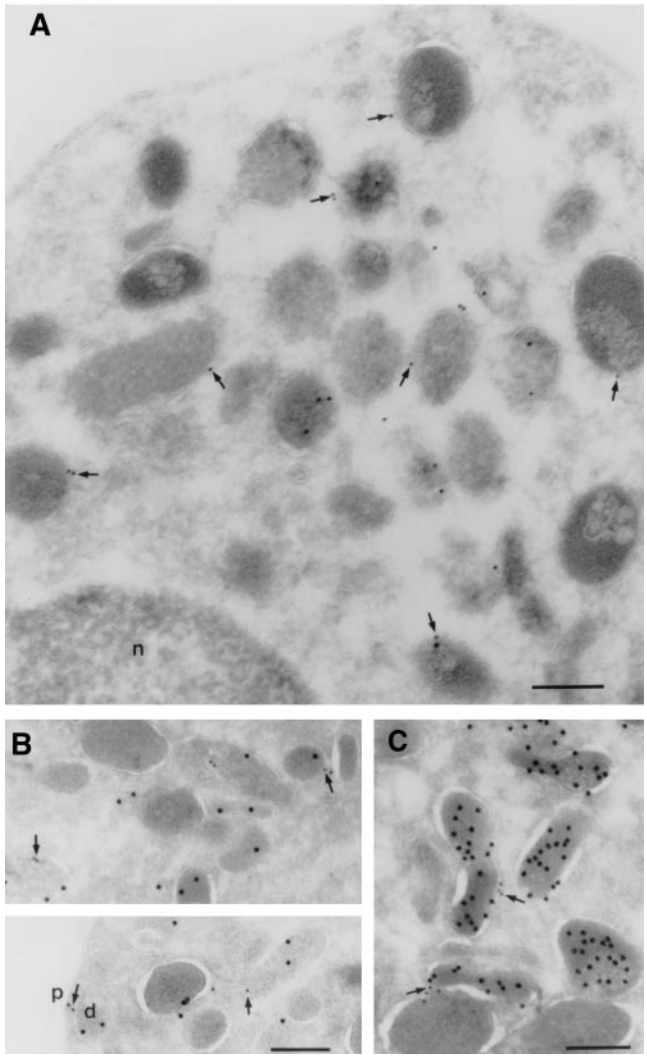

FIGURE 2. Localization of VAMP-2 in human resting neutrophils by electron microscopy. Cryosections of neutrophils were immunogold-labeled as described in *Materials and Methods* with: *A*, anti-VAMP-2 mAb (10-nm gold); *B*, anti-VAMP-2 mAb (10-nm gold) + anti-gelatinase Ab (15-nm gold); *C*, anti-VAMP-2 mAb (10-nm gold) + anti-lactoferrin Ab (15-nm gold). *A*, An area of a neutrophil showing a partly dissolved content of the cytosol with VAMP-2 localized at the membrane of cytoplasmic granules (arrows); n, nucleus. *B*, VAMP-2 labeling is detected in the membrane of gelatinase-positive granules (arrows). A gelatinase-positive granule docked (d) to the plasma membrane (p) is labeled for VAMP-2 (arrow) on the side of the granule membrane facing the plasma membrane. *C*, VAMP-2 labeling is observed on the membrane of lactoferrin-positive granules (arrows). Bars, 200 nm.

Following neutrophil activation with 1 μg/ml PMA for 10 min, two major ultrastructural changes are observed: 1) an increasing number of granules are contacting with the plasma membrane (we define the contact area as that in which granule and plasma membrane appear <10 nm apart each other); 2) large areas of cytoplasm with few or no granules due to exocytosis, and invaginations of the plasma membrane intruding into the cell (see Figs. 3 and 7). After immunolabeling in PMA-activated neutrophils, VAMP-2

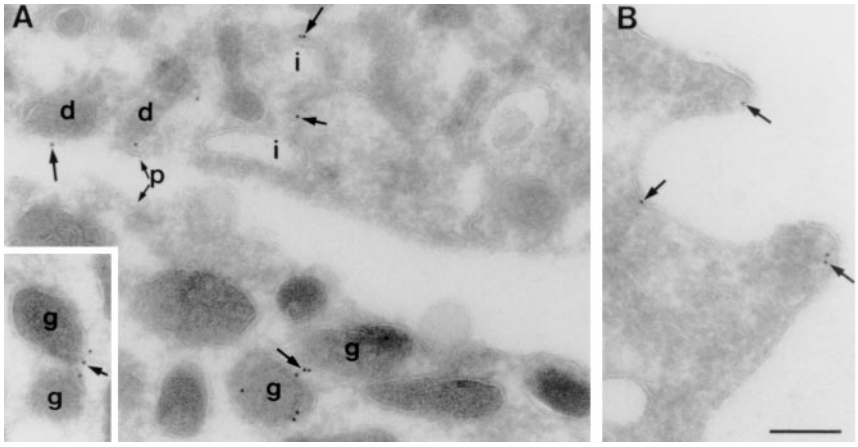

FIGURE 3. Localization of VAMP-2 in PMA-activated human neutrophils by electron microscopy. Sections of PMA-activated neutrophils were immunogold labeled with anti-VAMP-2 Ab (10-nm gold). *A,* An area of two neutrophils separated by their plasma membranes (p); the cell on the top shows two docking granules (d) contacting with the plasma membrane and labeled for VAMP-2 on the contact area (arrows); VAMP-2 is also present on membrane invaginations (i; arrows). The cell at the *bottom* and *inset* shows VAMP-2 labeling (arrows) in the adjoining granule membranes of two contacting granules (g). *B,* An area of a neutrophil devoid of granules with VAMP-2 labeling on the plasma membrane (arrows). Bar, 200 nm.

was found on the plasma membrane (Fig. 3*B*), on membrane invaginations (Fig. 3*A*), and also on the granule membrane side of docked granules contacting with the plasma membrane and on the adjoining sides of two contacting granules (Fig. 3*A*).

Effect of Abs directed against VAMP-2 on granule exocytosis in electropermeabilized human neutrophils

We next analyzed whether VAMP-2 had a functional role in neutrophil exocytosis. To this aim, we prepared electropermeabilized neutrophils that were able to undergo exocytosis of cytoplasmic granules upon cell activation with Ca^{2+} and GTP-γ-S (29, 38, 39). We have recently shown that electropermeabilized neutrophils, following two discharges of 5 kV/cm, allow rapid access of Abs into the cytoplasm in functional neutrophils, as assessed by both flow cytometry and confocal microscopy analyses (29), and >95% of the cells were rendered permeable (29). Incorporation of Abs into electropermeabilized neutrophils was also demonstrated by visualization of FITC-conjugated Abs inside the electropermeabilized neutrophils by confocal microscopy (29). Degranulation was analyzed by measuring up-regulation of the granule membrane markers CD63 and CD66b at the cell surface, as a measure of neutrophil degranulation in electropermeabilized neutrophils (29, 39). This method has been previously shown to monitor efficiently neutrophil degranulation in electropermeabilized neutrophils (29, 39). Following paraformaldehyde fixation of electropermeabilized neutrophils, we monitored the expression of both CD63 and CD66b only at the cell surface, allowing us to determine neutrophil degranulation (29). Most of the intracellular CD63 and CD66b pools (>80%) resulted incorporated into the cell surface of electropermeabilized neutrophils after cell activation (data not shown). Previous immunoelectron microscopy studies have shown that CD63 (also named as LIMP, gp55, melanoma-associated Ag ME491, Pltgp40, and LAMP-3) is present exclusively in the azurophilic granules (48), whereas CD66b (formerly named CD67) is located in the specific granules (49) of resting neutrophils. However, the putative localization of CD66b in gelatinase-containing tertiary granules has not been previously analyzed. To this aim, ultrathin cryosections of neutrophils were double labeled with anti-CD66b and anti-gelatinase Abs. CD66b was found on the membrane and gelatinase on the matrix of the same granules (Fig. 4). These data indicate that CD66b is also a marker for tertiary granules, in addition to specific granules. Thus, up-regulation of CD63 parallels secretion of azurophilic granules, whereas CD66b up-regulation parallels secretion of both tertiary and specific granules. Incubation of electropermeabilized neutrophils with the Cl 69.1 anti-VAMP-2 mAb that recognized specifically VAMP-2 (31) inhibited CD66b up-regulation in a dose-dependent manner, but had no effect on CD63 up-regulation after cell activation with Ca^{2+} and GTP-γ-S (Fig. 5). In contrast, incubation of electropermeabilized neutrophils with irrelevant mouse Igs, including P3×63 myeloma culture supernatant or an isotype-matched unrelated mouse mAb, such as anti-CD20 mAb, used as negative controls, had no effect on neutrophil degranulation (Fig. 5). These data indicate that anti-VAMP-2 mAb inhibits secretion of both tertiary and specific granules without affecting secretion of azurophilic granules.

Effect of TeTx on exocytosis of tertiary and specific granules in electropermeabilized human neutrophils

The clostridial toxin TeTx specifically cleaves VAMP proteins (47), and thereby constitutes an excellent tool to study functional

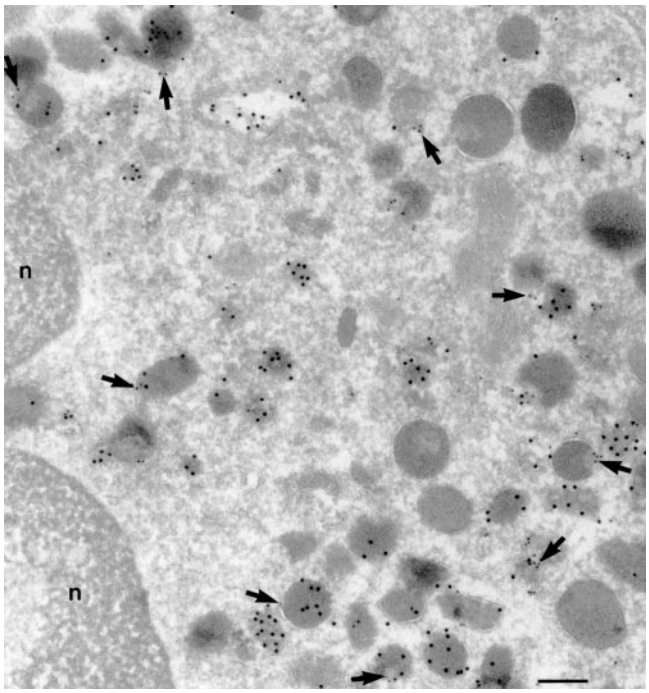

FIGURE 4. CD66b colocalizes with gelatinase in the same granules. Neutrophils were fixed and processed for ultrathin cryosections. Sections were labeled with anti-CD66b Ab (10-nm gold) + anti-gelatinase Ab (15-nm gold). CD66b and gelatinase are detected on the membrane and on the matrix, respectively, of the same granules (arrows); n, nucleus. Bar, 200 nm.

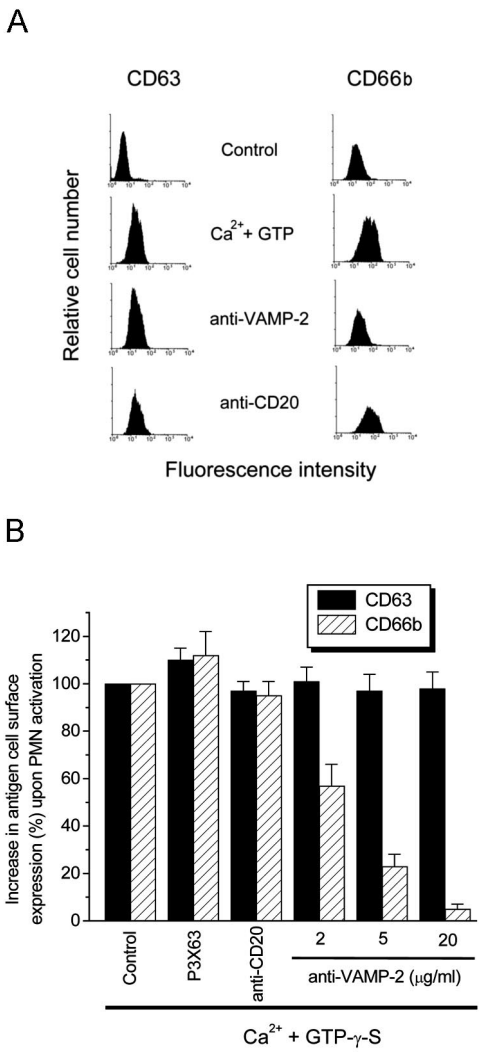

FIGURE 5. Effect of anti-VAMP-2 Abs on the up-regulation of CD63 and CD66b cell surface expression in activated electropermeabilized human neutrophils. *A*, Electropermeabilized cells were incubated with 20 μg/ml anti-VAMP-2 mAb or 20 μg/ml anti-CD20 mAb, activated with Ca^{2+} + GTP-γ-S, and assayed for CD63 and CD66b Ag expression by flow cytometry as described in *Materials and Methods*. Untreated electropermeabilized cells (Control) and electropermeabilized cells stimulated with Ca^{2+} + GTP-γ-S (Ca^{2+} + GTP) in the absence of Abs were run in parallel. The results shown are representative of three independent determinations. *B*, Electropermeabilized human neutrophils (PMNs) were incubated in the absence (Control), or in the presence of P3×63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), or of increasing concentrations of anti-VAMP-2 mAb, and then activated as above and analyzed for CD63 and CD66b cell surface expression. Data are expressed as percentage of the cell surface Ag increase upon electropermeabilized PMN activation compared with the CD63 and CD66b cell surface up-regulation detected in control Ca^{2+} + GTP-γ-S-stimulated electropermeabilized neutrophils in the absence of any Ab (Control), considered as 100% of increase in cell surface Ag expression. Mean values ± SD of three independent determinations are shown.

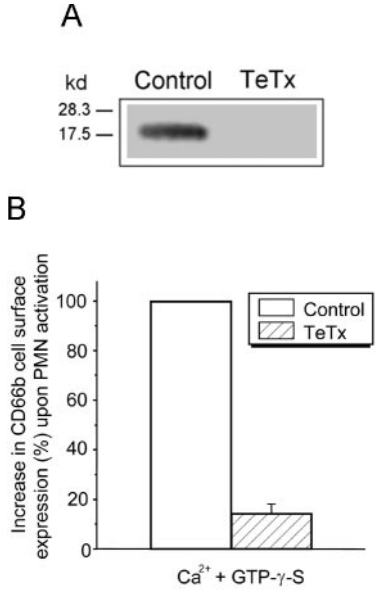

FIGURE 6. Effect of TeTx on VAMP-2 and on the up-regulation of CD66b cell surface expression in electropermeabilized human neutrophils. Electropermeabilized human neutrophils (PMNs) were incubated without (Control) or with preactivated TeTx (400 nM) and activated with Ca^{2+} + GTP-γ-S as described in *Materials and Methods*. Untreated control and TeTx-treated permeabilized cells were subjected to SDS-PAGE and immunoblotted for VAMP-2 to assay the susceptibility of VAMP-2 to TeTx cleavage (*A*), and analyzed for CD66b cell surface expression (*B*). The molecular masses (kilodaltons) of protein markers are indicated on the *left* in *A*. Data in *B* are expressed as the percentage of CD66b cell surface increase as in Fig. 5, and are shown as mean values ± SD of three independent determinations.

aspects of these SNARE proteins. To access the neutrophil cytoplasm in whole functional neutrophils with clostridial toxins, we prepared electropermeabilized neutrophils as indicated in *Materials and Methods*. Incubation of electropermeabilized neutrophils with TeTx cleaved VAMP-2 (Fig. 6*A*), further demonstrating the permeability properties of the electroporated cells, and inhibited CD66b up-regulation following cell activation with Ca^{2+} and GTP-γ-S (Fig. 6*B*), further supporting the involvement of VAMP-2 in the exocytosis of tertiary and specific granules.

In vivo interaction between VAMP-2 and syntaxin 4 in activated human neutrophils

We next investigated whether VAMP-2 could mediate exocytosis of tertiary and specific granules through its interaction with SNARE proteins located at the plasma membrane. A major location of syntaxin 4 in the plasma membrane of human neutrophils has been previously reported by subcellular fractionation studies (25), and interaction between VAMP-2 and syntaxin 4 has been reported in different systems (43–45, 50, 51). To get a better insight on the subcellular localization of syntaxin 4, we examined the subcellular location of syntaxin 4 by immunogold electron microscopy using an anti-syntaxin 4 mAb that recognized specifically the 36-kDa syntaxin 4 protein (data not shown). Gold label, corresponding to immunolabeled syntaxin 4, was observed on the cell membrane of resting human neutrophils (data not shown). After neutrophil activation with 1 μg/ml PMA for 10 min, syntaxin 4 was found on the plasma membrane facing the cytosol, with some locations at the membrane regions contacting with docked granules (Fig. 7, *inset*), and on membrane invaginations (Fig. 7).

Because we found that both VAMP-2 and syntaxin 4 were located in regions involved in the docking of granules to the plasma membrane, we examined whether VAMP-2 could interact with syntaxin 4 in vivo in human neutrophils by coimmunoprecipitation experiments. VAMP-2 was immunoprecipitated from resting and PMA-activated neutrophils, and after SDS-PAGE and blotting, the VAMP-2 immunoprecipitates were probed for VAMP-2 and syntaxin 4 (Fig. 8*A*). No interaction between VAMP-2 and syntaxin 4

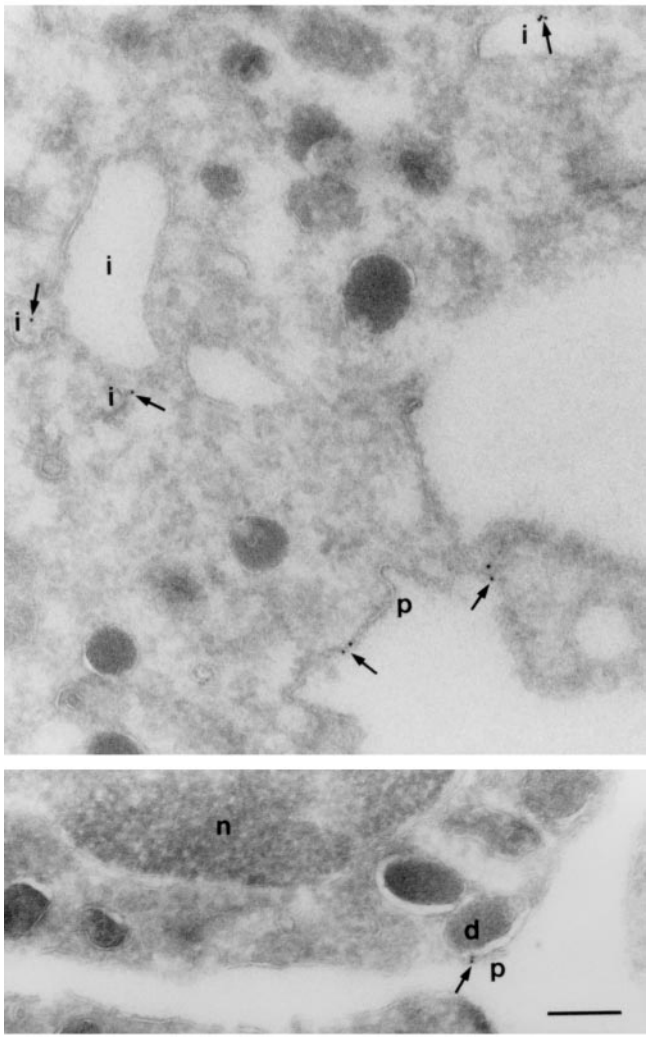

FIGURE 7. Localization of syntaxin 4 in PMA-activated neutrophils by electron microscopy. Sections of PMA-activated neutrophils were immunogold labeled with anti-syntaxin 4 Ab (10-nm gold). Arrows show labeling for syntaxin 4 on the plasma membrane (p) and on membrane invaginations (i). *Inset*, An area of another neutrophil with a docked granule (d) labeled for syntaxin 4 (arrow) on the contact area with the plasma membrane (p). Bar, 200 nm.

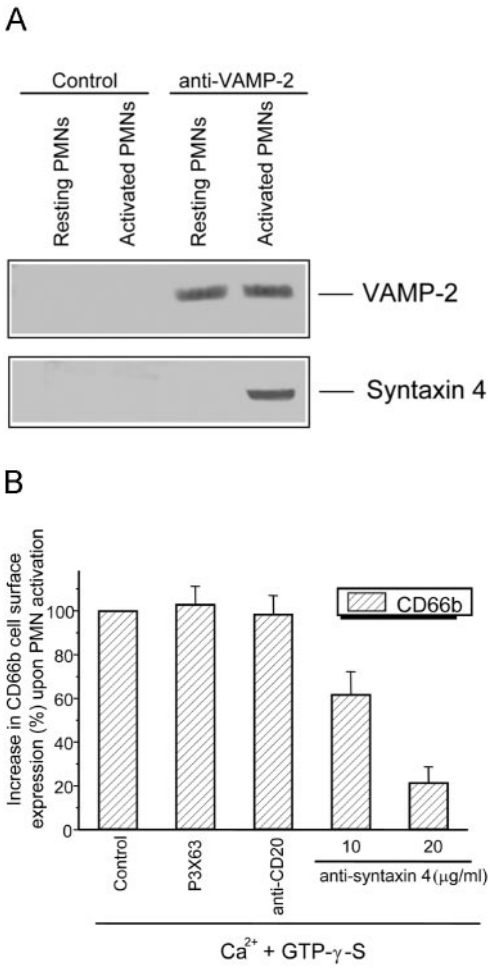

FIGURE 8. VAMP-2/syntaxin 4 interaction in neutrophil exocytosis. *A*, In vivo interaction between VAMP-2 and syntaxin 4 in activated human neutrophils. VAMP-2 was immunoprecipitated from detergent-solubilized cell extracts of resting and PMA-activated human neutrophils (PMNs), and immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-VAMP-2 or anti-syntaxin 4 mAbs. Lysates were also immunoprecipitated with P3×63 myeloma culture supernatant as a negative control (Control). The positions of VAMP-2 and syntaxin 4 are indicated. Experiment shown is representative of three performed. *B*, Effect of anti-syntaxin 4 mAb on the up-regulation of CD66b cell surface expression in activated electropermeabilized human neutrophils. Neutrophils (PMNs) were electropermeabilized, incubated in the absence (Control) or in the presence of P3×63 (20 μg/ml), anti-CD20 mAb (20 μg/ml), or of increasing concentrations of anti-syntaxin 4 mAb, activated with Ca^{2+} + GTP-γ-S and analyzed for CD66b cell surface expression as in Fig. 5. Data are expressed as the percentage of CD66b cell surface increase as in Fig. 5. Mean values ± SD of five independent determinations are shown.

was detected in resting neutrophils, but we observed that both VAMP-2 and syntaxin 4 coimmunoprecipitated after PMA activation (Fig. 8*A*). Similar data were obtained immunoprecipitating first with anti-syntaxin 4 Ab (data not shown). These findings indicate that both SNARE proteins, VAMP-2 and syntaxin 4, are brought together following neutrophil activation under experimental conditions that induce secretion of tertiary and specific granules.

Effect of anti-syntaxin 4 Abs on the exocytosis of CD66b-containing granules in electropermeabilized human neutrophils

Because both VAMP-2 and syntaxin 4 interacted each other under experimental conditions that induced exocytosis of tertiary and specific granules, we examined whether Abs directed against syntaxin 4 could block exocytosis of these organelles. We found that incubation of electropermeabilized neutrophils with anti-syntaxin 4 mAb blocked CD66b up-regulation following cell activation with Ca^{2+} and GTP-γ-S (Fig. 8*B*). However, irrelevant mouse Igs, such as P3×63 myeloma culture supernatant or the mouse isotype-matched anti-CD20 mAb, had no effect on neutrophil degranulation (Fig. 8*B*). These data indicate that syntaxin 4 is involved in neutrophil exocytosis.

Discussion

The data reported in this study demonstrate for the first time the involvement of the R-SNARE VAMP-2 through its interaction with the Q-SNARE syntaxin 4 in human neutrophil exocytosis.

Previous reports have shown that human neutrophils express VAMP-2, but not VAMP-1 or cellubrevin/VAMP-3 (25, 30). In this study, we have conducted a functional characterization of neutrophil VAMP-2. Cl 69.1 anti-VAMP-2 mAb, which specifically

recognizes VAMP-2 (31), blocks exocytosis of tertiary and specific granules, but not secretion of azurophilic granules, in electropermeabilized human neutrophils, indicating that VAMP-2 plays a key role in the secretion of tertiary and specific granules. The major localization of VAMP-2 in these two latter granules in resting human neutrophils, assessed both by subcellular fractionation and immunoelectron microscopy studies, and its translocation to the cell surface upon neutrophil activation, indicate that this protein serves as a v-SNARE in neutrophil secretion. These data extend previous subcellular localization studies by Brumell et al. (25) who reported that VAMP-2 was mainly located in tertiary granules using subcellular fractionation studies.

The data reported in this study also indicate that VAMP-2 interacts in vivo with the plasma membrane Q-SNARE syntaxin 4 following neutrophil activation that promotes exocytosis of tertiary and specific granules. The fact that anti-VAMP-2 mAb can immunoprecipitate SNARE complexes containing VAMP-2 and the Q-SNARE syntaxin 4 indicates that this Ab does not bind to the VAMP-2/Q-SNARE interacting region. Cl 69.1 anti-VAMP-2 mAb was generated by Edelman et al. (31) using a synthetic peptide corresponding to the N terminus region of VAMP-2 (residues 2–17), whereas the sequence of VAMP-2 involved in the formation of the SNARE complex (SNARE motif) lies between residues 31–85 (22). However, because anti-VAMP-2 mAb inhibits exocytosis of tertiary and specific granules, it can be suggested that the previous binding of the Ab to VAMP-2 hampers the subsequent binding of VAMP-2 with interacting Q-SNAREs required for membrane fusion, likely due to a steric hindrance or conformational change. TeTx that cleaves VAMP-2 and Abs against syntaxin 4 inhibit secretion of tertiary and specific granules in electropermeabilized neutrophils, suggesting that VAMP-2/syntaxin 4 interaction is mediating secretion of these granules in human neutrophils. Interestingly, we have also detected VAMP-2 at the granule membrane on the adjoining sides of two contacting granules (Fig. 3*A*). This suggests that VAMP-2 can be also involved in granule-granule fusion processes that can take place during either compound exocytosis (52) or endocytosis. Taken together, the experiments described in this study suggest that VAMP-2 functions as a v-SNARE in human neutrophils, mediating fusion of the specific and tertiary granules with the cell surface through its interaction with syntaxin 4, acting as a t-SNARE.

In contrast, we have also found in preliminary experiments that Abs against syntaxin 4 inhibited exocytosis of CD63-enriched azurophilic granules in electropermeabilized neutrophils activated with Ca^{2+} and GTP-γ-S (B. Martín-Martín and F. Mollinedo, unpublished observations), suggesting the involvement of syntaxin 4 in the exocytosis of azurophilic granules. Thus, syntaxin 4 could act as a t-SNARE in the exocytosis of distinct neutrophil granules, serving as a docking site for VAMP-2 in the exocytosis of specific and tertiary granules, and interacting with a still unknown structure in the exocytosis of azurophilic granules. However, this remains to be elucidated by further experimentation.

We have previously found that during neutrophil activation SNAP-23, mainly located in tertiary and specific granules in resting neutrophils, binds to syntaxin 6, mainly located in the plasma membrane of human neutrophils, regulating exocytosis of CD66b-positive granules in human neutrophils (29). As we have found in this study that CD66b is also a membrane-bound marker of tertiary granules, in addition to specific granules (49), the interaction between SNAP-23 and syntaxin 6 seems to mediate the exocytosis of both tertiary and specific granules. In this study, we have found that SNARE complexes, containing VAMP-2 and syntaxin 4, are formed during neutrophil activation that leads to exocytosis of tertiary and specific granules. Our present and previous findings (29) show that specific Abs against VAMP-2, syntaxin 4, syntaxin 6, and SNAP-23 are able to inhibit secretion of CD66b-positive granules, suggesting that VAMP-2 and SNAP-23, acting as v-SNAREs, and syntaxin 4 and syntaxin 6, acting as t-SNAREs, are involved in the regulation of exocytosis of both tertiary and specific granules. Current evidence suggest that neuronal SNARE complexes involved in membrane fusion consist of four-helix bundles, formed from three Q-SNAREs and one R-SNARE (3 (Q-SNARE)/1 (R-SNARE) type). Based on these data, it could be envisaged a hypothetical model in which two 3 (Q-SNARE)/1 (R-SNARE) type ternary complexes, containing syntaxin 4/SNAP-23/VAMP-2 and syntaxin 6/SNAP-23/VAMP-2, respectively, would be required for exocytosis of neutrophil tertiary or specific granules. These complexes could involve the interaction of Q-SNAREs (syntaxin 4, syntaxin 6, SNAP-23) with the R-SNARE VAMP-2, forming two different 3 (Q-SNARE)/1 (R-SNARE) ternary complexes. SNAP-23 would contribute with two SNARE motifs, and thereby two glutamines within the central part of the SNARE motifs, in the four-helix bundle of the SNARE core complex, whereas syntaxin 4, syntaxin 6, and VAMP-2 would contribute with one SNARE motif each. This model would explain our findings involving the participation of different SNARE proteins (VAMP-2, syntaxin 4, syntaxin 6, and SNAP-23) in neutrophil exocytosis (this report and Ref. 29), and would propose a key role for VAMP-2 in neutrophil exocytosis as it would be the R-SNARE constituent of both putative SNARE complexes required for exocytosis of tertiary and specific granules. This model represents a working hypothesis that must be proved with further experimentation. Preliminary data suggest that VAMP-2 can interact with the above mentioned SNARE proteins following neutrophil activation (J. Canchado and F. Mollinedo, unpublished observations), suggesting the putative requirement for the above postulated two SNARE complexes in neutrophil exocytosis. However, this must be tested with further experimentation.

The presence of SNAP-23 and VAMP-2 in tertiary and specific granules, but not in azurophilic granules (this report and Ref. 29), gives an explanation to the prone secretion of both tertiary and specific granules in contrast to the sluggish mobilization of azurophilic granules. Specific and tertiary granules share several constituents and are mainly involved in exocytosis, serving as intracellular reservoirs of membrane proteins normally required at the cell surface of activated neutrophils (1, 2), whereas azurophilic granules are mainly involved in phagocytosis, degrading phagocytosed microorganisms. This also explains that both tertiary and specific granules are secreted in a rather similar way, and in fact, exocytosis of specific granules cannot occur without exocytosis of tertiary granules. The differential location of SNARE proteins in readily mobilized granules (specific and tertiary granules) vs sluggishly mobilized granules (azurophilic granules) can be of major importance to differentially regulate mobilization of neutrophil granules with distinct exocytic capabilities and functions. Elucidation of the molecular mechanisms involved in granule secretion is not only of interest in neutrophil biology, but is also of major pharmacological interest inasmuch as proteins mediating neutrophil exocytosis may serve as appropriate targets for anti-inflammatory agents.

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