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The Platelet Release Reaction: Just when you thought platelet secretion was simple

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Abstract

Purpose of the Review—In response to agonists produced at vascular lesions, platelets release a host of components from their three granules: dense core, alpha, and lysosome. This releasate activates other platelets, promotes wound repair, and initiates inflammatory responses. While widely accepted, the specific mechanisms underlying platelet secretion are only now coming to light. This review focuses on the core machinery required for platelet secretion.

Recent Findings—Proteomic analyses have provided a catalogue of the components released from activated platelets. Experiments using a combination of *in vitro* secretion assays and knockout mice have lead to assignments of both v- and t-SNAREs (Soluble NSF Attachment Protein Receptors) to each of the three secretion events. SNARE knockout mice are also proving to be useful models for probing the role of platelet exocytosis *in vivo*. Other studies are beginning to identify SNARE regulators which control when and where SNAREs interact during platelet activation.

Summary—A complex set of protein-protein interactions control the membrane fusion events required for the platelet release reaction. SNARE proteins are the core elements but the proteins that control SNARE interactions represent key points at which platelet signaling cascades could affect secretion and thrombosis.

Keywords

SNAREs; exocytosis; VAMP-8/endobrevin; syntaxin

Introduction

The most prominent structural features of a platelet are its three granules: dense core, alpha, and lysosomal. Activated platelets release material from these granules; but, what is the biological role of platelet secretion? The most obvious is a paracrine role since released ADP directly activates other platelets in the immediate area and serotonin affects smooth muscle cells. Released fibrinogen and von Willebrand Factor (vWF) play roles in thrombus stability. However, these clearly defined examples account for only a fraction of the proteins and small molecules released by platelets. There are a host of cytokines, chemokines, and growth factors released by platelets upon activation. While platelet exocytosis is understood in general terms, the molecular mechanisms involved have only recently been elucidated. With that understanding comes the tools to directly address the preconceived notions about platelet secretion and the ability to probe the specific roles of different exocytosis events.

Platelet Secretion

In humans, granule release deficiencies present as mild to moderate bleeding diatheses. While generally not life-threatening, the condition does pose risks *e.g.* during surgeries, childbirth, dental extractions. The two major class of Storage Pool Deficiencies (SPD), Hermansky-Pudlak Syndrome (HPS, δ SPD) [1–5] and Gray Platelet Syndrome (GPS, α SPD) [6,7], are characterized by the loss of granule cargo from dense core granules for HPS and from α -granules for GPS. HPS patients show increased bleeding times, pigmentation defects (dense core granule biosynthesis is mechanistically similar to that of melanosomes), and lung fibrosis. There are 15 HPS-like mouse strains that have similar defects. Cloning of the defective genes and the subsequent analysis of their products has shown that the phenotypes are related to dysfunctional sorting of dense core granule proteins during biogenesis [3,4]. Most of the HPS genes encode proteins that participate in the sorting of cargo from and through endosomal compartments. GPS patients present with more heterogeneous bleeding defects but generally have normal pigmentation. Less is known about the underlying molecular defects that cause GPS, though one knockout mouse strain (transcription factor $Hzf^{-/-}$) does display a loss of α -granule cargo [8]. In this strain, initial bleeding times are normal, yet the incidence of rebleeding is increased. One striking feature of GPS patients is that all have myelofibrosis [7]. This is perhaps due to the inability of the megakaryocytes to retain synthesized growth factors. Consistently, plasma levels of Platelet Factor IV (PF4) and β -thromboglobulin (both are α -granule cargoes) are elevated in GPS patients. Little is known about the consequences of the inappropriate distribution of α -granule cargo. Patients with loss of both granules ($\alpha\delta$ SPD) are rare and do have bleeding defects [9,10]. These observations demonstrate the impact that release of platelet cargo has on hemostasis.

Platelet Granule Cargo

To date, more than 300 proteins and small molecules have been shown to be secreted from activated platelets [11]. This “secretome” can be classified by granular source and proposed function. Dense core granules mainly contain small molecules such as ADP, serotonin, and calcium. These components are critical for further platelet activation and vasoconstriction. As discussed above, failure to release these components does lead to defects in thrombosis. α -Granules contain proteinaceous components such as PF4, vWF, and platelet derived growth factor. These cargo proteins have the widest array of functions. Platelets also release lysosomal enzymes such as cathepsins and hexosaminidase which may play a role in clot remodeling or in further platelet activation. Functionally, the “secretome” can be divided into even more categories (reviewed in [12]). The most obvious class of proteins is the adhesive proteins such as fibrinogen, fibronectin, vitronectin, and thrombospondin which are released from α -granules and function in platelet-platelet binding and subsequent clot formation. Other membrane proteins, such as P-selectin, are sequestered in α -granules and exteriorized to the surface upon granule-plasma membrane fusion. Platelets also release fibrinolytic agents such as PAI-1 and TAFI which are important for clot remodeling. Perhaps the most diverse category is the mitogens such as IGF-1, VEGF, and bFGF. These proteins are thought to promote wound healing and angiogenesis through their stimulation of chemotaxis, cell proliferation, and maturation of the cells surrounding the wound site. Finally, platelets release a number of chemokines and cytokines such as RANTES, IL-8, and MIP1 α which promote activation of passing leukocytes and lead to a range of immune responses. This release catalog suggests that platelet secretion is pivotal in establishing the microenvironment at the wound site. Therefore, controlling release from the granule stores, either specifically or globally, may prove to be an effective strategy to manipulate this microenvironment.

Granule Heterogeneity

It is becoming increasingly clear that our old monolithic view of platelet granules is overly simplistic. Recent studies have suggested that α -granules are heterogeneous in content and that that heterogeneity may result in differential release of granule cargo. Italiano and colleagues have shown that α -granules can be distinguished based on the pro or anti-angiogenic factors that they contain [13]. Anti-angiogenic factors, such as endostatin, reside in different granules than do pro-angiogenic factors such as VEGF. In human platelets, these two classes of granules can be differentially induced to release, depending on which PAR-directed agonist is used [13,14]. As similar degree of granule heterogeneity was initially reported by Storrie and colleagues who examined the localization of fibrinogen and vWF [15]. Functionally relevant granule heterogeneity is seen in other hematopoietic cells such as mast cells and neutrophils. In those systems, the differential release in response to distinct agonists appears mediated by different SNARE proteins [16,17]. As discussed below, differential use of t-SNAREs may account, in part, for the differential release of cargo from different classes of platelet granules; however, this remains to be addressed.

Secretory Machinery

Since the original description of SNAREs in neurons [18] much has been learned. Recent models of SNARE function posit that vesicle/granule-target membrane fusion is governed, in part, by the matching of a vesicle SNARE (v-SNARE), with a heterodimeric or trimeric, protein complex in the target membrane (t-SNAREs). This *trans*-membrane complex is the minimal element required for membrane fusion [19]. v-SNAREs are type II integral membrane proteins of generally low molecular weight (15–25 kDa) which contain a characteristic 60–70 amino acid SNARE motif [20]. Human and mouse platelets contain VAMP-2/synaptobrevin, VAMP-3/cellubrevin, VAMP-7/TI-VAMP, and VAMP-8/endobrevin, with the later being the most abundant [21–24]. t-SNAREs are of two types: the SNAP-23/25 type (SNAP-23, SNAP-25, SNAP-29, Sec9p), and the syntaxin type (syntaxin 1–19, *etc.*). The SNAP-23-like proteins have two SNARE motifs, lack transmembrane domains, but are generally anchored to the membrane through thioester-linked acyl groups. The type II membrane protein, syntaxins, contain the characteristic SNARE motif and an N-terminal regulatory domain [25]. Human platelets contain syntaxin 2, 4, 7, and 11 [26–29] as well as SNAP-23 [30] and SNAP-29 [31]. The presence of SNAP-25 is perhaps controversial [31]. SNAREs employ the SNARE motifs to form a four-helix bundle arranged as a parallel coiled-coil [32]. This structure is stabilized by core hydrophobic interactions running the length of the bundle. Based on this inherent stability, the temporal and spatial control of SNARE complex formation and disassembly represent important tasks for the secretory machinery.

SNAREs in Platelets

Three groups have contributed to our present knowledge of the function of platelet SNAREs (for reviews see [33,34]). Our group [28] first showed that SNARE proteins are present in platelets and Flaumenhaft *et al.* [30] demonstrated a role for syntaxin 4 and SNAP-23 in α -granule release. Syntaxin 4 is also involved in lysosome release [27]. Subsequent work showed that SNAP-23 is involved in all three release events as is syntaxin 2 [26,27,35]. No role has been detected for syntaxin 7 and syntaxin 11's role remains to be addressed. Based on these data, syntaxin 2 solely mediates dense core granule release but functionally overlaps with syntaxin 4 in mediating α -granule and lysosome release. This dual usage of syntaxin 2 and 4 might explain how differential release of subclasses of α -granules could occur. Perhaps syntaxin 2 promotes release of pro-angiogenic α -granules while syntaxin 4 mediates release of anti-angiogenic granules. This would imply that t-SNARE regulation may be the essential discriminator of which classes of cargo are released. Given the general usage of the v-SNARE, VAMP-8 (discussed below), this is an attractive model to explain the observations of Italiano

et al. [13] and Ma *et al.* [14]; but, more direct analysis with knockout mice will be required to fully understand how the t-SNAREs could facilitate differential α -granule release.

Assigning roles for the v-SNAREs had been more ambiguous. Initial studies [30] showed that P-selectin exposure (α -granule release) was sensitive to Tetanus neurotoxin (a heterodimeric, zinc-dependent, endopeptidase specific for VAMP-1, -2, and -3 [36]). Subsequently, Feng *et al.* [37] showed that an anti-peptide antibody to the divergent N-terminus of VAMP-3 could inhibit α -granule release, suggesting a role for VAMP-3. However, these data were not consistent with the lack of a secretion defect in platelets from VAMP-3^{-/-} mice [23]. Polgar *et al.* [22] showed that the cytoplasmic domains (containing the SNARE motif) of VAMP-3 and VAMP-8 could inhibit release from dense core and α -granules. Since the isolated cytoplasmic domains are promiscuous in their associations *in vitro* [38,39], it was uncertain whether the inhibitory effects were illustrative of a v-SNARE's role or of the t-SNARE heterodimer's binding preferences.

Recent work [24] has shown that VAMP-8/endobrevin is the primary v-SNARE in mouse platelets. VAMP-8^{-/-} platelets have a clear secretion defect which is not seen in VAMP-3^{-/-}, VAMP-2^{+/-}, VAMP-2^{+/-}/VAMP-3^{-/-} platelets or from either permeabilized human or VAMP-3^{-/-} platelets that have been treated with the catalytic Tetanus toxin light chain. The secretion defect in VAMP-8^{-/-} platelets however was not complete. α -Granule and lysosome release were significantly affected even at high agonist doses and upon extended incubation; however, dense core granule release eventually approached wild-type levels. The VAMP-8-independent release could be eliminated by treatment with Tetanus toxin, suggesting that the secondary mechanism employed either VAMP-2 or -3. Preliminary experiments, using permeabilized human platelets and VAMP-8-specific Fab fragments, show that VAMP-8's roles in mouse and human platelets are similar (Ren Q and Whiteheart SW, unpublished data).

The redundancy in v-SNARE usage is analogous to chromaffin cells [40], where there is a primary v-SNARE (VAMP-2) required for rapid and efficient, agonist-induced release, and a secondary v-SNARE (VAMP-3) which functions, albeit less efficiently, in the absence of the primary v-SNARE. Full ablation of release from chromaffin cells required deletion of both VAMP-2 and VAMP-3. The redundancy in VAMP usage observed in *ex vivo* platelet secretion assays is consistent with the *in vivo* thrombosis phenotype of the VAMP-8^{-/-} mice. Upon laser injury, the VAMP-8^{-/-} mice show a delay in thrombus formation and an increase in embolization, though the overall extent of platelet accumulation is not statistically different from wild-type (Graham *et al.* submitted).

In light of VAMP-8's role in platelets, it is striking to note recent reports indicating that a Single Nucleotide Polymorphism (SNP) in the human VAMP-8 gene strongly correlates with early onset myocardial infarction (MI) [41,42]. The authors suggest that this SNP might affect VAMP-8 expression levels given its position in the gene's 3'-untranslated region. One could hypothesize that heightened VAMP-8 levels would increase a platelet's secretion ability, making it more likely to release its cargo and thus hyperthrombotic. However, further biochemical and functional analysis will be required to fully understand the ramifications of this genetic connection between VAMP-8 and early onset MI.

SNARE regulators

SNARE regulators control how and when SNARE proteins interact. Some are required to stabilize and sort SNAREs to appropriate membranes. Other regulators play key roles in promoting and selecting SNARE-SNARE interactions. Many of these regulators are sensitive to second messengers such as diacylglycerol and Ca²⁺ and others are substrates for kinases. While much is known about some of these SNARE regulators in other systems, relatively little is known about their precise roles in platelets. However, it has been possible to determine if

the families are represented and in some cases whether they are important for platelet secretion. Syntaxin-binding proteins of the Munc18 family (a, b, and c [43]) are present in platelets. Antibodies that affect Munc18c binding to syntaxin 4 have a positive effect on release from permeabilized platelets [44], while peptides based on regulatory sequences of Munc18a and c are inhibitory [43]. Munc18 family members are phosphorylated in platelets and, though the significance of this is not yet clear, phosphorylation does affect Munc18 binding to syntaxins [43,44]. At least two members of the Munc13 family are present in platelets (Munc13-1 and Munc13-4; Schraw TD, Ren Q, and Whiteheart SW, unpublished data). In neurons, chromaffin cells, and cytotoxic T-cells, these proteins are important for granule docking/priming and SNARE engagement through interactions with the syntaxin t-SNAREs [45–47]. Munc13-4 has been shown to be involved in dense core granule release *via* its interaction with the small GTP-binding proteins, Rab27a and b [48]. Other potential SNARE regulators such as DOC2a and tomosyn are present in platelets (Ye S, Ren Q, Schraw, TD, and Whiteheart SW, unpublished data) but their roles in secretion have not yet been probed.

Secretory Machinery as a Potential Therapeutic Target

The goal of anti-thrombotic therapies is a regimen that dampens thrombosis without inducing excessive bleeding. The secretory machinery might prove an effective target to accomplish this goal. *In vivo* analysis of the VAMP-8^{-/-} mice shows a clear defect in laser-induced arteriole thrombosis but one not as complete as in an HPS mouse (Graham *et al.* submitted). Thrombi in VAMP-8^{-/-} mice show a significant delay in initiation and well as a lack of stability. However, thrombi do form, and there is no significant difference in tail bleeding times in these animals. These data suggest that targeting the platelet's primary, secretory machinery might be an effect way to manage thrombosis *in vivo* and thus limit clot formation without inducing excessive bleeding. Future analysis of other mouse strains with secretion deficient platelets is ongoing to validate this concept and to probe the additional roles that platelet exocytosis might play in the vasculature.

Conclusions

It is clear that activated platelets release material from their granular store. Recent work has catalogued that releasate and elucidated the core machinery that mediates the membrane fusion events required for release. With that knowledge the focus now turns to how the SNARE protein interactions are regulated during platelet activation. These studies have also produced the tools needed to address the importance of platelet secretion not only to thrombosis but also to the normal and pathogenic *sequellae* of vascular damage.

Abbreviations

SNARE, Soluble NSF Attachment Protein Receptor; VAMP, Vesicle Associate Membrane Protein; SPD, Storage Pool Deficiencies; HPS, Hermansky-Pudlak Syndrome; GPS, Gray Platelet Syndrome; PF4, Platelet Factor IV; vWF, von Willebrand Factor; SNP, Single Nucleotide Polymorphism; MI, myocardial infarction.

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