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Proteolytic processing of platelet receptors

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Abstract

Platelets have a major role in hemostasis and an emerging role in biological processes including inflammation and immunity. Many of these processes require platelet adhesion and localization at sites of tissue damage or infection and regulated platelet activation, mediated by platelet adheso-signalling receptors, glycoprotein (GP) Ib-IX-V and GPVI. Work from a number of laboratories has demonstrated that levels of these receptors are closely regulated by metalloproteinases of the A Disintegrin And Metalloproteinase (ADAM) family, primarily ADAM17 and ADAM10. It is becoming increasingly evident that platelets have important roles in innate immunity, inflammation, and in combating infection that extends beyond processes of hemostasis. This overview will examine the molecular events that regulate levels of platelet receptors and then assess ramifications for these events in settings where hemostasis, inflammation, and infection processes are triggered.

KEYWORDS
glycoprotein, metalloproteinase, platelet, receptor, shedding

Essentials

- Metalloproteinases regulate release/shedding of bioactive membrane proteins.
- Shedding is critically important for normal cell function in the vasculature.
- Levels of platelet receptors GPIb-IX-V and GPVI are regulated by metalloproteolytic shedding.
- The premier platelet sheddases belong to the A Disintegrins And Metalloproteinase (ADAMs) family.
- Modulating ADAM activity may alter platelet adheso-signalling receptor density and function.

1 | INTRODUCTION

Along with red cells, platelets feature as a predominant cell type in the bloodstream. Platelet numbers in a healthy individual are usually maintained at a stable level ranging from 150 to 400 × 10^6 per mL of blood.[1,2] These levels of platelets vastly exceed the numbers required to mount a normal hemostatic response, and so they are consistent with the idea that the role of platelets in biology extends beyond hemostasis.[3–6] Within the critical biological process of hemostasis, platelets play a pivotal role in identifying injured or disrupted endothelium lining the vasculature. Through a number of different but highly integrated processes, platelets transition from a rapidly moving, nonactivated state to a situation where they roll slowly, adhere, and activate at a site of injury.[7] This enables recruitment of additional platelets to form a thrombus. This process requires the engagement of platelet receptors that mediate both the rolling and adhesion of platelets, as well as the intraplatelet signalling leading to platelet degranulation, Ca^{2+} flux, release of secondary

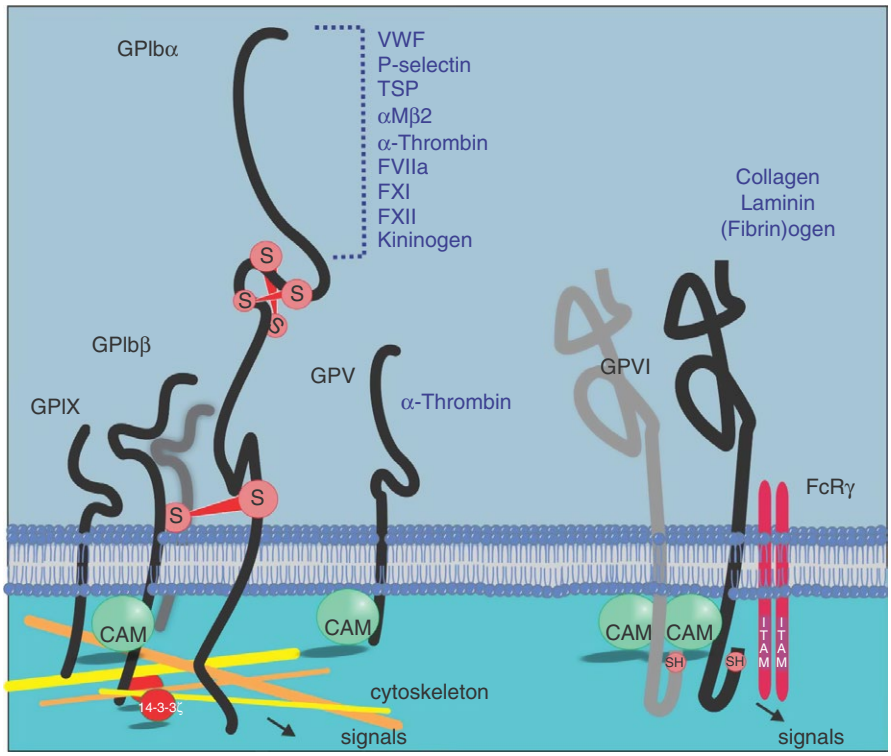

FIGURE 1 Platelet adhesion/signaling receptors and their ligands. Platelet glycoproteins GPIbα and GPVI can bind a variety of ligands, many of which trigger intracellular signals that lead to platelet activation, degranulation and increased affinity of αIIbβ3 for fibrinogen. Cytoskeletal proteins and 14-3-3ζ associate with the cytoplasmic tails of the GPIb-IX-V complex. GPIb-IX-V contains intersubunit and intrasubunit disulphide bonds, represented by S, symbolising the link between adjacent sulfhydryl moieties in GPIbα and between GPIbα and GPIbβ. Calmodulin (CAM) associates with the juxtamembrane cytoplasmic regions of GPIbβ, GPV, and GPVI. The penultimate residue of GPVI is cysteine, represented by a free sulfhydryl group, SH. Not all cytoplasmic components known to associate with cytoplasmic tails of each receptor are shown

agonists such as adenosine diphosphate (ADP) and thromboxanes, exposure of phosphatidylserine and upregulation of fibrinogen-binding capacity by the platelet-specific integrin αIIbβ3. This process has been described extensively in a number of recent reviews.[8–11]

The process of primary hemostasis is governed by the platelet-specific adhesion/signalling proteins glycoprotein (GP) Ib-IX-V and GPVI which predominantly bind von Willebrand factor (VWF) and collagen, respectively.[12] Both receptors can engage with other ligands, however, engagement of these receptors by VWF and collagen coordinate the platelet response to exposed subendothelial matrix across a range of vascular flow rates. As GPVI and the GPIb-IX-V complex cooperate and coordinate the platelet adhesion-signaling response, the relative densities of these receptors on the membrane are important for efficient and effective function.[13,14] This review will discuss molecular mechanisms that rapidly alter the densities of these primary platelet adhesion receptors and influence capacity of platelets to respond. The review will also consider how the same mechanisms modulating platelet and other vascular cell receptors may contribute to vascular inflammation.

1.1 | GPIb-IX-V

The GPIb-IX-V complex consists of GPIbα disulphide linked to GPIbβ and noncovalently linked to GPIX and to GPV. All four subunits of the complex are members of the leucine-rich repeat family of proteins however the N-terminal portion of GPIbα is the major ligand-binding region of the complex (Figure 1). Within the leucine-rich repeat domain of GPIbα, repeats 2-4 (amino acids 60-128) play a crucial role in regulating adhesion to VWF under shear conditions.[15,16] The ectodomain of GPIbα is essential for thrombus formation[17] and likely also for other aspects relating to the role of platelets in coagulation and innate immunity responses as this portion of the receptor complex is able to bind a number of key molecular players in these critical pathways.[18] Apart from both plasma and extracellular matrix VWF, other GPIbα binding partners include coagulation proteins factors XI and XII, thrombin, thrombospondin, and high-molecular-weight kininogen, the leukocyte integrin αMβ2, and P-selectin, found on activated platelets and endothelial cells. The ectodomain of GPIbα also associates with the extracellular portion of the platelet collagen receptor GPVI[19] (see below) and this interaction influences how collagen engages GPVI.[19,20] Whilst the binding sites within GPIbα for these ligands remain to be fully described, all of these binding proteins engage the extracellular region of GPIbα. The consequences of VWF binding to the GPIb-IX-V complex has remained a matter of some debate; however, if the A1 domain (the GPIbα-binding portion of VWF) is presented to the platelet in an appropriate context, this binding interaction can generate powerful intracellular phosphorylation events.[21] The cytoplasmic tail portions of GPIbα and GPIbβ associate directly with components of the platelet cytoskeleton including actin, and α-actinin, and engagement of GPIb-IX-V by VWF leads to actin polymerisation, an event that is sensitive to the level of shear stress to which the platelet surface is exposed.[22] GPIb-IX-V also contains sequences which bind 14-3-3ζ at the GPIbα C-terminus, as well as protein kinase A, tissue necrosis factor-alpha receptor-associated factor (TRAF)-4, and calmodulin binding sites on GPIbβ.[23–25] 14-3-3ζ in association with phosphoinositol 3-kinase[26] regulates the VWF-binding affinity of GPIb-IX-V and inhibiting 14-3-3ζ association blocks receptor signalling.[23]

1.2 | GPVI

GPVI is one of a number of platelet membrane proteins that can bind collagen (others include GPV, CD36 and α2β1)[27]; however, it is regarded as the major receptor for collagen as this receptor rapidly triggers powerful intracellular signalling events and activating pathways that enable the platelet to respond and adhere to collagen[28] (Figure 1). GPVI is a member of the immunoglobulin (Ig)-like superfamily of adhesion proteins and contains two extracellular Ig-like domains. Within the cytoplasmic tail region, GPVI has a calmodulin-binding sequence[29] and a sequence which binds TRAF-4.[25] Surface expression of GPVI requires the tandem expression of the Fc receptor (FcR) γ chain, an ~10-kD protein that links with GPVI via a salt bridge within the plasma membrane. The cytoplasmic domain of FcRγ contains an immunoreceptor tyrosine activation motif (ITAM) and together the GPVI/FcRγ complex transmits ligand-induced signalling events into the platelet by triggering phosphorylation of two tyrosine residues with the ITAM and subsequent activation of p72-spleen tyrosine kinase (Syk).[28] Along with collagen, GPVI can bind laminin,[30] fibrin,[31,32] fibrinogen,[33,34] histones,[35] adiponectin,[36] and the extracellular matrix metalloproteinase inducer (EMMPRIN)[37] expressed on monocytes and leukocytes. Intact GPVI is also essential for efficient generation of thrombin at the platelet surface.[31] The best characterized GPVI interaction is with collagen. When engaged by collagen, both the intracellular[38] and extracellular[39] regions of GPVI/FcRγ can dimerize and this is likely to aid and enhance the clustering of the receptor,[40] and bring ITAMs within the cytoplasmic tail of GPVI/FcRγ together. This triggers tyrosine phosphorylation of members of the Src family of kinases leading to upregulation of phosphoinositide (PI)-3 kinase activity and integrin activation. As the ectodomains of GPVI and GPIbα are co-associated on the platelet membrane,[19] it is reasonable to believe that these two adheso-signaling proteins display a level of functional cooperation and coordinated output across a range of shear and ligand exposure conditions. Interestingly, the ectodomain of GPIbα can modulate the rate and extent of activation of platelets by collagen[20] and collagen-related peptide.[19] In particular, anti-GPIbα monoclonal antibodies that target the anionic sulphated tyrosine region of GPIbα (amino acids 269-282) interfere with platelet responsiveness to these GPVI ligands. This was not related to any specific antibody property as specific removal of the GPIbα ectodomain by treatment with the snake venom protein mocarhagin also ablated collagen-related peptide-induced aggregation.[19] By altering one or both of the ligand binding regions of GPVI and GPIbα, platelet responsiveness particularly to collagen is likely to be modulated.

2 | PLATELET ADHESION RECEPTORS FUNCTION UNDER FLUID SHEAR STRESS

The engagement of the GPIb-IX-V complex by VWF and VWF/collagen occurs in flowing blood and is an exquisite example of a shear-sensitive interaction. The interaction occurs through immobilized VWF partially unfolding under fluid shear and enabling a region within the A1 domain of VWF to be accessible and interact with the N-terminal portion of the GPIbα subunits within the complex.[41] This complex interaction occurs and is sustained under a range of shear stress rates via specialized bonds that are sensitive to shear stress and this interaction directly impacts on the rate and extent of platelet activation.[42,43] GPIbα also senses and responds to changes in fluid shear stress and whilst the mechanisms by which this subunit of the GPIb-IX-V complex alters its affinity for VWF remain to be elucidated, regions within the GPIbα ectodomain that do not overlap with the VWF ligand binding region have been identified to influence both the affinity of the receptor for ligand and the ability of the receptor complex to maintain VWF binding under fluid shear stress. These include a region within leucine-rich repeats 6 and 7[44] and a mechanosensing domain within the extracellular juxtamembrane region of GPIbα.[45] The former was identified as binding a cyclic peptide termed OS-1, identified by phage display to act as an allosteric inhibitor of VWF-GPIbα interactions.[44] The latter is a region spanning ~60 amino acids lying between the macroglycopeptide and transmembrane domain of GPIbα, which unwinds in response to pulling of prebound VWF A1 domain, as demonstrated in experimental systems using optical tweezers.[45] Both studies illustrate the potential of nonligand binding ectodomain regions of the GPIbα subunit to influence and promote ligand binding capacity and affinity, and potentially stabilize receptor ligand interactions at fluid shear rates found in the vasculature.

GPVI plays an important role in hemostasis and thrombosis through integrin activation, supporting adhesion and the initial stages of platelet aggregation. However, patients and mice with platelets lacking GPVI show only a mild bleeding diathesis[46–48] most likely due to the existence of compensatory pathways that generate thrombin and that lead to platelet activation independent of GPVI.[49] However, the situation is different under thrombotic conditions where mice with platelets lacking GPVI are protected against arterial thrombosis and subsequent neointima formation[50] and demonstrate an impaired thrombus formation at high shear rates.[51] GPVI is likely to contribute significantly to stable thrombus formation as the ectodomain is important for efficient thrombin formation[31,52] and GPVI-fibrin interactions are likely to stabilize a forming thrombus under shear stress.[31,32]

Clinical therapies that target platelet responsiveness (antiplatelet therapy) can successfully reduce cardiovascular events, especially in people at higher risk; however, all current antiplatelet therapies carry an increased probability of bleeding. Because loss of GPVI does not result in major hemostatic complications, the therapeutic potential of targeting GPVI is an exciting area that is being actively explored.[53–55] Anti-GPVI antibodies, particularly single domain antibody clones and fragment antigen-binding (Fab) fragments may be useful candidate antithrombotic reagents[56,57] as they could potentially interfere with collagen-GPVI interactions and trigger metalloproteolytic GPVI shedding and/or internalization.

3 | METALLOPROTEINASE-MEDIATED RECEPTOR SHEDDING

Along with triggering fibrinogen binding to the major platelet integrin αIIbβ3, activation of pathways from both the GPIb-IX-V complex and

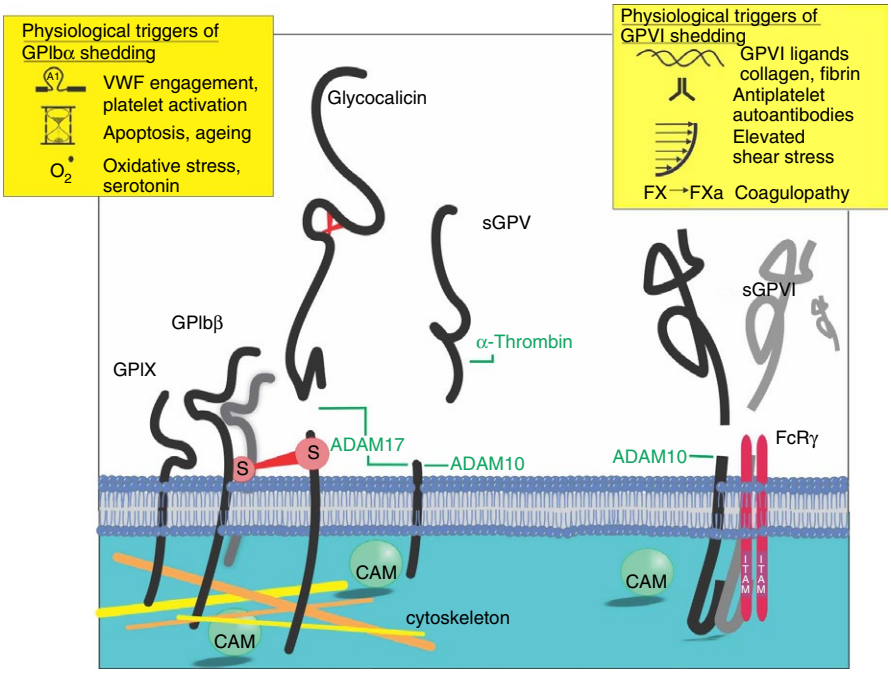

FIGURE 2 Ectodomains of GPIbα, GPV, and GPVI are shed under conditions of platelet activation. Calmodulin dissociation from the juxtamembrane cytoplasmic region of GPIbβ, GPV and GPVI is a common event also observed in metalloproteolytic shedding of a number of cell receptor ectodomains across vascular biology. GPIbα and GPIbβ intersubunit disulphide bonds which remain intact after shedding of the glycocalicin ectodomain are represented as S symbols

GPVI leads to a rapid and irreversible metalloproteinase-mediated cleavage of the ligand-binding ectodomains of GPIbα, GPV, and GPVI[58–60] (Figure 2). In a process that was initially characterized in murine platelets,[61–63] the ectodomains of these receptors are cleaved within extracellular juxtamembrane regions resulting in the release of an ~110-130-kDa fragment of GPIbα (termed glycocalicin) and an ~55-kDa GPVI fragment[59] from human platelets. The extracellular region of GPV is also released by the action of thrombin to produce an ~20-kDa platelet-associated fragment[59,64] and by metalloproteolytic cleavage of the complete extracellular region to leave an ~5-kDa remnant fragment.[59] This process is clearly different from other forms of receptor removal which involve either the export of receptors from the plasma membrane via packaging in extracellular vesicles[65] such as occurs with platelet and endothelial P-selectin, or internalization processes whereby receptors are either moved to ligand-inaccessible surface-connected canalicular storage pools or degraded.[66,67] In contrast to GPIbα which appears to be constitutively shed,[18,68] GPVI is stable on the surface of circulating nonactivated platelets[59,60] with no evidence of a platelet-associated 10-kDa remnant fragment. This supports the use of intact and soluble GPVI as platelet-specific markers of activation.[69]

4 | PLATELET METALLOPROTEINASES

The receptor and bioactive protein shedding process is mediated by members of the A Disintegrin And Metalloproteinase (ADAM) family with prominent roles for ADAM10 and ADAM17 across biology.[70–72] The ADAMs family of metalloproteinases (Figure 3) has more than 40 members and most members share a basic domain structure consisting of an N-terminal prodomain followed by a catalytic, a disintegrin, and a cysteine-rich domain. Most family members contain epidermal growth factor-like domains (although ADAM10 and ADAM17 do not) followed by a single pass transmembrane domain and a short cytoplasmic tail.[72,73] ADAM10 and ADAM17 are both found on the membrane of resting platelets and these enzymes mediate the cleavage of GPVI and GPIbα, respectively. In murine platelets, shedding of these receptors may involve contributions from both ADAM10 and ADAM17.[74] ADAMs proteinases, particularly ADAM10 and ADAM17 are broadly expressed across a variety of cell types, both at the cell surface and in intracellular granules as zymogens. The prodomain is removed from immature ADAMs prior to being brought to the cell surface as mature catalytically active proteins.[72] However, on platelets, mature ADAM10 and ADAM17 both seem to be constitutively present at the platelet surface, and in the case of ADAM10 at least, have detectable proteolytic activity.[75] The crystal structure of the ADAM10 ectodomain was recently solved[76] and revealed a compact arrangement of the domains permitting intrinsic autoinhibition of the catalytic domain within the mature protein by the disintegrin and cysteine-rich domains and preventing substrate access to the metalloproteinase active site. This suggests that there is a level of control of ADAM10 activity at a membrane surface, under resting conditions.

Platelet granules also contain a number of members of the matrix metalloproteinase (MMP) family.[77] These metalloproteinases generally do not have a transmembrane domain and so are released from storage granules of platelets and many other cell types where they are able to diffuse into extracellular and interstitial spaces. As their name suggests, MMPs cleave many different types of matrix proteins including collagens, laminins, and fibronectin. Platelet-associated MMP-1, MMP-2, MMP-9, and MMP-14 have been shown to differentially modulate and at times inhibit thrombus formation by exerting collagenolytic activity.[78] MMPs are also able to act at the platelet surface where, for example, MMP-1 cleaves the thrombin

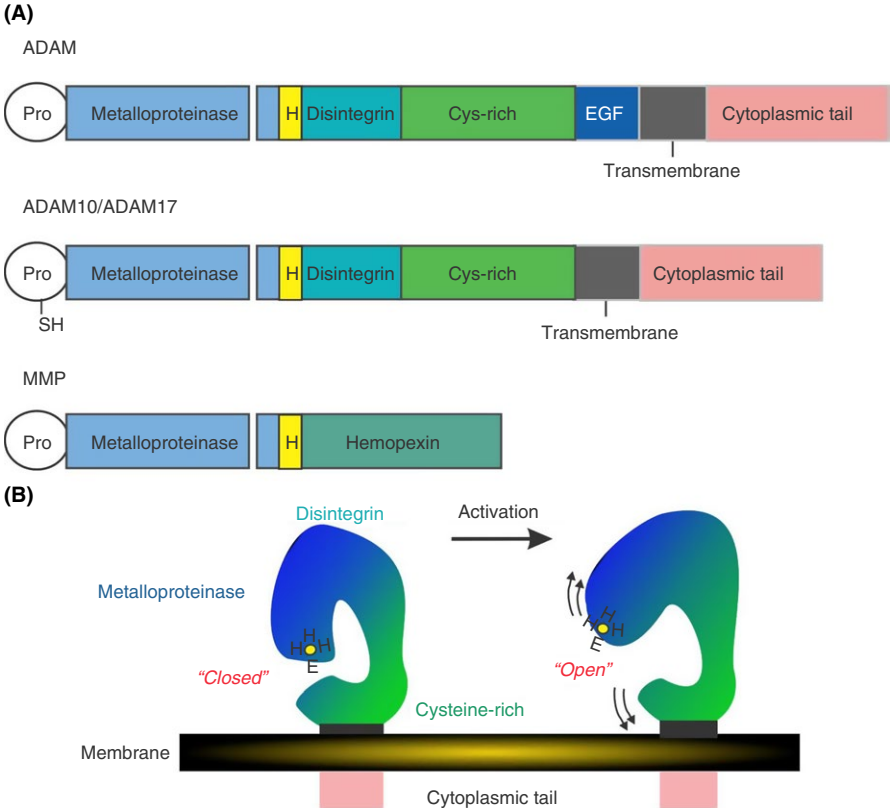

FIGURE 3 Domain structure of ADAMs. (A) ADAMs have a uniform domain structure with a prodomain that is removed prior to transport to the cell membrane, followed by a metalloproteinase, disintegrin,cysteine-rich and epidermal growth factor-like domains domains, a transmembrane domains and a cytoplasmic tail. ADAM10 and ADAM17 do not have EGF-like domains and do have a free sulfhydryl (SH) group within the prodomain which may coordinate with the HEHH Zn^{2+} binding sequence within the metalloproteinase domain. (B) Analysis of the crystal structure of human ADAM10 [76] suggests a closed conformation of the enzyme under resting conditions where the cysteine-rich domain occludes the metalloproteinase active site. Under conditions of activation, the metalloproteinase domain is freed and a substrate can gain access to the catalytic site

receptor protease activated receptor (PAR)-1 at a distinct site that strongly activates Rho-GTP pathways, signalling cell shape change and motility.[79] Similarly, MMP-2 engages with αIIbβ3 and is able to cleave PAR-1 at a noncanonical site resulting in the activation of phosphatidylinositol 3- kinase, enhanced aggregation, and a contribution to arterial thrombosis.[80] Dual roles for ADAMs and MMPs in platelet biology are likely, and it will be of great interest to examine how these metalloproteinase superfamily members cooperate and coordinate their respective activities to fully enable platelet function.

5 | REGULATORY MECHANISMS THAT MAY INFLUENCE PLATELET RECEPTOR SHEDDING

How platelet receptor levels are regulated on circulating platelets remains an open question. As the process is largely driven by metalloproteinases, control of receptor cleavage events is likely to be provided either by direct inhibition of the catalytic process or by controlling access of the enzyme to the substrate. In the case of GPIbα, roles for a membrane-proximal region of the GPIbβ cytoplasmic domain[81] and a 28-amino acid mechanosensory domain within the extracellular juxtamembrane region of GPIbα[82] in maintenance of stable surface levels of the GPIbα subunit have been identified. Both of these regions regulate the availability of the ADAM17 cleavage site within GPIbα to metalloproteases such as ADAM17 and so aid in control of GPIbα levels.

The endogenous inhibitors of both ADAMs proteins and the MMPs are members of the tissue inhibitors of metalloproteinase (TIMP) family.[83] There are four members of the TIMP family and studies have shown that megakaryocytes and platelets have mRNA transcripts and detectable levels of protein for all TIMPs.[84] Interestingly, the TIMP-2 transcript is actively transcribed in thrombin-stimulated platelets.[85] ADAM10 is primarily inhibited by TIMP-1 and ADAM17 by TIMP-3 although there is a significant amount of cross-inhibition amongst the family.[86] TIMPs are found in the plasma as well as in intracellular storage granules of most cell types including platelets. TIMPs are able to compete with endogenous ADAM substrates for binding sites within the catalytic and disintegrin-like domains of ADAMs, and so disrupt access of the catalytic domain for the substrate. However, little is known about the regulatory role of TIMPs in platelet ADAMs and MMP biology.

Tetraspanins featuring the TspanC8 subgroup (Tspan5, 10, 14, 15, 17, and 33)[87,88] and the iRhom subgroup of protease-inactive rhomboids (iRhom1 and 2)[89,90] have emerged as important regulators of ADAM10 and ADAM17, respectively. In nucleated cells, members of the TspanC8 subgroup are required for correct enzymatic maturation and trafficking of ADAMs to the cell surface. In certain cell types, there is evidence that cells can target the ADAMs to distinct substrates[91] and this may involve different TspanC8s and iRhoms.[88,92–96] Roles for iRhoms and tetraspanins (in particular Tspan14)[97] in regulating platelet ADAMs activity are exciting avenues of research enquiry that are likely to explain differential cleavage of GPVI and GPIbα in circulating platelets.

In response to ligand engagement, exposure to elevated shear or during coagulopathy, GPVI is proteolytically cleaved from the platelet surface. In a system that is reminiscent of the classical ADAM17-mediated shedding of leukocyte L-selectin,[98] detachment of calmodulin from the cytoplasmic juxtamembrane binding site, either by ligand engagement or by treatment of platelets with an inhibitor of calmodulin, triggers the release of the GPVI ectodomain.[60] In the following sections, the mechanisms most relevant to physiological shedding of platelet receptors will be discussed, with a focus on the regulation of platelet GPVI levels.

6 | TRIGGERS OF PLATELET RECEPTOR SHEDDING

6.1 | Laboratory approaches

There are various ways that proteolytic release of GPVI can be triggered involving either physiological or experimental tools and reagents that act either in intracellular and/or extracellular spaces. The standard means of activating ADAMs across cell biology involves treatment of cells with phorbol myristyl acetate (PMA) which crosses the plasma membrane and serves to activate protein kinase C and either trigger passage of mature ADAMs proteins to a membrane surface, or (as in the case of platelets) enhance the proteolytic activity of ADAMs present at the cell surface.[91,99] ADAMs activity can also be upregulated by treatment of cells with thiol-modifying reagents[100] such as *N*-ethyl maleimide (NEM) which is a very effective means to trigger almost complete release of GPVI from platelets.[59,101] While the mechanism of action of NEM is not clearly defined, this reagent may react with a cysteinyl group present within the prodomain of all ADAMs. This reactive "cysteine switch" sits within a divalent cation binding site and coordinates the binding of Zn^{2+} which is essential for catalytic activity of the metalloproteinase.[100] NEM and other thiol-modifying reagents may modify this cysteine group to release any inhibitory mechanism and drive the enzyme into a high affinity enhanced catalytic state. The calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) is a cell-permeable competitive antagonist which competes with intracellular calmodulin binding sequences for calmodulin binding. Dissociation of calmodulin from receptor cytoplasmic juxtamembrane sequences forms part of the ligand-mediated signalling process,[98] and so treatment with W7 circumvents the need to provide a ligand. In platelets, treatment with W7 results in detachment of receptor-bound calmodulin and triggers shedding of GPVI, GPV, and GPIbα.[102] Interestingly, the W7 mechanism of action does not increase the endogenous platelet ADAM10 activity,[75] suggesting that calmodulin dissociation alters the availability of the ADAM10 cleavage site within GPVI. These reagents are highly useful laboratory tools that have enabled rapid evaluation of ADAMs structure and catalytic potential for a huge range of substrates in both primary cell culture and in cell lines. In platelets, these reagents have broad utility, particularly NEM where treatment of washed platelets or platelet-rich plasma for 15-30 minutes with 5 mmol L^{-1} NEM is sufficient to liberate greater than 90% of GPVI and so permit an assessment of GPVI shedding potential across blood donor populations, as well as create a GPVI-deficient platelet to aid in the assessment of platelet-activating plasma components.[103]

An additional means to remove GPVI from the surface of platelets that has been put to excellent use in mouse models of thrombosis and hemostasis is the use of anti-GPVI antibodies.[104,105] Injection of mice with intact antibodies or Fab fragments that bound to GPVI epitopes either within the collagen-binding domain or outside, induced a transient thrombocytopenia and a down regulation of platelet GPVI. Antibody treatment in vitro did not achieve the same loss, and additional work demonstrated the existence of a pathway downstream of GPVI that efficiently led to internalization and irreversible loss of murine GPVI.[67] Whether the same process of GPVI internalization occurs in human platelets treated with anti-GPVI antibodies in vitro, or with anti-GPVI autoantibodies in vivo has not been reported, however, treatment of human platelets treated with anti-GPVI antibodies can induce GPVI shedding in vitro. In one study with eight monoclonal antibodies, this loss was independent of engagement of FcγRIIa (present on human but not mouse platelets) by the Fc portion of the antibody[106] indicating antibody binding to GPVI could directly trigger metalloproteolysis.

6.2 | Exposure to GPVI ligands

Fibrillar collagen type 1 is the major collagen type that engages GPVI. Together with collagen type 3, it is the predominant collagen found in the subendothelium in the blood vessel wall.[107] An assessment of GPVI binding of other collagen types has been made; however, the majority of studies of GPVI shedding induced by collagen exposure have utilized the type 1 form. Collagen and the chemically crosslinked collagen-related peptide (CRP), a GPVI-specific agonist, both induce shedding of GPVI in suspension assays and require activation of intracellular signalling events including phosphorylation of Src family kinases and Syk as well as activation of PI-3 kinase but do not require engagement or outside-in signalling from the integrin αIIbβ3. Ligand-induced GPVI shedding can proceed in the absence of integrin engagement.[60] Shedding triggered by other GPVI ligands which may engage and cluster GPVI through sites other than the collagen-binding site remains a field of discovery. At the International Society on Thrombosis and Haemostasis meeting in Berlin, a number of new GPVI interactions were discussed in both oral and poster presentations. Fibrin is a more recently described ligand for GPVI and studies have demonstrated that while fibrin–GPVI interaction will generate intracellular signals,[32,108] this signalling is not required for fibrin-induced GPVI shedding.[109] The fibrin interaction with GPVI is mediated by the D-dimer region of fibrin[33,108] and for GPVI shedding to occur, fibrin must be polymerized.[109] Whether fibrin can bind platelet GPVI monomer or dimer[33,108] remains a matter for debate; however, dimeric GPVI-Fc fusion proteins do not engage fibrin.[110] Similarly, the fibrin-binding site within GPVI is contentious.

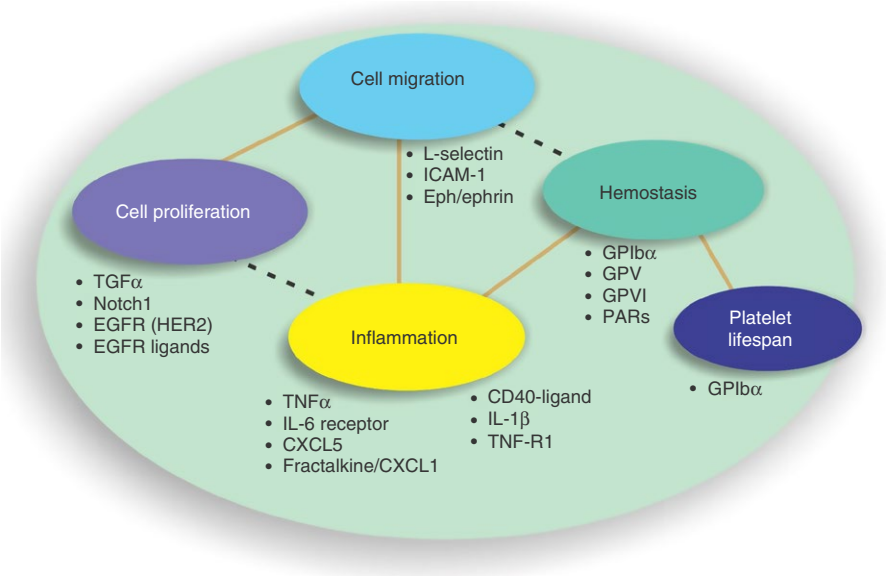

FIGURE 4 Extracellular proteolysis controls the active levels of a range of growth factors, chemokines and adhesion receptors. Levels of a range of active biological compounds are controlled by the action of members of the ADAMs family of metalloproteinases. Only a selection of these proteins are shown here. These bioactive proteins cooperate and influence a broad spectrum of critical biological processes. EGFR, epidermal growth factor receptor; GP glycoprotein; ICAM, intercellular adhesion molecule; IL, interleukin; TGF, transforming growth factor; TNF, tissue necrosis factor

In one study, the GPVI-fibrin interaction occurred only with GPVI in dimeric form and could be abrogated by pretreatment with collagen or CRP, implying at least partial overlap of the binding site for these ligands,[33] however, fibrin-GPVI monomer interactions and separate CRP and fibrin binding sites were proposed in another study.[108] Under certain experimental conditions and in collaboration with αIIbβ3, the fibrin monomer component fibrinogen also can engage GPVI.[33,34] Understanding how these two GPVI ligands intersect and contribute to GPVI function is important, as selective disruption of one type of GPVI-ligand interaction, either through competitive inhibition at the ligand-binding site, or at the level of GPVI dimerization represents an enticing new approach to develop antiplatelet agents with minimal effects on hemostasis.

6.3 | Activation of coagulation

Through comparison of sGPVI levels in matched plasma and serum samples from healthy donors, it emerged that GPVI shedding can be triggered by coagulation.[111] Through the use of direct inhibitors of thrombin and active factor X (FXa), together with other inhibitors of the coagulation pathway, a major role for thrombin in triggering the release of GPVI either directly by acting on GPVI or indirectly through activation of thrombin receptors on platelets has been ruled out.[60,109,111] Generation of FXa either through recalcification in the presence of thrombin inhibitors, or by treatment of platelet-rich plasma with Russell viper venom, a direct FX activator, resulted in the rapid release of GPVI that could be blocked by broad spectrum metalloproteinase inhibitors, and partially blocked by a specific inhibitor of ADAM10. Similar to fibrin-mediated GPVI shedding,[109] this mechanism of shedding did not require platelet activation, degranulation, or aggregation, implying that FXa can directly trigger ADAM10-mediated cleavage of GPVI. In the absence of a consensus sequence within GPVI that is recognized by FXa, FXa may either directly act on ADAM10 to enhance substrate cleavage or indirectly modulate an intermediary factor that is involved in GPVI stability at the platelet surface. Coagulation-induced shedding of platelet GPVI in human plasma via a metalloproteinase-mediated FXa-dependent mechanism may serve to down-regulate GPVI expression under procoagulant conditions independent of GPVI ligands. Monitoring levels of sGPVI in plasma from patients with high levels of FXa and/or fibrin deposition who are at risk of developing disseminated intravascular coagulation,[111] or sepsis[109] may be useful for clinical management of these complex patients.

6.4 | Exposure to elevated fluid shear stress

Human platelets normally circulate in a resting state and are exposed to shear rates within a physiologic range (~20-2000 s^{-1}).[11,112,113] Platelets may encounter shear rates well beyond 10 000 s^{-1} under pathologic conditions, for example, in a stenosed atherosclerotic artery or within mechanocirculatory support devices such as left ventricular assist devices (LVADs) or extra-corporeal membrane oxygenation (ECMO) devices, and become activated and begin to aggregate. Shear-dependent platelet activation is initiated by binding of plasma VWF to platelets primarily through GPIbα, leading to platelet activation, secretion of ADP, and other agonists, and αIIbβ3-dependent aggregation.[113,114] Additionally, when exposed to elevated fluid shear stress, metalloproteolytic shedding of GPVI is triggered.[101] In experimental systems shear-induced GPVI shedding was not as a consequence of VWF engagement of GPIb-IX-V or platelet activation as shear-induced shedding occurred in washed platelets where VWF engagement was blocked by anti-GPIbα or anti-VWF monoclonal antibodies, and in platelet-rich plasma isolated from a patient with Type 3 von Willebrand disease (where VWF was absent). Shear-induced shedding did not require platelet signalling pathways or activation of αIIbβ3 and appeared to be a direct effect of exposure to fluid shear stress. This shear-dependent instability of the platelet adhesion receptors is

likely to be of paramount importance in patient groups where risk of both thrombosis and of bleeding are heightened. Deployment of devices such as LVADs or ECMO necessitates the use of significant antiplatelet and anticoagulant medication however in cohorts of people in receipt of mechanocirculatory support, exposure to fluid shear stress levels approaching 50 000 s^{-1} was associated with loss of platelet adhesion receptors in conjunction with loss of VWF multimers.[115–117] This loss may combine with other disease-related vascular factors and contribute to the high rate of serious bleeding seen in this patient cohort.[118] Whether measurement of sGPVI levels in plasma samples taken prior to implantation will enable stratification of patients into low- and high-risk bleeding groups[115] and the opportunity to tailor antiplatelet and anticoagulant therapy is the subject of ongoing research.

6.5 | Antiplatelet antibodies and autoantibodies

In primary immune thrombocytopenia (ITP) and in heparin-induced thrombocytopenia (HIT), patients generate antibodies that are reactive with antigens on the surface of platelets and megakaryocytes. In ITP, these antibodies disrupt megakaryocytopoiesis, induce platelet apoptosis or opsonise the surface of the platelet enhancing the rate of clearance of platelets by the liver and spleen.[119] In at least a subset of patients, antibody binding to platelet surface antigens including GPIbα, αIIbβ3, and α2β1 leads to engagement of platelet FcγRIIa by the Fc portion of the autoantibody. In ITP patients with anti-GPVI autoantibodies, the loss of responsiveness to collagen by light transmission aggregometry, loss of platelet GPVI by flow cytometry, or enhanced GPVI shedding has been demonstrated.[103,120–122] In this pathological scenario, autoantibody-mediated GPVI loss may involve signalling contributions from both GPVI and FcγRIIa. In HIT, autoantibodies that recognise platelet factor-4 in combination with heparin, form immune complexes which also engage FcγRIIa.[123] FcγRIIa is a second ITAM-containing signalling receptor, and this binding can trigger significant platelet activation and platelet clearance[124] as well as activation of GPVI shedding pathways.[125]

7 | CONCLUSION

Metalloproteolysis of receptor ectodomains is a regulatory mechanism that is common to many cell types across cell biology (Figure 4). In some cases, this mechanism liberates a bioactive portion of a latent factor, while in others cases, it is a means of controlling the reactive or adhesive properties of a cell or enabling the cell to sense its surroundings. In platelets, the release of the ligand binding portions of GPVI and GPIbα are likely to modulate the densities of each of these cooperating receptors, parameters that are important for the adhesive properties of the platelet. When using platelets from mice deficient in their subtle receptor density changes act to limit thrombus growth and propagation of coagulation at the site of thrombus formation. However, beyond these outcomes that are critical for hemostasis, and in keeping with the burgeoning roles for platelets in innate immunity and inflammation, loss of these ectodomains are also likely to influence how platelets engage with other cells such as leukocytes and endothelial cells as well as tumor cells.[126] Indeed, modulation of receptor levels on the surface of platelets is likely to be critical for new avenues of research where platelets are demonstrated to undergo diapedesis[127] and in the utility of platelets for delivery of therapies to critical sites of injury, inflammation, and metastasis.[128,129]

RELATIONSHIP DISCLOSURE

The author has no conflicts of interest to declare.

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