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The Ins and Outs of Endocytic Trafficking in Platelet Functions

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Abstract

Purpose of review—While platelet endocytosis has been recognized in granule cargo loading and the trafficking of several platelet surface receptors, its acute physiological relevance is poorly understood as is its mechanism. This review discusses the current understanding of platelet endocytosis and its implications for platelet function.

Recent findings—Recent studies are beginning to identify and define the proteins that mediate platelet endocytosis. These studies have shown that platelets contain different endosomal compartments and may use multiple endocytic routes to take in circulating molecules and surface proteins. The studies have also shown that platelet endocytosis is involved in several aspects of platelet function such as signaling, spreading, as well as granule cargo loading.

Summary—Mechanistic studies of platelet endocytosis have shown it to be not only involved in granule cargo loading but also in various other platelet functions important for hemostasis and beyond.

Keywords

receptor-mediated; pinocytosis; endosomes; cargo trafficking

INTRODUCTION

Anucleate platelets contain many standard cellular compartments (*i.e.*, Golgi, endosomes, multivesicular bodies, autophagosomes, endoplasmic reticulum [1–5]), in addition to the functionally-relevant dense and α -granules. Dense granules contain small molecules important for recruiting platelets and augmenting coagulation. α -Granules contain polypeptides that modulate everything from adhesion to angiogenesis and inflammation [6]. Despite our knowledge of granules, the functions of these other “organelles” are relatively ill-defined in platelets. Platelet endocytosis has been long recognized; but, a mechanistic understanding of it, its molecular machinery, the trafficking routes, and their relevance to platelet function remain understudied, in part, due to a lack of experimental tools. This is changing with the identification of key regulators that affect cargo uptake and/or receptor

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trafficking in platelets (Table 1). Platelet endocytosis is important for loading some α -granule cargo (*e.g.*, fibrinogen (Fg) and Vascular Endothelial Growth Factor (VEGF)) [7–9]. Endocytosis can contribute to thrombosis by controlling surface expression of proteins, such as $\alpha_{IIb}\beta_3$ [10]. Integrin trafficking modulates spreading on surfaces and clot retraction [2]. Endocytosis of purinergic receptors *e.g.*, P2Y₁ and P2Y₁₂, mediates their resensitization [11]. Endocytosis of C-type Lectin Receptor-2 (CLEC-2) and Thrombopoietin receptor (Mpl/TPOR) regulates their surface levels and downstream signaling [12, 13]. These data clearly show that endocytosis has significant importance in platelet physiology.

HISTORICAL PERSPECTIVE

Clathrin-coated membranes were first noted in the 1980s [14]. They were found either as coated pits or vesicles, dotted around the plasma membrane (PM), α -granules, and lysosomes. Behnke *et al.* showed that coated pits lined the PM and open canalicular system (OCS) and coated vesicles either existed by themselves or fused with secretory granules [15]. These studies demonstrated that active endocytosis and intracellular trafficking occurs in platelets. Uptake of small particles and solutes into membrane structures (thought to be OCS) *via* a phagocytosis-like process was first seen in human platelets [16]. Using transmission electron microscopy (TEM) and cytochemistry, endocytosis of plasma proteins *e.g.*, albumin, IgG, and $\alpha_{IIb}\beta_3$ receptor-mediated uptake of Fg into megakaryocytes and platelets was shown by Bainton and colleagues [8, 17–20]. This demonstrated a role for endocytosis in platelet physiology, since Fg is a significant α -granule cargo. Klinger *et al.* showed the presence of clathrin-coated vesicles colocalizing with Fg, vWF, and fibronectin on the cytoplasmic faces of the α -granules, OCS, and the PM [21]. Studies of resting platelets using colloidal gold-tagged Fg, as a marker of receptor-mediated endocytosis and acid phosphatase as a lysosome marker, demonstrated that both clathrin-dependent and independent endocytosis occur in platelets [22]. While these data show that endocytosis occurs, the extent of the process, its mechanisms and routes, and its importance to platelet function were undefined.

CLATHRIN-MEDIATED ENDOCYTOSIS

In this process (Figure 1), specific extracellular molecules bind to the ectodomain of receptor proteins. This complex then accumulates in coated pits at the PM through interactions of adaptors and “coats” with the receptor’s cytoplasmic domain. These complexes enter the cell *via* coated vesicles [23]. This process utilizes clathrin, which is critical for the formation of clathrin-coated vesicles (CCVs) at specialized domains of the PM called clathrin-coated pits (CCPs) [24–26]. Adaptor proteins mediate the concentration of the receptors and a GTPase, called Dynamin, which promotes vesicle scission [27]. Quantitative proteomics shows that platelets contain most of the clathrin-mediated, endocytic machinery [28, 29] (Table 1).

Dynamin

All three isoforms of this mechanochemical GTPase, Dynamin1 (DNM1), Dynamin2 (DNM2) and Dynamin3 (DNM3), are detected in platelets [28, 29], with human platelets containing each and mouse platelets expressing predominantly DNM2. Interestingly,

Dynamins, especially DNM3, contribute to megakaryopoiesis [30, 31]. DNM3 co-localizes to the demarcation membrane system (DMS), which is thought to serve as a membrane reservoir during proplatelet formation [31]. Consistently, pan-DNM inhibitors, such as Dynasore, impair proplatelet formation [32]. Further, Genome Wide Association Studies (GWAS) linked DNMs with platelet size and formation showing that a single nucleotide polymorphism (SNP) within the *DNM3* gene promoter associates with variable mean platelet volume in humans [32].

Mutations in DNM2 have been associated with thrombocytopenia and hematopoietic diseases, most notably in Charcot-Marie-Tooth disease [33, 34]. Using platelet-specific-knockout mice, Bender *et al.* determined the role of DNM2 in thrombopoiesis [13]. These mutant mice have severe macrothrombocytopenia with giant platelets being cleared rapidly from circulation leading to thrombocytopenia. Bone marrow MKs have altered DMS, packed with abnormally high numbers of CCVs, in addition to increased emperipoiesis. Clathrin-mediated endocytosis is also impaired in DNM2^{-/-} MKs, as noted by mislocalization of early endosomal markers, such as Early Endosomal Antigen 1 (EEA1) and Adaptor protein, Phosphotyrosine interacting with PH domain and Leucine Zipper 1 (APPL1). Proplatelet formation from DNM2^{-/-} MKs is also impaired. As a potential mechanism for these defects, thrombopoietin receptor (TPO/Mpl) endocytosis is significantly affected in DNM2^{-/-} mice leading to constitutive phosphorylation of the downstream Janus Kinase 2 (JAK2). While mainly expressed in the brain, platelets contain low levels of DNM1 [35].

Dynamin-related protein-1 (Drp)

Dynamin-related protein-1 (Drp1) belongs to the DNM family of GTPases, and has a key role in mediating fission and fusion during mitochondrial biogenesis [36]. Platelets contain Drp1, which is phosphorylated upon activation [37]. Drp1 appears to affect fusion pore stability during granule exocytosis and, consistently, its inhibition affects platelet accumulation during thrombus formation *in vivo*. A specific role for Drp1 in platelet endocytosis is unknown.

Disabled-2 (Dab2)

Disabled-2 (Dab2) is a clathrin- and cargo-binding adaptor protein that is involved in endocytic trafficking of many cell surface receptors and in modulating intracellular signaling [38]. Alternate splicing generates two isoforms, p82-Dab2 and p59-Dab2 [39]. Dab2 is present in megakaryocytes and platelets and plays a key role in megakaryocytic differentiation [40, 41]. p82-Dab2 is mainly expressed in human while p59-Dab2 is predominant in mouse platelets [42]. In human platelets, p82-Dab2 is in the cytosol and on α -granules and regulates Fg binding and platelet aggregation [43]. In mouse, p59-Dab2 is required for platelet aggregation, Fg uptake, RhoA-ROCK activation, secretion of ADP, and $\alpha_{IIb}\beta_3$ -mediated platelet activation [42]. Platelet-specific, Dab2^{-/-} mice have a bleeding diathesis and defective thrombosis [42]. These data identify Dab2 as a modulator of integrin inside-out signaling in platelets and Fg uptake; however, its mechanism remains unknown.

CLATHRIN-INDEPENDENT ENDOCYTOSIS

Clathrin-independent (CI) endocytic pathways can be further classified based on DNM use. Some use DNM, such as those involving caveolae and/or Rho families of GTPases, while DNM-independent pathways use members of the Cdc42 and ADP-ribosylation factor (Arf) families of small GTPases to mediate vesicle scission (Figure 1) [44]. Several of these proteins are present in platelets (Table 1).

Caveolae-mediated endocytosis involves the formation of Caveolin-decorated, flask-shaped membrane invaginations at the PM [45, 46]. Human platelets contain Caveolin-1, -2, and -3; however, whether they are used for endocytosis is unknown [47]. The second type of DNM-dependent CI pathways utilizes RhoA, which recruits the actin machinery to produce membrane invaginations for endocytosis [46]. RhoA deficiencies cause macrothrombocytopenia and defective platelet activation, granule secretion, spreading, clot-retraction, thrombosis, and hemostasis. RhoA is also important for $\alpha_{IIb}\beta_3$ integrin signaling [48, 49]. Since RhoA has been shown to be involved in platelet activation, it is difficult to assess which defects are attributable to impaired endocytosis.

Dynamin-independent CI pathways use either the Rho family member, Cdc42 or the Arf family of small GTPases, chiefly Arf6. Of the six members of the Arf family, Arf1, Arf3, and Arf6 have been detected in platelets [28, 50]. The Rho family GTPase, Cdc42, is important for platelet aggregation in response to collagen and $\alpha_2\beta_1$ integrin activity [51].

ADP-ribosylation factor 6 (Arf6)

The role of Arf6 in endocytosis, endocytic trafficking, and receptor recycling in nucleated cells is well-established [52, 53]. Our group showed its presence and function in platelets [50]. In resting platelets, Arf6 is in the active, GTP-bound form. Upon platelet activation, it converts to the inactive, Arf6-GDP [50]. This transition is regulated by two waves of platelet signaling pathways, primary signaling (*e.g.*, PAR receptor, GPIIb/IIIa) and contact-dependent signaling (*e.g.*, $\alpha_{IIb}\beta_3$ integrin [54]), which is consistent with studies showing the involvement of Arf6 in regulating integrin function in platelets [55, 56]. Arf6 has also been shown to be involved in P2Y₁₂ receptor internalization, which is required for receptor desensitization and resensitization to ADP. P2Y activation by ADP stimulates Arf6 activation, and activated Arf6 stimulates Nm23-H1, a nucleoside diphosphate kinase, which in turn promotes Dynamin-dependent internalization of P2Y receptors [57].

To better understand the role of Arf6, platelet-specific Arf6 conditional knockout mice were generated [2]. Arf6^{-/-} platelets have defective $\alpha_{IIb}\beta_3$ -mediated Fg uptake *in vitro* and *in vivo* and demonstrated enhanced spreading on Fg-coated surfaces and thrombin-induced clot retraction. Hemostasis parameters such as secretion, aggregation, tail bleeding times, and FeCl₃-induced occlusion in carotid artery injury model of arterial thrombosis were normal. These mice will be a valuable tool to assess the importance of endocytic trafficking in platelets and megakaryocytes.

PHAGOCYTOSIS

Phagocytosis involves ingestion of large particles such as invading microbes or dead cells. This process can be mediated by opsonic receptors, such as Fc γ RIIa, on the platelet surface. Fc γ RIIa is crucial for immune complex clearance and platelets have been shown to phagocytose immune complexes in a Fc γ RIIa-dependent manner [58, 59]. Platelets can phagocytose bacteria such as *P. gingivalis* [60] and *S. aureus* [61, 62] and viruses such as HIV-1 [61]. These particles are taken up inside a membrane-bound compartment thought to be related to the OCS. Platelets can also phagocytose inert molecules, *e.g.*, latex beads, which can cause platelet aggregation due to release of ADP [63]. Longer incubations with latex beads erode granule integrity, though the mechanism of this process is unclear. Liposomes can also be phagocytosed by human platelets [64]. Once phagocytosed, it is unclear what happens to particles; however, acid phosphatase-positive phagosomes have been detected in human platelets, suggesting that trafficking for degradation could occur [65].

ENDOCYTIC ROUTES IN PLATELETS

Compartments

Platelets contain distinct membrane-bound compartments called endosomes that may be “way stations” for internalized cargo molecules [2]. Normally, endocytic cargo shuttles to early endosomes and can be either recycled back to the cell surface (*via* recycling endosomes) or sorted to degradative compartments *e.g.*, late endosomes or lysosomes (Figure 1). The platelet OCS was proposed as the epicenter for cargo uptake; however, recent evidence, from tomographic analyses of scanning transmission electron microscopic images of quiescent platelets, showed a distinct closed canalicular system of discrete membrane-bound structures, which is distinct from α -granules [66]. In the early stages of platelet activation, the closed canalicular system fuses with the PM and becomes open, while the α -granules fuse with the PM *via* long tubular connections [66]. One could speculate that the closed canalicular system represents endosomes that fuse with the PM during platelet activation. Immunofluorescence showed the presence of two distinct populations of early and recycling endosomes in platelets. Using Rab4 and Rab11 GTPases as markers for early and recycling endosomes, respectively, Huang *et al.* provided the first evidence for the time-dependent trafficking of Fg from early to recycling endosomes in platelets [2]. Previously, Heijnen *et al.* detected multi-vesicular bodies (MVBs) in platelets, which appear to be part of the route taken by endocytosed Fg on its way to α -granules [3]. What remains to be determined is which endocytic trafficking routes are active in platelets and how are they used.

SNARE Machinery

Platelets contain Soluble *N*-ethylmaleimide Sensitive Fusion Protein Attachment Protein Receptor (SNARE) proteins, which drive membrane fusion and cargo exocytosis [6]. They are known to mediate inter-compartmental trafficking in most cells [67], but no such role has been established in platelets. Platelets contain isoforms of the Vesicle-Associated Membrane Proteins (VAMPs) and Syntaxins, in addition to SNAP-23 [28, 68]. Of these, VAMP-8,

Syntaxin-11, and SNAP-23 are the major exocytotic SNAREs; others have secondary or no roles in granule cargo release [69–72]. VAMP-7, though less involved in secretion, interacts with actin cytoskeleton to modulate platelet spreading and thus, could affect integrin trafficking [73]. Immunofluorescence studies showed VAMP-7⁺ structures at the periphery, while VAMP-8⁺ and VAMP-3⁺ structures are more central within the granulomere [74, 75]. VAMP-3 deletion had no effect on secretion [76], thus these could be VAMP-3⁺ endosomes. Consistently, loss of VAMP-3 does cause defective Fg uptake, enhanced spreading and clot retraction thereby affecting $\alpha_{IIb}\beta_3$ integrin trafficking [Banerjee *et al.*, in revision].

SNARE regulators, which could also affect endocytosis, have been detected. Vacuolar Protein Sorting-associated protein 33A and B (VPS33A and VPS33B), which are members of the Sec/Munc family of syntaxin chaperones, are important for dense and α -granule biogenesis, respectively [77, 78]. VPS33B binds to the integrin β subunit to modulate $\alpha_{IIb}\beta_3$ -mediated Fg endocytosis, platelet activation, aggregation, spreading, clot retraction and *in vivo* thrombosis and hemostasis [79].

RECEPTOR TRAFFICKING IN PLATELETS

Studies of receptor recycling have mainly focused on $\alpha_{IIb}\beta_3$ and its movement in and out of platelets. Fg enters *via* binding to $\alpha_{IIb}\beta_3$, transits through the multivesicular bodies and ends up in α -granules [3, 8]. Thus, Fg content is an indicator of $\alpha_{IIb}\beta_3$ trafficking and its defects. Insights into this process came from studies using the c7E3 Fab fragment (abciximab), an antagonist of $\alpha_{IIb}\beta_3$ [80]. Rapid staining of the OCS within 1 min, post-addition of c7E3, showed that internalization of $\alpha_{IIb}\beta_3$ can be fast [80]; consistent with the widely-held theory that the OCS is the entry site for the endocytosis of various exogenous substances [81].

Once inside, these surface receptors may recycle back to the PM; however, this is unclear [82–86]. In unstimulated platelets, an internal pool of $\alpha_{IIb}\beta_3$ exists in steady-state equilibrium with the surface pools. Upon activation with agonists (*e.g.*, thrombin, ADP), the steady-state changes and the internal pool is trafficked to the PM, increasing surface expression [2]. Since ADP-stimulation enhances Fg internalization in platelets, it is possible that activation simply increases the rate of recycling and return of $\alpha_{IIb}\beta_3$ to the surface [83, 87]. Surface levels of other proteins, such as glycoprotein Ib and glycoprotein IV (CD36) may be controlled in a similar manner [88]. These studies suggest that endocytosis, by mediating alterations in the distribution of PM proteins, could have acute roles during the formation of a growing thrombus.

CONCLUSION

Endocytosis is a multi-step process that utilizes several routes of cargo entry/transit/exit. Many regulators, *e.g.*, Arf6, Dab2, *etc.*, facilitate $\alpha_{IIb}\beta_3$ -mediated Fg uptake, integrin trafficking, and the dynamic processes that affect contact-based signaling during spreading and clot retraction. Others, *e.g.*, DNM2, have more profound roles not only in platelets but also in megakaryocytes. Endocytosis probably allows platelets to modulate the receptor-mediated interactions with their immediate environment in a thrombus. Endocytosis may also enable platelets to interpret circulating signals in the form of circulating pathogens or

infectious agents. The platelets' ability to endocytose and potentially decipher content from their environment provides them with the ability to differentially respond to what they take in. Thus, platelets could function as vascular "vacuum cleaners", surveying for damage or pathogens and responding to what they detect. This is a novel concept that requires future studies to uncover what platelets can internalize and to define what they do with what they take up. The transgenic animals, discussed in this review, will be invaluable in this endeavor. Clearly, platelets are more complex "cells" with extensive endomembrane systems and a greater capacity for intracellular trafficking than previously imagined.

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KEY POINTS

- Platelets can endocytose and/or phagocytose a variety of molecules and particles.
- The platelet proteome contains many elements of the clathrin-dependent and -independent endocytic pathways.
- Imaging studies have shown that platelets contain several types of endosomal compartments.
- Endocytosis is integral to platelet function.

Figure 1. Potential Endocytic Routes in Platelets

Cargo can enter platelets either *via* clathrin-dependent endocytosis, requiring GTP hydrolysis by Dynamin and using specific surface receptors (*e.g.*, $\alpha_{IIb}\beta_3$ -mediated fibrinogen entry) or *via* clathrin-independent endocytosis that may require Dynamin (*via* caveolin- or RhoA-dependent pathways) or may not (Arf6- or Cdc42-dependent pathways). Internalized cargo then transits through Rab 4 GTPase-positive early endosomes, where it can be sorted to recycling endosomes (Rab 11-positive) for return to the plasma membrane or to multivesicular bodies and ultimately into α -granules for storage (*e.g.*, fibrinogen, vWF, thrombospondin-1). Alternatively, cargo can move into late endosomes, either directly from early endosomes or through multivesicular bodies. Cargo from late endosomes can transit into dense granules or to lysosomes where it may be degraded or stored. The complexity of these pathways in platelets has not been studied in sufficient detail.

Table 1

Endocytic and Sorting Machinery in Platelets.

Full name of Protein *	Aliases/Identifiers	Abundance (copies per human platelet)&
Clathrin-mediated endocytosis		
Clathrin Heavy Chain 1, 2	CLTC1; CLTC2	13,600; 4,300
Clathrin Light Chain A, B	CLTA; CLTB	2,800; 2,100
AP-2 complex (α , β , σ , μ)	AP-2	ND; 7,900; ND; 4,800
AP-3 complex	AP-3	3,500
AP-4 complex	AP-4	1,400
Dynamin 1, 2, 3	DNM1, DNM2, DNM3	5,200; 6,100; 3,600
Endophilin A2, B2	Endo A2; Endo B2	3,500; 2,900
Sorting Nexins (1, 2, 3, 4, 5, 6, 7, 8, 9, 24, 29, 30)	SXN	1,500; 5,100; 8,500; 1,400; 1,700; 2,300; 1,400; 730; 1,900; 1,800; 810; 1,100.
Clathrin-independent endocytosis		
Ras-related C3 botulinum toxin substrate 1, 2	Rac1; Rac2	32,900; 27,900
Cell division control protein 42 homolog	Cdc42	27,900
ADP-ribosylation Factor 1, 3, 4, 5, 6	Arf1, Arf3, Arf4, Arf5, Arf6	49,800; 44,300; 33,300; 36,200; 6,400
Flotillin-1, 2	FLOT-1; FLOT-2	7,500; 6,000
Rab GTPases		
Rab 1A, 1B, 2A, 2B, 3, 4, 5, 6A, 6B, 7, 8A, 8B, 9, 20, 21, 30, 31, 32, 33, 35, 37, 38		27,700; 26,000; 11,600; 7,400; 11,200; 23,200; 7,700; 20,900; 27,500; 18,800; 17,900; 16,100; 2,500; 1,500; 5,600; 4,700; 2,200; 8,900; 2,000; 5,600; 9,200; 4,400
Protein Sorting Complexes		
Vacuolar Protein Sorting-associated protein 4A, 4B, 11, 13A, 13B, 16, 26A, 26B, 28, 29, 33A, 33B, 35,	VPS 4A; VPS 4B; VPS11; VPS 13A, VPS 13C; VPS 16; VPS 26A, VPS 26B; VPS 28; VPS 29; VPS 33A; VPS 33B; VPS 35; VPS 36; VPS 37A; VPS 37B; VPS 41; VPS 45; VPS 52	2,900; 4,300; 1,100; 2,000; 1,600; 1,500; 1,100; 3,100; 3,600; 4,800; 1,700; 2,100; 4,600; 2,500; 2,900; 1,900; 810; 2,200; 900;
Neurobeachin-like 2 protein	NBEAL2	3,000
Other		
Early Endosome Antigen-1	EEA-1	1,700
Lysosomal Associated Membrane Protein-1, 2	LAMP-1; LAMP-2	3,000; 2,000
Vacuolar Protein Sorting-associated protein 33B Interacting Protein	VIPAR	1,200

Full name of Protein*	Aliases/Identifiers	Abundance (copies per human platelet)&
Calreticulin	CALR	20,300
Connexin	CANX	10,400
Calmodulin	CaM	15,600

* Listed are human platelet proteins that may contribute to endocytosis and/or subsequent cargo-sorting events.

& Included are copy numbers, estimated based on quantitative proteomic analyses (28).

ND= Not determined.