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COMMENTARY

Exocytosis of Neutrophil Granulocytes

Erzsébet Ligeti and Attila Mócsai*

DEPARTMENT OF PHYSIOLOGY, SEMMELWEIS MEDICAL UNIVERSITY, BUDAPEST, HUNGARY

ABSTRACT. Neutrophil granulocytes play an important role in the defense mechanisms of mammalian organisms against bacterial invaders. The combat arsenal of neutrophils consists of engulfing and endocytosing the foreign particle, producing toxic oxygen compounds, and liberating substances stored in intracellular vesicles. At least four different types of granules are formed during maturation of neutrophil granulocytes in the bone marrow. Functional properties of release from the different granule populations differ in several respects from characteristics of neurotransmitter release, the best understood secretory process in mammals. The available data indicate that several key proteins of the exocytotic machinery identified in neural tissue either are absent from neutrophil granulocytes or their subcellular localization is different. Furthermore, in a human disease (Chédiak-Higashi syndrome), the defect of the secretory pathway affects mainly the cells of the haemopoietic lineage. Taken together, these data suggest that regulated exocytosis from neutrophil granulocytes (or perhaps also from other haemopoietic cells) may represent a specific case of the general mechanism of secretion. BIOCHEM PHARMACOL **57**;11:1209–1214, 1999. © 1999 Elsevier Science Inc.

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GENERAL MECHANISM OF SECRETION

The current concept holds that membrane fusion proceeds by the same mechanism from "yeast to man" and from the endoplasmic reticulum to the plasma membrane [1–6]. According to the SNARE† hypothesis [5], the specificity of the process is provided by integral membrane proteins, residing in the vesicular and in the target membranes (*SNAP-Re*ceptors, v-SNAREs, and t-SNAREs, respectively), which are able to interact with each other, allowing the correct docking of the vesicle to be fused. Different sets of v-SNARE and t-SNARE proteins function at the various fusion steps, such as the fusion of ER-derived vesicles to the Golgi membrane or Golgi-derived secretory vesicles to the plasma membrane. Additional proteins (the Rab family of small GTP-binding proteins, the family of Sec1 proteins) seem to be responsible for the "supervision" of the specificity of docking [7]. In addition to the membrane-specific SNARE proteins, efficient fusion requires also the participation of ubiquitous cytosolic proteins: NSF (*N*-ethylmaleimide-*s*ensitive *f*usion protein) and the SNAPs (soluble *N*SF-*a*ttachment *p*roteins: α-, β-, and γ-SNAPs). NSF is an ATPase that binds firmly to SNAPs attached to SNARE proteins but not to soluble SNAPs. The energy of ATP hydrolysis is required for the dissociation of the fusion complex and probably for the preparation of the SNARE proteins for the next round of membrane fusion [8]. In some specialized fusion processes, NEM-sensitive homologues of NSF have been shown to function as ATP-hydrolyzing enzymes [9, 10].

The generality of the above mechanism of membrane fusion is firmly supported by similarities of the secretion process in yeast and in mammalian tissues. For most proteins participating in the fusion event in mammalian cells (v-SNAREs, t-SNAREs, SNAP, NSF, and Rabs), homologous proteins have been identified in yeast [4]. A molecular complex consisting of stoichiometric amounts of the relevant t- and v-SNAREs, α- and γ-SNAP, and NSF could be isolated from both synaptic vesicles [5] and yeast ER [11]. Furthermore, in *in vitro* fusion assays purified yeast proteins could substitute for missing or mutated mammalian homologues [4]. On the other hand, mechanisms of vesicular transport different from the above detailed one seem to exist, too. As an example, vesicular transport to the apical membrane of epithelial cells has been suggested to proceed without the involvement of NSF, α-SNAP, SNAREs, and Rab proteins [12].

The first mammalian SNARE proteins have been identified in neural tissue responsible for the release of neurotransmitters [5–7]. The membrane of the synaptic vesicles contains the 18 kDa protein synaptobrevin or VAMP, whereas the 35 kDa protein syntaxin and the 25 kDa protein SNAP-25 are localized in the presynaptic plasma membrane (note that SNAP-25 and the NSF-binding

* Corresponding author: Dr. Erzsébet Ligeti, Department of Physiology, Semmelweis Medical University, P.O. Box 259, H-1444 Budapest 8, Hungary. Tel. (361) 266-7426; FAX (361) 266-7480; E-mail: Ligeti@puskin.sote.hu

†*Abbreviations:* ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; fMLP, formyl-methionyl-leucyl-phenylalanine; MAP kinase, mitogen-activated protein kinase; MEK, MAP/ERK kinase; NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF-attachment protein; SNARE, SNAP-Receptor; and VAMP, vesicle-associated membrane protein.

SNAP are different proteins). In synaptic membranes, synaptobrevin, syntaxin, and SNAP-25 form a stable complex with α-SNAP and NSF that can be isolated from detergent-solubilized brain tissue, provided the hydrolysis of ATP is prevented by using ATPγS [5]. The neural SNARE proteins are specific targets of various clostridial toxins: synaptobrevin is cleaved by tetanus toxin and botulinal toxins B, D, and F, whereas syntaxin is proteolyzed by botulinal toxin C1 and SNAP-25 by botulinal toxins types A and E [4].

The release of transmitter substances at the nerve terminals is induced by a sudden rise of intracellular [Ca^{2+}]. Accordingly, Ca^{2+}-sensors have been suggested to control the assembly/disassembly of the SNARE complexes. The Ca^{2+}-binding protein synaptotagmin has been shown to associate with the SNARE complex but to be released by an excess of α-SNAP. The competitive nature of binding of synaptotagmin and α-SNAP to the SNARE complex gave rise to the hypothesis that in the resting state molecular "clamps" prevent the fusion step, and exocytosis is initiated only after removal of these "clamps," e.g. by the sudden increase of intracellular [Ca^{2+}]. The Ca^{2+}-sensitive protein synaptotagmin could operate as one of these "clamps" [6]. In this view, constitutive fusion (such as fusion of ER-derived vesicles to Golgi membranes, or fusion steps within the Golgi) would proceed without the intervention of any "clamps."

The same or homologous SNARE proteins as in neural tissue were shown to be present in the vesicle and plasma membrane of other secretory cells, such as pituitary, adrenal chromaffin, or pancreatic β-cells [13]. Specific cleavage of the SNARE proteins by clostridial toxins allowed the assessment of the functional role of these proteins in the exocytotic process. The presence of NSF, α- and/or γ-SNAP, synaptotagmin, and Rab3 has also been demonstrated in the cells investigated. These mammalian cells function as "professional" secretory cells, where the proteins packaged in the secretory vesicles are synthesized continuously throughout the lifetime of the cell, and a sudden rise of the intracellular [Ca^{2+}] is sufficient for the initiation of the release of the content of the secretory vesicles.

SECRETION FROM NEUTROPHIL GRANULOCYTES

Neutrophil granulocytes provide an interesting model for studies on exocytosis, as they exhibit a few specific features differing from the general pattern of secretion. These cells possess several types of granules, which are formed during different periods of maturation in the bone marrow. The soluble proteins packaged into these granules are synthesized in the same maturation phase as the granule itself, but later the corresponding genes are gradually turned off [14, 15], and in mature, circulating granulocytes the synthesis of the granule proteins is either absent or very low [16].

Generally, four types of granules are distinguished. The most ancient population, the primary or azurophilic granules, appear in the promyelocytic phase and contain the neutrophil-specific protein myeloperoxidase and many hydrolytic enzymes. Due to their high content of acidic hydrolases, they are often regarded as modified lysosomes, although the characteristic lysosomal marker proteins (LAMPs) are not present on azurophilic granules [17, 18]. The other three types of granules are peroxidase-negative, and they are classified as secondary (specific) granules, tertiary (gelatinase) granules, and secretory vesicles. They appear in the myelocytic stage or later. The secretory vesicles contain plasma proteins, and they arise on the endosomal pathway, but similar to the other granule populations, secretory vesicles are not reformed in mature cells. Classification of the granule population of the neutrophil granulocytes is based mainly on the results of high-resolution cell fractionation techniques, but inhomogeneities have been reported within each group [19, 20]. Recent reports suggest that granules of the neutrophil granulocytes should rather be regarded as a continuum from the earliest azurophilic granules to the latest gelatinase granules, with overlaps in the protein content [15].

Exocytosis of the different granule populations results in liberation of the soluble proteins packaged in the given granules and translocation of specific proteins from the granule membrane to the plasma membrane. Both processes have a significant role in the defensive functions of the neutrophils, e.g. the migration of the cells through the vessel wall is supported both by the appearance of additional adhesion proteins in the plasma membrane and by release of collagen-degrading enzymes.

There are characteristic differences in the conditions required for the exocytosis of the different granule populations.

(1) The kinetics of release of the different granule populations show strict hierarchy under both *in vitro* conditions and *in vivo*. Secretory vesicles are released the fastest, followed by gelatinase granules, whereas the liberation of specific granules occurs considerably slower [21].

(2) Significant differences were reported in the response to elevation of intracellular [Ca^{2+}]. An increase of intracellular [Ca^{2+}] by 40–50 nM already induces release of proteins from secretory vesicles, and complete release of this granule fraction can be achieved in Ca^{2+} medium by ionomycin alone. Gelatinase granules can also be released completely by Ca^{2+} plus ionomycin, but only at higher intracellular [Ca^{2+}]. On the other hand, exocytosis of specific granules requires intracellular [Ca^{2+}] of approximately 1 μM, whereas the release of myeloperoxidase-positive granules occurs only at extreme values of intracellular [Ca^{2+}] [21, 22]. In line with these differences, Ca^{2+} *per se* (entering via ionomycin) is only able to induce the liberation of approximately 50 and 20% of the protein content of specific and azurophilic granules, respectively [21]. Differences in the Ca^{2+} sensitivity of the various granule populations may be related to the reported variations in binding and processing of annexins, a group of

Ca^{2+}-regulated proteins to the various granule types [23, 24].

(3) Stimulation of various plasma membrane receptors induces the exocytosis of the majority of secretory vesicles and a significant part of the gelatinase granules [20, 21], whereas the release of the contents of specific and azurophilic granules into the extracellular space only occurs in the presence of cytochalasin B, i.e. when microfilaments are disrupted.

In contrast to neuroendocrine cells, the exocytotic machinery of neutrophil granulocytes seems to be controlled both by Ca^{2+}-dependent mechanisms and by other regulatory steps initiated by occupation of plasma membrane receptors. As detailed above, exocytosis of all types of granules is dependent on elevation of intracellular Ca^{2+}. However, under various experimental conditions, a significantly larger release of primary and secondary granules was detected upon stimulation of plasma membrane receptors than upon addition of ionomycin [21, 22, 25]. Furthermore, almost complete release of secretory vesicles could be achieved with the chemoattractant fMLP when both extra- and intracellular Ca^{2+} have been chelated [21]. These observations point to the importance of signalling pathways independent of, or parallel to, increase of intracellular [Ca^{2+}]. The pathways of signal transduction from the various plasma membrane receptors to the exocytotic machinery of the different granules are at present unknown. The involvement of tyrosine kinases, one group of the possible messengers, is discussed below.

The idea that secretion from neutrophil granulocytes (and perhaps other haematopoietic cells) involves mechanisms partially differing from other secretory cells is supported also by a natural mutation, appearing in the form of a human disease. Patients suffering from Chédiak-Higashi syndrome have increased susceptibility to infection, pointing to the impairment of their antibacterial defense system. Release of the granule contents from neutrophil granulocytes (and cytotoxic lymphocytes) is defective, whereas secretion from neuroendocrine cells seems not to be affected [26].

PROTEINS OF THE SECRETORY MACHINERY IN NEUTROPHIL GRANULOCYTES AND OTHER HAEMATOPOIETIC CELLS

In view of the considerable differences in the functional properties of secretion from neutrophil granulocytes, it is surprising how little is known about the molecular mechanism of exocytosis and the proteins participating therein. A detailed study based on immunodetection of proteins [27] showed that the classic, neural-type SNARE proteins—syntaxin 1, VAMP-1, and SNAP-25—are not detectable in circulating granulocytes. This observation has been confirmed by screening the cDNA library of HL-60 cells by using polymerase chain reaction (PCR) [28]. Syntaxin 4 and VAMP-2, homologs of the neural SNARE proteins, have been demonstrated in neutrophil granulocytes on both the protein and the mRNA level [27, 28]. Similar to neural tissue, syntaxin 4 was localized mainly to the plasma membrane, but VAMP-2 could be detected on only tertiary granules and secretory vesicles, whereas it was absent from the membrane of primary and secondary granules [27].

Recently SNAP-23, a protein homologous to the almost exclusively neurally localized SNAP-25, has been cloned in B lymphocytes [29]. This protein was shown to be present in numerous non-neural tissues, and the majority of it was associated with the plasma membrane [30]. In neutrophil granulocytes, two isoforms of SNAP-23 have been detected on the mRNA level [31], although no data are available about the subcellular localization of the corresponding proteins. SNAP-25 also has been described in neutrophils; however, differently from neural tissue, it was localized to the membrane of peroxidase-negative granules [32].

Up to the present, no data have been published about the existence of NSF or one of its homologs in neutrophil granulocytes, although exocytosis of primary granules definitely is inhibited by NEM*. Similarly, data are lacking concerning α-, β-, or γ-SNAP, the neural Ca^{2+}-sensor synaptotagmin, or the participating Rab proteins. In one study, the screening of a cDNA library from HL-60 cells gave negative results for NSF and synaptotagmin†. Thus, homologs of the neural SNARE proteins have been demonstrated in neutrophil granulocytes, but no positive information is available about the other core proteins of the secretory machinery.

Recent experimental data indicate that in some cell-free systems and in reconstituted phospholipid vesicles containing v-SNARE and t-SNARE proteins, one round of fusion can occur without the participation of NSF and SNAPs. These observations gave rise to the suggestion that the SNARE proteins alone are sufficient for the fusion event, whereas NSF and the SNAP proteins are required for the separation of the stable complex of v- and t-SNAREs, allowing repeated docking-fusion cycles [8]. At this point, it should be recalled that mature granulocytes do not reproduce their granules, and exocytosis from these cells may be limited to one single round of fusion.

Concerning the proteins of the exocytotic machinery in other haematopoietic cells, sporadic data are available on eosinophil granulocytes [33], macrophages [34–38], and platelets [39]. The information collected in Table 1 shows that up to now, in no cell of the haematopoietic lineage could be found a whole set of proteins that–according to the SNARE hypothesis–would be required for granule release in a similar way as described for neuroendocrine tissues.

* Horváth O, Mócsai A and Ligeti E, unpublished observation.
† Smolen J, personal communication, cited with permission.

TABLE 1. Presence of proteins of the exocytotic machinery in different cells of the haemopoietic lineage

Secretory protein	Neutrophils	Eosinophils	Macrophages	Platelets
Syntaxin	Syntaxin-4	Syntaxin-3	Syntaxin-2 Syntaxin-3 Syntaxin-4	Syntaxin-2 Syntaxin-4
VAMP	VAMP-2	∅	"VAMP-like"	∅
SNAP-25	∅/+	∅	?	∅
SNAP-23	+	?	?	?
NSF	?/∅	?	+	+
SNAP	?	?	α-SNAP β-SNAP	α-SNAP γ-SNAP
Synaptotagmin	?/∅	∅	?	∅
Synaptophysin	∅	∅	?	∅
Rab 3a	?	∅	?	?
Reference	27, 28, 31, 32	33	34–38	39

Explanation of symbols: (+) the protein or its mRNA has been detected; (∅) the protein or its mRNA could not be detected; and (?) no data available.

ROLE OF TYROSINE KINASES IN DEGRANULATION

Tyrosine kinases have been shown to be activated upon stimulation of the neutrophil granulocytes by various receptor agonists, and they have been attributed a role in activation of the superoxide-producing NADPH oxidase [40, 41]. However, controversial data have been published concerning their participation in the organization of the degranulation response of the neutrophils [25 and references therein, 42].

As a first attempt, the effect of the broad-specificity tyrosine kinase inhibitors was investigated [25, 42]. The release of the primary and secondary granules induced by ligands of chemotactic receptors (in the presence of cytochalasin B) or by serum-opsonized zymosan was powerfully blocked. These results clearly indicated that tyrosine kinases do participate in the organization of the exocytotic response.

The role of kinases belonging to the Src family has been addressed by both pharmacological and genetic approaches. PP1, a recently described inhibitor of the Src-family tyrosine kinases, inhibits the release of secondary granules triggered by tumor necrosis factor (TNF) from adherent granulocytes or by the chemotactic ligand fMLP from suspended cells [43]. In accordance with this observation, exocytosis of secondary granules was impaired severely in neutrophil granulocytes obtained from $Fgr^{-/-}$, $Hck^{-/-}$ double knockout mice. As degranulation from these cells could be successfully induced by phorbol ester or by the Ca^{2+}-ionophore ionomycin, it is suggested that Fgr and Hck may be involved in the signalling pathway of adhesion molecules rather than in a final common step of the exocytotic process. It has to be noted, however, that in human neutrophil granulocytes Fgr and Hck were shown to be localized mainly to the membrane of secondary and primary granules, respectively [44, 45]. Upon stimulation of the cells with serum-opsonized zymosan, Hck became activated and it was translocated to the phagosomes [45, 46]. In the case of Fgr, a similar effect was obtained by the chemoattractant fMLP [44]. The recent observation that phagocytosis of certain Mycobacteria inhibits Hck activity and degranulation in neutrophil granulocytes provides further support for the participation of Hck in the process of regulated exocytosis [47].

Another family of tyrosine kinases shown to become active upon stimulation of neutrophil granulocytes are the MAP kinases [48–50]. Their possible role in degranulation was investigated by a pharmacological approach. The recently described MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059) had no effect on the release of either primary or secondary granules induced by either chemotactic agents or phorbol esters, although inhibition of MEK could be clearly demonstrated in the cells investigated [25]. In our experiments, partial inhibition of spreading of the granulocytes on fibrinogen could be observed after treatment with PD98059*. Other authors reported partial inhibition of phagocytosis and prevention of fMLP-induced apoptosis [48, 51], but the data concerning chemotaxis are controversial [52–54]. Thus, we suggest that activation of ERK may be involved in organization of the shape changes and regulation of apoptosis of granulocytes, rather than in exocytosis. In contrast to ERK, inhibition of p38 MAP kinase by SB 203580 resulted in a partial inhibition of fMLP-induced exocytosis of primary granules†.

Taken together, pharmacological and genetic data suggest that tyrosine kinases may be involved at different steps in the complex pathway leading from stimulation of a surface receptor to the final fusion event. Kinases of the Src family play a definitive role, whereas from the MAP kinase family only p38 seems to be involved. ERK probably does not participate in the organization of the exocytotic response of neutrophil granulocytes. Elucidation of the specific roles of the individual kinases will require further biochemical, pharmacological, and genetic approaches.

* Mócsai A and Ligeti E, unpublished observation.
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