

Control of granule exocytosis in neutrophils

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1. ABSTRACT

Neutrophils are granulocytes derived from bone marrow that circulate through the blood and become recruited to tissues during infection or inflammation. They are the most abundant white blood cell and comprise the first line of defence in the innate immune system. However, they are also capable of causing tissue damage in a wide range of diseases. Release of chemotactic signals from inflamed or infected tissues trigger neutrophil migration from the bloodstream to inflammatory foci, where they contribute to inflammation by undergoing receptor-mediated respiratory burst and degranulation. Degranulation from neutrophils has been implicated as a major causative factor in numerous inflammatory diseases. However, the mechanisms that control neutrophil degranulation are not well understood. Recent observations indicate that receptor-mediated granule release from neutrophils depends on activation of distal signaling pathways that include the *src* family of tyrosine kinases, β -arrestins, the tyrosine phosphatase MEG2, the kinase MARCK, Rabs and SNAREs, and the Rho GTPase, Rac2. Some of these pathways are specifically required for membrane fusion between the granule and plasma membrane, leading to exocytosis. This review focuses on the understanding of distal molecular mechanisms controlling exocytosis from neutrophils.

2. INTRODUCTION

Neutrophils are highly mobile, short-lived white blood cells that are densely packed with secretory granules. They emigrate from the bone marrow into the blood and infiltrate tissues in response to injury or infection. In healthy individuals, peripheral blood neutrophils make up the majority of white blood cells (40-80%). The lungs form the largest marginated pool of neutrophils in the body, where these cells fulfill an important sentinel role in maintaining alveolar sterility. As a major effector cell in innate immunity, neutrophils act as a double-edged sword. If neutrophils are absent, for example, in congenital neutropenia, or the more common cyclic neutropenia, opportunistic infections result from overgrowth of normally resident skin and gut bacteria and fungi at sites of injury, or exposed mucosal tissues. At the other extreme, accumulation and overactivation of neutrophils can be fatal in disorders such as in septic shock or acute respiratory distress. The tissue-damaging effects of neutrophils are completely dependent on activation of degranulation.

Degranulation is defined as the secretion by receptor-mediated exocytosis of granule-derived substances. Upon receptor-mediated stimulation of neutrophils, granules are mobilized triggering their docking and content secretion which can occur intracellularly with

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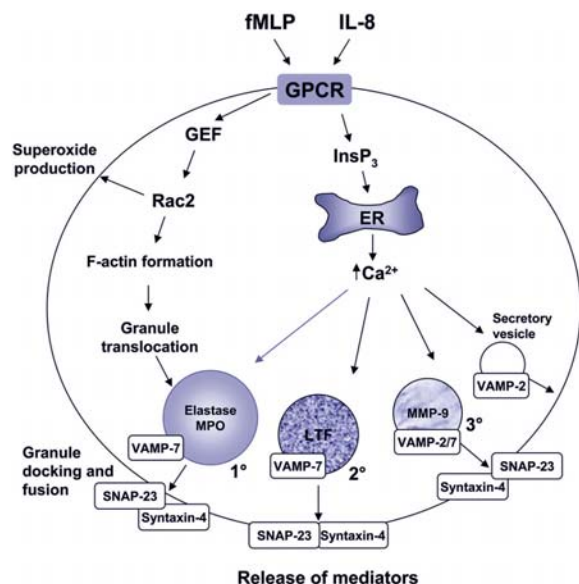


Figure 1. Rho GTPase and SNARE signaling pathways involved in Ca²⁺-dependent neutrophil degranulation. Receptor binding by a chemoattractant leads to G-protein-coupled signal transduction (GPCR) through multiple overlapping intracellular pathways to regulate the selective release of neutrophil granules. Some of these pathways may be non-redundant, for example, through G protein-activated guanine nucleotide exchange factors (GEF) to activate Rac2, which selectively mobilizes primary granules.

microbe-laden phagosomes, or extracellularly at the plasma membrane. Upon exocytosis of granules, neutrophils release a diverse array of antimicrobial proteins and enzymes, many of which also possess tissue-damaging properties. At the same time, neutrophils release reactive oxygen species by respiratory burst, to aid in pathogen clearance, and cytokines, to recruit additional leukocytes to the region of infection or inflammation.

A recent study by Brinkmann *et al.* (1) described a novel mechanism allowing elimination of microbial pathogens by neutrophils. Upon activation by ligands such as interleukin-8 (IL-8), lipopolysaccharide, and interferon- α with complement 5a (2), neutrophils generated a web of extracellular fibres known as “neutrophil extracellular traps” (NETs), composed of DNA, histones, and antimicrobial granule proteins, which were highly effective at trapping and killing invasive microbes. The authors proposed that NETs amplified the effectiveness of antimicrobial components by concentrating them in a fibrous, net-like structure and reducing their exposure to host tissues. While this report did not describe the molecular mechanisms responsible for NET formation and its association with granular protein, it opened a new horizon in the field of neutrophil biology in relation to mediator release and bactericidal activity. Moreover, NET formation may be an important mechanism in sepsis (3).

In many inflammatory disorders, such as acute lung injury, ischemia/reperfusion injury, severe asphyx

asthma, rheumatoid arthritis, and septic shock, excessive neutrophil degranulation is a common feature (4). Recent findings have identified a number of important signaling pathways in neutrophils that may be essential for neutrophil exocytosis.

3. GRANULE POPULATIONS IN NEUTROPHILS

Neutrophils contain at least four different types of granules identified by subcellular fractionation and transmission electron microscopy. These are: (1) primary granules, also known as azurophilic granules, (2) secondary granules, also known as specific granules, (3) tertiary granules, and (4) secretory vesicles (Figure 1). The primary granules are the main storage site of the most toxic neutrophil-derived mediators, including elastase, myeloperoxidase, cathepsins, and defensins. The secondary and tertiary granules contain lactoferrin and matrix metalloproteinase-9 (MMP-9, also known as gelatinase B), respectively (5). The secretory vesicles in human neutrophils contain human serum albumin, suggesting that they derive from endocytosis. The secondary and tertiary granules have overlapping contents but can be discriminated by their intrinsic buoyant densities when centrifuged on gradient media (6). All of these granule types are retained in the cell cytoplasm and are not released until receptors in the plasma membrane or phagosomal membrane signal to the cytoplasm to activate their movement for secretion of their contents by exocytosis. Thus, the receptor-linked secretory pathway is an important control mechanism, as the neutrophil is highly enriched in tissue-destructive proteases and other enzymes.

4. DEGRANULATION MECHANISMS IN NEUTROPHILS

Receptor stimulation of neutrophils by a secretagogue triggers granule translocation to the phagosomal or plasma membrane, where they dock and fuse, releasing their contents. The release of granule-derived mediators from neutrophils occurs by a tightly controlled receptor-coupled mechanism termed “regulated exocytosis”. Exocytosis is postulated to take place in four discrete steps (7). The first step of exocytosis is granule recruitment from the cytoplasm and translocation to the target membrane, which is dependent on actin cytoskeleton remodeling and microtubule assembly (8). This is followed by the second step of granule tethering and docking, leading to contact of the outer surface of the granule lipid bilayer membrane with the inner surface of the target membrane. Granule priming then follows as the third step to make granules fusion-competent and ensures that they fuse rapidly, in which a reversible fusion pore structure develops between the granule and the target membrane. The fourth and final step, granule fusion, occurs by formation and rapid expansion of the fusion pore, leading to complete fusion of the granule membrane with the target membrane and expulsion of release granular contents to the outside of the cell. This increases the total surface area of the cell, and exposes the interior surface of the granule membrane to the exterior. Tethering and docking steps require the sequential action of Rab and SNARE proteins

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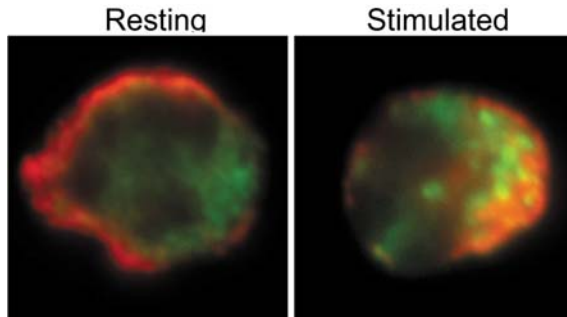


Figure 2. F-actin remodeling during receptor-mediated exocytosis in neutrophils. Resting and cytochalasin B/f-Met-Leu-Phe-stimulated neutrophils were fixed while in suspension, then attached to poly-L-lysine-coated slides and stained with rhodamine-phalloidin (F-actin, red) and FITC-conjugated anti-CD63 (primary granules, green). Cells were stimulated with 10 μ M cytochalasin B for 5 min at 37°C, followed by 15 min of 5 μ M f-Met-Leu-Phe. Images were collected by wide-field microscopy (original magnification, 63X).

(9); whether these factors also are needed for subsequent steps of fusion remains actively debated.

Translocation and exocytosis of granules in neutrophils require, as a minimum, increases in intracellular Ca^{2+} , guanosine triphosphate (GTP) as well as hydrolysis of adenosine triphosphate (ATP) (10,11). Not surprisingly, there are numerous target molecules for these effectors, including Ca^{2+} -binding proteins such as annexins and calmodulin, and GTP-binding proteins such as heterotrimeric and small monomeric proteins G proteins. The role of ATP in exocytosis, being utilized by ATP-hydrolyzing enzymes (i.e., the SNARE-binding *N*-ethylmaleimide-sensitive factor [NSF] AAA-ATPase) as well as kinases, is to act as an energy source for SNARE complex reorganization and as a phosphate donor for phosphorylation of downstream effector molecules.

4.1. Actin cytoskeletal dynamics in exocytosis

Actin remodeling is clearly a prime downstream target of activated effector molecules during receptor-mediated exocytosis. The actin cytoskeleton forms a mesh around the periphery in many different kinds of secretory cells (i.e., neutrophils, mast cells, neurons and endocrine cells). This may act as a shield against aberrant granule docking and fusion at the plasma membrane. Theoretically, then, the actin cytoskeletal mesh must be disassembled during exocytosis (12-14). However, some studies in neutrophils suggest that F-actin is normally diffuse in resting neutrophils, and only assembles into a cortical ring upon stimulation by f-Met-Leu-Phe (15,16). The reason for this discrepancy is likely to be methodological. In the latter studies, live neutrophils were first adhered to poly-L-lysine-coated glass slides (which itself leads to activation), before stimulation with f-Met-Leu-Phe for a period of time prior to fixation. Therefore, this procedure may lead to F-actin remodeling prior to stimulation. We have recently developed a method of visualizing neutrophils that were fixed in suspension immediately after f-Met-Leu-Phe

stimulation, followed by adherence of fixed cells to poly-L-lysine-coated slides (Mitchell *et al.*, manuscript in submission). This led to the detection of an actin cortical ring in resting cells which disassembled upon stimulation of exocytosis (Figure 2). Our findings suggest that resting, unstimulated neutrophils may contain an actin cortical ring that forms a barrier against granule docking and fusion that is rapidly disassembled during receptor activation and exocytosis.

Several studies report of actin remodeling to direct neutrophil migratory responses (reviewed in Affolter and Weijer, 2005) (17). Directed neutrophil movements, or chemotaxis towards sites of infection, are driven by polarized F-actin formation, with the leading edge of cells showing enhanced levels of actin polymerization. Interestingly, neutrophils produce a polarized response even when subjected to uniform concentrations of chemoattractants, and maintain this “front-ness” via continued production of activated effector molecules such as 3'-phosphoinositol lipids in the cell membrane (18,19). A recent comprehensive study of neutrophil granular proteins revealed that actin associates with all granule subsets, which suggests that actin may play an active role in regulating neutrophil exocytosis (20). Results from our own studies support this hypothesis; however, a simple model of actin depolymerization to facilitate granule mobilization does not suitably explain our observations. We found that cytoplasmic F-actin formation is also required for primary granule exocytosis, and that granules co-localize with polarized F-actin formed during chemoattractant stimulation (Figure 2). Therefore, polarized chemotactic responses that drive cytoskeletal remodeling may trigger polarized exocytosis, likely through the formation of “actin tracks” along which granules are driven to the plasma membrane for exocytosis.

4.2. Ca^{2+} signaling in exocytosis

Increases in intracellular Ca^{2+} alone are sufficient to induce the release granules from neutrophils, particularly if the concentration of Ca^{2+} is elevated to sufficiently high levels by the use of Ca^{2+} ionophores such as A23187 or ionomycin. A hierarchy of granule release exists in response to elevating concentrations of Ca^{2+} (21,22). The order of release is secretory vesicles > tertiary granules > secondary granules > primary granules (22,23). The release of each type of granule appears to be differentially regulated by unique intracellular signaling pathways. Activation of many neutrophil receptors causes elevated Ca^{2+} levels, including the seven transmembrane-spanning G protein-coupled receptors such as the formyl peptide receptor (that binds the bacterial tripeptide, f-Met-Leu-Phe) and chemokine receptors (such as the interleukin-8 receptor, CXCR1) (24,25). Although Ca^{2+} is a crucial second messenger in the activation of exocytosis, the specific target molecules for Ca^{2+} in neutrophil degranulation have not yet been identified. Several candidates have been suggested, notably annexins, protein kinase C, and calmodulin, all of which bind Ca^{2+} to modulate their activities (26-30). Neutrophils have been shown to require these Ca^{2+} -binding proteins for granule translocation and exocytosis.

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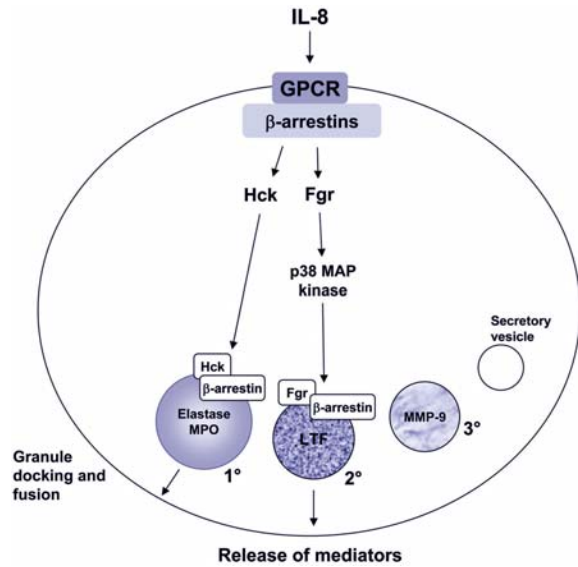


Figure 3. Tyrosine kinases associated with chemokine-induced neutrophil degranulation. Receptor binding leads to direct binding of the G-protein-coupled receptor (GPCR) by β -arrestins, which also translocate to primary and secondary granules along with *src* family kinases Hck and Fgr.

4.3. Phospholipid signaling in degranulation

Numerous studies have indicated a role for phospholipids, particularly polyphosphoinositides, in regulation of neutrophil degranulation. Polyphosphoinositide production, such as phosphatidylinositol 4,5-bisphosphate (PIP₂), induced by the activation of the hematopoietic cell-specific isoform phosphatidylinositol 3-kinase- γ (PI3K γ) has been shown to be required for granule exocytosis in permeabilized neutrophilic-like cells, HL-60 cells (31). Intracellular sites of PIP₂ formation in neutrophils are not known, but are likely to occur both at the plasma and granule membranes. Regions of PIP₂ enrichment in the membrane form essential binding sites for many intracellular signaling molecules, particularly those that contain pleckstrin homology domains, such as Rho guanine exchange factors including Vav, which signal downstream to Rho guanosine triphosphatases (GTPases) (see below) (32-34). Phosphatidylinositol transfer protein (PI-TP) has been shown to be essential for the transport of phosphatidylinositol to cellular membranes as a substrate for PI3K activity to generate PIP₂, and is also capable of restoring exocytotic responses in HL-60 cells (31). In addition, a role for phospholipase D has been indicated in neutrophil degranulation, particularly for primary and secondary granule release, as its product, phosphatidic acid, induces the release of these granules (35). Phosphatidic acid and its lipid derivative, lysophosphatidic acid, may act as a second messengers for downstream reactions, but they clearly aid fusion through the generation of membrane curvature (36). Thus, membrane lipid remodeling is also an essential component of degranulation in neutrophils.

4.4. Role for SRC family kinases in neutrophil degranulation

Protein phosphorylation is a critical event in neutrophil activation leading from receptor stimulation to exocytosis. Phosphorylation is carried out by kinases which are themselves frequently activated by phosphorylation by upstream molecules. This specifically involves the attachment of a phosphate molecule, donated by intracellular ATP, to a key site in the effector molecule, leading to conformational changes that cause activation. Receptor stimulation through the formyl peptide receptor by f-Met-Leu-Phe leads to phosphorylation of a wide range of kinases which then activate their respective effector pathways. Kinases can be discriminated based on their affinity for different amino acid residues in effector molecules. Thus, serine/threonine kinases and tyrosine kinases have been characterized as two distinct types of kinases involved in receptor signaling. Tyrosine kinases are further differentiated for their intrinsic association with the intracellular domain of receptors (receptor tyrosine kinases) or as cytosolic enzymes (nonreceptor tyrosine kinases).

The *src* family of nonreceptor tyrosine kinases have been implicated in the control of exocytosis of granule products from neutrophils. Three *src* family members, Hck, Fgr, and Lyn, are expressed in neutrophils and activated by f-Met-Leu-Phe receptor stimulation. Hck translocates to the myeloperoxidase-positive primary granule population following cell activation (37), while Fgr instead becomes associated with the lactoferrin-containing secondary granules during exocytosis (38). The selective recruitment of *src* kinases indicates that different signaling pathways exist in neutrophils to induce the release of each granule population. Treatment of human neutrophils with the *src* family inhibitor PP1 led to inhibition of the release of primary granules, secondary granules, and secretory vesicles in response to f-Met-Leu-Phe (39). Neutrophils isolated from *hck*^{-/-}*fgr*^{-/-}*lyn*^{-/-} triple knockout mice also showed a deficiency in secondary granule release of lactoferrin, although it was not possible to determine primary granule release of β -glucuronidase from murine cells (39). Interestingly, Hck and Fgr were recently shown to regulate the activation of the Rho GEF, Vav1, to signal through the Rho GTPase, Rac, and induce actin polymerization and superoxide release (34). The deficiency in secondary granule release in triple knockout neutrophils correlated with reduced p38 mitogen-activated protein (MAP) kinase activity, suggesting that *src* kinases act upstream of p38 MAP kinase. Indeed, treatment of human neutrophils with the p38 MAP kinase inhibitor, SB203580, led to reduced primary and secondary granule exocytosis in response to fMLP (39). However, another MAP kinase inhibitor, PD98059, which blocks ERK1/2 activity, did not affect release of primary and secondary granules or secretory vesicles (39). These findings indicate that *src* kinases, Hck, Fgr, and Lyn, along with p38 MAP kinase, but not ERK1/2, play a role in regulating the release of granules in response to f-Met-Leu-Phe stimulation in neutrophils, and probably act at an early signaling step proximal to the formyl peptide receptor in this process (Figure 3).

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4.5. β -arrestin function in regulating exocytosis

A group of scaffolding proteins known as β -arrestins have been shown to be required for activating signaling pathways leading to exocytosis of primary and secondary granules in neutrophils (40). β -arrestins are cytosolic phosphoproteins that were previously characterized for their role in endocytosis of ligand-bound chemokine receptors, particularly CXCR1, which is the high affinity receptor for the neutrophil chemotactic factor, IL-8. β -arrestins act by uncoupling activated G protein-coupled receptors from their associated heterotrimeric G proteins, and binding directly to the cytoplasmic tail of the CXCR1 receptor (40,41). Dominant negative mutants of β -arrestin were shown to inhibit the release of granules from transfected rat basophilic leukemia (RBL) cells, a cell line resembling mast cells and basophils (40). Interestingly, β -arrestins also associate with the primary and secondary granules in IL-8-activated neutrophils, and they do so by binding to Hck and Fgr, respectively (40). Thus, β -arrestins act at two sites in the cell during chemokine activation; one site at the receptor in the plasma membrane and a second on granule membranes (Figure 3).

4.6. Requirement for GTPases in exocytosis

Neutrophil granule exocytosis also requires binding of GTP to intracellular effector molecules, as the addition of the non-hydrolyzable analog GTP γ S to permeabilized or patch-clamped neutrophils leads to secretion of granule-derived mediators (42). This suggests that GTP-binding proteins, including GTPases, may be involved in granule translocation and exocytosis. To date, over 100 different types of GTPases have been identified in eukaryotic cells, with heterotrimeric G proteins and *ras*-related monomeric GTPases being two of the most comprehensively studied families of regulatory GTPases. *Ras*-related GTPases can be divided into several subfamilies based on their homology at the amino acid level. While heterotrimeric G proteins typically bind to the plasma membrane to transduce receptor signals to the cytoplasm, the superfamily of *ras*-related GTPases may reside in the cytoplasm, actin cytoskeleton, or on membranes to fulfill multiple regulatory roles in cell activation. *Ras*-related GTPases function by serving as important switches for turning on or off a signaling event. They are switched on by binding to high energy GTP, which is usually hydrolyzed to form GDP in order to activate the next effector molecule in the signaling pathway. Binding to GTP induces the association of many cytosolic GTPases to membrane or cytoskeletal sites within the cell.

Interestingly, exocytosis from neutrophils and other myeloid cells does not require hydrolysis of GTP to GDP, evident in the effects of GTP γ S which is poorly hydrolyzable and yet potently stimulates exocytosis (10,43). This finding suggests that there is a requirement only for GTP-bound forms of GTPases in exocytosis. The function of Rab GTPases in facilitating membrane fusion, for example during homotypic fusion in endoplasmic reticulum, is strongly inhibited by GTP γ S (44). This observation suggests that Rab GTPases may be precluded

from being essential for the final steps of exocytosis, and that other *ras*-related GTPases may fulfill this important function.

4.6.1. Rho GTPases

One particular group of *ras*-related GTPases is the Rho subfamily of GTPases, which serve a role in regulating actin cytoskeletal rearrangement and in the release of reactive oxygen species. Rho GTPases are potently activated by GTP γ S and do not require GTP hydrolysis and membrane/cytoskeleton dissociation for their effector function. Remodeling of the actin cytoskeleton by Rho GTPases is critical for allowing a diverse range of cellular activities to occur, including cell motility (chemotaxis), phagocytosis, and exocytosis. The three prototypical members of the Rho GTPase subfamily are Rho, Rac, and Cdc42 (45-47). Rac is present in three different isoforms: Rac1, Rac2, and Rac3. The functions of Rac1 and Rac2 in superoxide generation and chemotaxis is well established in neutrophils (48). Evidence that Rho GTPases are critically important in cellular regulation is provided in their availability as substrates for a number of bacterial toxins, including *Clostridium difficile* Toxin B and *Clostridium sordellii* lethal toxin, which inhibit Rho GTPase function by glucosylation of specific residues (49,50).

Rac1 and Rac2 share 92% identity in their amino acid sequences, and mainly differ in the final 10 amino acids of their carboxyl termini. Both isoforms are expressed in neutrophils, although human neutrophils express more Rac2 than Rac1 (51). It is because of this high homology that they serve functionally interchangeable roles in actin cytoskeletal remodeling and regulation of the release of reactive oxygen species by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in neutrophils (52-54). Intriguingly, because of the small degree of sequence variation, they exhibit distinct intracellular functions (16,55,56). Thus, Rac2 is the preferential activator of NADPH oxidase in neutrophils (57). Human neutrophils translocate most of their Rac2 to intracellular sites of NADPH oxidase activation following stimulation of respiratory burst (58), suggesting that the neutrophil oxidase preferentially produces reactive oxygen species at intracellular sites. The activation of the oxidase is clearly dependent on Rac binding; however, the requirement for GTP binding by Rac to activate the oxidase complex is the subject of some controversy. For example, one study showed that while phorbol myristate acetate (PMA) potently stimulates superoxide release in neutrophils, it does not induce significant Rac-GTP formation (59). Conversely, PMA-induced Rac-GTP formation was demonstrated in a separate study which was suggested to be critical for superoxide release in neutrophils (60). Our findings using the newly described Rac inhibitor, NSC23766 (61), showed that while it strongly inhibited f-Met-Leu-Phe-induced Rac1-GTP and Rac2-GTP formation in neutrophils, it was unable to block superoxide release induced by PMA or f-Met-Leu-Phe (Mitchell *et al.*, manuscript in submission). These observations suggest that Rac does not require activation (by binding to GTP) for assembly and activation of NADPH oxidase.

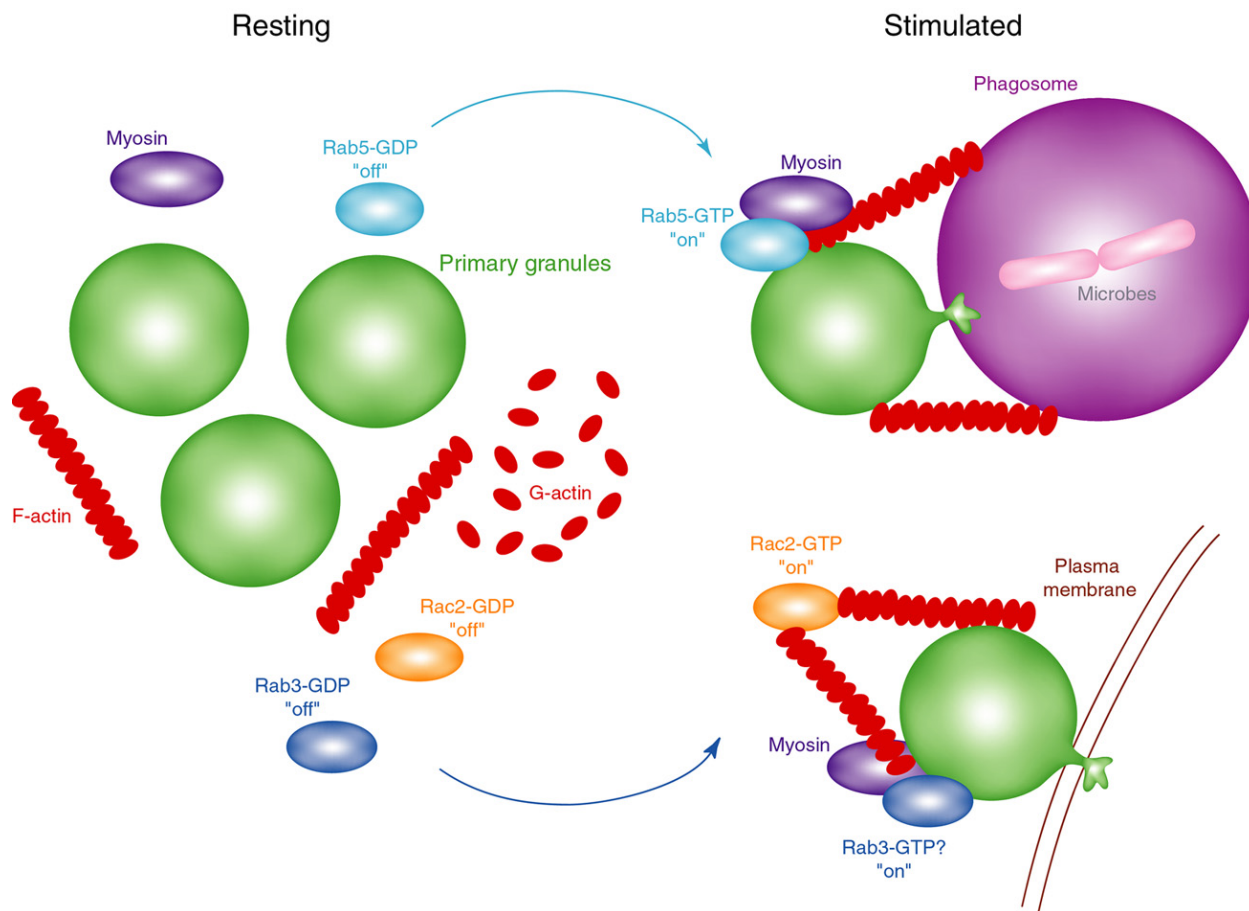


Figure 4. Postulated mechanisms for Rab and Rho GTPases in cross-talk for granule translocation and exocytosis. F-actin formation drives the transport of granules and aids their fusion to phagosomal or plasma membranes. Rab3 and Rab5 are postulated to differentiate between the target membranes, while myosin may act as an unconventional actin motor to propel granules along actin filaments.

We have determined that Rac2 serves a crucial and selective role in degranulation from neutrophils. Gene deletion of Rac2 led to a profound exocytotic defect in neutrophils, with a complete loss of primary granule myeloperoxidase release from murine bone marrow neutrophils (56). Release of granule enzymes from secondary and tertiary granule in response to a variety of stimuli was normal in *Rac2^{-/-}* neutrophils, indicating a selective role for Rac2 in primary granule exocytosis. *Rac2^{-/-}* neutrophils express normal or even elevated levels of Rac1 (57,62,63), suggesting that Rac2 serves a distinct role from Rac1 in regulating translocation and exocytosis of granules. Although *Rac2^{-/-}* neutrophils showed a loss of primary granule release, p38 MAP kinase phosphorylation was still evident in response to f-Met-Leu-Phe stimulation. This is in contrast to the findings of Mocsai *et al.*, who demonstrated an important role for p38 MAP kinase in primary granule release (39).

Our findings suggest that activated Rac2 functions to induce F-actin-induced granule translocation. *Rac2^{-/-}* neutrophils failed to translocate primary granules to the cell membrane during f-Met-Leu-Phe stimulation (56). Thus, the defect in primary granule exocytosis in *Rac2^{-/-}*

cells lies in the translocation machinery required to move the granules to the membrane for docking and fusion. This is likely to require actin polymerization, and Rac2 has been shown to induce the formation of F-actin which is required for chemotaxis (62). This may be the pivotal reaction which allows Rac2 to simultaneously translocate granules to the cell membrane and direct the movement of neutrophils at the leading edge of the cell (62). Identification of downstream effector molecules of Rac2 that are responsible for regulating actin cytoskeletal remodeling will be important in identifying the pathway(s) associated with Rac2-mediated primary granule release (Figure 4).

4.6.2. Rab GTPases

Distal signaling events must also activate factors that actively catalyze granule translocation and fusion at the plasma membrane. Whereas Rho GTPases may facilitate mobilization indirectly through actin remodeling, Rab GTPases (and SNAREs, see below) are directly involved in catalyzing these downstream events of exocytosis. The Rab family of GTPases constitute a large family (> 60) of small monomeric GTPases, which have been shown to attach vesicles to myosin V-type motors to actively drive their transport (64,65). Rabs also perform long-range vesicle

tethering reactions (as opposed to SNARE-mediated docking) to exocytosis sites via engagement of “velcro” effector complexes. The final choice of granule docking sites for exocytosis is directed by Rab proteins, which is achieved by distinct recruitment of Rab proteins and their effector complexes to granule subpopulations (64,66). Although Rab3 and Rab27 have not been studied in neutrophil exocytosis, by inference from other excitable secretory cells, a role for Rab proteins in granule fusion in neutrophils will undoubtedly be established (Figure 4).

Three Rab proteins, Rab3, Rab4 and Rab5, co-purify with neutrophil granules (67). It was shown that Rab5a directs the intracellular fusion of granules with pathogen-containing phagosomes in neutrophils (68) and other phagocytic cells (69-71). However, Rab5 is not known to direct granules to dock at the plasma membrane. Rabs of at least two different classes direct vesicle docking at the plasma membrane in excitatory cells; Rab3 in neuronal and endocrine cells (72,73) and Rab27 in cytotoxic T lymphocytes (74). Indeed, Rab3D expression is upregulated during myeloid differentiation into granulocytes, and therefore this Rab3 isoform may be a specific factor in myeloid cell degranulation (75). Rab3D also interacts with Rab3-associated kinase in mast cells, which can phosphorylate the plasma membrane t-SNAREs syntaxin-4, rendering it inactive (76). This confers a negative regulatory step between Rabs and SNAREs which might explain why Rab must go through GTP cycling (binding and hydrolysis) for fusion (Figure 4). However, later studies with a Rab3D knockout mouse model showed no deficiencies in granule exocytosis in mast cells as well as endocrine tissues, but exhibited defects in granule maturation (77). Taken together, the roles of Rab3D and Rab27 in neutrophil degranulation are currently unknown, and this area of research deserves further investigation.

It is interesting to also speculate whether Rho and Rab GTPase cross-talk during degranulation. Cross-talk mechanisms have already been shown to occur within the Rho family, which coordinate cell migration towards stimuli in neutrophils (19,46) and other cell types (78,79). Multi-GTPase cross-talk would also be a clever mechanism for granules to spatially and temporally coordinate the activation of Rho GTPases, to induce the formation of “actin tracks” followed by Rab GTPase association, and to recruit actin-based motors to self-regulate their exocytosis.

4.7. SNARE molecule binding in exocytosis from neutrophils

The final step of exocytosis has been postulated to involve the mutual recognition of secretory granules and target membranes, associated with the existence of intracellular receptors that guide the docking and fusion of granules. This led to the formation of the SNARE (soluble NEM-sensitive-factor attachment protein (SNAP) receptor) paradigm, which states that secretory granules possess membrane-bound receptor molecules that bind in trans another set of membrane-bound receptors on target membranes (80).

Studies on yeast and neuronal cells have yielded significant insights into the highly conserved components

of SNARE complexes which are membrane-bound proteins essential for vesicular docking and fusion in all cell types (80,81). The prototypical members of this complex are vesicle-associated membrane protein (VAMP-1, also known as synaptobrevin-1), syntaxin-1, and synaptosome-associated protein of 25 kDa (SNAP-25). The exocytotic SNARE complex consists of a vesicular SNARE (v-SNARE) VAMP, which binds to plasma membrane target SNAREs (t-SNAREs) syntaxin-1 and SNAP-25. These SNARE isoforms were originally characterized in neuronal cells (80). The fusion of membranes is proposed to depend on cytosolic NSF (*N*-ethylmaleimide-sensitive factor) and α , β , or γ -SNAP (soluble NSF-attachment protein)-mediated disassembly of the SNARE complex (80).

During binding, SNARE molecules form a four-helix (α -helices) coiled-coil structure contributed by three different molecules (one v-SNARE and two t-SNAREs). The binding region on each SNARE molecule associated with the four α -helices is known as the SNARE motif. The stability of the bonds within the SNARE structure is such that it is resistant to treatment with the detergent, sodium dodecyl sulphate (82).

SNARE molecules are exquisitely sensitive to cleavage by clostridial neurotoxins containing zinc endopeptidase activity, in particular, tetanus toxin (TeNT) and botulinum toxin serotypes (BoNT/A, B, C, D, E, F, and G) (83). The effects by these toxins on intracellular SNARE molecules are likely to be the molecular basis of spastic and flaccid paralysis induced by tetanus and botulinum toxin poisoning, respectively. TeNT and BoNT holotoxins are only able to enter neuronal cells, since their heavy chain components require a ganglioside-binding site on the cell surface, lacking in non-neuronal cells (83).

Other isoforms of SNAREs have been identified in cells outside of the neuronal system (syntaxin-4 and SNAP-23) (84), while VAMP-2 expression is widely distributed between neuronal and non-neuronal tissues (85). In addition, VAMP-4 (86), VAMP-5 (87), and the tetanus toxin-insensitive isoforms, VAMP-7, formerly known as tetanus toxin-insensitive VAMP (TI-VAMP) (88-91), as well as VAMP-8 have been characterized in non-neuronal tissues (92-94).

Neutrophils have been reported to express many of the SNARE isoforms so far identified. In an early report, neutrophils were shown to express syntaxin-4 and VAMP-2 (95). VAMP-2 was localized to tertiary granules and CD35⁺ secretory vesicles, and VAMP-2⁺ vesicles translocated to the plasma membrane during Ca²⁺ ionophore stimulation. By reverse transcriptase-polymerase chain reaction, mRNA encoding syntaxins 1A, 3, 4, 5, 6, 7, 9, 11, and 16 have been identified in human neutrophils and the neutrophil-like cell line HL-60 (96).

The functional role of SNAREs has been determined in studies using permeabilized neutrophils. SNAP-23 and syntaxin-6 appear to be important in regulating neutrophil secondary granule exocytosis using

antibodies against these molecules in electropermeabilized cells stimulated with Ca^{2+} and $\text{GTP}\gamma\text{S}$ (97). Addition of antibodies to VAMP-2 and syntaxin-4 to electropermeabilized neutrophils blocked Ca^{2+} and $\text{GTP}\gamma\text{S}$ -induced exocytosis (98). Exocytosis in the latter two papers was measured by flow cytometric analysis of granule markers CD63 (primary granules), CD66b (secondary granules), which are upregulated on the cell surface during stimulation. While anti-VAMP-2 blocked secondary granule CD66b upregulation in response to Ca^{2+} and $\text{GTP}\gamma\text{S}$, there was no inhibition of CD63⁺ primary granule release with this antibody. In summary, although VAMP-2 was shown to be involved in secondary granule exocytosis, there are no reports describing a VAMP isoform associated with primary granule exocytosis. This would appear to be a significant gap in our understanding of the mechanisms of degranulation in these cells, as primary granules are specifically enriched in bactericidal and cytotoxic mediators, including elastase and myeloperoxidase.

We and others have recently determined that VAMP-7 is highly expressed in all neutrophil granule populations, and that it may be an essential component for SNARE-mediated exocytotic release of primary, secondary, and tertiary granule release (43,99). Inhibition of VAMP-7 by low concentrations of specific anti-VAMP-7 antibody prevented the release of myeloperoxidase, lactoferrin, and matrix metalloprotease-9 in SLO-permeabilized human neutrophils. These findings indicate that VAMP-7 may play a promiscuous role in controlling regulated exocytosis of numerous granule populations. This is compatible with the recent observations that SNARE molecules are capable of binding multiple cognate and non-cognate partners (100). Thus, SNARE isoforms are likely to play a crucial role in the regulation of granule fusion in neutrophils.

4.8. Other potential regulatory molecules of exocytosis in neutrophils

Recent findings have suggested a role for a protein tyrosine phosphatase MEG2 in regulation of neutrophil degranulation. Neutrophils express MEG2 in their primary, secondary, and tertiary granules, which translocates to the phagosomal membrane upon phagocytosis of serum-opsonized iron beads (101). MEG2 was recently shown to be a phosphatase required for dephosphorylation of NSF, the cytosolic ATPase required to cycle SNARE proteins between bound and unbound conformations in order to allow repeated membrane fusion events (102). This study demonstrated for the first time that NSF possesses a tyrosine residue that is phosphorylated, and that dephosphorylation triggers the binding of another cytosolic protein, α -SNAP, that is also required for SNARE cycling, in order to promote vesicular fusion. Cells expressing a dephosphorylated form of mutant NSF exhibited substantial enlargement of their granules, suggesting that the dephosphorylated NSF remained bound to α -SNAP to allow repeated homotypic granule fusion and enlargement of the granules in the cells. Transfection of a phospho-mimicking mutant of NSF was shown to inhibit the secretion of interleukin-2 from Jurkat T cells (102). In addition, MEG2 was shown to be activated by

polyphosphoinositides, particularly PIP_2 (101), suggesting that MEG2 is directly associated with the membrane fusion event in granule fusion.

An additional regulatory protein is myristoylated alanine-rich C kinase substrate (MARCKS), which may be required for neutrophil primary granule release (103). This is an interesting discovery since MARCKS was originally characterized for its ability to cross-link actin filaments and is regulated by protein kinase C and Ca^{2+} -calmodulin (104). This may provide a novel mechanism for Ca^{2+} -induced exocytosis in connection with actin polymerization in neutrophils.

5. SUMMARY

These recent experimental observations reveal that a large group of intracellular signaling molecules exists in neutrophils to regulate translocation of granules to the cell membrane for docking and fusion to release their contents. Many of these molecules are already natural targets for bacterial toxins to inhibit their function, which highlights their important role in regulating bactericidal mediator release. It may be possible to exploit the use of bacterial toxins as a tool to prevent or modulate neutrophil degranulation. Neutrophil exocytosis is an important event in numerous inflammatory diseases. Neutrophil-derived granule products, including the high molecular weight form of matrix metalloprotease-9 specific to neutrophils, have been shown to increase in proportion to asthma severity in the airways of asthmatic patients (105). Moreover, neutrophils and their granule products are strongly associated with the pathogenesis of numerous inflammatory diseases (4). Further analysis of the signaling pathways that specifically activated to induce the release of different granule populations in neutrophils may create opportunities for the development of drugs that will prevent degranulation from neutrophils in airway diseases as well as inflammatory disorders.

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Abbreviations: ATP: adenosine triphosphate; BoNT: botulinum neurotoxin; F-actin: filamentous actin; GTP: guanosine triphosphate; GTP γ S: guanosine 5'-(3-O-thio)triphosphate; GTPases: guanosine triphosphatases; MAP: mitogen-activated kinase; MARCKS: myristoylated alanine-rich C kinase substrate; MMP: matrix metalloprotease; NADPH: nicotinamide adenine dinucleotide phosphate; NET: neutrophil extracellular trap; NSF: N-ethylmaleimide-sensitive factor; PI3K: phosphatidylinositol 3-kinase; PIP $_2$: phosphatidylinositol 4,5-bisphosphate; PMA: phorbol myristate acetate; SLO: streptolysin-O; SNAP: soluble NSF attachment protein; SNAP-23/25: synaptosome-associated protein of 23/25 kDa; SNARE: SNAP receptor; TeNT: tetanus neurotoxin; VAMP: vesicle-associated membrane protein

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