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Insights into platelet-based control of coagulation

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ABSTRACT

The coagulation process is activated by tight control mechanisms, in which platelets play prominent and unique roles. In thrombosis and hemostasis, activated platelets regulate the coagulation system in various ways: by exposing a phosphatidylserine surface for thrombin formation, by supporting fibrin formation, and by regulating the retraction of a fibrin clot. In this review we discuss the involvement of platelet receptors, other membrane proteins, downstream signaling proteins, cytoskeleton-linked proteins and plasma proteins in these procoagulant functions. Studies with both genetically modified mice and pharmacological inhibitors indicate that, for collagen-adhered platelets, in part common signaling pathways lead to phosphatidylserine exposure, generation of thrombin and fibrin, and retraction of the fibrin clot. However, prolonged Ca^{2+} elevation leads to thrombin generation, whereas integrin-dependent signaling stimulates fibrin clot retraction. Contact-dependent signaling pathways, triggered by homotypic platelet-platelet interactions, act in particular via the integrin route.

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Introduction

The coagulation process is regulated by tight control mechanisms. During thrombosis and hemostasis, platelets provide prominent and unique roles in supporting and confining the coagulant activity [1,2]. Particularly studies with genetically modified mice and with inhibitors using human platelets have provided detailed insights into the molecular determinants of platelet-based coagulation. In this review, we discuss these findings regarding three mechanisms, how platelets: (i) form a procoagulant surface with exposed phosphatidylserine; (ii) provide sites for thrombin and fibrin formation; and (iii) steer fibrin clot retraction (Figure 1).

Regulation of platelet phosphatidylserine (PS) exposure

Flow chamber devices have extensively been used to study the process of thrombus formation at arterial shear conditions in order to evaluate not only platelet aggregation, but also platelet procoagulant activity (PS exposure) and fibrin formation within the same experiment. The current methodology, using small-size chambers in combination with fluorescence microscopy, has revealed key roles of many platelet receptors and signaling proteins in the buildup of a thrombus and the appearance of procoagulant platelets [3]. Table 1 provides an overview of published studies, where thrombus formation together with PS

exposure is assessed under arterial shear conditions using blood from genetically modified mice. Table 2 shows corresponding data with human blood and specific pharmacological inhibitors. Note that the earlier literature concentrated on understanding the role of collagen receptor-induced Ca^{2+} fluxes in PS exposure, while recent papers revealed the contribution of various downstream signaling events and of autocrine products secreted from platelet granules. In addition, adhesive receptors other than collagen receptors have been examined for a role in procoagulant activity.

Collagen-induced Ca^{2+} signaling as the classical route

At high, arterial wall-shear rate, platelet adhesion to von Willebrand factor (vWF) via glycoprotein Ib-V-IX (GPIb), and to collagen via the receptors glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$ leads to strong activation and to the formation of a stable thrombus [1,4]. A subpopulation of the platelets in the thrombus, particularly those platelets in direct contact with the collagen and the platelets that are co-activated by thrombin assume a round morphology, which is accompanied by PS exposure and microparticle shedding [5,6]. In contrast to the aggregated platelets in the thrombus core, the PS-exposing cells lack activation of the fibrinogen receptor, integrin $\alpha_{\text{IIb}}\beta_3$ [7]. The main mechanism underlying PS exposure is a prolonged elevation in cytosolic Ca^{2+} level [8,9]. Hence, signaling molecules downstream of the GPVI-Fc receptor γ chain (FcR γ) complex and involved in Ca^{2+} mobilization via phospholipase C γ (PLC γ) and phosphatidylinositol 3-kinase (PI3K) will contribute to PS exposure [1]. As indicated in Table 1, platelets from knockout mice deficient in several signaling molecules show a decreased ability to thrombus formation as well as PS exposure. These signaling proteins include the receptors GPIb (via vWF), GPVI,

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Fig. 1. Platelet-based coagulation processes in a thrombus. The extrinsic and intrinsic coagulation systems are initiated after vascular disruption via tissue factor (TF) and collagen, respectively. Collagen-adhered platelets and patches of platelets in a thrombus expose PS, which serves as an efficient surface for assembly of the tenase (factors IXa/VIIIa) and prothrombinase (factors Xa/Va) complexes, resulting in thrombin generation and fibrin formation. At low shear rates, GPIb interaction with vWF mediates focal fibrin formation at the platelet surface via PS exposure. The aggregated platelets in a thrombus display activated $\alpha_{IIb}\beta_3$ and accomplish clot retraction by contracting fibrin fibers in the presence of thrombin.

Table 1

Mouse proteins and genes involved in platelet procoagulant activity and fibrin formation under high-shear flow conditions

Target protein	Perturbation	Effect on thrombus formation	Effect on coagulation	Reference
<i>Platelet receptors and membrane proteins</i>				
Axl	<i>Axl</i> ^{-/-}	TS ↓ (instability)	PS =	[14]
Cyclophilin D	<i>Ppid</i> ^{-/-}	TS ↑	PS ↓	[7]
Glycoprotein Ib	p0p/B Ab	TS ↓	PS ↓	[82]
Glycoprotein VI	<i>Fcer1g</i> ^{-/-} , JAQ1 Ab	TS ↓ (no thrombi)	PS ↓	[34,83,84]
Gqα	<i>Gnaq</i> ^{-/-}	TS ↓	PS ↓	[83]
Integrin $\alpha_2\beta_1$	α_2 -null, β_1 -null	TS ↓	PS ↓	[83–85]
Integrin $\alpha_{IIb}\beta_3$	JON/A mAb	TS ↓	PS ↓	[82]
LAT	<i>Lat</i> null, <i>Lat</i> ^{+/-} , <i>Lat</i> ^{-/-}	TS ↓	PS ↓	[33,34]
Mer	<i>Mer</i> ^{-/-}	TS ↓ (instability)	PS =	[14]
Orai1	<i>Orai1</i> ^{-/-} , 2-APB	TS ↓	PS ↓, fibrin ↓	[17,18]
P2Y ₁	MRS-2179	TS ↓	PS ↓	[83]
P2Y ₁₂	<i>P2yr12</i> ^{-/-} , cangrelor	TS ↓	PS ↓	[12]
STIM1	<i>Stim1</i> ^{-/-}	TS ↓	PS ↓, fibrin ↓	[17]
	<i>Stim1</i> (LOF)	TS =	PS =	[23]
Tyro3	<i>Tyro3</i> ^{-/-}	TS ↓ (instability)	PS =	[14]
<i>Platelet signaling proteins (kinases)</i>				
PI3 kinases	<i>Pik3r1</i> ^{-/-} , wortmannin	TS ↓	PS ↓	[27]
PI3 kinase α/β	PIK-75, TGX-221, <i>Pik3r2</i> (LOF)	TS ↓	PS ↓	[27,48,86]
Protein kinase Cα	<i>Prkca</i> ^{-/-}	TS ↓	PS ↓	[87,88]
Protein kinase Cβ	<i>Prkcb</i> ^{-/-}	TS ↓ (no thrombi)	PS ↓	[87]
Protein kinase Cδ	<i>Prkcd</i> ^{-/-}	TS ↑	PS =	[87]
Protein kinase Cθ	<i>Prkcg</i> ^{-/-}	TS ↑/↓	PS ↑	[26,87]
Protein kinase D2	<i>Pkd2</i> ^{-/-}	TS ↓	PS =	[89]
Src family kinases	PD0173952	TS ↓ (no thrombi)	PS ↓	[33,85]
<i>Platelet signaling proteins (non-kinases)</i>				
α-Granules	<i>Nbeal2</i> ^{-/-}	TS ↓	PS ↓	[30]
CalDAG-GEF1	<i>Rasgrp</i> ^{-/-}	TS ↓	PS ↓	[23]
Calpain-1	<i>Capn1</i> ^{-/-}	TS =	PS ↓	[7]
CLP36	<i>Clp36</i> (LOF)	TS ↑	PS ↑	[31]
Gas6	<i>Gas6</i> ^{-/-}	TS ↓ (instability)	PS =	[14]
Phospholipase Cγ2	<i>Plcg2</i> (GOF), <i>Plcg2</i> ^{-/-} , <i>Plcg2</i> ^{-/-}	TS ↑	PS ↑	[90]
Phospholipase D1	<i>Pld1</i> ^{-/-}	TS ↓	PS ↓	[54]
<i>Plasma proteins</i>				
Factor XI	<i>F11</i> ^{-/-}	TS ↓	PS ↓, fibrin ↓	[34]
Factor XII	<i>F12</i> ^{-/-} , CTI	TS ↓	PS ↓, fibrin ↓	[34]
Factor VII	FVIIai	TS ↓	PS ↓, fibrin ↓	[34]
(Pro)thrombin	melagatran	TS ↓	PS ↓, fibrin ↓	[35]
VWF	saratin	TS ↓	PS ↓	[33,82]

Ab, antibody; GOF, gain-of-function; LOF, loss-of-function; PS, phosphatidylserine, TS, thrombus size; = unchanged; ↑ increased; ↓ decreased.

Table 2

Human proteins involved in platelet procoagulant activity and fibrin formation under high-shear flow conditions

Target protein	Intervention	Effect on thrombus	Effect on coagulation	Reference
<i>Platelet receptors and membrane proteins</i>				
Glycoprotein Ib	6B4, 12G1 Abs	TS ↓	PS ↓	[91]
Glycoprotein VI	9O12, 10B12 Abs	TS ↓ (no thrombi)	PS ↓	[11,92,93]
Integrin $\alpha_2\beta_1$	6F1, 15D7 Abs	TS ↓	PS ↓	[85,92–94]
Integrin $\alpha_{IIb}\beta_3$	agastat, latrofiban	TS ↓ (no thrombi)	PS ↓	[85,94]
Orai1	2-APB	TS ↓	PS ↓	[18]
P2Y ₁₂	ticagrelor, cangrelor	TS ↓ (instability)	PS ↓, fibrin ↓	[11,12,48]
<i>Platelet signaling proteins (kinases)</i>				
PI3 kinases	wortmannin	TS ↓ (instability)	PS ↓	[27,48]
PI3 kinase α/δ	PIK-75, YM-024	TS ↓	PS ↓	[27]
PI3 kinase β	TGX-221	TS ↓	PS ↓	[27]
PI3 kinase γ	AS-252424	TS =	PS =	[27]
PI3 kinase δ	IC-87144	TS =	PS =	[27]
Protein kinase C	GF-109203X, RO-318425	TS ↓ (no thrombi)	PS ↑	[25]
Src family kinases	PD-0173952	TS ↓ (no thrombi)	PS ↓	[85]

Abbreviations as for Table 1.

FcR γ , $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$; various adapter and scaffold proteins, e.g. linker for activated T-cells (LAT); effector proteins such as the PI3K isoforms α/β , Src family kinases, and PLC γ 2. Inhibitory studies with human blood indicate a similar involvement of receptors (GPIb, GPVI, $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$) and signaling molecules (PI3K isoforms α and β and Src family kinases) in thrombus formation and PS exposure (Table 2). These findings support the concept that the reduced PS exposure is a direct consequence of diminished GPVI signaling to elevated Ca²⁺, rather than to a reduced platelet deposition per se [9].

Collagen- and GPVI-dependent PS exposure is greatly enhanced by co-stimulation of platelets with thrombin (acting via the Gq α protein), at both static and flow conditions, an effect that is explained by the more prolonged Ca²⁺ mobilization in response to two agonists [10]. Other soluble agonists, in particular ADP, have been shown to enforce GPVI-induced Ca²⁺ signaling, PS exposure and thrombin generation, particularly via the P2Y₁₂ receptors, and hence contribute to platelet procoagulant activity. Consequently, knock-out (mouse) or inhibition (human) of P2Y₁₂ resulted in a smaller thrombus size and decreased PS exposure [11–13]. However, thrombus formation not always coincides with PS exposure. For instance, the interaction of plasma-derived Gas6 with the platelet Gas6 receptors (TAM receptors), while enforcing integrin activation and contributing to thrombus stability, did not contribute to platelet PS exposure [5,14].

Insights into the role of elevated Ca²⁺

Platelets contain well-developed Ca²⁺ entry mechanisms for inducing and maintaining high cytosolic Ca²⁺ levels [15]. Recent work has pointed to a role of the so-called store-operated and receptor-operated Ca²⁺ entry pathways in PS exposure. The integral membrane protein, stromal interaction molecule (STIM)1, has been identified as the central Ca²⁺ sensor in the platelet reticular membrane that regulates store-operated Ca²⁺ entry [16]. Via a direct coupling mechanism, STIM1 activates the Ca²⁺ entry channel Orai1. Deficiency in either STIM1 or Orai1 resulted in decreased thrombus formation and PS exposure on collagen (Figure 2A) [17,18]. Interestingly, in the presence of high thrombin concentrations, STIM and Orai1 appeared to be less important for PS exposure [17]. This was recently explained by the finding that thrombin-dependent Ca²⁺ signaling in part relies on receptor-operated Ca²⁺ entry via TRPC channels [19].

The Ca²⁺ signal in procoagulant platelets is enhanced by the release of mitochondrial Ca²⁺ via mitochondrial permeability transition pore formation (Figure 2A) [20]. A key

regulator in this pore formation is the mitochondrial localized peptidylprolylisomerase, cyclophilin D [21]. Accordingly, GPVI-induced PS exposure was found to be reduced in platelets from mice lacking cyclophilin D [7]. However, in these platelets ionomycin-induced PS exposure is unchanged [22], designating that mitochondrial collapse as such is not a strict requirement for PS exposure.

Redundancies downstream of diacylglycerol and Ca²⁺

Isoforms of PLC produce two second messengers, diacylglycerol (DAG) and inositol-trisphosphate serving as an intracellular Ca²⁺ mobilizer. Recent investigations have examined the roles in platelet procoagulant activity of two sets of Ca²⁺- and DAG-dependent effector proteins, which are activated downstream of PLC γ 2, namely CalDAG-GEF1 and isoforms of protein kinase C (PKC). Interestingly, these effector proteins can also modulate the Ca²⁺ signal itself, in a not yet completely understood way (Figure 2A). Platelets from mice lacking CalDAG-GEF1 are greatly impaired in thrombus formation as well as in PS exposure [23]. However, the situation for PKC is more complex as described next.

Human and mouse platelets express both the conventional PKC isoforms α and β and the novel PKC isoforms δ and θ [24]. In human blood, addition of a general inhibitor for all PKC isoforms resulted in greatly abolished thrombus formation, but in increased PS exposure of the remaining adhered platelets [25]. Studies with knockout mice indicated a positive role in both thrombus formation and PS exposure of the two conventional PKC isoforms α/β , whereas the two novel PKC isoforms δ/θ were inhibitory [26,27]. The precise phosphorylation events through which these PKC isoforms operate are still unclear [24]. Jointly, these data point to non-redundant and positive roles of CalDAG-GEF1 and PKC α/β , and to antagonistic and negative roles of PKC δ/θ in the regulation of thrombus buildup and PS exposure.

Calpain is another Ca²⁺-dependent effector protein controlling platelet activation. While deficiency in mouse calpain-1 had no effect on thrombus size, this resulted in a markedly lower PS exposure [7,22].

Additive roles of dense- and alpha-granule secretion

Platelet dense (δ) and alpha (α) granule constituents are known to amplify platelet responses, such in agreement with the bleeding phenotype seen in patients with platelets lacking these granules [28]. Novel data indicate that granule secretion

Fig. 2. Signaling pathways implicated in platelet-dependent procoagulant activity, thrombin generation and clot retraction. (A) Platelets on collagen signal to second messenger generation (PLCγ2, CalDAG-GEF1, PKC isoforms) and Ca²⁺ mobilization combined with store-operated Ca²⁺ entry (STIM1, Orai1) and release of mitochondrial Ca²⁺ via mPTP formation, resulting in sustained high cytosolic Ca²⁺ levels, granule secretion and PS exposure. These processes are supported by α- and δ-granule constituents. The PS-exposing platelets show procoagulant activity by catalyzing tenase and prothrombinase activities (see also Figure 1). (B) Continuous inside-out signaling via PI3K, Rap1b and calpain leads to sustained α_{IIb}β₃ activation, enabling integrin outside-in mediated actin cytoskeleton reorganization via Rac1, RhoA, WASP and talin resulting in clot retraction. Homotypic interactions via connexins, ESAM and JAM-A differentially regulate clot retraction, while tetraspanins may directly interact with α_{IIb}β₃.

supports not only platelet aggregation, but also platelet procoagulant activity. The δ-granules contain calcium, serotonin, ADP, ATP and polyphosphates. The observed impairment of collagen/thrombin-induced PS exposure of platelets deficient in P2Y₁₂ or in the presence of P2Y₁₂ inhibitors [12,13], can well be explained by inability of secreted ADP to interact with this receptor on neighboring platelets and thereby diminished signaling to platelet procoagulant activity. Lack of δ-granules is the underlying cause of the Hermansky-Pudlak bleeding disorder [28]. Mice deficient in one of the Hermansky-Pudlak genes (*Hsp3*) show an impaired thrombosis tendency *in vivo*, but PS exposure has not yet been assessed.

The α-granules store multiple proteins, several of which can support thrombus formation, e.g. vWF, fibrinogen, factor V and thrombospondin [29]. Mutations in the *Nbeal2* gene associate with gray platelet syndrome, a rare bleeding disorder characterized by macrothrombocytopenia and the absence of platelet α-granules. Mice lacking expression of the *Nbeal2* gene have been reported to be decreased in both thrombus formation and PS exposure [30]. These mice provide an excellent model for further determination of the roles of α-granule proteins in hemostasis, thrombosis and inflammation.

Secretion of both α- and δ-granules is increased in CLP36-deficient mice, thus explaining a larger thrombus size and

higher PS exposure of these platelets under flow [31]. CLP36 is an adaptor protein that binds to α -actinin-1 and associates with actin filaments and stress fibers [32]. Jointly, these findings seem to indicate that both types of secretion granules have additive roles in PS exposure along with thrombus formation (Figure 2A).

Regulation of platelet-dependent thrombin generation and fibrin formation

Platelet membranes with exposed PS provide an efficient surface for assembly of the tenase (factors IXa, VIIIa) and prothrombinase (factors Xa, Va) complexes, accomplishing high thrombin generation and subsequent fibrin formation [1]. Interestingly, genetic ablation or pharmacological inhibition of factors of either the extrinsic (VII) or the intrinsic (XII, XI) coagulation pathways diminished thrombin generation under stasis (Tables 3–4). Several of the signaling proteins that contribute to GPVI-induced PS exposure under flow have also been shown to control GPVI-mediated thrombin generation under stasis, as depicted for GPVI, PI3K β , Syk, Orai1, STIM1 and P2Y₁₂.

Strikingly, platelets bind almost all coagulation factors, including thrombin and fibrin [8]. At high shear rate in the presence of tissue factor, fibrin is predominantly confined to the core of platelet thrombi, which implies platelet support of the extrinsic coagulation system, triggered by the tissue factor/factor VIIa complex [33]. Studies carried out in the absence of tissue factor also point to a role of the intrinsic coagulation system via factors XI and XII. In flow studies on collagen with recalcified blood, collagen fibers were found to play a dual role, *i.e.* as a ligand for GPVI resulting in platelet activation, and as a substrate for the binding and activation of factor XII [34]. Consequently, deficiency in factor XII or XI resulted in a decreased thrombus size, reduced PS exposure, and delayed fibrin formation (Table 1). Inhibition of thrombin was even more suppressive [35]. Of note, signaling downstream of P2Y₁₂ amplified these processes [12,13]. Factor XIIa may also play another role, *i.e.* by directly binding to fibrin and regulating clot structure [36]. Jointly, these findings point to involvement of both the intrinsic and extrinsic coagulation systems in thrombin generation and ensuing fibrin formation at high-shear flow conditions. Furthermore, the formed thrombin generates a positive feed-forward loop to enhanced PS exposure via activation of the PAR receptors on platelets [8].

Classically, insufficiently controlled coagulation is regarded as a cause of thrombosis under venous shear conditions. Recently, several insights also point to a role of platelets, in interplay with vWF and coagulation factors, in the thrombotic process under flow at low, venous wall-shear rates. In the absence of anticoagulants and low shear rate (implying accumulation of thrombin), a fibrin network is formed on the platelet surface with an outward-directed star-like appearance [37]. This focal fibrin formation relies on vWF-GPIb interactions, causing rises in cytosolic Ca²⁺ and PS exposure [37]. The signaling mechanism may involve GPIb-dependent changes of the actin cytoskeleton [38,39]. Interestingly, the fibrin fibers incorporate thrombin that seems to be relocating from PS-exposing and factor Xa-binding platelets [40].

The role of neutrophils in murine venous thrombosis models recently received much attention. It is reported that neutrophils, which are trapped in murine veins subjected to partial stasis, produce neutrophil extracellular traps (NETs), *i.e.* large extracellular DNA strands fibers surrounded by histones and neutrophil antimicrobial proteins [41]. In venous and microvascular thrombosis models, NETs formation can be a stimulus and scaffold for the formation of large thrombi of platelets, fibrin and erythrocytes [41,42]. Furthermore, NETs can be formed in the vasculature during sepsis, where they

may help to reduce microbial infections [43,44]. The underlying mechanisms of platelet responses to neutrophils and NETs have to be elucidated.

Towards understanding platelet-dependent fibrin clot retraction

In hemostasis, the process of platelet-dependent clot retraction provides a means to tighten wound edges after clotting [45]. Especially under conditions of coagulation and fibrin formation, thrombi that are formed under flow can undergo a mechanism of retraction, which can be considered as an equivalent process as clot retraction [8]. However, clot retraction as such has mostly been examined under static conditions, *e.g.* by determining the condensation of a platelet/fibrin clot in thrombin-stimulated platelet-rich-plasma. In general, clot retraction is regarded as a platelet response evoked by $\alpha_{IIb}\beta_3$ inside-out signaling, thus implying dependency of (thrombin-induced) $\alpha_{IIb}\beta_3$ activation and binding of fibrinogen to platelets [46]. Table 3 provides an overview of published effects of genetic knockout in mouse on clot retraction, while Table 4 gives corresponding data for human blood. Below we describe new insights into the molecular mechanisms with emphasis on integrin inside-out and outside-in signaling, homotypic platelet-platelet interactions, and the role of the actin cytoskeleton.

Enforcement of $\alpha_{IIb}\beta_3$ inside-out signaling

While clot retraction for long has been recognized as a process that reflects integrin outside-in signaling, new data provide insight into the underlying signaling events in platelets via activated integrins. The ADP receptor P2Y₁₂ prolongs integrin activation and platelet aggregation [47]. Continued signaling via this receptor (via Gi and PI3K β) maintains $\alpha_{IIb}\beta_3$ in the active conformation and thus prevents platelet disaggregation [48,49]. Likely, similar signaling mechanisms are implicated in clot retraction, since mouse platelets lacking integrin β_3 or PI3K β are impaired in this process (Table 3, Figure 2B).

An enforcement loop that acts in a similar, though weaker way is that of (plasma-derived) Gas6 interacting with the platelet Gas6 receptors, also called TAM receptors (Tyro3, Axl and Mer). Activation of these receptors by Gas6 supports platelet aggregation and tyrosine phosphorylation of the β_3 integrin chain, again in a PI3K-dependent way [47,50]. Overall, TAM receptor signaling can enforce the activation of $\alpha_{IIb}\beta_3$, and thus help to prevent platelet disaggregation. Platelets from mice deficient in one of the TAM receptors have a decreased ability to clot retraction, which supports a role for Gas6 and its receptors in integrin outside-in signaling (Figure 2B) [50].

Calcium also play a central role in integrin activation via the Ca²⁺- and DAG-dependent effector proteins CalDAG-GEF1 and isoforms of PKC, which are activated downstream of PLC γ 2. Activation of Rap1b – via CALDAG-GEF or PKC – promotes the assembly of an activation complex with RIAM (Rap1 interacting adaptor molecule) and talin. Cleavage of talin mediated by RIAM, calpain and phosphatidylinositol 4,5-bisphosphate (PIP₂) enables talin binding to the integrin β_3 chain, thereby activating the integrin and enabling ligand binding [51]. An additional level of regulation is provided by phospholipase D (PLD) 1 and 2. Both isoforms are present in platelets and translocate to the plasma membrane during platelet activation and produce phosphatidic acid to stimulate the production of PIP₂ [52,53]. Mice deficient in PLD1 display impaired thrombus formation and PS exposure due to impaired integrin $\alpha_{IIb}\beta_3$ inside-out signaling [54]. Fibrin clot retraction is also reduced in these mice, similarly as in mice lacking Rap1b or talin [54,103,104].

Table 3

Mouse proteins and genes involved in platelet-dependent thrombin generation and clot retraction under stasis.

Target protein	Perturbation	Effect on coagulation	Reference
<i>Platelet receptors and membrane proteins</i>			
Axl	<i>Axl</i> ^{-/-}	CR ↓	[50]
Connexin 37	<i>Gja4</i> ^{-/-}	CR ↓	[59]
Connexin 40	<i>Gja5</i> ^{-/-} , ⁴⁰ Gap27	CR ↓	[60]
* Cyclophilin D	<i>Ppid</i> ^{-/-}	CR ↓	[22]
ESAM	<i>ESAM</i> ^{-/-}	CR ↓	[67]
Galectin-1	<i>Lgals1</i> ^{-/-}	CR ↓	[55]
* Integrin $\alpha_{IIb}\beta_3$	β_3 -null	CR ↓	[95]
JAM-A	<i>F11r</i> ^{-/-}	CR ↑	[61]
Mer	<i>Mer</i> ^{-/-}	CR ↓	[50]
* Orai1	<i>Orai1</i> ^{-/-}	TG ↓	[17]
* P2Y ₁₂	cangrelor, ticagrelor	TG ↓	[11–13]
PECAM-1	<i>Pecam1</i> ^{-/-}	CR =	[72]
* STIM1	<i>Stim1</i> ^{-/-}	TG ↓	[17]
Tetraspanin 32 (TSSC6)	<i>Tspan32</i> ^{-/-}	CR ↓	[70]
Tetraspanin CD151	<i>Cd151</i> ^{-/-}	CR ↓	[71]
TMEM16F (anoctamin 6)	<i>Ano6</i> ^{-/-}	TG ↓	[96]
Tyro3	<i>Tyro3</i> ^{-/-}	CR ↓	[50]
<i>Platelet signaling proteins (kinases/phosphatases)</i>			
AMPK α 2	<i>Prkaa2</i> ^{-/-}	CR ↓	[97]
PKD1	<i>Pdpk1</i> ^{-/-} , <i>Pdpk1</i> (LOF)	CR ↓	[98]
* PI3 kinase	LY-294002	CR ↓	[76]
* PI3 kinase β	<i>Pik3cb</i> -null, TGX-221	CR ↓	[75]
Protein phosphatase 1 γ	<i>Ppp1cc</i> ^{-/-}	CR ↓	[99]
PTP1B	<i>Ptpn1</i> ^{-/-}	CR = / ↓	[100,101]
<i>Cytoskeleton linked proteins</i>			
Rac1	EHT-1864	CR ↓	[102]
Rap1b	<i>Rap1b</i> ^{-/-}	CR ↓	[103]
RhoA	<i>Rhoa</i> ^{-/-}	CR ↓	[79]
Talin-1	<i>Tln1</i> -null	CR ↓	[104]
WASP	<i>Wasp</i> ^{-/-}	CR ↓	[81]
<i>Platelet signaling proteins (other)</i>			
* Calpain	<i>Capn1</i> ^{-/-}	CR ↓	[100]
Cbl	<i>Cbl</i> ^{-/-} , <i>Cbl</i> (LOF)	CR ↓	[105]
Gas6	<i>Gas6</i> ^{-/-}	TG =	[14]
Lnk	<i>Sh2b3</i> ^{-/-}	CR ↓	[106]
mTOR	rapamycin	CR ↓	[107]
NF- κ B	BAY-117082	CR ↓	[108]
PER2	<i>Per2</i> -null	CR ↓	[109]
Phosphodiesterase 3	milrinone	TG ↓	[110]
* Phospholipase C γ 2	<i>Plcg2</i> ^{-/-}	CR ↓	[111]
SHIP1 (PIP ₃ phosphatase)	<i>Inpp55</i> -null	CR ↓	[112]
<i>Plasma proteins</i>			
* Factor XII	<i>F12</i> ^{-/-} , CTI	TG ↓	[34]
* Factor XI	<i>F11</i> ^{-/-}	TG ↓	[34]
* Factor VII	FVIIai	TG ↓	[34]
* (Pro)thrombin	melagatran	TG ↓	[35]

Abbreviations: see Table 1. In addition: CR clot retraction; TG thrombin generation; * also involved in PS exposure, see Table 1.

Recently, galectin-1 has been recognized as a new platelet agonist perhaps acting by direct activation of $\alpha_{IIb}\beta_3$. Galectin-1 binding to platelets evokes common activation signals, such as Ca²⁺ mobilization, phosphorylation of PLC γ 2, Syk and mitogen-activated protein kinases [55]. Mice lacking galectin-1 were found to be impaired in clot retraction, likely because of diminished outside-in signaling.

Homotypic interactions regulating $\alpha_{IIb}\beta_3$ activity

Platelets contain a whole repertoire of receptors and signaling molecules that regulate the formation of tight platelet-platelet contacts and can trigger contact-dependent signaling events via homotypic interactions [56]. Among these are proteins forming gap junctions via the inter-cellular coupling of two hemichannels between adjacent cells [57,58]. Two members of the family of connexins – implicated in hemichannel formation – i.e. connexins

37 and 40, are highly expressed in platelets. Knockout (mouse) and inhibitor (human) studies indicated that both connexins participate in platelet-dependent clot retraction [59,60]. Surprisingly, however, these connexins also appeared to contribute to other activation processes, such as platelet aggregation, integrin activation, Ca²⁺ signaling and α -granule secretion [59]. Hence, it seems that connexins have an overall role in the platelet activation process, rather than a specific role in tight junction formation during clot retraction. Clearly, more research is needed to elucidate the precise action mechanisms of platelet connexins.

Junctional adhesion molecule (JAM)-A is another established molecule exhibiting homotypic interactions in platelets [61,62]. Recently, JAM-A was identified as a negative regulator of platelet activation and, hence, of thrombus formation [61]. Along the same line, integrin outside-in signaling and clot retraction were increased in mouse platelets lacking JAM-A. Earlier data suggested that JAM-A acts as a co-receptor for stimulatory antibodies via

Table 4

Human proteins involved in platelet-dependent thrombin generation and clot retraction coagulation under stasis

Target protein	Intervention	Effect on coagulation	Reference
<i>Platelet receptors and membrane proteins</i>			
IP receptor	prostaglandin E ₁	TG ↓	[113, 114]
G13 protein	mSRI peptide	CR ↓	[115]
Gap junctions	carbenoxolone, Gap27, 18β-GA	CR ↓	[59]
* Glycoprotein VI	9O12 Ab	TG ↓	[113, 114]
* Integrin α ₂ β ₁	6F1 Ab	TG ↓	[113]
* Integrin α _{IIb} β ₃	Glanzmann patients, abciximab	CR ↓, TG ↓	[113, 114, 116]
* Orai1	2-APB	TG ↓	[18]
* P2Y ₁₂	cangrelor, ticagrelor	TG ↓	[12, 113, 114, 117]
<i>Platelet signaling proteins (kinases)</i>			
* PI3 kinase β	TGX-221	TG ↓	[117]
SFK	PD0173952	CR ↓	[78]
Syk	Syk inhibitor II	TG ↓	[118]
<i>Platelet signaling proteins (non-kinases)</i>			
Cbl	OXS1-2, PP2	CR ↓	[105]
Cyclooxygenase	aspirin	TG ↓	[113]
WASP	WAS patients	CR ↓	[81]
<i>Plasma proteins</i>			
Factor XII	Corn trypsin inhibitor	TG ↓	[34]
Factor VII	FVIIa	TG ↓	[34]

For abbreviations, see Table 3.

interaction with the FcγRIIA receptor on human platelets [63,64]. The mechanism proposed is suppression by JAM-A of integrin outside-in signaling through downstream protein kinases, such as Src, Syk and focal adhesion kinase [65,66]. An earlier suggestion, that JAM-A interferes with fibrinogen binding to the activated integrin, could not be confirmed in recent work [61].

In spite of the examples above, integrin outside-in signaling and clot retraction are not always affected in the same ways. In platelets lacking endothelial cell specific adhesion molecule (ESAM), spreading on immobilized fibrinogen (reflecting outside-in signaling) was unaffected, while fibrin clot retraction was delayed [67]. Similarly to connexins and JAM-A, also ESAM is considered to cluster platelets via homotypic interactions.

Platelet integrins form multi-molecular complexes with other membrane proteins such tetraspanins. Reported are high expressions of tetraspanin-32, CD151, CD9 and CD63 [68]. It has been suggested that tetraspanins support integrins in the regulation of contact-dependent signaling [69]. Although the precise molecular mechanism is still unclear, tetraspanin interaction with integrins is likely, since deficiency in tetraspanin-32 or CD151 was found to lead to impaired clot retraction [70,71].

Another 'difficult' membrane protein in terms of platelet function is PECAM-1. Platelets from mice lacking PECAM-1 have been reported as no more than slightly impaired in agonist-induced responses, with unchanged clot retraction [72]. This is in contrast to an earlier detected inhibitory role of PECAM-1 in human platelet function [73,74]. The reason for this discrepancy is unclear. Taken together, the various platelet proteins implicated in homotypic interactions seem to contribute in a balanced way (positive and negative) to platelet clot retraction, often in a similar way as to integrin activation (Figure 2B).

Actin cytoskeleton rearrangement

Traditionally, clot retraction is thought to be a thrombin- and fibrin-dependent process initiated by fibrin binding to α_{IIb}β₃. Subsequent actin and myosin cytoskeleton rearrangements generate contracting forces that are transmitted to the fibrin clot (Figure 2B) [75, 76]. More recent findings point to the existence of a clot retraction mechanism involving Rho kinase and Src family kinases, which precedes the actin-dependent process [77,78].

In agreement with this, genetic ablation of RhoA was found to diminish clot retraction [79]. Furthermore, integrin-dependent phosphorylation by Src and Syk kinases of the ubiquitin ligase Cbl leads to the recruitment of SH2-domain containing proteins that mediate platelet spreading and clot retraction [80].

The Wiskott-Aldrich syndrome, caused by a deficiency in the Wiskott-Aldrich syndrome protein (WASP), is accompanied with thrombocytopenia and a small platelet size [28]. Platelets from mice deficient in WASP are decreased in ability to clot retraction, likely because of abnormal actin filament formation [81]. A similar phenotype is observed in platelets from Wiskott-Aldrich syndrome patients.

In human (thrombin-stimulated) platelet-rich plasma, weakened clot retraction is measured upon pharmacological inhibition of α_{IIb}β₃, G13 protein, Cbl or gap junction proteins (Table 4). Taken together, the findings with mouse and human platelets may indicate that especially impairments in sustained integrin activation, in platelet-platelet contacts or in actin filament formation lead to diminished clot retraction. Whether deficient clot retraction is a marker for impaired hemostasis (bleeding), is an attractive hypothesis. However, this has not systematically been studied.

Conclusion

Studies with both genetically modified mice and pharmacological inhibitors indicate that, in collagen-adhered platelets, in part common signaling pathways lead to PS exposure (procoagulant activity), generation of thrombin and fibrin, and retraction of the fibrin clot. However, signaling events causing prolonged Ca²⁺ elevation particularly stimulate PS exposure and thrombin generation, whereas integrin-dependent signaling events rather enforce fibrin clot retraction. Contact-dependent signaling pathways, triggered by homotypic platelet-platelet interactions, seem to act via the integrin route.

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Conflict of interest statement

The authors report no relevant conflicts of interest.

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