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Localized Exocytosis of Primary (Lysosomal) Granules During Phagocytosis: Role of Ca$^{2+}$-Dependent Tyrosine Phosphorylation and Microtubules$^{1}$

Hans Tapper,$^{2*}$ Wendy Furuya,† and Sergio Grinstein †

The uptake and killing of bacteria by human neutrophils are dependent on the fusion of secretory granules with forming phagosomes. The earliest component of exocytosis was found to precede phagosome closure, so that granular membrane constituents were detectable on the plasmalemma. We show that during phagocytosis of IgG-opsonized particles, this early secretory response is highly polarized in the case of primary granules, but less so for specific granules. The vectorial discharge of primary granules was dependent on calcium, but no evidence was found that calcium is involved in determining the polarity of exocytosis. In particular, a redistribution of endomembrane calcium stores toward forming phagosomes could not be detected. Polarized granule exocytosis was accompanied by focal tyrosine phosphorylation and actin polymerization, although the latter was not required for the response. Instead, microtubules seemed to contribute to the vectorial nature of the response. During particle ingestion, the microtubule-organizing center relocated toward forming phagosomes, and colchicine treatment altered the pattern of exocytosis, reducing its directionality. We hypothesize that the focal activation of tyrosine kinases generates localized signals that induce exocytosis in a calcium-dependent manner, and that reorientation of microtubules facilitates preferential delivery of granules toward the forming phagosome. The Journal of Immunology, 2002, 168: 5287–5296.

Professional phagocytes, e.g., macrophages and neutrophils, play an important role in our innate defense against invading pathogens, in the resolution of inflammation, and in the general maintenance of tissue homeostasis (1, 2). These functions are highly dependent on the ability of these cells to internalize particles and on other events that are coupled to or triggered by phagocytosis (e.g., secretion, oxidative burst). Unlike most other cells, neutrophils are endowed with several distinct types of secretory organelles. They are intended to secrete content proteins, as well as deliver integral proteins to the cell surface and phagosomal membranes. At least four distinct types of secretory organelles are currently recognized (1, 3). Primary or azurophilic granules are lysosomal in nature. They contain proteases, myeloperoxidase, etc. Their membranes express CD63, which is a useful marker for immunofluorescence and flow cytometry. Secondary granules contain lactoferrin, vitamin B$_{12}$-binding protein, lysozyme, etc. They are smaller and less dense than primary granules and possess CD66b on their membranes. Tertiary granules are related to secondary granules, and share some of their contents, but are distinct in that they contain gelatinase and lack CD66b. Finally, neutrophils possess small secretory vesicles, which are filled with albumin and express on their membranes latent alkaline phosphatase and CD35. All four types of granules coexist in each cell. They are, however, secreted under different conditions and with distinct sensitivity toward stimuli (3, 4). Secretory vesicles are the most responsive and are secreted first, even before the neutrophil reaches the site of infection. Tertiary granules are more sensitive than secondary granules, which are in turn more responsive than primary granules. In permeabilized cells or in cells treated with ionophores, this progression can be mimicked in vitro as the concentration of Ca$^{2+}$ is increased (5, 6). Clearly, the function of the individual granule types is distinct, as are the signal transduction pathways leading to their release.

As part of the microbial killing process, the contents of secretory granules are emptied into the phagosomal space. Concurrently, granular membrane proteins are inserted into the phagosomal membrane, where they play an essential role in killing (e.g., the H$^{-}$-ATPase and some membrane-bound forms of cathepsin). Clearly, such delivery of granular components must be targeted: random insertion throughout the cell membrane would not only be wasteful, but potentially deleterious. Indeed, uncontrolled release of elastase and other proteases is among the major causes of lung damage in cystic fibrosis and of joint pathogenesis in arthritis as well (1, 7). In the present study, studies have been performed to spatially locate secretion of primary and secondary granules during phagocytosis of yeast (zymosan) coated with Ig to engage the FcR. Early studies (8–10) have described exocytosis occurring during neutrophil phagocytosis, before closure of the phagosome. Using a combination of flow cytometry and dual-wavelength confocal immunofluorescence, we found that exocytosis of both primary and secondary granules precedes sealing of the phagosomal space. More importantly, CD63 was found to be highly localized to the prephagosomal cup, while CD66b was more randomly distributed. Because the phagosomal membrane is formed by invagination of
the plasma membrane, it is not immediately apparent how exocytosis can be selectively targeted to the phagosome. Polarized exocytosis exists in other cell types (11). Neurons have well-defined presynaptic nodules in which vesicles accumulate and fuse, at the exclusion of other areas of the surface membrane. Similarly, acinar cells of exocrine tissues deliver their secretory contents to the lumens of the duct via the apical membrane, without occurrence of basolateral secretion. Yet these systems differ from the neutrophil in that polarization is a permanent, preestablished feature of these cells. By contrast, neutrophils are seemingly symmetric cells before their acute activation.

Increased cytosolic Ca$^{2+}$ is essential for exocytosis of primary and secondary granules in neutrophils (3, 6). Thus, removal of external Ca$^{2+}$ and preloading with bis(2-aminophenoxy)ethane-N,N',N'-tetraacetate (BAPTA)$^3$ eliminates exocytosis. It is well established that release of internal Ca$^{2+}$ stores suffices for the secretory response and that omission of external Ca$^{2+}$ has little effect. There is some discrepancy regarding the pattern of Ca$^{2+}$ release from stores, but some authors have pointed out that cytosolic free calcium concentration ([Ca$^{2+}$]i) is preferentially elevated in the vicinity of the phagosome (12, 13). This could readily account for the occurrence of localized exocytosis near the phagosomal cup. It has been also suggested that the Ca$^{2+}$ stores themselves rearrange, concentrating around the phagosome (13). Obviously, this could account for the observed [Ca$^{2+}$]i localization. It is not clear whether this repositioning of the stores is sufficiently fast to account for the exocytosis that precedes closure of the phagosome. Another early response to phagocytic stimuli is cascades of tyrosine phosphorylation of multiple cellular targets (14–16). In the present work, we will present data on the role of Ca$^{2+}$ and phosphorylation on tyrosine in the targeting of secretory responses toward early phagosomes.

Regulated changes in the actin and microtubule cytoskeletal networks are required for phagocytosis to proceed normally, and might be necessary also for focal targeting of secretory responses. Conditions have been reported that produce either centripetal or centrifugal movement of lysosomal organelles along microtubules in phagocytes and other cells (17, 18). These include acute treatment with CAMP, changes in pH, and others. Such vectorial displacement, if directed to one pole of the cell, could also contribute to focal exocytosis induced by phagocytic stimuli.

Materials and Methods

Materials

Unlabeled and Texas Red-conjugated zymosan particles, rhodamine phalloidin, and the acetoxymethyl esters of Indo-1 and BAPTA were obtained from Molecular Probes (Eugene, OR). RPMI 1640, HEPES, human IgG, cytochalasin B, colchicine, fibrinogen, and poly-L-lysine were from Sigma-Aldrich (St. Louis, MO). Ionomycin was from Calbiochem (La Jolla, CA). Erbstatin analog was obtained from Biornol Research Laboratories (Plymouth Meeting, PA). Powdered PBS was obtained from Pierce (Rockford, IL). BSA was from Boehringer Mannheim (Mannheim, Germany). mAbs to both CD63 (CLB-CD63) and CD66b (CLB-CD66b) were the generous gift of A. J. Verhoeven (Red Cross Blood Transfusion Center, Central Laboratory of The Netherlands). Rabbit polyclonal anti-peptide Abs raised against calreticulin and SERCA2 were the generous gift of K. H. Krause (Division of Infectious Diseases, University Hospital, Geneva, Switzerland). A rabbit polyclonal Ab to calcxin was the generous gift of D. Williams (University of Toronto). For some experiments, rabbit polyclonal Abs were precleared of zymosan-reactive Abs by incubation with zymosan particles for 1 h at 37°C. mAb to SERCA2 (clone I2D8) was obtained from Affinity Bioreagents (Golden, CO). mAbs against phosphorylated Src were obtained from Zymed Laboratories (clones PY20, PY-7E1, PY-Plus- cocktail; San Francisco, CA) and from Upstate Biotechnology (clone G10; Lake Placid, NY). mAbs to tyrosine tubulin (clone TUB-1A2) and α-tubulin (clone DM 1A) were obtained from Sigma-Aldrich. Donkey serum and the secondary Abs used for immunofluorescence (Cy3-conjugated donkey anti-rabbit and FITC- and Cy3-conjugated donkey anti-mouse) were from Jackson Immunoresearch (West Grove, PA).

Experimental media

Nominally bicarbonate-free solution RPMI 1640 was buffered to pH 7.3 with 25 mM HEPES. Na$^+$-based solution (Na medium) was also buffered to pH 7.3 and contained (in mM): 127 NaCl, 1.2 KH$\_2$PO$\_4$, 5.4 KCl, 0.8 MgSO$\_4$, 1.8 CaCl$\_2$, 5.6 glucose, and 10 HEPES. Calcium medium, CaCl$\_2$ was replaced by EGTA (1 mM). All media were adjusted to 290 ± 5 mMsm with the major salt.

Coating of coverslips

Glass coverslips were washed with methanol and overlaid with 0.25 ml poly-L-lysine (0.2 mg/ml in water). After evaporating the added fluid at 50–65°C, the poly-L-lysine-coated coverslips were washed twice with distilled water. Coverslips were used within 1 day of coating.

Neutrophil isolation, preparation of zymosan, and protocol for stimulation of cells

Human neutrophils (>98% pure) were isolated from fresh heparinized blood of healthy volunteers by dextran sedimentation, followed by Ficoll-Hypaque gradient centrifugation. Contaminating red cells were removed by NH$_4$Cl lysis. Neutrophils were counted using a model ZM Coulter counter (Hialeah, FL), resuspended in HEPES-buffered RPMI 1640 medium at 10$^7$ cells/ml, and maintained in this medium at room temperature until use. The viability of the cells was greater than 97%, as judged by trypan blue exclusion. All experiments were performed within 3 h of neutrophil isolation.

Zymosan particles are baker’s yeast (Saccharomyces cerevisiae) that has been subjected to boiling and acid and to trypsin treatment (19). The zymosan particles used were relatively homogeneous in size with an average particle diameter of 3 µm. Zymosan particles were dispersed in PBS by vortexing and gentle sonication. After one wash, the particles were opsonized by incubation for 1 h at 37°C with 2 mg human IgG/mg zymosan. After two subsequent washes, the opsonized particles were counted using the Coulter counter. That the particle suspension was well dispersed was routinely verified by phase contrast microscopy, and freshly prepared particles were used in all experiments.

Synchronization of the interaction between neutrophils and zymosan was achieved by rapidly sedimenting the cells together with zymosan in a microcentrifuge tube, followed by resuspension. This process was complete within 30 s.

Fluorescence microscopy

Fixation of neutrophils with 1.6% paraformaldehyde in PBS was initiated at 4°C for 30 min and continued at room temperature for 1 h. In some experiments, fixed cells were permeabilized by treatment with a buffer containing 0.01% Triton X-100, 100 mM PIPES (pH 6.8), 5 mM EGTA, 100 mM KOH, and 2 mM MgCl$\_2$ for 5 min. Similar results were obtained using higher concentrations of Triton X-100 (up to 0.1%). Blocking was performed with 5% donkey serum in PBS for 2 h. After washing with PBS, cells were incubated at room temperature with the indicated primary Ab for 2–4 h in PBS containing 1% BSA. Following washing, incubation with secondary Ab was for 1 h, also in PBS containing 1% BSA. Cells were adhered to poly-L-lysine-coated coverslips after incubation with secondary Ab. After washing, the samples were overlaid with Slow Fade or ProLong (Molecular Probes) before mounting.

To label F-actin, rhodamine phalloidin (6 µM stock solution in methanol) was evaporated and redissolved in PBS, according to the instructions of the manufacturer (final concentration 165 nM). This staining solution was applied to fixed cells for 20 min at room temperature, after which the coverslips were washed twice with PBS and mounted for fluorescence microscopy. Samples stained with phalloidin or with fluorescent Abs were analyzed using a Leica (Deerfield, IL) TCS 4D laser confocal microscope. At least 100 cells were examined in each experiment.

Flow cytometry

Neutrophils were fixed and stained, as described above for immunofluorescence microscopy. After washing, the cells were diluted in PBS and analyzed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). For every sample, at least 10,000 ungated cells were counted.

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$^3$ Abbreviations used in this paper: BAPTA, bis(2-aminophenoxy)ethane-N,N',N'-tetraacetate; [Ca$^{2+}$]i, cytosolic free calcium concentration; MTOC, microtubule-organizing center; PK2, proline-rich tyrosine kinase 2; OZ, IgG-opsonized zymosan particle.
Selection of cell populations of interest was done after the acquisition of raw data, using the Lysis II analysis software, as described previously (20).

**Measurement of cytosolic Ca\(^{2+}\) concentration**

\([\text{Ca}^{2+}]\), was measured using Indo-1 and a Hitachi Model F-4000 spectrophluorometer, set at 37°C and equipped with magnetic stirring. Neutrophils were loaded with Indo-1 by incubation with 2 \(\mu M\) of the acetoxy-methyl ester form of the dye for 15 min at 37°C. After washing twice, 10⁶ cells were allowed to interact with IgG-opsonized zymosan particles (OZ) by cosedimentation at room temperature. The cells were rapidly resuspended in a phosphate-free Na medium at 37°C, and recording of Indo-1 fluorescence was initiated within 30 s of cosedimentation. Indo-1 fluorescence was monitored using excitation and emission wavelengths of 331 and 410 nm, respectively, and was calibrated using ionomycin and \(\text{Mn}^{2+}\), as described (21). A dissociation constant of 250 nM for the indo-1-Ca\(^{2+}\) complex was used to calculate [Ca\(^{2+}\)].

**Results**

**Exocytosis of primary and secondary granules during phagocytosis: detection by confocal immunomicroscopy**

Exocytosis during the course of FcR-mediated phagocytosis was studied using immunocytochemical detection of luminal epitopes on membrane proteins of primary and secondary granules (CD63 and CD66b, respectively). These epitopes, which are inaccessible in intact, unstimulated cells, become exposed to extracellular Abs upon insertion of the granule membrane into the plasmalemma (5). Using this approach, we had earlier shown that the secretory response induced by IgG-opsonized particles precedes the formation of sealed phagosomes (20). We now extend these studies, using confocal microscopy, to define the site of exocytosis with respect to the forming phagosome. By immunostaining of permeabilized neutrophils during various stages of phagocytosis, we could not demonstrate an obvious accumulation of either primary or secondary granules in the part of the cell closer to the zymosan (not shown). However, a rapid fusion of granules with the phagosome might preclude such an accumulation. Next, human neutrophils were exposed for short (1-min) periods to OZ particles, fixed, and subjected to immunostaining without permeabilization. As shown in Fig. 1C, the site of attachment of OZ, where phagosomes are being formed, can be defined readily by differential interference contrast microscopy (Fig. 1, C and F). Shortly after induction of phagocytosis, CD63 was found to accumulate preferentially in the vicinity of the opsonized particles (Fig. 1, A and B), while CD66b distributed more homogeneously on the neutrophil surface (Fig. 1, D–F). These differential distribution patterns were evident when analyzing single confocal sections (Fig. 1, cf A and D), as well as after reconstructing serial sections of the entire cell (Fig. 1, cf B and E). Similar results were obtained in >10 preparations using blood from different donors.

**Role of calcium in the polarized exocytosis of CD63: extracellular vs intracellular sources**

Calcium is widely accepted to have an essential role in neutrophil granule secretion (3, 6). It is therefore conceivable that the polarized exocytosis of CD63 is generated by a localized increase in Ca\(^{2+}\). In fact, cytosolic Ca\(^{2+}\) gradients have been reported to form in the vicinity of the phagosome (12). To determine the role of cytosolic Ca\(^{2+}\) in the targeting of CD63 toward the forming phagosome, we first defined the source of the divalent cation utilized during exocytosis induced by OZ. As illustrated in Fig. 2A–C, addition of a calcium ionophore in the absence of OZ sufficed to induce exocytosis, which was substantially inhibited by omission of extracellular Ca\(^{2+}\) (Fig. 2D–G). Importantly, the distribution of CD63 on the surface of ionomycin-activated cells was random, indicating that there are no preexisting regions of higher fusogenic activity. Also, when cells were stimulated by phorbol ester + ionomycin or with cytochalasin B + fMLP, was the induced exocytosis nonlocalized (not shown). In contrast to the effects of the ionophore, exocytosis of CD63 in response to OZ was not only restricted to the membrane of the forming phagosome, but was largely independent of the presence of extracellular Ca\(^{2+}\) (Fig. 2D–G). This suggests that endomembrane stores may be the primary source of the Ca\(^{2+}\) required for OZ-induced exocytosis of CD63.

The increased Ca\(^{2+}\) found in the vicinity of the phagosome has been attributed to a redistribution of intracellular Ca\(^{2+}\) stores, which ostensibly accumulate near the phagosomal membrane (13). Preferential Ca\(^{2+}\) release from endomembranes attracted to the region of the forming phagosome could readily account for the observed focalized exocytosis of CD63. We therefore sought to confirm the redistribution of Ca\(^{2+}\) stores following phagocytosis, by analyzing the localization of calcineurin, calreticulin, and SERCA2b, acknowledged as resident proteins of the endoplasmic reticulum, the primary endomembrane Ca\(^{2+}\) store. Unlike Stendahl et al. (13), we repeatedly failed to see accumulation of the markers in the vicinity of the phagosome (Fig. 3, A–H). Concentration of fluorescence around the opsonized particles was only seen when crude polyclonal IgG preparations were used (insets in

**FIGURE 1.** OZ induces differentially localized expression of CD63 and CD66b at the surface membrane of human neutrophils. Suspended human neutrophils were mixed with Texas Red-labeled OZ at a particle-cell ratio of 2:1 and rapidly cosedimented. Cells and particles were resuspended and incubated at 37°C for 1 min before cooling on ice. This was followed by fixation, immunostaining, and attachment of the stained cells onto poly-L-lysine-coated coverslips. Cells were stained with Abs against CD63 (A–C) or CD66b (D–F), followed by FITC-labeled secondary Ab. Single confocal x-y-sections are shown in A and D, while composites of serial sections (slices spaced ~0.5 μm apart) are shown in B and E. The corresponding Nomarski images are shown in C and F. Arrowheads identify OZ in contact with cells. No labeling of cells or particles was seen with secondary Ab alone. Bars = 10 μm. Similar results were obtained in at least 10 separate experiments.
**FIGURE 2.** OZ-triggered primary granule exocytosis is not dependent on calcium influx. A–C, Suspended human neutrophils were stimulated with ionomycin (0.5 μM, 5 min at 37°C in calcium-containing Na medium). D–F, Cells were rapidly sedimented with Texas Red-labeled OZ, resuspended, and incubated for 1 min at 37°C in calcium-free Na medium (containing 1 mM EGTA). Stimulation was followed by cooling on ice, fixation, immunostaining, and attachment of the stained cells onto poly-L-lysine-coated coverslips. Cells were stained with Abs against CD63, followed by FITC-labeled secondary Ab. Single confocal xy-sections are shown in A and D, while composites of serial sections (slices spaced ~0.5 μm apart) are shown in B and E. The corresponding Nomarski images are shown in C and F. Arrowheads identify OZ in contact with the cells. No labeling of cells or particles was seen with secondary Ab alone. Images are representative of at least five separate experiments. G, Cells were stimulated as above (in Na medium with or without calcium, as indicated) and immunostained for CD63, and their fluorescence was quantified using flow cytometric analysis, as described in Materials and Methods. IgG-Z, IgG-zymosan. Experimental data are expressed as percentage of the highest fluorescence observed in each experiment, and are presented as means of eight separate experiments.

Even though the redistribution of endomembrane Ca$^{2+}$ stores to the vicinity of the forming phagosome. We therefore devised an experimental protocol that enabled us to assess the exocytosis of CD63 in the absence of localized Ca$^{2+}$ release. To this end, we obliterated the endogenous changes in Ca$^{2+}$ elicited by OZ, yet allowed exocytosis to occur by artificially elevating Ca$^{2+}$ in a diffuse manner, using an ionophore. The data justifying this approach are compiled in Fig. 4. As reported earlier, phagocytosis of OZ is associated with a large, transient elevation of cytosolic Ca$^{2+}$ (Fig. 4A, upper trace). This increase could be completely prevented by prior loading of the cells with the Ca$^{2+}$-buffering agent BAPTA (Fig. 4B, lower trace). While OZ was ineffective under these conditions, subsequent addition of ionomycin produced a concentration-dependent, sustained increase in Ca$^{2+}$ (Fig. 4C), as anticipated from the finite buffering power of BAPTA. Because the ionophore is likely to distribute homogeneously in the plasmalemma, the Ca$^{2+}$ change recorded should have occurred diffusely throughout the cell, i.e., without preferential accumulation of the cation near the forming phagosome.

In parallel flow cytometric experiments, pretreatment of the cells with BAPTA was shown to inhibit OZ-triggered expression of CD63 at the cell surface, even when extracellular Ca$^{2+}$ was reintroduced (Fig. 4C); see also Fig. 5A). This is consistent with the notion that Ca$^{2+}$ is required for primary granule exocytosis (3, 6). Accordingly, the addition of a low concentration of ionomycin (0.5 μM) to BAPTA-loaded cells restored their ability to up-regulate CD63 in response to OZ in Ca$^{2+}$-containing medium (Fig. 4C). Importantly, this concentration of the ionophore was a relatively poor inducer of CD63 exocytosis in cells not exposed to OZ, most likely due to the attenuated magnitude and retarded kinetics of the...
Ca$_2^+$ increase. It therefore appears that the phagocytic event facilitated exocytosis of primary granules at lower, otherwise sub-threshold Ca$_2^+$ levels.

As shown in Fig. 5, while little surface CD63 was detectable by immunofluorescence in BAPTA-treated cells exposed to OZ (Fig. 5, A and B), surface epitope was readily detectable when 0.5 μM ionomycin was also added. Strikingly, the fluorescence was again concentrated in the region of the forming phagosomes (Fig. 5C). Similar observations were made in three separate experiments. Under the conditions used, any calcium released locally by OZ was neutralized by BAPTA (Fig. 4A). We therefore conclude that additional signals, unrelated to Ca$_2^+$, are generated locally by the FcR, inducing polarized exocytosis of primary granules. Although the process is Ca$_2^+$ dependent, localized calcium gradients may not be essential to polarization. However, such gradients could certainly contribute to the response in a more physiological setting.

**Accumulation of phosphotyrosine is spatially restricted and calcium dependent**

Phosphotyrosine accumulation near phagosomes has been demonstrated previously (14). Because tyrosine phosphorylation is thought to be an important early event in FcR-mediated signaling (15, 16), it could be a determinant of the spatial localization of exocytosis in neutrophils. As shown in Fig. 6, A and B, phosphotyrosine accumulation was also clearly observed adjacent to forming OZ phagosomes in neutrophils. The specificity of the immunostaining is indicated by the inhibitory effects of erbstatin, a tyrosine kinase antagonist (Fig. 6, C and D). Removal of extracellular Ca$_2^+$ had no discernible effect on the extent or localization of the phosphotyrosines (Fig. 6, E and F), but chelation of intracellular Ca$_2^+$ with BAPTA greatly reduced the staining (Fig. 7, A and B). This reduction in phosphotyrosine labeling was due to the divalent cation-binding properties of BAPTA and is not due to nonspecific effects of the chelator or the loading process. This was concluded from the observation that staining reappeared upon addition of ionomycin, to impose an elevated level of cytosol Ca$_2^+$ (Fig. 7, C and D). Restoration of tyrosine phosphorylation by the ionophore was also detectable by flow cytometry (Fig. 7E). As for CD63 exocytosis, the concentration of ionomycin used was not a potent inducer of tyrosine phosphorylation in the absence of OZ (Fig. 7E). Remarkably, the reappearance of phosphotyrosine accumulation was restricted to the nascent phagosome (Fig. 7C), despite the diffuse influx in Ca$_2^+$ caused by ionomycin. This observation parallels the effects described for CD63 exocytosis and suggests that the focal accumulation of phosphotyrosine is an important step in dictating the polarization of exocytosis. It also implies that a localized signal promotes the Ca$_2^+$-dependent accumulation of phosphotyrosine.
near the nascent phagosome. This signal could be a low, barely detectable level of Ca\(^{2+}\)-independent tyrosine phosphorylation or another, as yet undefined response.

**Role of OZ-triggered actin polymerization in targeting exocytosis**

Completion of phagocytosis requires the integrity of the actin cytoskeleton (23, 24). Because actin polymerizes actively in the region of the nascent phagosome, we considered the possibility that the cytoskeleton may be involved in targeting secretory granules to this region. In cells stained with rhodamine phalloidin, typical actin cups can be observed during the early stages of OZ internalization, as illustrated in Fig. 8, A and B. As reported, we found that cytochalasin inhibited phagocytosis and reduced the number of OZ particles associated with the surface of neutrophils. Nevertheless, because of the cosedimentation protocol utilized, sufficient numbers of interacting particles could be found for systematic analysis by confocal immunofluorescence microscopy. Fig. 8, C and D, shows that, despite intimate contact between the cell and particle, F-actin staining was reduced and not preferentially concentrated in a characteristic cup. However, failure of actin to polymerize in the presence of cytochalasin did not preclude the accumulation of phosphotyrosine in the region of contact with the particle (Fig. 8, E and F). More importantly, exocytosis of primary granules persisted in cells that associated with particles (Fig. 8, G and H). Remarkably, CD63 was found to accumulate preferentially in the area of contact with the OZ. Thus, actin polymerization appears not to be critically required for the phagosomal targeting of primary granules.

**Role of microtubules in polarization during exocytosis**

Microtubules are involved in the intracellular transport and localization of a variety of organelles, including secretory granules (25). Recently, it was demonstrated that polarized exocytosis of secretory lysosomes by dendritic cells involves microtubule-mediated recruitment of granules (26). It was therefore conceivable that polarized exocytosis of primary granules during phagocytosis is similarly mediated by microtubules. In suspended (nonadherent) neutrophils, well-defined microtubules are very difficult to discern by immunostaining. However, the localization of the microtubule-organizing center (MTOC) can be readily defined. As shown in Fig. 9, A and B, the MTOC was consistently localized very close to nascent phagosomes in neutrophils. To quantify this phenomenon, the cells were arbitrarily subdivided into three regions: the third closest to the forming phagosome, the middle third, and the distal third (see Fig. 9G). After staining for tubulin, the location of the MTOC with respect to the phagosome was defined in 500 cells, from three separate experiments. The compiled data, shown in Fig. 9G, demonstrate that the MTOC preferentially locates in the immediate vicinity of the phagocytic cup.

We next investigated whether the focal accumulation of phosphotyrosine and the localized exocytosis of primary granules were...
microtubule-dependent processes. Initial experiments revealed that microtubule-disrupting agents such as colchicine induced a relocation of the MTOC, which was less frequently associated with nascent phagosomes. In fact, the MTOC was often seen on the opposite side of the cells (not shown). Despite the disruption of microtubules, tyrosine-phosphorylated proteins still accumulated close to phagosomes, as determined by immunostaining (Fig. 9, C and D). However, primary granule exocytosis was much less localized in cells treated with colchicine (Fig. 9, E and F), implying that microtubules may be responsible, at least in part, for targeting the secretory response to the area of the phagocytic cup.

Discussion

Role of calcium in targeting of exocytosis in neutrophils

It is well established that Ca$^{2+}$ triggers exocytosis in neurons and neuroendocrine cells (27), and neutrophil degranulation is generally accepted to be also a Ca$^{2+}$-dependent process (6). However, at least four types of secretory granules and vesicles exist in neutrophils, and these display distinct profiles of Ca$^{2+}$ sensitivity (3, 6). Secretory vesicles have the lowest Ca$^{2+}$ threshold and are also most readily mobilized in vivo (3, 4). In decreasing order of Ca$^{2+}$ sensitivity, neutrophils also possess gelatinase-containing (tertiary) granules, specific (secondary) granules, and lysosome-like (primary) granules. The subcellular Ca$^{2+}$ gradients reported to appear during the course of phagocytosis in neutrophils (12) could in principle dictate the preferential exocytosis of granules near the phagosome. Moreover, those granules with the lowest Ca$^{2+}$ sensitivity
would be expected to polarize most effectively, since other granules would be more likely to reach the secretory Ca\(^{2+}\) threshold not only near the phagosome, but elsewhere as well.

The involvement of Ca\(^{2+}\) in polarized exocytosis was tested using BAPTA. As reported before for specific granules (28), exocytosis of primary granules induced by opsonized particles was inhibited by chelation of Ca\(^{2+}\). This stresses the differences between neutrophils and macrophages, in which phagosome-lysosome fusion was recently demonstrated to be a Ca\(^{2+}\)-independent process (29). Although essential for secretion, Ca\(^{2+}\) does not appear to be the main determinant of the polarized exocytosis occurring during neutrophil phagocytosis of IgG-opsonized zymosan, as shown in the present study. It has earlier been shown that the initial Ca\(^{2+}\) elevation observed upon cell-particle contact is not localized, and that a nonuniform distribution of Ca\(^{2+}\) can be observed only after formation of a phagosome (12). Such delayed gradient formation could not account for the rapid polarization of primary granule exocytosis reported in this work. Also, migration of human neutrophils, a process that is ostensibly dependent on polarized exocytosis of endomembranes (30), appears to proceed normally when Ca\(^{2+}\) gradients are eliminated (31, 32). Jointly, our results suggest that, while Ca\(^{2+}\) plays a permissive role in exocytosis, it appears not to be the sole determinant of focal exocytosis of primary granules in neutrophils.
Role of tyrosine phosphorylation in the targeting of exocytosis

An early event likely to partake in signaling the spatial restriction of primary granule exocytosis is a localized tyrosine phosphorylation. Human neutrophils express FcγRII (CD32) and FcγRIII (CD16), and ligation of these receptors leads to protein tyrosine phosphorylation. Kinases of the src family are thought to phosphorylate the receptors, which in turn promote the recruitment and activation of p72^{src}. Clustering of this kinase has been shown to be a sufficient signal to trigger phagocytosis (15). In accordance with an important role for tyrosine phosphorylation in phagocytosis, it has been demonstrated that the tyrosine phosphatase YopH of *Yersinia* can inhibit its own phagocytic uptake, as well as FcγR-mediated phagocytosis of other phagocytic prey (33).

The initial round of tyrosine phosphorylation triggered by FcR leads to the activation of phospholipase C and the subsequent Ca^{2+} release from inositol trisphosphate-sensitive stores. Our experiments revealed that a sizeable fraction of the tyrosine phosphorylation depended on the elevation of Ca^{2+}. This finding is not necessarily incompatible with the sequence of events postulated above, but instead reflects the existence of two components of tyrosine phosphorylation: an early phase that activates phospholipase C and is presumably Ca^{2+}-independent, and a secondary phase that requires prior elevation of Ca^{2+}. A component of Ca^{2+}-dependent tyrosine phosphorylation has been reported earlier in neutrophils (34, 35) and also in platelets (36–39). Recently, a Ca^{2+}-dependent tyrosine kinase, known as proline-rich tyrosine kinase 2 (PYK2), cell adhesion kinase β, or related focal tyrosine kinase, was described to exist in a variety of cell types, including neutrophils (31–33). This type of nonreceptor tyrosine kinase may well be responsible for the secondary, Ca^{2+}-dependent wave of phosphorylation during phagocytosis and may partake in the activation of primary granule exocytosis. This would account for the Ca^{2+} dependence of exocytosis, while some process other than Ca^{2+} changes would accumulate PYK2 near the phagosome. In this regard, it is noteworthy that PYK2 is related to focal adhesion kinase, a tyrosine kinase that clusters in focal adhesions in a Ca^{2+}-independent manner (40).

The role of the cytoskeleton in the targeting of exocytosis

Actin polymerization is an absolute requirement for completion of phagocytosis, but the exocytosis induced by opsonized particles persists when polymerization at the barbed end is prevented by cytochalasin (41). Remarkably, while actin is thought to be a central element in the establishment of neutrophil polarity, focal exocytosis of CD63 was observed in the presence of cytochalasin, at doses that obliterated phagocytosis. Moreover, tyrosine phosphorylation remained concentrated in the area of contact between the OZ particle and the leukocyte. Thus, while actin polymerization and/or cross-linking may be needed for optimal stimulation by FcR and for formation of phagosomal cups, the actin cytoskeleton does not appear to mediate the targeting of exocytosis to the area of the phagosome.

Microtubules had been implicated earlier in the process of phagosomal maturation. Late phagosomes in neutrophils were found to localize adjacent to the centriole (42), and movement of phagosomes along microtubules was documented directly (43). In addition, fusion of phagosomes with lamp 2-containing vesicles was shown to be a microtubule-dependent process in J774 cells (44). Jointly, these findings can be interpreted to mean that fusion of phagosomes with lysosomes is promoted by the centripetal movement of the phagosome. Our data suggest that an additional mechanism may be involved, namely the directed movement of secretory granules toward the nascent phagosome. This notion is supported by the following observations: 1) kinesin, a motor protein that displaces organelles toward the plus end of microtubules, is found associated with secretory granules of neutrophils (45); 2) the MTOC appears to relocate during phagocytosis to the vicinity of the nascent phagosome; and 3) disruption of microtubules with colchicine, which alters the relocation of the MTOC, is associated with a more disperse pattern of exocytosis. Thus, coalescence of phagosomes with lysosomes may be facilitated by two concomitant microtubule-mediated events: centripetal movement of granules and centripetal displacement of sealed phagosomes. Colchicine, which had been reported earlier to partially inhibit secretion in neutrophils (42), may decrease the efficiency of the exocytic process by reducing the rate at which the granules collide with the plasma membrane, where they presumably dock and fuse via a soluble N-ethylmaleimide-sensitive factor attachment receptor-mediated process.

Experimental data from yeast and mammalian cell systems (46, 47) suggest that a large protein complex known as the exocyst, which includes Sec6/8, plays a role in the polarization of secretory pathways through the recruitment of vesicles to specific domains on the plasma membrane. We therefore investigated the localization of Sec6/8 in phagocytosing human neutrophils by immunoblotting of plasma membrane and early phagosomal fractions. In preliminary experiments, no accumulation of Sec6/8 was observed in early (2- to 5-min) phagosomal membranes (not shown), suggesting that factors other than the exocyst dictate the targeting of azurophil granules to phagosomes.

Rapid repositioning of the MTOC upon stimulation has been reported in a few cell types, including macrophages challenged with immobilized immune complexes (48). At present, comparatively little is known about the underlying mechanism. In T cells, the displacement of the MTOC was found to require extracellular calcium and most likely involves elevated cytosolic Ca^{2+} (49). This displacement is of likely relevance for the formation of receptor signaling complexes and the activation of tyrosine phosphorylation events (50). Also, during ingestion of *Chlamydiae*, tyrosine phosphorylation of host cell proteins is required to trigger a microtubule-dependent redistribution of the microorganism (51). Of note, phosphorylation of tyrosine residues on tubulin (52), as well as tubulin tyrosination (53), are felt to be important in microtubule remodelling. The linkage between FcR engagement and the activation of these processes, and their precise role in directed exocytosis remain to be defined.

In summary, our findings indicate that in neutrophils, fusion of lysosomal granules with the phagosome is an early, vectorial event. While exocytosis requires calcium, this cation appears not to be directly responsible for the polarization of exocytosis, nor is de novo actin polymerization required. Instead, the localized activation of tyrosine kinases may promote relocation of the MTOC, possibly directing microtubules toward the phagosome. Kinesin-mediated movement of granules toward the phagosome would then favor preferential exocytosis at or near the forming phagosome.

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