Platelet Receptor Proteolysis
A Mechanism for Downregulating Platelet Reactivity

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Abstract*—**The platelet plasma membrane is literally at the cutting-edge of recent research into proteolytic regulation of the function and surface expression of platelet receptors, revealing new mechanisms for how the thrombotic propensity of platelets is controlled in health and disease. Extracellular proteolysis of receptors irreversibly inactivates receptor-mediated adhesion and signaling, as well as releasing soluble fragments into the plasma where they act as potential markers or modulators. Platelet-surface sheddases, particularly of the metalloproteinase-disintegrin (ADAM) family, can be regulated by many of the same mechanisms that control receptor function, such as calmodulin association or activation of signaling pathways. This provides layers of regulation (proteinase and receptor), and a higher order of control of cellular function. Activation of pathways leading to extracellular shedding is concomitant with activation of intracellular proteinases such as calpain, which may also irreversibly deactivate receptors. In this review, platelet receptor shedding will be discussed in terms of (1) the identity of proteinases involved in receptor proteolysis, (2) key platelet receptors regulated by proteolytic pathways, and (3) how shedding might be regulated in normal physiology or future therapeutics. In particular, a focus on proteolytic regulation of the platelet collagen receptor, glycoprotein (GP)VI, illustrates many of the key biochemical, cellular, and clinical implications of current research in this area. (Arterioscler Thromb Vasc Biol*. 2007;27:1511-1520.)**

Key Words: glycoprotein Ib-IX-V ■ glycoprotein VI ■ platelets ■ thrombosis ■ shedding

This review focuses on proteolytic pathways that regulate the function and surface expression of platelet receptors involved in hemostasis and thrombotic disease such as heart attack or stroke. In particular, platelet-specific receptors, glycoprotein (GP)VI and the GPIb-IX-V complex that bind collagen or von Willebrand factor (vWF), respectively, form a physical and functional complex[1] initiating platelet thrombus formation at high shear stress in flowing blood.[2–6] Major consequences of signal transduction following engagement of GPVI or GPIb-IX-V include (1) activation of platelet integrins, primarily $\alpha_{IIb}\beta_3$ that binds vWF or fibrinogen and mediates platelet aggregation, and (2) activation of pathways leading to metalloproteinase-dependent receptor ectodomain shedding. Recent biochemical and cellular studies on shedding pathways[7–10] and surface expression[11–13] of GPVI illustrate some of the key mechanisms underlying platelet receptor proteolysis, and recent preclinical studies[14] show how significant these findings may be in the context of thrombotic risk. The endogenous platelet-surface sheddases that cleave GPVI and/or other platelet receptors are also being revealed.[15–17] The first part of the review, therefore, will briefly discuss the metalloproteinase-disintegrin (ADAM) family, matrix metalloproteinase (MMP) family, and the intracellular proteinase calpain, followed by a description of their potential receptor substrates on platelets. Finally, the way in which proteinases and platelet receptor expression might be regulated physiologically and/or therapeutically will be considered. Using GPVI as the main example, this review will discuss recent studies on proteolytic pathways in platelets with respect to (1) the proteinases involved, (2) key adhesion receptors, and (3) how both receptors and proteinases might be regulated. Together, the findings may be broadly applicable to other cell types, and may provide new approaches for antithrombotic therapies or assessment of thrombotic risk.

Platelet Proteinases/Sheddases

Members of 2 main families of proteinases are expressed on the cell surface, the ADAM family of metalloproteinase-disintegrins and the MMP family of matrix metalloproteinases, and may act as sheddases by cleaving the ectodomain of surface receptors. One of the major families of intracellular enzymes involved in regulating platelet surface receptors is the cysteinyl proteinase, calpain. There are common features in the regulation of both ectodomain sheddases and cytoplasmic domain-cleaving proteinases such as calpain. For example, both proteolytic events are inhibited by EDTA, and both may be regulated by calmodulin (below). This suggests there may be common mechanisms for switching on both extracel-

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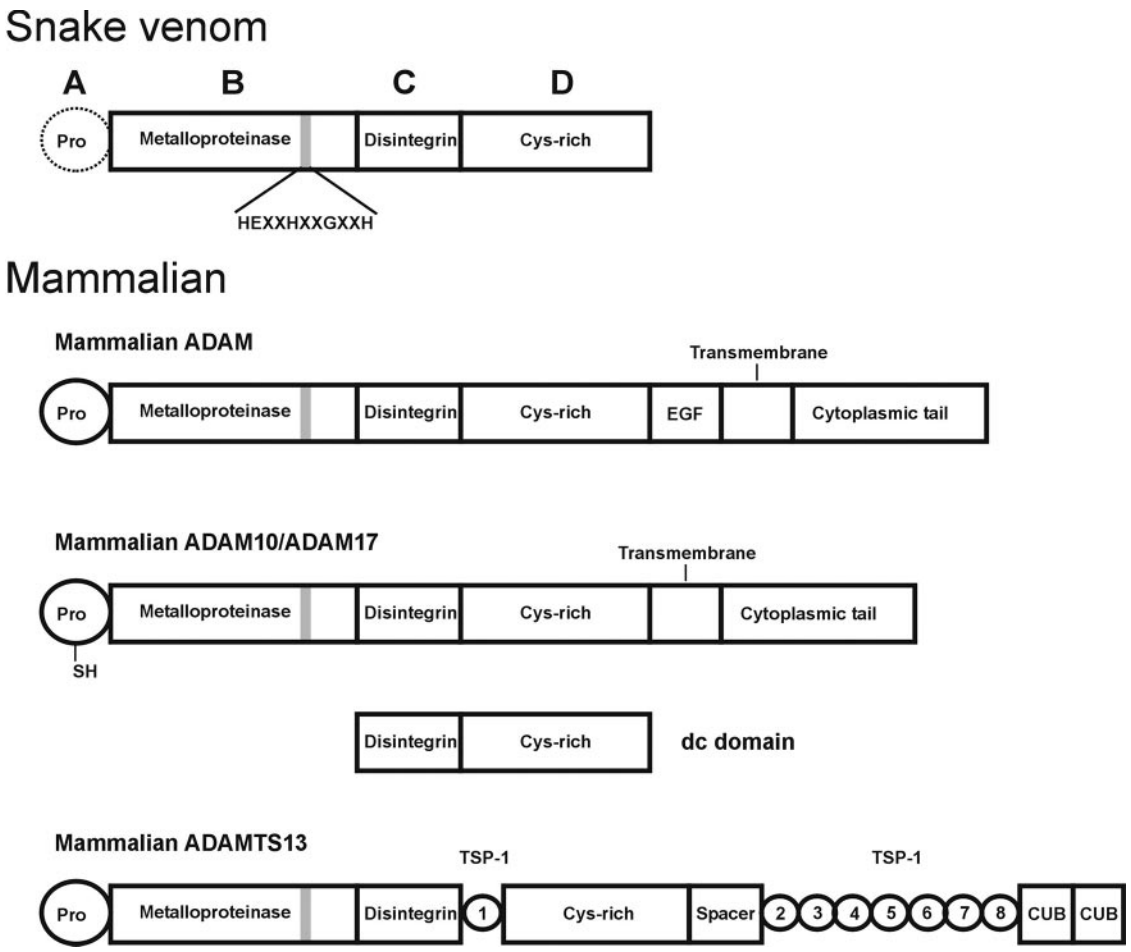

Figure 1. Metalloproteinase-disintegrins. Snake venom or mammalian metalloproteinase-disintegrin family proteins, consist of propeptide domain (that is usually cleaved from snake venom metalloproteinase-disintegrins) (A), Catalytic domain containing the Zn^{2+}-coordination motif (HEXXHXXGXXH) (B), the disintegrin domain (C), and the Cys-rich domain (D). A representative snake venom metalloproteinase-disintegrin is shown. Mammalian metalloproteinases shown are typical members of the membrane-associated ADAM (a disintegrin and metalloproteinase) family, containing an epidermal growth factor-like (EGF) domain and a transmembrane domain. ADAM10 or ADAM17 (TACE; TNF-α convertase) lack the EGF domain, but contain a free thiol (SH) within the prodomain that regulates catalytic activity (Cys-switch). ADAMTS13 (that controls vWF multimer size) is shown as a representative of the ADAMTS family, which contain thrombospondin type-1 (TSP-1) and *c*omplement components C1r/C1s, *U*egf (sea urchin fibropellins), and *b*one morphogenic protein-1 (CUB) domains. See the text for references.

lular and intracellular proteolytic pathways, to regulate adhesion and signaling in platelets or other cells.

ADAM Family: ADAM10 and ADAM17

ADAM10 and ADAM17 are members of the ADAM (a disintegrin and a metalloproteinase) family of cell surface proteinases,[18–20] and consist of modular domains: a propeptide domain, a catalytic domain containing a conserved metal ion-coordination motif (HEXXHXXGXXH), a disintegrin domain, a Cys-rich domain, transmembrane domain, and cytoplasmic tail (Figure 1). Other members of the ADAM family contain an additional epidermal growth factor (EGF)-like domain before the transmembrane domain. Cellular, biochemical, and bioinformatic studies indicate that there are 29 forms of ADAM family metalloproteinases, and 19 forms of related ADAMTS metalloproteinases (that also include one or more thrombospondin-1 type domains; Figure 1).[18–22] The main categories of ADAM function are cell adhesion, receptor shedding, and extracellular matrix degradation.[21] ADAM-mediated biological roles include fertilization, reproduction, neuromuscular development, angiogenesis, inflammation, cancer, and as intimated below, thrombosis.[18–21] On platelets, ADAM10 is involved in the proteolytic processing of amyloid precursor protein (APP), and expression levels may be a marker for Alzheimer disease.[23] ADAM family metalloproteinases contain a signal peptide, and a propeptide domain, the removal of which after furin-mediated or other processing produces an active form of the enzyme. The prodomain of ADAM10 and ADAM17 contains a cysteine switch, that is, a free sulfydryl involved in maintaining the metalloproteinase in an inactive form, with activation of the enzyme occurring after proteolytic removal of the prodomain or on modification by thiol-modifying reagents such as *N*-ethylmaleimide (NEM; refer below).[20,24–27] As shown in Figure 1, mammalian ADAM family metalloproteinases are structurally related to snake venom metalloproteinase-disintegrins which in their mature form consist of a catalytic domain, a disintegrin domain, and a Cys-rich domain.[28]

Vascular apoptosis factor-1 (VAP-1), a rattlesnake venom metalloproteinase-disintegrin from *Crotalus atrox*, was recently used to obtain the first crystal structure for an intact ADAM (metalloproteinase domain, disintegrin domain, and Cys-rich domain), revealing how these domains are configured. VAP-1 forms a C-shaped structure, with the metalloproteinase and Cys-rich domains forming globular regions at either end of the 'C', with the intervening disintegrin linking these two regions.[29] The Cys-rich domain is situated near the catalytic site, suggesting it acts as a regulatory domain.[29] Previous studies involving mammalian ADAM10 show that a recombinant disintegrin/Cys-rich (dc) fragment regulates ADAM10-dependent proteolysis of the ephrin receptor complex.[30]

ADAM10 and/or ADAM17 regulate ectodomain shedding of receptors on vascular cells including endothelial cells, leukocytes or platelets, and other cell types.[18–20] A lot is known about the functional importance of these sheddases in mammalian biology, but relatively little is known about the detailed mechanisms for their mode of action and regulation. As discussed in detail elsewhere,[19] selective inhibitors are currently not widely available and there is minimal conservation within cleavage sites. Both ADAM10 and ADAM17 are expressed on resting platelets,[15–17,23] and one or both have been directly linked to shedding of platelet receptors (below). In this regard, two common properties of ADAM10 and ADAM17 are relevant to their role as platelet sheddases. First, as originally shown by Kahn et al for leukocyte L-selectin,[31] ADAM-mediated shedding is regulated by the cytosolic regulatory protein, calmodulin. Calmodulin binds to a juxtamembrane, positively-charged/hydrophobic sequence within the cytoplasmic tail of L-selectin, analogous to calmodulin-binding sequences that form an amphipathic α-helix in other proteins.[32] Disruption of this interaction by mutagenesis of the calmodulin-binding site of the receptor, or by treating L-selectin–expressing cells with calmodulin inhibitors, induces ectodomain shedding.[31] This implies calmodulin associated with the receptor acts as an inhibitor of shedding. Further evidence shows that calmodulin also regulates ADAM function. For example, calmodulin regulates proteolytic processing and activation of ADAM10, because the cytoplasmic domain of pro-ADAM10 interacts directly with calmodulin in human glioma cells, and calmodulin inhibitors increase conversion of pro-ADAM10 to active ADAM10 (and promote ADAM10-mediated proteolysis of CD44).[33] Second, activation of intracellular signaling pathways may be involved in inside-out activation of surface sheddases.[18–20] The cytoplasmic tails of ADAM10 and/or ADAM17 contain motifs that interact with signaling molecules, including consensus sequences for binding Src homology (SH)-2 and SH-3 domains, even though precise pathways are far from fully understood. As discussed further below, ADAM-dependent ectodomain shedding may be induced by the phorbol ester, PMA, which activates protein kinase C (PKC),[16–20] or alternately, blocked by inhibitors of cell signaling pathways.[8] On other cells, ADAM10 or ADAM17 may be differentially regulated, by Ca^{2+}/calmodulin or PKC-dependent pathways,[31] and related mechanisms may control shedding of platelet receptors.

MMP

Members of the MMP family exist as secreted or surface-expressed forms, which include a transmembrane domain or are GPI-linked.[34] They contain a propeptide (inhibitory Cys-switch) domain, a catalytic, and other domains. Four members of the MMP family, MMP-1, MMP-2, MMP-4, and MMP-9 have been identified on platelets,[35–38] and can play a role in platelet aggregation. MMP-2, and the colocalized metalloproteinase inhibitor, tissue inhibitor of metalloproteinase-4 (TIMP-4), are released from collagen- or thrombin-stimulated platelets; the relative concentrations of MMP-2 and TIMP-4 regulate MMP-2-dependent platelet aggregation.[39] A role for MMP family metalloproteinases in regulating platelet receptor ectodomain shedding has been suggested[9] but not yet definitively shown.

Calpain

Calpain is a ubiquitous intracellular cysteinyl proteinase, switched on in activated platelets and regulated, in part, by intracellular Ca^{2+} levels.[40,41] The 3 isoforms of calpain, acting on >100 intracellular substrates,[41,42] play a role in regulating cytoskeletal rearrangements associated with cell motility, adhesion, and division. Notably in the context of activation of sheddases discussed here, the ability of a protein to bind calmodulin confers a strong likelihood that this protein is a substrate for calpain.[43] In other words, mechanisms for activating extracellular shedding pathways of proteolysis also have the potential to activate intracellular calpain-dependent proteolytic pathways *via* a calmodulin-dependent mechanism. Interestingly, EDTA inhibits both metalloproteinase-mediated receptor shedding and calpain activity,[44] presumably, in the latter case, by chelating extracellular calcium and interfering with Ca^{2+} flux. In platelets, the most prevalent μ-calpain isoform is localized to focal adhesions, where it regulates shape change, motility, and adhesion mainly through modulation of integrin clustering and function.[45–49] Calpain also regulates $\alpha_{IIb}\beta_3$ on activated platelets by cleavage of the β_3 cytoplasmic tail, resulting in the removal of 2 NXXY motifs that are important for ligand binding, bidirectional signaling, and cytoskeletal attachment.[50–52] Four calpain-dependent cleavage sites flanking the NXXY motifs have been identified.[50] One of the critical functions of the cytoplasmic domain of $\alpha_{IIb}\beta_3$ is the regulation of clot contraction, involving association of the receptor with contractile actin filaments of the cytoskeleton in activated platelets. Proteolysis of the cytoplasmic tail of β_3 by calpain abolishes $\alpha_{IIb}\beta_3$-dependent clot contraction. Both calmodulin and calpain have been implicated in regulating the intracellular proteolysis of the cytoplasmic domain of platelet-endothelial cell adhesion molecule-1 (PECAM-1) in platelets,[53,54] supporting the possibility of dual extracellular (sheddase) and intracellular (calpain) proteolytic pathways operating in tandem in platelets (below).

Platelet Receptors

GPVI

Glycoprotein (GP)VI (≈65 kDa) is a member of the immunoglobulin (Ig) superfamily, with two extracellular Ig domains, a mucin-like domain, transmembrane domain, and

cytoplasmic tail.[5,6] In addition to the major physiological ligand, collagen, GPVI also binds laminin[55] and nonphysiological ligands including cross-linked collagen-related peptide (CRP) and the snake toxins, convulxin, and alborhagin, which bind at overlapping but distinct binding-sites within the extracellular Ig domains.[56–58] GPVI signals by Fc receptor γ-chain (FcRγ)-dependent and FcRγ-independent pathways[5,6,59–61]: the former involves an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail of FcRγ, whereby ligand-induced cross-linking of GPVI/FcRγ leads to activation of the ITAM-dependent Syk kinase; this involves direct interaction of Src family kinases, Fyn and Lyn, with a consensus Pro-rich sequence in the GPVI cytoplasmic tail,[62] whereas a juxtamembrane calmodulin-binding sequence is involved in GPVI-dependent Ca^{2+} signaling.[61,62] These signaling pathways result in secretion of agonists such as ADP, and inside-out activation of platelet integrins, primarily $\alpha_{IIb}\beta_3$ that binds fibrinogen or vWF and mediates platelet aggregation.

Another major consequence of GPVI signaling induced by the GPVI ligands, collagen, CRP, and convulxin, is the activation of proteolytic pathways leading to GPVI ectodomain shedding,[7–10] and the release of a soluble ≈55-kDa fragment into the platelet supernatant, with an ≈10-kDa remnant fragment that remains platelet-associated. GPVI shedding is metalloproteinase-dependent, and inhibitable by EDTA, by a broad-specificity metalloproteinase inhibitor GM6001, and by tumor necrosis factor (TNF)-α protease inhibitor (TAPI).[9] Ligand-induced shedding also requires GPVI signaling, and is blocked by inhibitors of GPVI-related signaling proteins, Src family kinases (PP1 or PP2), PI3-kinase (wortmannin), and Syk kinase (piceatannol).[8] Signaling also leads to rapid dissociation of calmodulin from the cytoplasmic tail, and calmodulin inhibitors such as W7 that dissociate calmodulin, also induce GPVI ectodomain shedding.[8,10,63] Alternately, GPVI shedding is induced by NEM,[1] which can directly activate sheddases.[20,21] Importantly, W7- or NEM-induced shedding[1,8] is independent of platelet activation, and is unaffected by blocking Src (PP1 or PP2) or Syk (piceatannol) (unpublished result, 2007). Recent evidence suggests that the sheddase involved in GPVI proteolysis is ADAM10.[64] This implies, therefore, that shedding of GPVI and GPIbα (see below) may involve distinct mechanisms, and that potentially, their expression may be differentially regulated. In this regard, in a platelet fully activated by a GPVI agonist, all of the detectable GPVI is shed while GPIbα and other platelet receptors remain almost fully expressed on the cell surface.[8] The time-frame for shedding of GPVI from human platelets in vitro is minutes to hours, whether induced by NEM, W7, or GPVI ligands[1,8,9]; in general, more potent agonists such as convulxin induce more rapid shedding than CRP or collagen, consistent with signaling-dependent activation of metalloproteinases. In vivo, it is possible that shedding may occur within minutes, particularly under shear conditions or on platelet aggregation (particularly if, like ADAM10-mediated shedding of the ephrin receptor complex,[30] GPVI shedding occurs in *trans*; Figure 2A). It is notable, however, that although shedding of platelet CD40 ligand is regulated by $\alpha_{IIb}\beta_3$-dependent signaling (see below), GPVI shedding is unaffected by $\alpha_{IIb}\beta_3$ blockade.[8] There is increasing evidence that expression levels of GPVI regulate in a graded fashion platelet reactivity toward collagen and other ligands.[11–13] There is a correlation between GPVI levels on human or murine platelets and adhesion to collagen under flow conditions. In clinical studies,[14] the levels of human platelet GPVI correlate with acute coronary syndrome, suggesting that elevated levels of platelet GPVI may act as a marker of impending acute coronary events.

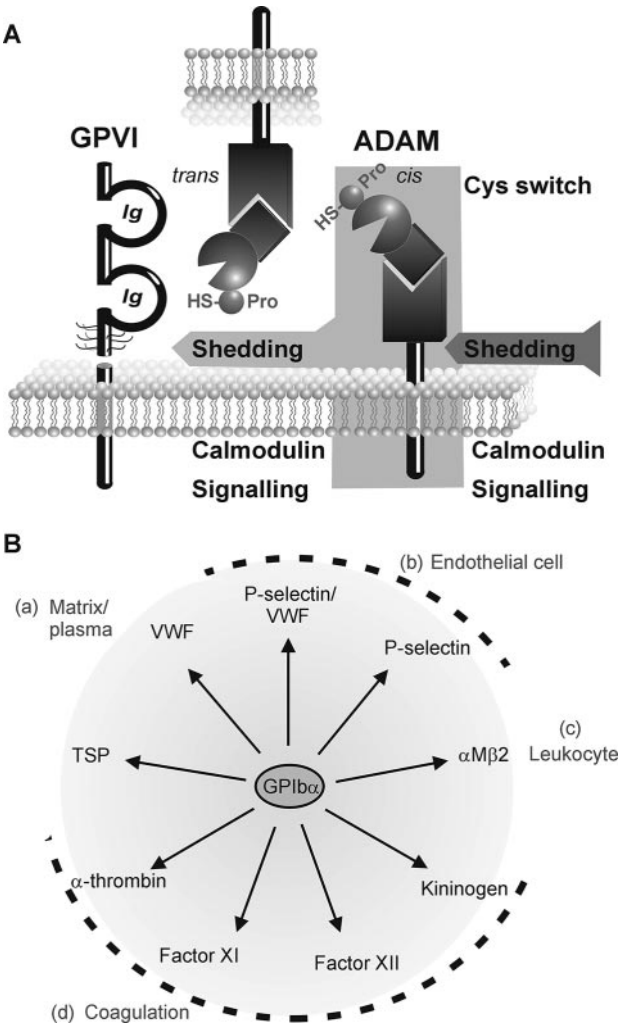

Figure 2. A, Glycoprotein VI and ADAM regulation. Platelet GPVI expression and function regulated by calmodulin association, signaling, and ADAM-mediated sheddases on the same (*cis*) or contacting (*trans*) cell. A Cys residue in the propeptide domain of ADAM10 or ADAM17 acts as a Cys-switch, whereby the thiol coordinates the active-site metal ion and inactivates the proteinase; removal of the prodomain enhances metalloproteinase activity. Ig, immunoglobulin domain; Pro, propeptide domain. B, Ligands of glycoprotein Ibα. The N-terminal domain of GPIbα (His1-Glu282) the major ligand-binding subunit of the platelet GPIb-IX-V complex, regulates platelet interaction with (a) adhesive ligands in the subendothelial matrix or plasma, (b) endothelial cells, (c) leukocytes, or (d) coagulation factors via overlapping, but not identical binding sites. Surface levels of GPIbα on platelets are likely to be critical in controlling platelet interactions under high shear.

GPIb-IX-V

Platelet GPIb-IX-V is a complex of GPIbα disulfide-linked to GPIbβ and forming a noncovalent complex with GPIX and GPV.[2,3] GPIbα is the major ligand-binding subunit of the receptor complex, and consists of a globular ≈45-kDa N-terminal ligand-binding domain, a sialomucin core, a transmembrane domain, and a cytoplasmic tail. Immediately N-terminal to the transmembrane domain is a pair of Cys residues, one or both of which form a disulfide-bond with GPIbβ.[65] The ligand-binding domain (His1-Glu282) consists of 7 leucine-rich repeats, their N- and C-terminal flanking sequences, and an anionic sequence with sulfation at Tyr276, Tyr278, and Tyr279.[2,3] Glycocalicin, a soluble ectodomain fragment of GPIbα (N-terminal and sialomucin domains), is present in plasma at 1 to 3 μg/mL,[66] and is formed by cleavage N-terminal to the disulfide bond linking GPIbα to GPIbβ. GPIbα shedding occurs in aging platelets, or platelets treated with the mitochondrial-targeting reagent, CCCP (carbonyl cyanide 3-chlorophenylhydrazone), and is mediated by ADAM17, because purified recombinant ADAM17 cleaves glycocalicin from platelets, and dysfunctional ADAM17 in genetically-modified mice leads to decreased plasma glycocalicin and decreased ectodomain shedding.[16,17] Like GPVI,[63] the cytoplasmic domain of GPIb-IX-V directly binds calmodulin at juxtamembrane sequences of GPIbβ and GPV.[67] In the case of GPV, calmodulin inhibitors also induce ectodomain shedding, by a mechanism that, like GPIbα, involves activation of ADAM17.[15]

Shedding of GPIbα is likely to have multiple effects on platelet function. There are multiple ligands for GPIbα[2] (Figure 2B) that regulate platelet adhesion to subendothelial matrix (vWF or thrombospondin[68]), activated endothelial cells (P-selectin or vWF/P-selectin),[69–71] or leukocytes ($\alpha_M\beta_2$).[72] Recent studies show that whereas thrombus formation is markedly impaired in transgenic mice where platelets express a GPIb-IX-V complex lacking the GPIbα ligand-binding ectodomain, vWF-deficient mice are similar to control mice[72,74]; the authors suggest GPIbα binding to ligand(s) other than vWF is critical in these models. GPIbα is known to play a key role in regulating coagulation at the surface of activated platelets, by binding to coagulation factors XI[75,76] and XII,[77] high molecular weight kininogen,[78] and thrombin.[79–83] The interaction of thrombin with GPIbα (1) facilitates thrombin-dependent activation of PAR-1,[83,84] (2) promotes activation of GPIbα-associated factor XI (potentially regulated by factor XII),[75–77] and (3) enables direct signaling *via* GPIbα in the absence of GPV.[85] Shedding of GPV,[15] therefore, is likely to control thrombin-dependent platelet activation *via* GPIbα; GPV also supports platelet adhesion to collagen.[86]

GPIb-IX-V and GPVI are physically and functionally coassociated on the platelet plasma membrane,[1] and are thought to act synergistically as mutual accessory signaling receptors, particularly at low agonist concentration.[87] The ectodomains of GPIbα (glycocalicin) and GPVI (≈55-kDa fragment) directly interact,[1] and this could account for their functional coassociation. Therefore, shedding of either receptor is likely to adversely affect activation thresholds of the companion receptor. Both GPVI (FcRγ)[60,61] and GPIb-IX-V (FcγRIIa)[88] indirectly use ITAM-bearing receptors, such that decreased ligand binding to either receptor as a result of shedding would attenuate ITAM-dependent signaling in platelets. In other pathology where receptor expression levels may be relevant, GPIbα binds β2-glycoprotein I, and GPIbα-mediated platelet activation by autoimmune complexes involving this ligand provides a mechanism for thrombus associated with antiphospholipid syndrome.[89]

PECAM-1

Like GPVI, PECAM-1 is a member of the Ig superfamily, with 6 extracellular Ig domains, transmembrane domain, and cytoplasmic tail. Unlike GPVI, the cytoplasmic domain of PECAM-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). PECAM-1 plays a role in attenuating thrombus formation involving GPVI or other receptors[90–92]; in this regard, PECAM-1 is physically and functionally associated with FcγRIIa on human platelets, and also negatively regulates collagen-dependent stimulation of GPVI/FcRγ.[93] Interestingly, like GPVI, PECAM-1 contains a calmodulin-binding sequence in the juxtamembrane region of the cytoplasmic tail, and calmodulin inhibitors induce proteolysis of PECAM-1.[53] Other studies show that the cytoplasmic domain of PECAM-1 is cleaved by calpain in activated platelets at a site upstream of the ITIM, thereby deactivating the receptor.[54] This suggests that the intracellular proteinase, calpain, is activated under the same conditions that activate ectodomain metalloproteinases in platelets (in response to calmodulin inhibitors), implying both extracellular and intracellular sites of receptors are targeted when proteolytic pathways are switched on.

Sema4D

One recent article shows that human platelets express the immune cell receptor, semaphorin 4D (Sema4D), and its binding partners, CD72 and plexin-B1.[94] Sema4D, CD72 (associated with the tyrosine phosphatase, SHP-1) and plexin-B1 contribute to the regulation of thrombus formation, and mice deficient in Sema4D show decreased occlusive thrombi in arterial thrombosis models.[94] Surface expression of these proteins increases on platelets activated with PMA, collagen, or other agonists, and metalloproteinase-mediated shedding of Sema4D releases a soluble ectodomain fragment. Shedding of Sema4D is inhibited in ADAM17-defective mice, supporting a role for ADAM17 in cleavage of platelet receptors following activation.

CD40L

Platelet CD40 ligand regulates stability of $\alpha_{IIb}\beta_3$-dependent thrombi.[95,96] It is shed from activated platelets by an $\alpha_{IIb}\beta_3$-dependent mechanism (shedding is blocked by inhibition of ligand binding to $\alpha_{IIb}\beta_3$).[96] This is in contrast to GPVI shedding, which is independent of $\alpha_{IIb}\beta_3$.[8] The physiological relationship between shedding of CD40L and other adhesion receptors and the effects of this on thrombus formation in vivo are not yet resolved.

G Protein–Coupled Receptors: PAR-1

G protein–coupled receptors may also be regulated by extracellular proteolytic pathways. Precedence for this is the

metalloproteinase-mediated cleavage of the ligand-binding extracellular N-terminal domain of the thrombin protease-activated receptor-1 (PAR-1) from the surface of human endothelial cells.[97] ADAM17 (or a related proteinase) is implicated in mediating PAR-1 cleavage, because it is inhibited by TNF-α protease inhibitor-2 (TAPI-2) and tissue inhibitor of metalloproteinase-3 (TIMP-3), but not TIMP-1 or -2. A specific role for calmodulin in this process is not established, but other G protein–coupled receptors including the platelet ADP receptor, $P2Y_1$,[98] directly bind calmodulin. The generality or physiological role of G protein–coupled receptor shedding on platelets is currently uncertain, but presumably occurs at the same time as adhesion receptor shedding involving ADAM17 or other sheddases.

Platelet Integrin Shedding

The function of both platelet and leukocyte integrins is directly relevant to the function of platelet GPIb-IX-V and GPVI. Primarily, the platelet-specific integrin, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), plays a key role in thrombus formation, by binding vWF, fibrinogen, or other ligands to mediate platelet aggregation, after ligand binding to GPIb-IX-V, GPVI, or other receptors.[2] Platelets also express the vitronectin receptor, $\alpha_v\beta_3$, and β_1 integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, which bind adhesive ligands including collagen, laminin, and/or fibronectin.[99] The leukocyte integrin, $\alpha_M\beta_2$ (Mac-1), is involved in leukocyte adhesion to the vessel wall, and is activated after initial contact of leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) with P-selectin expressed on activated endothelial cells or activated mural platelets. This enables $\alpha_M\beta_2$-mediated firm adhesion either to the endothelial immunoglobulin family receptor, intercellular adhesion molecule-1 (ICAM-1), or to platelet GPIb-IX-V.[73] The inserted (or I) domain of α_M mediates binding of $\alpha_M\beta_2$ to GPIbα, ICAM-1 or other ligands.

Whereas the proteolytic regulation of β_3 integrins by calpain has been reported (refer above),[50–52] the possibility that ectodomain shedding plays a role in regulating the function of platelet integrins has not been widely explored. There is, however, precedence for integrin shedding on other cells. For example, recent studies involving another leukocyte β_2 integrin, $\alpha_L\beta_2$, show that an as yet unidentified cell surface proteinase(s) cleaves both a membrane-proximal region of β_2 and a more upstream site of α_L, releasing a soluble ectodomain fragment of α_L (containing the I domain) in complex with the ectodomain of β_2, from the surface of blister-fluid neutrophils.[100,101] Cell surface-expressed ADAM family receptors are known to interact via their distintegrin domains with integrins on other cells. For example, ADAM15, principally via its RGD-containing disintegrin domain, acts as a counter-receptor for $\alpha_{IIb}\beta_3$.[102] It is tempting to speculate a broader role for ADAM family metalloproteinases in platelet integrin shedding, as suggested for shedding of platelet GPIb-IX-V, GPVI, and other receptors.

Regulation of Proteinases/Receptors

Calmodulin

Despite evidence that calmodulin binds directly to juxtamembrane cytoplasmic sequences of sheddase substrates, including GPV, GPVI, L-selectin, and other receptors, and that dissociation facilitates ectodomain shedding,[8,15,31,61,63] the mechanism for precisely how this occurs is obscure. Calmodulin might maintain the receptor in a protected, noncleavable state on resting cells by conformational changes across the membrane affecting ectodomain cleavage sites. Calmodulin-binding sequences consisting of positively-charged/hydrophobic amphipathic helix in the cytoplasmic juxtamembrane position may be involved in stable membrane insertion. In this case, disruption of calmodulin by mutagenesis or by treatment with calmodulin inhibitors such as W7 or trifluoperazine, might enable increased exposure of extracellular juxtamembrane sheddase-cleavage sites above the lipid bilayer facilitating hydrolysis. Calmodulin-binding sequences occur on 3 key platelet receptors that initiate thrombosis at arterial shear rates,[74,103–105] GPIb-IX-V, GPVI, and $P2Y_1$, and may play additional roles other than regulating ectodomain shedding. The calmodulin-binding sequence of GPVI, for example, is critical for initiating ligand-induced Ca^{2+} signaling in GPVI-transfected cell lines.[61] Similarly a major functional consequence of ADP binding to $G_{\alpha q}$-linked $P2Y_1$ is elevation of cytosolic Ca^{2+} upstream of signaling by the other platelet ADP receptor, $G_{\alpha i}$-linked $P2Y_{12}$.[98,103] Analogous calmodulin-binding sequences in epidermal growth factor receptor (EGFR), MARCKS, and other proteins interact strongly with the plasma membrane and sequester phosphatidylinositol bisphosphate (PIP_2), a multifunctional mediator, which is also upstream of inositol triphosphate (IP_3)-dependent release of Ca^{2+} from internal stores.[106] Finally, calmodulin inhibitors may also act, in part, by activation of ADAM10 and/or ADAM17, and there is some evidence for a direct interaction between calmodulin and pro-ADAM10, which may regulate proteolytic processing.[18–20,33] Nevertheless, in platelets, calmodulin inhibitors induce shedding of GPIbα, GPV, and GPVI (without the requirement for platelet activation or signaling), suggesting that calmodulin-receptor interaction could be therapeutically targeted to decrease functional receptor expression.[10]

NEM

As mentioned earlier, ADAM10 and ADAM17 contain a Cys switch in the prodomain, allowing these proteinases to be directly activated by thiol-modifying reagents such as NEM.[20,24] MMP sheddases may also contain a regulatory Cys switch.[34] Treating human platelets with NEM has been shown to induce shedding of the ≈55-kDa ectodomain fragment of GPVI,[1] in the absence of GPVI ligands or platelet activation. Like calmodulin inhibitors, therefore, NEM treatment provides a mechanism for directly inducing loss of platelet surface GPVI (or other receptors shed by ADAM10 or ADAM17) without accompanying platelet activation. Although using thiol-modifying agents such as NEM in vivo would presumably be limited, this supports the concept that direct activation of sheddases could be exploited therapeutically.

Aging

Platelet receptor expression levels have also been investigated with respect to platelet aging and clearance; anucleate

platelets circulate in the bloodstream for ≈1 week. In dogs, platelet responsiveness to collagen decreases with age, correlating with an age-dependent decrease in GPVI-dependent platelet activation.[107] In zebra fish, an age-related decrease in adhesion receptor levels on nucleated thrombocytes also correlates with decreased participation in thrombus formation; younger thrombocytes are more adhesive, and have a higher propensity to form thrombi.[108] A specific role for platelet sheddases in regulating age-related changes in adhesion receptor levels has not been directly demonstrated. However, as discussed above, mimicking aging, by treating platelets with the mitochondrial poison CCCP, induces ADAM17-mediated GPIbα shedding.[16] The key question of whether shedding is a determinant or a consequence of platelet aging has also not been addressed.

Nonphysiological Ligands

Another mechanism for inducing shedding of receptors is binding of ligands. Nieswandt and colleagues originally showed that the anti-mouse GPVI monoclonal antibody, JAQ1, also selectively decreased GPVI surface expression,[109] involving, at least in part, metalloproteinase-mediated shedding. Similarly, human platelets in a NOD/SCID mouse model can be depleted of GPVI in an activation-independent manner by anti-human GPVI antibodies.[110] Whether proteolysis of GPVI in this study was the result of ADAM-mediated shedding was not directly assessed, however Western blots with antibodies against the GPVI cytoplasmic tail indicate that the soluble plasma form of human GPVI generated in this model lacks the cytoplasmic domain.[110] Anti-GPVI antibody treatment provides a mechanism for selectively downregulating platelet GPVI surface expression in vivo (GPVI is essentially platelet-specific).

PMA

The phorbol ester, phorbol 12-myristate 13-acetate (PMA) activates platelet protein kinase C (PKC), and also induces metalloproteinase-mediated ectodomain shedding of GPIbα and GPV.[16,17] Although treatment with PMA stimulates platelet activation, it is possible that dissecting this pathway leading to activation of ADAM sheddases could identify targets allowing activation-independent downregulation of platelet adhesion receptors.

Aspirin

One recent report shows that aspirin promotes ADAM17-mediated shedding of GPIbα and GPV from human or mouse platelets, with increased levels of the respective ectodomain fragments in plasma.[17] The classical target for aspirin as an antithrombotic, cyclooxygenase-1 (COX-1), was unlikely to be responsible for aspirin-related metalloproteinase-mediated shedding; however, because shedding appeared normal in COX-1–deficient mice,[17] the mechanism may instead involve acylation of ADAM17 and/or substrate(s).

Diagnostics/Exposure of Cryptic Sites by Proteinases

There are at least 3 areas where an increased understanding of platelet receptor shedding mechanisms might be applied diagnostically. First, although platelet levels of GPVI have been correlated to risk of acute coronary syndrome,[14] little evidence exists on whether plasma GPVI levels are indicative of thrombotic risk. One study shows that GPVI is present in normal human plasma,[111] but further developments are needed to facilitate analytical screening of patient samples. Second, although platelet levels of intact GPVI have been assessed by flow cytometry, the ≈10-kDa remnant fragment of GPVI on platelets has not been directly measured. Antibodies against the GPVI cytoplasmic domain have been used to analyze this fragment by Western blot,[109] however antibodies against the new N terminus of cleaved GPVI could be used in flow cytometry to determine ratios of intact versus shed receptor as an index of thrombotic exposure. The exploitation of cryptic postproteolytic sites and fragments has recently been reviewed elsewhere.[112] Third, proteinase activity assays, for example, based on consensus substrates developed for ADAM10 and ADAM17,[113] could be used to assess sheddase activity levels on resting versus stimulated platelets as an additional measure of surface expression status of GPVI (or other platelet receptors). Total ADAM10 or ADAM17 levels are measurable by flow cytometry,[23] and determining specific activity of surface metalloproteinase could indirectly comment on thrombotic potential. Given the clinical importance of GPVI levels,[14] development of these and other methods is warranted.

Clinical Implications of Platelet Proteolysis

The pathology of thrombus formation in thrombotic disease can be described as a series of steps. Atherosclerotic plaque rupture or vascular injury exposes the subendothelial matrix, supporting platelet translocation, then rapid adhesion via exposed vWF and collagen, initiated by binding to platelet GPIb-IX-V and GPVI/FcRγ, respectively.[1–6,74,105,114,115] Adhesive interactions involving these receptors enables signal transduction and platelet activation, with secretion of agonists such as ADP, and activation of platelet integrins, in particular $\alpha_{IIb}\beta_3$ that binds vWF or fibrinogen, resulting in firm adhesion, spreading, and recruitment of additional platelets from the bloodstream into the developing thrombus.[73,116–118] Arterial thrombus formation in vivo is a dynamic process, with developing thrombus at the site of injury gradually building up over time but not without large fragments becoming detached from the thrombus mass, and subsequent rebuilding by additional incoming platelets.[118,119–121] That is, after initiation, the degree of thrombus stability is a determinant of the overall rate and progression of thrombus build-up, before either entirely occluding the blood vessel, or reaching a state of surface passification where thrombus growth is limited and thrombus size is stabilized.[121] Ectodomain shedding of platelet adheso-signaling receptors, GPIbα and GPVI, would provide an attractive mechanism for controlling thrombus growth and stability. Several lines of evidence support this contention. First, platelet spreading on prothrombotic surfaces bearing vWF and/or collagen would require the detachment (then possibly reattachment) at the tips of filopodia or the leading edge of lamellipodia. Receptor shedding would facilitate this process. Second, shedding of GPIbα could limit vWF at the surface of the developing thrombus and limit

platelet translocation. Receptor shedding would thus enhance passification of the thrombus surface. Third, because both GPIb- and GPVI-dependent signaling involve receptor cross-linking, shedding of GPIbα and GPVI could lower surface density of intact receptor and thereby attenuate platelet signaling, activation, and secretion. Ectodomain shedding of GPIbα and GPVI would also conceivably facilitate thrombus embolization by decreasing the number of platelet-matrix contacts. Future studies should evaluate these possibilities in vivo, as the underlying molecular mechanisms are determined.

Disclosures

None.

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