Thrombin and Platelet Activation*

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The accumulation of thrombin at sites of vascular injury provides one of the chief means for recruiting platelets into a growing hemostatic plug. Studies completed over the past 10 years show that platelet responses to thrombin are mediated by a subset of G protein-coupled receptors known as protease-activated receptors. These receptors are activated on cleavage by thrombin, initiating the intracellular signaling events needed to transform mobile, nonadhesive platelets into cells that can participate in the growth of an immobile hemostatic plug. How this is accomplished is the subject of this review. (CHEST 2003; 124:188–25S)

Key words: G proteins; G protein-coupled receptors; phospholipase C; platelet; protease-activated receptors; thrombin

Abbreviations: ADP = adenosine diphosphate; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; GP = glycoprotein; GPCR = G protein-coupled receptor; GTP = guanosine triphosphate; NO = nitric oxide; PAR = protease-activated receptor; PG12 = prostaglandin 12; TAa = thromboxane A2; VWF = von Willebrand factor

Human platelets normally circulate in a quiescent state, prevented from premature activation by the presence of the endothelial cell monolayer, by the signal-inhibiting effects of prostaglandin I2 (PGI2) and nitric oxide (NO), and by limitations on the local accumulation of platelet agonists. It is only when these barriers are overcome that platelets can become activated. That can happen after local trauma or in response to the rupture of an atherosclerotic plaque. Thrombin plays an essential role in activating platelets, just as it does in the formation of the fibrin clot. When added to human platelets in vitro, thrombin causes platelets to change shape, stick to each other, and secrete the contents of their storage granules. How this is accomplished is still not fully understood, but a major step forward occurred in 1990\(^2\) with the identification of a G protein-coupled receptor (GPCR) that can be activated proteolytically by thrombin. Until that receptor, now known as protease-activated receptor (PAR)-1, was identified, there was no clear paradigm for the initiation of intracellular events by an extracellular protease. In the dozen years since then, a family of protease-responsive receptors has been identified and steadily

progress has been made toward understanding how they work and how thrombin activates platelets. The results have provided insights into normal platelet biology and opened an avenue for the development of new antiplatelet agents—a promise that has yet to be fulfilled. To place what is known about the activation of platelets by thrombin into context, this article begins with an overview of the events encompassing platelet plug formation and then focuses on the role of thrombin.

STAGES IN THE FORMATION OF A STABLE PLATELET PLUG

Platelet plug formation requires a coordinated series of events that can overcome local resistance to platelet activation long enough for bleeding to stop. This is not a trivial task, particularly if unwarranted platelet activation is to be avoided. The barriers to platelet activation are substantial. Part of the barrier is formed by the endothelial cell monolayer, which physically separates platelets from agonists embedded in the vessel wall, especially collagen and von Willebrand factor (VWF). VWF is a multimeric protein synthesized by endothelial cells that plays an essential role in the adhesion of platelets to collagen under the high-flow conditions found in arteries. It is secreted in an ultrahigh-molecular-weight form that is normally cleaved by the metalloprotease, ADAMTS13, preventing it from binding to platelets spontaneously and causing a thrombotic microangiopathy.\(^3\) The shear stresses produced when blood flows over VWF anchored to collagen exposes platelet-binding sites, allowing the VWF to support platelet/collagen and platelet/platelet interactions (Fig 1). In addition to collagen and VWF, tissue factor is also present in the vessel wall, on the surfaces of activated endothelial cells and monocytes, and on circulating microparticles that stick to activated platelets, so injuries that alter or remove the endothelial barrier result in the local generation of thrombin as well as the exposure of collagen.

In addition to serving as a physical barrier, endothelial cells release PGI2 and NO, whose net effect is to globally depress the intracellular signaling events needed to support platelet activation by raising cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate levels (Fig 1).\(^4\) The ability of cyclic nucleotides to inhibit platelet activation has been exploited in the development of antiplatelet agents, such as dipyridamole, which work by inhibiting the phosphodiesterases that would otherwise metabolize cAMP within platelets. The importance of PGI2 and NO as barriers to platelet activation is indicated not only by the effectiveness of molecules that mimic PGI2 as antiplatelet agents, but also by the prothrombotic effects of deleting the gene encoding the platelet PGI2 receptor in mice.\(^5\) As a further barrier to platelet activation, some endothelial cells express CD39 on their luminal surface. CD39 can hydrolyze small quantities of adenosine diphosphate (ADP) released from damaged red cells and activated platelets, preventing the ADP from activating additional platelets.\(^6\) The use of CD39 as an antithrombotic is being explored in animal models. Other barriers to platelet activation include the diluting effects of blood...
flow, the presence of inhibitors of thrombin, and the short half-life of the platelet-derived agonist, thromboxane A₂ (TxA₂).

Given all of these barriers, platelet activation should ideally occur only after substantial injuries. If this were always the case, there would be little need for antiplatelet agents in clinical settings. Formation of the platelet plug in response to vascular injury can be thought of as occurring in three stages: initiation, extension, and perpetuation (Fig 1). Initiation begins with the tethering, rolling, and arrest of moving platelets on collagen and their subsequent activation to form a platelet monolayer. Large VWF multimers are essential to this process, particularly under high shear conditions in the arterial circulation, but thrombin can also help to initiate platelet activation. Extension refers to the recruitment of additional platelets through the local accumulation of thrombin, ADP, and TxA₂. Perpetuation refers to the events that stabilize the platelet plug until wound healing can occur, some of which involve molecules on the platelet surface that are capable of generating intracellular signals only after platelets have come into sustained contact with each other. The net result is the formation of a fibrin-anchored platelet plug, a structure in which platelet/platelet interactions are supported by the binding of fibrinogen and fibrin to the integrin αIβ3 (also known as glycoprotein [GP] IIb-IIIa) and by VWF bound to GP Ib and α₂β₃ (Fig 2).

**Initiation**

The arrest and eventual activation of moving platelets by collagen plus VWF requires several receptors on the platelet surface, including those that can bind directly to collagen (GP VI and the integrin α2β₁) and those that bind to collagen indirectly via VWF (the GP Ib/IX/V complex and the integrin α₂β₃). The presence of binding sites for collagen and VWF on GP VI, GP Ib, α₂β₁, and αIβ3 allows platelets to stop their forward movement in the arterial circulation long enough to become activated and fully adherent. This can happen rapidly, but only selectively. Videomicroscopy of blood vessels following focal injuries shows that most platelets moving by the site of injury too quickly to stop. In a manner that is very much reminiscent of how leukocytes escape from the circulation, a small proportion of platelets rolling along the vessel wall is able to react initially to injury and form the nidus for a platelet plug. This requires platelets to both adhere to collagen and be activated by it. The binding of collagen to GP VI on the platelet surface causes the clustering of GP VI and its associated γ-chain within the plane of the
The sequence of these domains and in part by the sequence of proteins interact with the cytoplasmic domains of the platelets by discharging Ca$^{2+}$solic-free Ca$^{2+}$ again during the perpetuation phase of platelet plug signaling through tyrosine kinases is a theme that occurs of a cell-surface receptor (GP VI in this case) followed by clustering mediated by the tyrosine kinase, Syk, and the activation of creating a tandem phosphotyrosine motif that is recognized by the tyrosine kinase, Syk, and the activation of phospholipase C$_{\gamma2}$ (Fig 3).$^8$ PLC$_{\gamma2}$ hydrolyzes PI-4,5-P$_2$ to produce 1,4,5-IP$_3$ and diacylglycerol, raising the cytosolic Ca$^{2+}$ concentration within the adherent platelets by discharging Ca$^{2+}$ stores from within the dense tubular system and activating protein kinase C. Clustering of a cell-surface receptor (GP VI in this case) followed by signaling through tyrosine kinases is a theme that occurs again during the perpetuation phase of platelet plug formation.

**EXTENSION**

The extension phase of platelet plug formation occurs when activated platelets accumulate on top of the initial monolayer of platelets bound to collagen (Fig 1). Key to the extension phase is the presence on the platelet surface of receptors that can respond rapidly to soluble agonists, including thrombin, ADP, and TxA$_2$. The local accumulation of these agonists allows circulating platelets to be recruited into the growing hemostatic plug even when they cannot arrest on collagen. Extension of the platelet plug requires the activation of $\alpha_{IIb}\beta_3$ through what is commonly called “inside-out” signaling, promoting the formation of stable platelet/platelet contacts mediated by bridges comprised of fibrinogen and VWF. The receptors involved in these events are typically members of the superfamily of GPCRs (Fig 3), which are membrane proteins with an extracellular N-terminus, an intracellular C-terminus, and seven transmembrane domains. Agonists bind to the surface-accessible domains of GPCRs, causing a conformational change that activates G proteins associated with the intracellular surface of the receptor.$^9$ G proteins interact with the cytoplasmic domains of the receptor with specificity determined in part by the sequence of these domains and in part by the sequence of the $\alpha$ subunit of the G protein. G proteins are heterotrimers comprised of a single $\alpha$, $\beta$, and $\gamma$ subunit. The $\alpha$ subunit contains a guanine nucleotide-binding site that is occupied by guanosine diphosphate (GDP) in the off state and GTP in the on state. Activation of the receptor causes exchange of guanosine triphosphate (GTP) for GDP, after which partial dissociation of the GTP-bound $\alpha$ from GB$\gamma$ exposes effector interaction sites on both. Fatty acylation of $\alpha$ and prenylation of $\beta$, cause them to remain associated with the plasma membrane until hydrolysis of the GTP bound to $\alpha$, allows the original heterotrimer to reform.

Mammalian G proteins fall into four families that are typically referred to by the designation of the $\alpha$ subunit. Human platelets express at least one member of the $G_a$ family and four members of the $G_i$ family ($G_{i1}$, $G_{i2}$, $G_{i3}$, and $G_{i4}$), which respectively stimulate and inhibit cAMP formation by adenyl cyclase, among other functions. In addition, platelets express one or more members of the $G_q$ family, which stimulate $\beta$ isoforms of phospholipase C, and two members of the $G_{12}$ family ($G_{12}$ and $G_{13}$), which help to regulate the platelet actin cytoskeleton.$^{10-14}$ Based on evidence from knockout and reconstitution studies, the abundance of G protein types in platelets appears to be necessary to support the actions of multiple dissimilar platelet agonists.$^{15}$

The GPCRs that respond to platelet agonists differ in their potency and their preferences for intracellular effector pathways. Some, such as the receptors for thrombin (PAR-1 and PAR-4), TxA$_2$ (TP), and ADP (P2Y12), cause phosphoinositide hydrolysis and raise the cytosolic Ca$^{2+}$ concentration by activating G$_q$, (Fig 3).$^{12}$ Others, such as the P2Y12 receptor for ADP and the $\alpha_{2A}$-adrenergic receptor for epinephrine, are coupled by G$_i$ or G$_z$ to the inhibition of adenylyl cyclase and to the activation of PI 3-kinase and the Ras family member, Rap1.$^{15-18}$ Optimal platelet activation via GPCRs is thought to require activation of both a $G_q$-coupled receptor and a $G_i$-coupled receptor.$^{19}$ The ability of the Gi family members in platelets to inhibit cAMP formation by adenylyl cyclase is most relevant when PGI$_2$ secreted by endothelial cells has inhibited platelet activation. In the absence of PGI$_2$, other G$_i$ effectors are more relevant.$^{15}$ The essential role of the $G_{12}$-coupled P2Y12 receptor for ADP is suggested by the phenotypes of the G$_{12}$ and P2Y12 knockout mice and by the proven utility of the P2Y12 antagonists ticlopidine and clopidogrel as antithrombolytic agents.$^{15,17,20-22}$ Thrombin and TxA$_2$ receptors can also cause the rearrangement of the actin cytoskeleton that underlies platelet shape change by coupling to guanine nucleotide exchange factors for Rho via G$_{12}$ and G$_{13}$.$^{10}$

**PERPETUATION**

The third phase of platelet plug formation, perpetuation, occurs at a point when direct interactions between platelets are of sufficient duration to make contact-dependent signaling feasible. Ultimately, these late events are thought to stabilize the platelet plug, helping to prevent premature disaggregation and regulating retraction of the clot. A number of recent events have helped to define some of the signaling events that are involved. The
best-described of these events involves outside-in signaling through integrins. Other examples include the binding of Eph kinases to ephrins, and the binding of CD40 ligand (CD40L) to \(\alpha_{IIb}\beta_3\). Each of these depends on one or more molecules expressed on the platelet surface, but the details differ in essential ways. Eph kinases and ephrins can engage with each other whenever platelets are in sufficiently close contact for a long enough time. Interactions with \(\alpha_{IIb}\beta_3\) require inside-out signaling before the integrin can bind to its ligands, particularly soluble ligands. CD40L is not expressed on the surface of resting platelets, but appears there after platelets have been activated. Once on the surface, CD40L can bind to activated \(\alpha_{IIb}\beta_3\) and (perhaps) to CD40. Evidence exists for a role for each of these contact-depending signaling mechanisms, but their relative contributions are still being studied.

**THROMBIN RECEPTOR STRUCTURE AND FUNCTION**

For years after thrombin was shown to activate platelets, little was known about how this might be accomplished. A variety of approaches established that the proteolytic activity of thrombin was required, and biochemical studies showed that G proteins are activated by thrombin, but there was no precedent for G protein activation by a protease. Binding studies identified high-affinity interactions with several sites on the platelet surface, including GP Ib, but efforts to show that any of these constituted a receptor in the signaling sense were not entirely successful. Substrates for thrombin were identified on the platelet surface, including GP V. However, cleavage of GP V did not appear to be required for platelet activation by thrombin. Before discussing the receptors that have been identified, it is worth briefly considering criteria that proved useful for establishing a protein as a true signaling receptor for thrombin. Such criteria included the following: (1) demonstrating the presence of the candidate receptor on the surface of resting platelets, (2) showing that it is a substrate for thrombin or closely associated with a substrate for thrombin, (3) demonstrating a link to intracellular signaling cascades, (4) showing that expression of the candidate receptor could render a cell that was otherwise unresponsive to thrombin capable of responding, and (5) showing that blocking, dismantling, or otherwise removing the

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**Figure 3. Overview of platelet activation.** Most platelet agonists activate platelets via G protein-coupled receptors on the platelet surface. Critical responses include G\(_q\)-mediated activation of mitochondrial Ca\(_{\text{2+}}\), activation of phospholipase A\(_2\) and protein kinase C, and G\(_12\)-mediated activation of Rho family members to support rearrangement of the platelet cytoskeleton (shape change). The increase in cytosolic Ca\(_{\text{2+}}\) is initially caused by the P\(_3\)-stimulated release of Ca\(_{\text{2+}}\) from within the dense tubular system of the platelet, which in turn triggers Ca\(_{\text{2+}}\) influx across the platelet plasma membrane. Activated PGI\(_2\) receptors (not shown) stimulate adenylyl cyclase, raising platelet cAMP levels and causing a generalized inhibition of platelet responses to agonists. G\(_i\) family members support the suppression of adenylyl cyclase by platelet agonists and also couple receptors to other critical effector pathways. TP = thromboxane receptor.
candidate receptor would reduce platelet responses to thrombin. So far, PAR family members are the only receptors that meet all of these criteria. However, it remains possible that other proteins on the platelet surface also play a role, either by initiating signaling themselves or by facilitating the activation of PARs.

Four members of the PAR family have been identified to date. Three (PAR-1, PAR-3, and PAR-4) are thrombin receptors. The fourth, PAR-2, is activated by serine proteases other than thrombin. All four of the PAR family members have a structure similar to other GPCRs, including an exposed N-terminus (Fig 4). Studies on PAR-1 established the paradigm that applies (with certain exceptions) to the other three family members. In each case, receptor activation begins when thrombin cleaves the N-terminus of the receptor, exposing a new N-terminus that serves as a tethered ligand. Given sufficient opportunity, proteases other than thrombin can also activate PAR-1 or render the receptor unresponsive to thrombin by cleaving the N-terminus in the “wrong” place. The binding site for the tethered ligand has been mapped to the extracellular loops of the receptor. Because the ligand is not free to diffuse away, it presents a highly effective local concentration at the receptor. As is the case for other GPCRs, contact between PAR ligands and the receptor is thought to initiate signaling because of an induced conformational change in the receptor that is transmitted across the plane of the plasma membrane to promote exchange of GTP for GDP on associated G proteins. In the case of PAR family members, the activation paradigm that was initially established for PAR-1 includes the ability to respond to peptides based on the sequence of the tethered ligand. The one exception to the rule is PAR-3, for which no activating peptide agonist has been identified.

What about the other three members of the PAR family? PAR-2 is expressed by a number of tissues, including endothelial cells, but not by platelets. PAR-2 can be cleaved and activated by trypsin and tryptase, but not by thrombin.26,29 It can also be activated by the tissue factor/VIIa complex and factor Xa, which may be particularly relevant for endothelial cells.30–32 PAR-3 was identified after a gene ablation study33 showed that platelets from mice lacking PAR-1 were still responsive to thrombin. PAR-3 is a major regulator of thrombin responses in rodent platelets,34 but little else is known about it. When overexpressed, human PAR-3 can respond to thrombin. However, on murine platelets PAR-3 serves solely to facilitate cleavage of PAR-4 by thrombin.35 The fourth family member, PAR-4, was identified by database searches using conserved domains of the other three family members.36,37 PAR-4 is expressed on human and mouse platelets and accounts for the continued ability of platelets from PAR-3 knockout mice to respond to thrombin.36,37 Simultaneous inhibition of human PAR-1 and PAR-4 with blocking antibodies or a small-molecule antagonist completely abolishes platelet responses to thrombin,38 as does deletion of the gene encoding PAR-4 in mice.39

Thus, the four PAR family members have some features in common, but also have differences. Of the three that can be activated by thrombin, two (PAR-1 and PAR-3) have similar dose/response curves. The third, PAR-4, requires 10- to 100-fold higher concentrations of thrombin, apparently because it lacks the hirudin-like sequences that can interact with the anion-binding exosite and facilitate receptor cleavage of thrombin.35–37 This distinction is important for understanding the role of PAR-4 in human and mouse platelets.

Platelet Activation by Thrombin

Putting all of this together, current evidence suggests that thrombin activates human platelets by cleaving and activating PAR-1 and PAR-4 (Fig 5). In turn, these receptors activate Gq, G12, and perhaps G13 family members, leading to the activation of PLCβ, PI 3-kinase, and the monomeric G proteins, Rho, Rac, and Rap1, and also causing an increase in the cytosolic Ca2+ concentration and inhibiting cAMP formation. This process is supported by released ADP and TxA2, which bind in turn to their own GPCRs on the platelet surface (Figs 3, 4). Cleavage of human PAR-4 requires a higher concentration of thrombin than does cleavage of PAR-1, and it is likely that PAR-1 is the predominant signaling receptor at low thrombin concentrations, but PAR-4 activation may be more sustained.41,42 Mouse platelets provide an interesting contrast to human platelets. Where human platelets express two functional PAR family members (PAR-1 and PAR-4), mouse platelets express PAR-3 and PAR-4, but signaling appears to be mediated entirely by PAR-4, with

**Figure 4. Structure and features of PAR-1.** Cleavage of PAR-1 by thrombin between arginine 41 and serine 42 exposes a new N-terminus that serves as a tethered ligand. Activation of PAR-1 is followed by a rapid burst of signaling before the receptor is desensitized and, in some cases, cleared from the cell surface.40
PAR-3 serving solely to facilitate the cleavage of PAR-4 at low thrombin concentrations.\textsuperscript{35,39}

One issue that remains unresolved is the contribution of other thrombin receptors on platelets, particularly the members of the GP Ib/IX/V complex. GP Ib is a heterodimer comprised of an $\alpha$ and a $\beta$ subunit, which are disulfide linked to each other. The heterodimer forms a complex with GP IX and GP V that serves as both a binding site for VWF and an anchor for the platelet cytoskeleton.\textsuperscript{43} A high-affinity binding site for thrombin located at approximately residues 268–287 on GP Ib$\alpha$ is thought to interact with domains other than the active site.\textsuperscript{44} Deletion of the extracellular domain of GP Ib$\alpha$ or blockade of the thrombin-binding site decreases platelet responses to thrombin.\textsuperscript{45–48} In theory, the binding of thrombin to GP Ib$\alpha$ could facilitate the cleavage of a PAR family member on human platelets, much as the binding of thrombin to PAR-3 is thought to facilitate cleavage of PAR-4 on mouse platelets (Fig 5).\textsuperscript{49} Thus, although it is very clear that PAR-1 and PAR-4 provide the primary response elements for thrombin on human platelets, it remains possible that interactions with one or more members of the GP Ib/IX/V complex may facilitate cleavage/activation of PAR-1 or otherwise regulate platelet activation by thrombin.

**Conclusion**

In summary, there is no simple answer to the question “how does thrombin activate human platelets?” At least two GPCRs, several heterotrimeric G proteins, and a long list of intracellular signaling molecules are involved. Other surface molecules, including GP Ib$\alpha$, may play an accessory role. The identification of PAR-1 and the recognition of its novel mechanism of action suggested that it might be possible to develop small-molecule antagonists of platelet activation by thrombin. A number of efforts to do so have been launched over the past 10 years. As might be expected (at least in hindsight), such efforts have met with qualified success. Antagonists for PAR-1 have been identified and tested \textit{in vitro} and in animal trials. Despite the competitive advantage of having a tethered ligand, it has proved possible to block PAR-1 activation by agonist peptides and, in some cases, thrombin as well. One problem is that platelets also express functional PAR-4 and full blockade of thrombin responsiveness requires inhibition of both receptors. One might imagine administration of a combination of PAR-1 and PAR-4 antagonists. Whether such a combination will be more useful as antiplatelet therapy than aspirin or an ADP receptor antagonist awaits demonstration. Orally active inhibitors of
thrombin are currently under investigation as potential substitutes for warfarin in patients needing long-term anticoagulation. Whether they offer an advantage based in part on their ability to block platelet activation via PAR-1 and PAR-4 remains to be seen.

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