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REVIEW ARTICLE

Platelet secretory behaviour: as diverse as the granules . . . or not?

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Summary. Platelets play a central role in the arrest of bleeding after damage to a blood vessel and in the development of thrombosis. Platelets rapidly respond after interaction with sub-endothelial components and release cargo from their storage granules. The three principal granule types of platelets are α-granules, dense granules and lysosomes. Timed release of granule contents and regulated expression of critical receptors are essential for maintenance of the platelet thrombus, yet also have important functions beyond hemostasis (i.e. inflammatory reactions and immune responses). α-granules store adhesive molecules such as von Willebrand factor and fibrinogen, growth factors and inflammatory and angiogenic mediators, which play crucial roles in inflammatory responses and tumor genesis. The α-granules comprise a group of subcellular compartments with a unique composition and ultrastructure. Recent studies have suggested that differential secretory kinetics of α-granule subtypes is responsible for a thematic release of adhesive and inflammatory mediators. In addition, new results indicate that activation-dependent synthesis and release of cytokines also contribute to the inflammatory role of platelets. We will discuss the various methods that platelets use to regulate secretory processes and how these relate to potential differential secretion patterns, thereby promoting adhesiveness and/or inflammatory functions. We will focus on the heterogenic granule population, open canalicular system (OCS) plasticity, the role of contractile and mechanobiological forces, and the fusogenic machinery.

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Introduction

Platelets circulate at variable flow rates in our bloodstream and are activated following binding to components such as von Willebrand factor (VWF) and collagen that are exposed during vascular injury [1]. Platelet secretion is tightly regulated and the result of various signaling pathways, which increase cytosolic Ca^{2+}, thereby promoting fusion of platelet granules with the plasma membrane (PM) and release of their contents. Secretion dynamics are crucial for stabilizing platelet adhesion, progression of platelet platelet interactions and building of a stable platelet thrombus [2]. Besides a crucial role in primary hemostasis, the release of platelet contents also has an important role in inflammatory reactions. Platelets are a rich source of chemokines, cytokines and growth factors, which are predominantly packaged in the main storage compartment, the α-granules [3]. These mediators are released at the sites of injury, thereby promoting wound repair and vascular remodeling [4] through signaling of target cells and leukocyte tethering [5]. As a consequence, platelet dysfunction can lead to bleeding disorders or thrombosis, but is also associated with inflammation, immunological responses [6] and cancer progression [7]. In contrast to previous perceptions, recent studies showed that platelets can synthesize and release IL-1β, suggesting that newly synthetized proteins also contribute to platelet function in inflammation [8]. Here we highlight recent progress in understanding the platelet secretory response, and discuss subjects such as granule subtypes, cargo distribution, platelet contraction, mechanobiology and OCS plasticity, and SNARE-dependent fusion.

Platelet secretory granules

α-granules

α-granules are the most abundant secretory organelles in platelets (50–80 per platelet). They contain a large

variety of adhesive proteins that are important for primary hemostasis, including the adhesive proteins VWF, fibrinogen, fibronectin, vitronectin and thrombospondin [9,10]. These proteins are important for platelet adhesive properties and building of a stable thrombus. Platelet α-granules also contain a large number of mediators that have a function in coagulation, wound repair, inflammation and angiogenesis. These include, amongst others, platelet factor 4 (PF4), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), transforming growth factor–β (TGF-β) and vascular endothelial growth factor (VEGF) [3]. PF4, β-thromboglobulin (CXCL7) and Rantes (CCL5) are the most abundant chemokines in platelet α-granules. PF4, CXCL7 and IL-8 released from activated platelets modulate inflammation by attracting neutrophils [7,11].

The mechanism by which adhesive and inflammatory cargo is packaged in storage granules is incompletely understood. α-granules are formed from multivesicular precursor organelles that acquire a cargo from the biosynthetic route (VWF, β-thromboglobulin and PF4) and endocytosis (fibrinogen, albumin and IgG) [12]. α-granules frequently harbor clathrin coats, an attribute shared with sorting organelles such as the trans-Golgi network (TGN) and endosomes. α-granules thus have properties of secretory organelles as well as late endosomes, which qualify them as lysosome-related organelles (LROs). LROs include also the platelet dense granules, Weibel-Palade bodies (WBPs) in endothelial cells, melanosomes in melanocytes, and lamellar bodies in type II lung cells [13].

The classical view that platelet α-granules represent a homogeneous population of organelles has been challenged by several groups. Italiano reported that anti-angiogenic factors, such as endostatin, reside in different granules to pro-angiogenic VEGF [14], while electron tomography showed the presence of different classes of α-granules [15], including spherical, multivesicular and tubular subtypes (Fig. 1). The multivesicular class probably reflects the endosomal connection. The biogenesis of the tubular α-granule population remains unclear. Platelet α-granules resemble the WPBs in several ways. Both organelles are involved in hemostasis, inflammation and angiogenesis and share crucial proteins, including VWF, P-selectin and CD63. Similar to WPBs, VWF in α-granules is compartmentalized and organized in tubular helix assemblies (Fig. 1). The tight packing of VWF in tubular structures is responsible for the tubular cigar shape of WPBs [16]. The VWF tubules in α-granules, however, are much shorter and occur far less frequently, making it unlikely that VWF packing is responsible for shaping the tubular α-granules [15].

Dense granules

Platelet dense granules (three to eight per platelet) form the second major secretory compartment. They contain

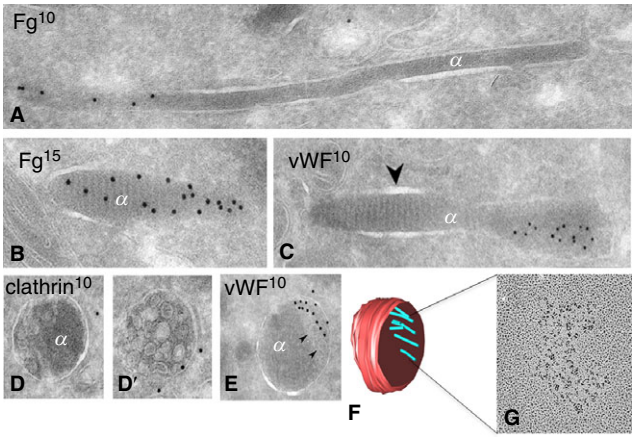

Fig. 1. Platelet α-granule subtypes: (A) tubular α-granule; (B,C) protein segregation in α-granules; (D,D') luminal membrane domains in α-granules; (E,F) VWF cargo assembled in peripheral tubules, arrowheads in (E) indicate luminal vesicles; (G) rotary shadow image of VWF multimeric protein. This research was originally published in Blood 2010; 116: 1147–56, © the American Society of Hematology [15].

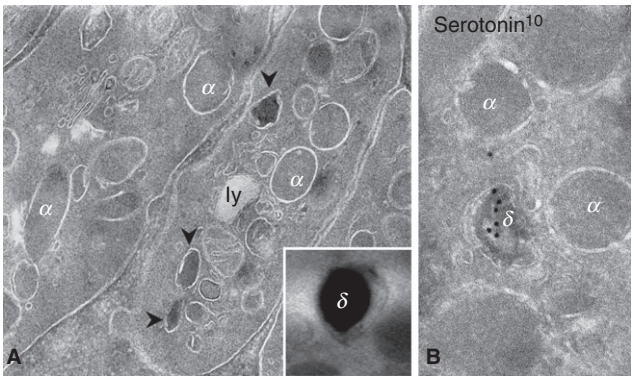

Fig. 2. The platelet secretory compartment. (A) Platelet α-granules (α), dense granules (arrowheads and δ) and lysosomes (ly). (B) Localization of serotonin in dense granules.

mainly small molecules such as ADP, ATP, serotonin, calcium, pyrophosphate and polyphosphate. Dense granules originate from endosomal precursors rather than TGN [17], and play a crucial role in primary hemostasis by acting as a feedback mechanism, stimulating the platelet P2Y12 receptor via release of ADP. Dense granules have characteristics in common with acidocalcisomes and chromaffin granules [18]. They are acidic in nature and accumulate acidophilic dyes such as acridine orange and mepacrine. Based on their high nucleotide content, dense granules also possess a high affinity for typical nuclear stains (dapi) and uranium ions [19] (arrow heads in Fig. 2A). The predominant calcium and phosphate content is also a reason for their electron opaque appearance in whole-mount electron microscopy (EM) and cryo-EM preparations (Fig. 2A, inset). Dense granules are difficult to preserve in frozen thin sections, where the dense core content is frequently lost, leaving only their limiting membranes (Fig. 2B). Dense granules contain the lysoso-

mal membrane proteins CD63 and LAMP1/2 [20], but also non-lysosomal proteins such as P-selectin, GPIb and αII-β3 integrin [20]. These membrane proteins are translocated to the cell surface upon platelet activation. Dense granules contain rab27a and b [21,22], two small GTPases required for content release.

Lysosomes

Platelet lysosomes contain acid hydrolases (cathepsins, hexosaminidase, β-galactosidase, arylsulfatase, β-glucuronidase and acid phosphatase) as their most important cargo, and similarly to dense granules they express CD63 and LAMP-1/2. Platelet lysosomal functions have not been well studied. Lysosomes serve a role in the digestion of phagocytic and cytosolic components, similar to that in nucleated cells. Secretion of the lysosomal content may have important extracellular functions, such as supporting receptor cleavage, fibrinolysis and degradation of extracellular matrix components, and remodeling of the vasculature. Platelet lysosomes may also contribute to a process termed unconventional secretion, described in nucleated cells [23]. Platelets have a constitutively active autophagy pathway, which is up-regulated by starvation [24]. The cytosolic substrates that are a target for autophagic sequestration in platelets are unknown. Platelets have been shown to synthesize proteins (tissue factor, IL-1β and Bcl-3) using mature and pre-messenger RNA. These proteins are probably produced in the cytoplasm and are released upon activation [8] via an incompletely understood mechanism. An interesting possibility could be that selective autophagic targeting and (auto)-lysosomal secretion may provide a way for these cytosolic proteins to exit the cell.

T-Granules

Recently, a novel type of secretory granule has been identified, termed T-granules, given their tubular morphology. T-granules contain TLR9, PDI and VAMP-8 [25]. Platelet spreading on glass and stimulation with type IV collagen increase the surface expression of TLR9, possibly via the SNARE proteins VAMP-8 and VAMP-7. The study suggests that T-granules are recruited to the cell surface and contribute to secretion. However, PDI is a resident ER protein and is exclusively localized to the dense tubular system (DTS) [26]. Electron tomography has shown that the DTS belongs to a reticular membrane network and not an isolated granule population [15]. Hence, the term T-granule is somewhat misleading when referring to a reticular compartment. Several studies have shown that PDI is released from activated platelets [27,28] and that increased cell surface expression contributes to platelet thrombus formation [29]. However, a direct fusion of DTS membranes with the cell surface has so far not been established.

Platelet activation and thematic secretion

Platelets harbor both inflammatory mediators and angiogenic factors with antagonistic function. They are mainly stored in α-granules, although the precise location of some of them has not been explored [3]. The specific intracellular distribution of molecules with apparent antagonistic function raises questions regarding α-granule secretory behavior. Rapid release of adhesive cargo (VWF and fibrinogen) is required to quickly respond to vascular injury. Slow and prolonged release is more likely to be essential for long-term processes such as recruitment of inflammatory cells and platelet contribution to vascular repair. Recent studies have suggested that circulating platelets sequester angiogenesis regulatory proteins and distribute these to different α-granule subsets [30]. This selective delivery to granule subpopulations was thought to be responsible for differential and thematic secretion behavior upon PAR1 and PAR4 stimulation [14]. Other studies have shown that fibrinogen and VWF are differentially packaged, which possibly causes different release rates [31]. This is plausible because VWF and fibrinogen reach the α-granules via distinct itineraries (cf. above). Given these features, subpopulations of α-granules may respond to specific agonists and hence release granule contents in a thematic fashion [32]. Earlier studies have shown that cargo is heterogeneously distributed within α-granules [33,34]. Recently, quantitative super-resolution immunofluorescence microscopy was used to map the protein co-distribution in resting platelets [35], demonstrating that cargo within individual α-granules is spatially segregated, apparently without thematic selectivity. Ultrastructural approaches confirmed that a heterogeneous α-granule population exists where cargo is spatially packaged into distinct zones. This raises the following important questions: (i) to what extent is thematically diverse cargo released at different kinetics and (ii) how is this regulated, considering that molecules with antagonistic function may originate from the same granule?

In a systematic analysis of the human platelet secretome, Jonnalagadda *et al*. investigated the time-dependent release of many cargo molecules and found distinct secretion rates but limited differences in thematic release [36]. In a recent proteomic study van Holten *et al*. evaluated platelet granule secretion after PAR-1 and PAR-4 stimulation and reported comparable release [37]. Together, these results suggest that secretion is regulated by other factors such as intragranular segregation, contraction, or alternative routes to and fusion with the OCS and PM. Interestingly, recent studies have demonstrated that a subset of the α-granules migrate towards the platelet periphery during spreading [38]. It was suggested that different VAMP isoforms may associate with discrete α-granule subpopulations, thereby contributing to differential secretion. Importantly, flow-adherent platelets release vWF strings in a polarized fashion, providing an array of

binding sites for incoming platelets (H. Heijnen, unpublished results), suggesting that granule dynamics and polarized secretion are probably tightly connected.

Timed release of thematically different cargo could also stem from a dilution of the protein gradient within the compartment once the granule membrane has fused with the PM or OCS. Early studies using cryo-fixation methods have shown that α- and dense granules form transient fusion pores with the PM [39,40]. Such fusion pores may deliver small molecules, while larger proteins are retained. Dense granules and α-granules have different secretory properties, but harbor the same fusogenic protein profile. Possibly distinct signaling pathways contribute to the differential release properties of both types of granules (see below).

Comparison of cargo release between platelets and endothelial cells

Platelet α-granules resemble WBPs in many ways. Both organelles are involved in the same processes and share content and trafficking proteins [41,42]. It has been well established that VWF is stored as helical tubules in WPBs, a characteristic organization also seen in platelet α-granules. WPB secretion may be regulated by several simultaneously operating mechanisms [43]: (i) differential release as a result of the heterogeneous nature of the organelle, (ii) time-dependent release via formation of fusion pores and kiss and run exocytosis, (iii) the involvement of selective fusogenic proteins (SNAREs), and (iv) actin-anchoring-based mechanisms (rab27a/b). A special type of homotypic fusion has recently been demonstrated in WPBs prior to exocytosis, which generates so-called secretory pods. This pathway might represent a selective way to generate unfurled VWF strings [44]. A variant of this secretory theme may operate in platelets. Stimulus-dependent compound fusion occurs when α-granules fuse with each other and/or the OCS [40,45]. This process may create a secretory pod variant for unwinding VWF polymers, as described for WPBs.

Platelet secretion and the cytoskeleton

Microtubules and the membrane skeleton are essential for maintenance of the platelet discoid shape and the integrity of the PM. Upon activation platelets exhibit an actin-myosin-based contraction, triggered by an increase in cytosolic calcium [46]. Inter-platelet transmission of platelet contractile forces and release of adhesive cargo are both essential for stabilization of the thrombus. Early *in vitro* observations have shown that platelet secretion is accompanied by granule centralization, implying that actin-myosin-dependent contractile forces facilitate the release of granule contents [47,48]. The exact mechanism by which reorganization of the actin cytoskeleton affects granule fusion events remains poorly understood. Contraction-induced targeting of secretory granules to the cell center may help to closely position them toward OCS and/or PM for secretion. Cytochalasins and latrunculin-A have been shown to increase cargo release from dense granules, indicating that actin depolymerization promotes dense granule exocytosis [49,50]. A direct relationship between actin and exocytosis has been demonstrated in pancreatic acinar cells and chromaffin cells [51,52]. F-actin and myosin are associated with platelet α-granules [49,53], and cortical actin has been reported to provide a natural barrier as well as playing a facilitative role in regulated exocytosis in other nucleated cells [51,52,54]. Thus far no data are available with respect to potential differences in the actin barrier between OCS and PM or whether these contribute to differential fusion behavior.

Open canalicular system remodeling, shear forces and cargo release

Although it is clear that the platelet cytoskeleton is linked to platelet shape changes and modulation of granule release, it is not clear to what extent forces generated by the flowing blood affect granule release. A key cytoarchitectural feature in platelet secretion is the OCS, a conduit through which plasma components can 'enter' the platelet and an intermediate for secretion [55]. Upon activation and spreading secretory granules migrate to the cell center, a process that is under the control of actin-myosin. This centralization enables α-granules to become closely apposed, supporting delivery to the OCS and PM [45]. Secretion of cargo via the OCS is generally slower than direct delivery at the cell surface. A key determinant for the rate of this process is the degree to which soluble cargo freely diffuses within the OCS or is retained by counter receptors. During platelet activation granule membrane proteins (P-selectin, GLUT3 and GPIIb-IIIa) are incorporated into both the OCS and PM [56,57] while other cell surface components (i.e. GPIb) are (partially) redistributed to the platelet interior [58]. Local receptor availability and changes in avidity may contribute to temporal retention of secreted products in the OCS, thereby delaying cargo release.

The OCS provides the circulating platelet with a unique membrane reservoir that is essential for platelet remodeling during adhesion. Platelet spreading requires consumption of the OCS membrane, whereby OCS-retained cargo becomes externalized. Platelets sense and respond to changes in their hemodynamic environment. Shear forces generated by the flowing blood have a high impact on membrane dynamics and release behavior. Platelet interaction with VWF under high shear induces the formation of long membrane tethers [59]. In addition, calcium-dependent uncoupling of the PM and the cytoskeleton, and shear forces have been shown to generate so-called flow-induced protrusions [60]. An interesting question in this context is whether local shear forces regulate receptor-cargo binding properties. Shear-exposed

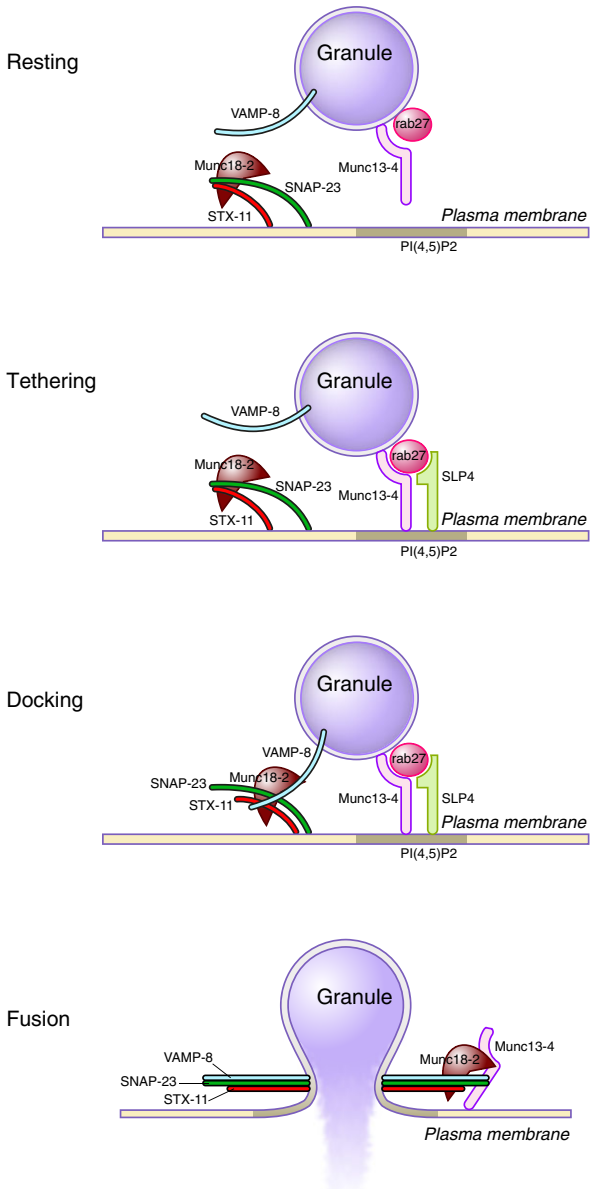

Fig. 3. Molecular model for platelet granule secretion. Schematic depicting the various stages and proteins involved in the release of platelet granule content. Once a resting platelet is activated, granules become tethered to plasma membrane regions enriched in PI(4,5)P2. The small GTPase rab27a in the active form associates with the tethering factors munc13-4 and synaptotagmin-like protein 4, both of which can associate with PI(4,5)P2 of the opposing plasma membrane. The tethering process limits the distance between the granule and plasma membrane, which allows SNARE proteins of granules and the plasma membrane to pair and engage in the formation of (pre)fusion SNARE complexes. The association with NSF causes zippering of the opposing SNARE proteins and subsequent fusion of the granule membrane with the plasma membrane. Molecular details of the proteins and events are described in the text.

soluble cargo is rapidly redistributed by the flow, while OCS-retained cargo fulfills a prolonged function. Flow-directed release and unwinding of platelet-bound VWF, derived from expelled OCS, may function in a similar fashion to that described for Weibel-Palade bodies.

Mechanism of granule release

Exocytosis of the platelet granule content is initiated and regulated through activation of cell surface receptors and involves granule tethering and docking followed by the fusion of the granule with the PM (Fig. 3).

Features of SNARE proteins

Fusion of the granule membrane and PM is driven by interactions between members of the soluble N-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs). The formation of specific trans complexes between cognate partners present on a granule (v-SNARE) and the PM (t-SNARE) provides the energy required to fuse two opposing membranes [61]. v-SNAREs and most t-SNAREs (except SNAP-23, 25 and 29) are type II transmembrane proteins. The cytoplasmic tails of each contain a highly conserved helical SNARE motif of 60–70 amino acids, while SNAP-23, 25 and 29 have two copies of this motif. The typical four-helical bundle at the core of the SNARE complex is thus formed by a t-SNARE of the SNAP-23, -25 and -29 subfamily together with a transmembrane v-SNARE and transmembrane t-SNARE [61].

SNAREs regulating platelet secretion

Over the years the SNARE paradigm of membrane fusion in eukaryotes has been firmly established. In accord with the highly specialized function and architecture of platelets, their repertoire of SNARES is limited. Platelet SNAREs include the v-SNAREs VAMP-2 (vesicle associated membrane protein), VAMP-3, VAMP-7 and VAMP-8, SNAP-23, SNAP-29 and t-SNAREs, syntaxin-2, syntaxin-4, syntaxin-7 and syntaxin-11 [62]. In spite of extensive biochemical and biophysical characterization, many SNARE protein complexes withstood elucidation of their precise biological role, in part because of redundancy, or promiscuous interactions *in vitro* or assays that rely on ectopic expression of soluble truncation constructs. Two converging conditions greatly changed this situation, especially in hemopoietic cells. Through the discovery of mutations in SNAREs and accessory factors, as causative agents for a number of human syndromes, and the development of knock-out animal models we now have a much better understanding of release pathways in platelets. In a VAMP-8 knock-out mouse model mild agonist-evoked release was impaired for all three granule types. Interestingly, although biogenesis of dense granules, lysosomes and α-granules was not affected by the VAMP-8 deletion, the secretion phenotype of dense granules was less affected than that of lysosomes and α-granules [63]. Because high doses of agonist bypass the VAMP-8 requirement, alternative VAMP-8-independent pathways need to be considered. Indeed,

VAMP-2 and VAMP-3 take on this task, albeit not as efficiently. In mast cells the same hierarchy for granule release has been observed, confirming the function of VAMP-8 and emphasizing the relationship between the granule types and LROs in different types of hemopoietic cells [64].

The principal t-SNARE in platelets is syntaxin 11, which associates with membranes via palmitoylation of C-terminal cysteines. Its significance for LRO release became apparent after the discovery that mutations in STX-11 cause familial hemophagocytic lymphohistiocytosis type 4 (FHL4), an autosomal recessive, life-threatening immune disorder characterized by uncontrolled activation of macrophages and cytotoxic T lymphocytes (CTL) [65]. In this inherited form of hemophagocytic lymphophistiocytosis, the lytic granules of CTL cannot fuse with the PM and therefore fail to release their lytic molecules and kill target cells. In platelets isolated from a FHL4 patient devoid of functional syntaxin-11, agonist-induced exocytosis of dense granules and α-granules is severely inhibited, while that of lysosomes is mildly affected. The recently established mouse STX-11 knock-out strains greatly enhance the availability of syntaxin-11-deficient platelets and will probably allow for establishing its precise role in platelet exocytosis [66].

The characterization of knock-out animals clearly established a requirement for VAMP-8 and syntaxin-11 in secretory events in a variety of cells, including platelets. Even though VAMP-8 and syntaxin-11 are the most abundant platelet SNAREs and do co-immunoprecipitate, we do not know whether they act together in fusion of granules with the PM. This issue is important because VAMP-8 serves as a regulator of fusion between late endocytic organelles [67], as well fusion of autophagosomes with endosomes and lysosomes [68]. As these are typical fusion reactions between intracellular organelles, the question then is what precise fusion event in platelets is critically dependent on VAMP-8. A likely locale for VAMP-8 action would be the PM or OCS, where it could drive fusion with granules. It is also possible that during platelet formation VAMP-8 regulates a fusion event in the maturation of the granules. This could represent a necessary step that delivers (exocytic) machinery to the granule membrane for subsequent fusion at the cell surface, a mechanism that is similar to lytic granule maturation in CTL [69]. Like VAMP-8, syntaxin-11 is also on endo-lysosomal membranes. Consistent with that localization, it regulates fusion between endo-lysosomal organelles in macrophages, together with the v-SNARE Vti1b [70]. As Vti1b is expressed in platelets as well, further experiments are needed to pinpoint the fusion event that syntaxin-11 regulates in platelet release.

Regulation of SNARE complexes

FHL3 and FHL5 are two other forms of FHL and are caused by mutation in UNC13-D and STX-BP2 [71]. Platelets of FHL3 patients exhibit strongly impaired capacity for exocytosis of all the granule content [72], a phenotype that is recapitulated in the Jinx mouse, a functional munc13-4 null [73]. In FHL5 platelets, dense granule and α-granule release is severely inhibited in the absence of STX-BP2 (munc18-2), while lysosome secretion is moderately jeopardized. Munc18-2 is a member of the family of sec1/munc18-like proteins that includes amongst others munc18, vps33 and vps45. They are essential to membrane transport and cell physiology. Genetic deficiencies in munc18-1, munc18-2, vps33b and vps45 all cause serious human disease, including infantile epileptic encephalopathy, FHL5, ARC syndrome, bleeding disorders and neutropenia. Sec1/munc18 proteins have an arch-shaped structure that clasps trans-SNARE complexes and directs their fusion activity in a spatial and temporal manner [71]. The interaction with sec1/munc18 proteins also contributes in an important manner to the stability of syntaxins. For instance, point mutations of munc18-2 in FHL5 patients are accompanied by impaired expression of syntaxin-11 [74], while a knock-out of munc18-1 in mouse brain strongly reduces syntaxin-1 levels [75]. Munc13-4 is an effector of rab27b, a small GTPase that is highly expressed in platelets [21]. Platelets of a rab27b knock-out mouse are impaired in dense granule secretion. As these platelets also contain fewer dense granules, the secretory phenotype seen in the absence of rab27b could be more complex and perhaps reflect a role in the biogenesis of dense granules [24]. In addition, platelets express slp1 and slp4, two rab27 effectors of the synaptotagmin-like protein family, which regulate dense granule release via rap1GAP2 and rab8, respectively [76,77].

In mast cells, rab27 and munc13-4 localize on LRO and the interaction between rab27 and munc13-4 is required for tethering LRO to the cell surface prior to fusion [78]. Munc13-4 contains an N-terminal and a C-terminal C2 domain connected by a long α-helical MUN domain. The MUN domain is conserved in all munc13 proteins and is involved in vesicle priming. The MUN domain is structurally homologous to the characteristic two-stacked helical bundle found in sec6 and sec15 subunits of the exocyst tethering complex. The N terminal C2 domain binds in a Ca^{2+}-dependent manner to PI(4,5)P2 [79]. This propensity allows Munc13-4 to interact with PI(4,5)P2-enriched regions on the PM, thereby facilitating tethering of secretory LRO to the PM. An important conceptual advance regarding the role of munc13 and munc18 came from recent studies on neuronal SNAREs in synaptic vesicle fusion, where Rizo showed a dependency of fusion on munc18 and munc13 [80]. Although this was accomplished with a specific SNARE complex, its significance will probably transpire beyond the case and provide a general explanation for the role of munc13 and munc18 proteins in membrane fusion. The closed form of syntaxin is clamped in a tight

complex with munc18 and not available for other SNAREs. Munc13 can extract syntaxin through an interaction of the MUN domain with syntaxin and possibly munc18, thereby catalyzing the formation of the full syntaxin-SNAP-25-VAMP-1 complex and fusion [80].

In electron microscopy studies, platelet and granule morphology in mutant cells appears very similar if not identical to that in wild-type cells. Equally unaffected are the signaling pathways that ultimately drive the exocytic release process. At the ultrastructural level, the mutant cells occasionally appear to contain more granules, whose precise origin and composition usually have not been rigorously established. Nevertheless, this phenotype is suggestive for the accumulation of the granules due to impaired fusion with the PM, an observation that has been made before in CTLs of FHL3 and Griscelli syndrome type 2 patients.

In interactor screens for proteins that co-regulate secretion with syntaxin in platelets or endothelial cells, the Whiteheart [81] and Lowenstein [82] laboratories discovered tomosyn-1. Tomosyn-1 (STX-BP5) is the founding member of a small family of syntaxin binding proteins [83]. Initially identified as a partner of neuronal syntaxin-1, it is now clear that tomosyn-1 is expressed beyond the brain and interacts with several syntaxins. Interestingly, single nucleotide polymorphisms in STX-BP5 constitute a risk factor for venous [84] and arterial [85] thrombosis, and have implicated tomosyn-1 as a regulator of VWF plasma levels. Direct evidence for this notion was obtained in platelets from STX-BP5 knock-out mice, which revealed a dependence of α-granule, dense granule and lysosome secretion on tomosyn-1 function [81]. Tomosyn-1 also has a non-essential role in the biogenesis of granules or sorting of cargo in granules, because several soluble content markers are present at altered levels in the granules of knock-out animals [81]. Intriguingly, endothelial cells of STX-BP5 knock-out animals are more efficient in stimulated secretion of histamine and VWF [82]. Consistent with the knock-out phenotype in these cells, ectopic expression of wild-type tomosyn-1 reduced VWF release. How tomosyn-1 can have opposing functions in platelets and endothelial cells remains to be established. Tomosyn-1 contains a C-terminal VAMP-like motif that could compete with a cognate v-SNARE and thereby block fusogenic trans SNARE complex formation, as seen after overexpression in neurons [86]. Expression of a tomosyn-1 deletion construct missing the v-SNARE domain, however, also inhibits secretion, suggesting that transactions with other proteins are relevant as well [83]. Tomosyn-1 and its homologs have been described as positive regulators of secretion in yeast [87] and endocrine cell lines [88]. In contrast, at the neuromuscular junction, tomosyn-1 is thought to be needed for the spatial organization of vesicle fusion, perhaps via mechanisms involving accessory proteins for tethering and docking [89]. A candidate could be the rab27 effector synaptagmin-like protein 4 (slp4) that was co-isolated with tomosyn-1 from platelets [81]. In epithelial cells slp4 acts as a tethering factor for transport vesicles at the apical PM, together with syntaxin-3, which in this cell type is the major munc18-2 partner [90].

Post-translational modifications in platelet exocytosis

Although SNARE proteins and accessory proteins are the main characters in membrane transport and fusion, their activity needs to be tightly controlled. A central question is how signals generated after receptor ligation plug into the membrane traffic machinery, as the pathways for these cells extensively employ post-translational modifications. Most of the published work concerns the effect of SNARE phosphorylation in a variety of cells and experimental systems [91].

Phosphorylation of SNARE proteins

Cross-linking of the high-affinity FcεRI receptor in mast cells is the trigger for LRO exocytosis and involves phosphorylation of SNAP-23 by IκB kinase 2, through a PKC pathway that is conserved in lymphocytes [92]. Phosphorylation of SNAP-23 is essential in degranulation as ectopic expression of the phospho-mimetic SNAP-23 mutant partially rescued impaired IgE-mediated degranulation in IκB kinase2-deficient mast cells [92]. In platelets, the agonist-induced activation with physiological stimuli such as collagen or thrombin also results in Ser95 phosphorylation of SNAP-23 via a PKC-IκB kinase 2 pathway [93]. Platelets from IκB kinase 2 −/− mice fail to phosphorylate SNAP-23 after thrombin stimulation and release from each of the platelet granules is inhibited. The importance of SNAP-23 phosphorylation in platelet secretion correlates with its propensity to increase SNARE complex formation with syntaxin-11 and VAMP-8, the two major SNARES in platelets that are essential in exocytosis [93]. Several syntaxins are modified by phosphorylation, including syntaxin-1, syntaxin-3B, syntaxin-17, and in platelets syntaxin-4 [91,94]. Activation of platelets by a physiological stimulus such as thrombin causes PKC-dependent phosphorylation of syntaxin-4. A general physiological role of phosphorylation in syntaxin function (if possible at all) has not yet emerged. For syntaxin-1 and syntaxin-4 it is clear that the phospho-forms have reduced binding to SNAP-25 and SNAP-23, respectively, and thereby might affect cognate fusogenic SNARE complexes and secretion.

Phosphorylation of SNARE regulators

Because munc18 proteins serve to direct the fusogenic action of trans-SNARE complexes, they are good candidates for regulation in response to intra- or extracellular signals. Munc18-1 is phosphorylated by PKC in neurons

and neuro-endocrine cells, where phosphorylation impairs association with syntaxin and correlates with increased exocytosis. Molecular dynamics simulations suggest that reduced affinity of phosphorylated munc18 drives a conformation, which makes syntaxin binding energetically and sterically unfavorable [95]. Thrombin-mediated platelet activation increases phosphorylation of a munc18, the identity of which remains to be established. Munc18-2 is ubiquitously expressed and essential for exocytosis in platelets. Though not yet formally shown in platelets, munc18-2 is phosphorylated during stimulatory conditions for regulated secretion in epithelial cells. As with munc18-1, phosphorylation decreases the interaction of munc18-2 with syntaxin-3 and enhances formation of a functional munc18-2-syntaxin 3 – SNAP-25-VAMP-2 fusion complex [96]. As syntaxin-3 can substitute for syntaxin-11, we may speculate that phosphorylation of munc18-2 in platelets regulates the munc18-2 – syntaxin-11 – SNAP-23 complex.

Platelet microparticles (PMPs) and exosomes

Platelet adhesion and activation are accompanied by the release of membrane-bound vesicles. The small-sized population (40–100 nm) of these membranes, termed exosomes, is secreted from the multivesicular alpha granule population [97], while the larger PMPs (100 nm–1 μm) are derived directly from the PM by mechanisms that require shearing forces generated by the flowing blood and calcium-dependent uncoupling of the membrane skeleton from the lipid bilayer [63,64]. PMPs and exosomes are implicated in cell to cell communication, and are an important vehicle for interaction with leukocytes [64]. PMPs have been observed in several diseases with an inflammatory component [98]. Platelet activation induces the synthesis and release of IL-1β [8]. IL-1β is synthesized on poly-ribosomes in the cytoplasm and can exit the cell via shedding of membrane-bound vesicles or release of exosomes. Indeed, IL-1β associated with PMPs supports leukocyte recruitment and the progression of arthritis [99].

Perspective

Overall, the pleiomorphic nature of and heterogeneity in cargo distribution of the α-granule population provide a challenge for further study. It is evident that there is a need for precise characterization of the platelet secretory pathway in order to define the criteria that control degranulation and secretion of selective mediators that promote or counteract adhesive and inflammatory responses. Platelets are non-polar circulating cells but regain polarity as soon as they adhere to the sub-endothelium and each other. Changes in membrane microdomains may generate specialized areas where secretion becomes highly polarized. How non-polar platelets control selective secretory domains under flowing conditions remains an important issue for further study. Critical for progress in this area is a full appreciation of the extent to which shear forces control membrane fission and fusion processes, which SNARE subsets are involved, and how the activity of SNARE regulation is coordinated. Also the role of shear forces in actin dynamics, particularly in the regulation of late stages of secretion, is an important area for further exploration, as well as the recently attributed role of platelet-derived vesicles and their heterogeneous content.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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