Effects of Thrombin on Interactions Between $\beta_3$-Integrins and Extracellular Matrix in Platelets and Vascular Cells

G.A. Stouffer, S.S. Smyth

Abstract—The $\beta_3$-integrin family consists of $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb/IIIa) and $\alpha_\beta V$. $\alpha_{IIb}\beta_3$ is found on platelets and megakaryocytes and has an essential role in hemostasis. $\alpha_\beta V$ has a broader distribution, and it functions in angiogenesis, neointimal formation after vascular injury, and leukocyte trafficking. There are important interactions between thrombin and $\beta_3$-integrins relative to both “inside-out” (integrin activation) and “outside-in” (modification of cellular events by ligand binding to integrins) signaling. Thrombin, by binding to G protein–coupled, protease-activated receptors, is a potent activator of $\alpha_{IIb}\beta_3$. Conversely, outside-in signaling through $\alpha_{IIb}\beta_3$ amplifies events initiated by thrombin and is necessary for full platelet spreading, platelet aggregation, granule secretion, and the formation of a stable platelet thrombus. In smooth muscle cells, $\alpha_\beta V$-integrins influence various responses to thrombin, including proliferation, c-Jun NH$_2$-terminal kinase-1 activation, and focal adhesion formation. Other interactions between $\beta_3$-integrins and thrombin include $\beta_3$-integrin promotion of the generation of thrombin by localizing prothrombin to cellular surfaces and/or enhancing the formation of procoagulant microparticles and the requirement of $\beta_3$-integrin function for platelet-dependent clot retraction. In summary, there is increasing evidence that interactions between $\beta_3$-integrins and thrombin play important roles in the regulation of hemostatic and vascular functions. (Arterioscler Thromb Vasc Biol. 2003;23:1240–1249.)

Key Words: $\beta_3$-Integrins

Integrins are heterodimeric cell surface receptors with noncovalently associated $\alpha$- and $\beta$-subunits. They are the predominant receptors for the extracellular matrix, binding ligands in both soluble and immobilized form, and thus serve as a critical link between the extracellular and intracellular environments. Integrins transduce signals through interactions of their cytoplasmic tails with cytoskeletal and signaling proteins, and ligation of integrins elicits a variety of signaling events. In addition to mediating responses to agents that directly bind to them, integrins also influence cellular responses to hormones, growth factors, and peptide mediators through cross-talk with growth factor and G protein–coupled receptors.

The $\beta_3$-integrin family consists of $\alpha_{IIb}\beta_3$ (also known as glycoprotein IIb/IIIa), which is found on platelets and megakaryocytes, and $\alpha_\beta V$, which has a broader distribution and is found on endothelial cells, smooth muscle cells (SMCs) and in small numbers, on platelets. The genes for $\alpha_{IIb}$ and $\beta_3$ are physically linked on the proximal portion of the long arm of chromosome 17; the gene for $\alpha_\beta V$ is on chromosome 2. In their mature forms, $\alpha_{IIb}$ and $\beta_3$ consist of 2 disulfide-bonded subunits (a heavy chain with $M_r \approx 125$ kDa and a light chain with $M_r \approx 25$ kDa). The overall protein sequences of $\alpha_{IIb}$ and $\alpha_\beta V$ exhibit 36% identity and 50% homology. The apparent molecular weight of the $\beta_3$-subunit, as detected by nonreduced and reduced polyacrylamide gel electrophoresis, is $\approx 90$ and 110 kDa, respectively, owing to the presence of extensive, intrachain, disulfide bonds that maintain the protein in a relatively compact form.

$\alpha_{IIb}\beta_3$ is the major platelet integrin (80 000 to 100 000 copies per platelet), and its essential role in hemostasis is well established. Variably severe mucocutaneous bleeding disorders occur in individuals with Glanzmann thrombasthenia, an inherited disease in which 1 or more genetic defects lead to impairment in the function of $\alpha_{IIb}\beta_3$ and sometimes $\alpha_\beta V$. Pharmacologic inhibitors of $\alpha_{IIb}\beta_3$ have been developed and, in intravenous form, are widely used to prevent coronary artery thrombosis, especially in patients undergoing percutaneous coronary artery interventions. Mice lacking either $\alpha_{IIb}$ or $\beta_3$, as a consequence of targeted gene ablation, display bleeding diathesis and platelet abnormalities similar to patients with Glanzmann thrombasthenia. $\beta_3$-Deficient mice also display protection from thrombosis in certain models. $\alpha_\beta V$ has a much broader distribution and serves multiple functions on vascular cells. Among other effects, $\alpha_\beta V$ has been proposed to play a role in endothelial cell function during angiogenesis, the vascular response to injury and neointimal formation, and leukocyte trafficking. Unlike $\alpha_{IIb}$ and $\beta_3$, genetic deficiency of $\alpha_\beta V$ has not been reported in humans, and targeted gene ablation of $\alpha_\beta V$ in mice is 100%
lethal, suggesting an essential developmental role for αv integrins. αv-Knockout mice develop normally until midgestation (E9.5), at which time the majority die, apparently due to placental defects. Those that survive (≈ 20% to 30%) develop cerebral blood vessel dilatation and hemorrhage and die perinatally.13,14

Activation of αIIbβ3

αIIbβ3 exists in “active” and “inactive” conformations. Activation is regulated by “inside-out” signaling, in which the conformation of the extracellular binding domain is controlled by interactions between the cytoplasmic domain of the integrin and intracellular mediators and cytoskeletal proteins (the Figure).15,16 The active form of αIIbβ3 binds several proteins that exist in soluble form in plasma (eg, fibrinogen, von Willebrand factor, and fibronectin) and thus, activation of αIIbβ3 is an important regulatory point in the control of thrombosis. Activation of αIIbβ3 to a ligand-competent state is accomplished primarily by a change in the receptor’s conformation (affinity modulation) and to a lesser extent, by receptor clustering (avidity modulation). A wide variety of agents that are released or exposed at sites of vascular injury (including thrombin, collagen, and ADP) promote the activation of αIIbβ3. After exposure to thrombin, platelets bind many soluble ligands and cells in an αIIbβ3-dependent manner (Table 1). The inactive form of αIIbβ3 does not bind most physiologic ligands, with the exception of prothrombin and immobilized fibrinogen.

Recent work17–22 has shown that αβ3, like αmβ3, has active and inactive conformations. In particular, this work has shown that the basal affinity state of αβ3-integrins varies among cell-types and that the affinity of αβ3-integrins for ligands is subject to short-term modulation by inside-out signals. Furthermore, there are activation-dependent (eg, prothrombin) and activation-independent (eg, fibrinogen) ligands. In vascular cells, the affinity of αβ3 can be modulated by phorbol esters, Mn2+, ADP, vascular endothelial growth factor, basic fibroblast growth factor, and elevations in intracellular cyclic AMP (cAMP).18,20,21,23

Two recently reported crystal structures of αβ3, without and with bound ligand, are shedding light on structural changes that occur with integrin activation.24,25 In both, αβ3 assumes a bent conformation, with the ligand-binding site oriented toward the membrane. Although the authors pointed out that this structure might not be the form found on cell surfaces, others believe that, in its resting state, αβ3 might assume a bent conformation and that a switchblade-like opening of the headpiece-tailpiece interface might occur with activation and/or ligand binding.26,27 Such an activation-dependent outward swing of αmβ3 would explain the long-standing observation that larger ligands (eg, fibrinogen or the monoclonal antibody 7E3 F(ab’2)2) preferentially recognize activated over resting αmβ3, whereas smaller ligands (eg, peptides or 7E3 Fab) bind equally well to both forms of the receptor.28

**TABLE 1.** Substrates and Cells to Which Platelet Adherence via αmβ3 is Enhanced by Thrombin100–104

<table>
<thead>
<tr>
<th>Cells</th>
<th>Extracellular Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monolayer of HUVEC</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Monolayer of HUVEC under flow conditions</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>Denuded rabbit aortic segments</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td></td>
<td>Vitronectin</td>
</tr>
</tbody>
</table>
TABLE 2. Selected Ligands for $\beta_3$ Integrins that Function in Hemostasis and/or Vascular Pathology

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Binds $\alpha_\text{IIb}\beta_3$</th>
<th>Binds $\alpha_\text{v}\beta_3$</th>
<th>Primary Source</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>Composed of 3 pairs of nonidentical chains; MW@340 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Soluble plasma protein</td>
<td>Converted to fibrin during clot formation; functions in platelet aggregation</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Glycoproteins; MW@235−270 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Plasma and extracellular matrix</td>
<td>Promotes cellular adhesion and migration; also functions in platelet aggregation.</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>Glycoprotein; MW@75 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Plasma and extracellular matrix; concentrated at sites of vascular injury</td>
<td>Adhesive protein; also functions in regulation of coagulation and fibrinolytic system</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>Family of 5 proteins; TSP1 is a trimer of identical subunits; MW of TSP1@450 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Platelet $\alpha$-granules and extracellular matrix; concentrated at sites of vascular injury</td>
<td>Modulate migration and proliferation of vascular cells; also involved in activation of latent TGF$\beta$</td>
</tr>
<tr>
<td>Von Willebrand Factor</td>
<td>Multimeric glycoprotein; MW@275 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Plasma–synthesized by endothelial cells and megakaryocytes</td>
<td>Mediates platelet adhesion at sites of injury; carrier for Factor VIII in blood</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Glycoprotein; MW@44 kD</td>
<td>No</td>
<td>Yes</td>
<td>Sites of vascular injury</td>
<td>Promotes migration and survival of vascular cells</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Glycoprotein; MW@72 kD</td>
<td>Yes</td>
<td>Yes</td>
<td>Plasma and sites of vascular injury</td>
<td>Promotes adhesion of vascular cells; precursor of thrombin</td>
</tr>
</tbody>
</table>

The effects of thrombin on activation of $\alpha_\text{IIb}\beta_3$ are not as well understood as thrombin-induced activation of $\alpha_\text{v}\beta_3$. Several years ago, Bennett and colleagues \(^{30}\) demonstrated that thrombin, ADP, and other platelet agonists markedly enhanced $\alpha_\text{IIb}\beta_3$-mediated platelet adhesion to osteopontin, suggesting that $\alpha_\text{IIb}\beta_3$, like $\alpha_\text{v}\beta_3$, undergoes affinity modulation in platelets. Recently, the same investigators demonstrated that ADP-induced activation of $\alpha_\beta_3$ requires receptors coupled to both G$\alpha_5$ and G$\alpha_1$, and that cytoskeletal constraints might maintain $\alpha_\beta_3$ in a resting state on platelets. \(^{30}\) Thrombin cleaves osteopontin near an Arg-Gly-Asp-Ser (RGDS) motif, which might enhance accessibility of the integrin-binding site, depending on the activation state of $\alpha_\beta_3$. It should be noted, however, that WOW-1, an engineered monoclonal antibody that recognizes activated $\alpha_\beta_3$, does not bind detectably to resting or thrombin-stimulated platelets. \(^{19}\) This might be because the low levels of $\alpha_\beta_3$ present on the platelet surface (50 to 150 copies per platelet; 50- to 100-fold less than $\alpha_{\text{IIb}\beta_3}$)\(^{30}\) are below the threshold for detection of WOW-1 binding. Moreover, a substantial intracellular pool of $\alpha_\beta_3$ has been reported, and it is possible that thrombin and other agonists upregulate platelet surface expression of $\alpha_\beta_3$ in addition to altering its affinity.

Analogous to the situation in platelets, we have found that thrombin enhances SMC adhesion to osteopontin via an $\alpha_\text{IIb}\beta_3$-dependent mechanism (authors’ unpublished observations). Others have shown that treatment of endothelial cells with thrombin enhances the binding of activated platelets and monocytes via an $\alpha_\beta_3$-dependent mechanism. In 1 study with plasma from patients with acute myocardial infarction, adhesion of platelets to the luminal surface of activated human umbilical vein endothelial cells was inhibited by $\approx 50\%$ by various $\alpha_\beta_3$ antagonists. Thus, endothelial cell–platelet interactions are enhanced by thrombin treatment of platelets (via activation of $\alpha_\text{IIb}\beta_3$) and thrombin treatment of endothelial cells (an effect inhibited by $\alpha_\beta_3$ antagonists). Treatment of endothelial cells with thrombin also enhanced the $\alpha_\beta_3$-mediated binding of monocytes isolated from peripheral blood and adhesion of cells from a monocytic cell line. \(^{30}\)

**Ligands for $\alpha_\text{IIb}\beta_3$ and $\alpha_\beta_3$**

Fibrinogen, fibronectin, thrombospondin, von Willebrand factor, and vitronectin are matrix proteins that bind 1 or more conformations of $\alpha_\beta_3$ and $\alpha_\text{v}\beta_3$, whereas osteopontin binds $\alpha_\beta_3$ but not $\alpha_\text{IIb}\beta_3$ (Table 2). These proteins in general tend to be large, exist in both soluble and immobilized states, and function in cellular adhesion processes. They circulate in blood and can also be found in the vascular wall (especially at sites of vascular injury) and/or in the $\alpha$-granules of platelets. Ligand binding to $\alpha_\beta_3$ and $\alpha_{\text{IIb}\beta_3}$ tends to occur via RGD sequences; however, other mechanisms might be involved. For example, binding of fibrinogen to $\alpha_{\text{IIb}\beta_3}$ is primarily mediated by a non-RGD binding site in the carboxy terminus of the $\gamma$-chain.

Prothrombin binds $\alpha_\beta_3$ on endothelial cells and SMCs and $\alpha_{\text{IIb}\beta_3}$ on platelets. On platelets, binding of prothrombin to $\alpha_{\text{IIb}\beta_3}$ accelerates prothrombin activation and thrombin formation. \(^{32,33}\) Interestingly, it binds both the inactivated and activated forms of $\alpha_{\text{IIb}\beta_3}$ but only the activated form of $\alpha_\beta_3$. \(^{18,19}\) In contrast, fibronectin and von Willebrand factor bind only the activated form of $\alpha_{\text{IIb}\beta_3}$, whereas fibrinogen binds the activated form of $\alpha_{\text{IIb}\beta_3}$ when soluble but binds the inactivated forms of $\alpha_\beta_3$ and $\alpha_{\text{IIb}\beta_3}$ when immobilized.

Thrombin can bind $\alpha_\beta_3$ under specific conditions but has not been shown to bind $\alpha_\text{v}\beta_3$. \(^{35}\) Soluble $\alpha$-thrombin binds $\alpha_\beta_3$ on endothelial cells via a cryptic RGD site that is exposed in the presence of low concentrations of plasmin and...
cell-associated heparan sulfate proteoglycans.\textsuperscript{36} Immobilized α-thrombin has been shown to bind purified α,β, and α,β, on endothelial cells.\textsuperscript{37}

**Thrombin Signaling and Integrin Activation in Platelets**

Thrombin, 1 of the most potent platelet agonists ex vivo, elicits its effects, at least in part, via G protein–coupled protease-activated receptors (PARs).\textsuperscript{38} Human platelets express PAR1 and PAR4.\textsuperscript{39–42} PAR1 is thought to mediate platelet activation at low thrombin concentrations, whereas PAR4 requires higher concentrations to elicit its effects. Platelet PAR1 couples to Gα, and PAR4 to Gα, but not Gα, pathways. Although their activation kinetics are different, engagement of either PAR1 or PAR4 can trigger platelet activation,\textsuperscript{43} suggesting that both receptors are capable of generating the intracellular signals necessary for activation of α,β3. Mouse platelets also express 2 PARs, PAR3 and PAR4.\textsuperscript{42,44} Unlike PARs 1 and 4, PAR3 does not appear to be capable of mediating transmembrane signaling; instead, it is thought to function as a cofactor by binding thrombin and promoting activation of PAR4.\textsuperscript{45} Interestingly, mice deficient in either PAR3 or PAR4 are protected against ferric chloride–induced arteriolar thrombosis.\textsuperscript{46,47}

The adhesive GPⅡbα in the GPⅡb/Ⅲa receptor complex also serves as a major platelet receptor for α-thrombin. The crystal structure of the extracellular, amino-terminal domain of GPⅡbα bound to thrombin has been solved by 2 groups,\textsuperscript{48,49} and it was revealed that 2 thrombin molecules interact with 1 GPⅡbα. The interactions with GPⅡbα are mediated by exosite I of 1 thrombin molecule and exosite II of the other thrombin molecule. Although there are discrepancies between the structures reported by the 2 groups with respect to contact sites and orientations,\textsuperscript{50} the results of both groups suggest that the interaction is bivalent and that thrombin binding might serve to bridge GPⅡbα receptors on the same or adjacent platelets. Thus, thrombin bound to GPⅡbα might promote platelet aggregation through receptor multimerization and/or enhanced PAR cleavage.

The first step in activation of α,β, by thrombin most likely involves Gα,-dependent phosphoinositide hydrolysis, because platelets from mice that lack Gα, fail to aggregate in response to thrombin.\textsuperscript{51} However, the important signals downstream of phosphoinositide hydrolysis are well understood. Moreover, α,β, can be activated in the absence of Gα, by concomitant engagement of receptors coupled to Gα, and Gα,\textsuperscript{52} suggesting that other initial pathways can trigger the common outcome of α,β, activation.

Several intermediate signaling molecules downstream of G protein–coupled receptor engagement have been implicated in inside-out activation of α,β, including isoforms of protein kinase C, Ca\textsuperscript{2+}, and phosphatidylinositol 3-kinase (PI-3K; the Figure).\textsuperscript{53–55} Syk is a nonreceptor tyrosine kinase activated by thrombin in mouse platelets,\textsuperscript{56,57} and Syk\textsuperscript{−/−} mice display modest reductions in fibrinogen binding in response to weak agonists (eg, ADP) but not in response to direct activation of protein kinase C by phorbol myristate acetate.\textsuperscript{58} The Ras family GTPase, Rap1b, is highly expressed in platelets and is activated in response to thrombin in a manner that depends on Ca\textsuperscript{2+} influx and protein kinase C.\textsuperscript{59} Expression of constitutively active Rap1b in megakaryocytes augments fibrinogen binding to α,β, induced by a PAR4 agonist,\textsuperscript{60} and expression of a Rap1b guanine-nucleotide exchange factor, CalDAG-GEF1, in megakaryocytes derived from embryonic stem cells also enhances agonist-induced fibrinogen binding.\textsuperscript{61} In addition, the platelet cytoskeleton appears to regulate activation of α,β, because inhibition of actin polymerization by low doses of cytochalasin D or latrunculin A promote fibrinogen binding to α,β,.\textsuperscript{62} Engagement of the adhesion receptor complex GPIb/IX also results in activation of α,β,.\textsuperscript{63} Both prostacyclin and nitric oxide, which mediate effects via cAMP and cGMP respectively, can negatively regulate α,β, activation in platelets.\textsuperscript{64} However, pathways involving cGMP and cGMP-dependent protein kinase might also stimulate α,β, activation under particular conditions, such as after engagement of the GPIb/IX complex.\textsuperscript{65}

Although the signaling pathways involved in activation of α,β, have only been partially elucidated, more is understood about the molecular rearrangements that regulate integrin affinity states. Affinity modulation appears to result from long-range conformational rearrangements in the integrin’s cytoplasmic tails that are transmitted through the membrane-spanning portion of the molecule to the extracellular domain.\textsuperscript{66} The α- and β-tails are thought to clasp 1 another to maintain an inactive state, and disruption of the “handshake” appears to promote receptor activation.\textsuperscript{66,67} Several proteins are known to interact with the cytoplasmic domains of α and β, and both the calcium- and integrin-binding protein CIB (which binds α) and talin (which binds β) have been reported to activate α,β, in vitro.\textsuperscript{68,69} The FERM domain in the talin head, in particular, binds with high affinity to the integrin β3 tail and in doing so, activates the ligand-binding properties of the receptor.\textsuperscript{70} Like the membrane receptor-binding sites in ezrin, radixin, and moesin, the integrin-binding site in talin appears to be exposed by proteolytic cleavage or binding to phosphoinositides. However, the physiologic mechanism that couples thrombin stimulation with talin-integrin binding has not been elucidated.

**Amplification of Thrombin Signaling in Platelets by ‘Outside-In’ Signaling Through α,β,3**

On ligand binding, α,β, elicits a series of outside-in intracellular events that includes activation of kinases and phosphatases, changes in cytoskeletal organization, and regulation of protein synthesis. Outside-in signaling through α,β,3 amplifies events initiated by thrombin and other agonists and is necessary for full platelet spreading, platelet aggregation, granule secretion, and the formation of a stable platelet thrombus.\textsuperscript{15,53} α,β,3-Dependent signaling appears to occur in at least 2 waves. One set of signals is triggered by ligand binding alone, and a second series of events requires ligand-induced integrin clustering and aggregation. Because integrin cytoplasmic tails lack known catalytic activity, it is thought that ligand-induced conformational changes in α,β, are propagated to the integrin cytoplasmic domains in a manner
that alters their ability to interact with protein adaptors, signaling molecules, and cytoskeletal proteins. In addition, receptor clustering might promote the assembly of localized signaling complexes.

The best characterized outside-in signaling involves the nonreceptor tyrosine kinases Src, Syk, and focal adhesion kinase (FAK). Emerging evidence indicates that Src constitutively associates with \( \alpha_\beta_3 \), and a pool of Src is activated by fibrinogen binding to \( \alpha_m \beta_3 \). The mechanism controlling Src activation is not entirely clear but might involve dissociation of an Src-regulatory kinase Csk from the \( \alpha_m \beta_3 \) complex. Syk directly binds to the \( \beta_3 \)-cytoplasmic tails and becomes activated in an Src-dependent manner after ligand binding to \( \alpha_m \beta_3 \). Syk then phosphorylates downstream targets (Vav1, Vav2, and SLP-76) that are involved in cytoskeletal reorganization.\(^72\) FAK phosphorylation and activation are late events that require \( \alpha_m \beta_3 \) clustering and platelet aggregation.\(^73\)

The \( \beta_3 \)-integrin cytoplasmic domain contains 2 tyrosine residues separated by 11 residues (NPLY\(^77\) and NITY\(^79\)). Platelet aggregation is accompanied by tyrosine phosphorylation of the \( \beta_3 \)-tail,\(^74\) which promotes association of the adaptor proteins Shc and Grb2 and the cytoskeletal protein nonmuscle myosin A with \( \alpha_m \beta_3 \).\(^75-77\) Neither talin nor Syk binds to \( \beta_3 \) when both cytoplasmic tyrosine residues are phosphorylated,\(^70,78\) whereas nonmuscle myosin A only binds to \( \beta_3 \) when both cytoplasmic tyrosine residues are phosphorylated.\(^79\) These observations suggest that tyrosine phosphorylation might serve as a molecular switch to dictate the association of specific adaptor/signaling proteins with the \( \beta_3 \)-cytoplasmic tail. In mice, mutation of both tyrosine residues to phenylalanine (diYP) impairs platelet aggregation in response to low-dose thrombin and results in unstable hemostasis and a tendency of the mice to rebleed.\(^78\) When expressed in the hematopoietic cell model K562, both Tyr 747 and 759 in \( \alpha_m \beta_3 \) are phosphorylated on ligand binding, whereas only Tyr 747 is phosphorylated in \( \alpha_m \beta_3 \).\(^79\)

The importance of tyrosine phosphorylation of \( \beta_3 \) when complexed with \( \alpha_m \) is less well studied. In Chinese hamster ovary cells, the \( \beta_3 \)-diYP mutation appears to disrupt signaling through \( \alpha_m \beta_3 \).\(^80\) Some investigators have found that tyrosine phosphorylation of \( \beta_3 \) negatively regulates \( \alpha_m \beta_3 \) ligand-binding strength,\(^81\) whereas others have reported that tyrosine phosphorylation of \( \beta_3 \) is required for \( \alpha_m \beta_3 \)-dependent cell adhesion.\(^79,82\)

\( \alpha_m \beta_3 \) is also required for the sustained accumulation of particular D3-phosphoinositides, which serve as intracellular messengers. In thrombin-stimulated platelets, PI-3K is activated rapidly, resulting in transient increases in PtdIns(3,4,5)\(_3 \).\(^43,84\) In contrast, PtdIns(3,4)\(_3 \) accumulation increases steadily over time. The synthesis/accumulation of PtdIns(3,4)\(_3 \) depends on outside-in signaling through \( \alpha_m \beta_3 \), but the mechanism is unknown. D3-phosphoinositides have been implicated in multiple biologic responses. PtdIns(3,4)\(_3 \) appears to be required to strengthen platelet aggregation. Thus, a positive feedback loop, in which integrin engagement facilitates thrombin-dependent generation of PtdIns(3,4)\(_3 \), might be required for irreversible platelet aggregation.

**Effects of Outside-In Signaling Through \( \alpha_m \beta_3 \) on SMC Responses to Thrombin**

Thrombin has a multitude of effects on SMCs, including, under specific conditions, stimulation of migration, proliferation, and production of cytokines and growth factors.\(^85\) Most of these effects are mediated by activation of PAR-1. Treatment of SMCs with thrombin stimulates the expression of \( \alpha_m \beta_3 \)-integrins\(^86\) and also stimulates phosphorylation of tyrosine residues within \( \beta_3 \)-integrins. Effects of outside-in signaling through integrins on responses to thrombin are not as well studied in SMCs as in platelets. We found that \( \alpha_m \beta_3 \)-antagonists partially inhibited thrombin-induced proliferation and that the effect was independent of the matrix on which the cells were grown.\(^87\)

This inhibitory effect was observed in SMCs derived from the rat and SMCs derived from humans and was not caused by detachment or apoptosis. \( \alpha_m \beta_3 \) Antagonists also partially inhibited thrombin-induced activation of c-Jun NH\(_2\)-terminal kinase-1 (JNK1; also known as stress-activated protein kinase-1), a member of the mitogen-activated protein kinase superfamily that has been implicated in integrin-mediated activation of the cell cycle.\(^88\) JNK1 is activated by dual phosphorylation at a Thr-Pro-Tyr motif and, once activated, functions to phosphorylate c-jun at amino-terminal serine regulatory sites, which increases activity of the transcription factor activator protein-1. \( \alpha_m \)-Thrombin stimulates a rapid, time-dependent increase in JNK1 activity in rat aortic SMCs,\(^89\) and recent studies\(^90\) have shown that integrin activation is necessary for JNK1 activation in some systems.

One possible mechanism to explain these effects is that \( \alpha_m \beta_3 \)-integrins play an important role in focal adhesion formation. Treatment of cultured SMCs with thrombin results in rapid formation of stress fibers, reorganization of the actin cytoskeleton, and assembly of focal adhesions.\(^91\) The current paradigm for cytoskeletal rearrangement is that focal adhesions form after activation of the small GTPase Rho, which activates myosin, resulting in F-actin bundling. Seasholtz et al\(^92\) showed that thrombin and thrombin receptor–activating peptide activated Rho and that C3 exoenzyme, which ADP-ribosylates and inactivates Rho, fully inhibited both thrombin-stimulated proliferation and migration in rat aortic SMCs. We found that \( \alpha_m \beta_3 \)-antagonists impaired focal adhesion formation in SMCs in response to thrombin treatment, although it is unknown whether this is mediated by blockage of access of a soluble ligand to \( \alpha_m \beta_3 \) or prevention of integrin clustering and/or signaling. We have also found that focal adhesion formation in response to thrombin treatment is impaired in SMCs isolated from \( \beta_3 \)-integrin–deficient mice.\(^88\)

**\( \beta_3 \)-Integrins and Platelet-Dependent Thrombin Generation and Clot Formation**

Platelets play a major role in controlling thrombin generation by providing necessary procoagulant factors.\(^93\) \( \beta_3 \)-Integrins have been implicated in platelet-dependent thrombin generation, because blocking ligand binding to \( \alpha_m \beta_3 \) and/or \( \alpha_m \beta_3 \) reduces thrombin generation by 40% to 70%.\(^34,59\) \( \beta_3 \)-Integrins contribute to the production of procoagulant microparticles as well as serving as platelet receptors for prothrombin, and thus, inhibition of ligand binding to platelet \( \alpha_m \beta_3 \) and \( \alpha_m \beta_3 \)
might decrease thrombin generation through multiple mechanisms.

Localized thrombin generation on the platelet surface results in fibrin production and the generation of a platelet-and fibrin-rich clot. Within minutes to hours of forming, these clots undergo platelet-dependent contraction, which might decrease the efficiency of thrombolysis. This process is analogous to matrix contraction exhibited by other vascular cells and appears to require actin-myosin contractility. αgrβv is required for clot retraction, because platelets from patients suffering from Glanzmann thrombasthenia do not support clot retraction. It should be emphasized, however, that the process of clot retraction does not simply reflect fibrinogen binding to αgrβv. Variants of fibrinogen that lack the γ-chain αgrβv-recognition site are capable of supporting clot retraction. In addition, whereas some agents that block fibrinogen binding to αgrβv also inhibit clot retraction, the ability to block clot retraction is not correlated with the ability to inhibit fibrinogen binding. Recently, αgrβv-dependent, protein tyrosine dephosphorylation has been observed to parallel clot retraction, suggesting that αgrβv engagement might generate and/or transmit the force necessary for clot retraction by promoting protein tyrosine dephosphorylation.

Summary

G protein–coupled receptors and integrins mediate pleotropic effects on vascular cell function and can induce spreading, migration, proliferation, differentiation, and survival. Both classes of receptors exist in low- and high-affinity states. Signals initiated by G protein–coupled receptors, such as the PAR receptors for thrombin, can trigger intracellular pathways that result in activation of integrins. Engagement of integrins by ligands, in turn, can modulate or amplify signals generated by thrombin. Less commonly, integrin–ligand interactions might be agonists in close proximity with their G protein–coupled receptor, eg, as might occur when, under certain circumstances, αgrβv binds thrombin. β3-Integrins also promote the generation of thrombin by localizing prothrombin to cellular surfaces and/or in the formation of procoagulant microparticles and platelet-dependent clot retraction requires βν-integrin function. Thus, βν-integrins play an important role in the regulation of many of thrombin’s diverse effects on the vascular wall, and therefore, cellular responses to thrombin can be regulated by modifying integrin-dependent events.[100–104]

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