Physiol Rev 93: 327–358, 2013
doi:10.1152/physrev.00016.2011

NEW FUNDAMENTALS IN HEMOSTASIS

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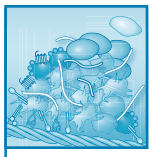

Versteeg HH, Heemskerk JWM, Levi M, Reitsma PH. New Fundamentals in Hemostasis. *Physiol Rev* 93: 327–358, 2013; doi:10.1152/physrev.00016.2011.— Hemostasis encompasses the tightly regulated processes of blood clotting, platelet activation, and vascular repair. After wounding, the hemostatic system engages a plethora of vascular and extravascular receptors that act in concert with blood components to seal off the damage inflicted to the vasculature and the surrounding tissue. The first important component that contributes to hemostasis is the coagulation system, while the second important component starts with platelet activation, which not only contributes to the hemostatic plug, but also accelerates the coagulation system. Eventually, coagulation and platelet activation are switched off by blood-borne inhibitors and proteolytic feedback loops. This review summarizes new concepts of activation of proteases that regulate coagulation and anticoagulation, to give rise to transient thrombin generation and fibrin clot formation. It further speculates on the (patho)physiological roles of intra- and extravascular receptors that operate in response to these proteases. Furthermore, this review provides a new framework for understanding how signaling and adhesive interactions between endothelial cells, leukocytes, and platelets can regulate thrombus formation and modulate the coagulation process. Now that the key molecular players of coagulation and platelet activation have become clear, and their complex interactions with the vessel wall have been mapped out, we can also better speculate on the causes of thrombosis-related angiopathies.

I. INTRODUCTION

Hemostasis enables an organism to *1*) close off damaged blood vessels, *2*) keep the blood in a fluid state, and *3*) remove blood clots after restoration of vascular integrity. The hemostatic system is a highly conserved machinery, from zebrafish to human, in which blood clotting, also referred to as coagulation, has a prominent role. Two millennia ago, the Greek philosopher Plato already described that the blood forms fibers once it leaves the heat of the body. He was also the first one to coin the term *fibrin*, which nowadays refers to a key blood clotting protein composing those fiber structures. Interestingly, Plato's view on blood clotting, which was shared by other early philosophers, such as Aristotle and Galen, remained the leading concept until the end of the 18th century. In the course of the 19th century, groundbreaking discoveries were made on the biological mechanism of coagulation. Around 1865, platelets were discovered as well as their critical function in hemostasis (30). It was proposed that a hypothetical protein termed "thrombin" could induce the formation of fibrin. The majority of the key players in coagulation were discovered during the course of the 20th century. In 1905, Morawitz constructed the first coagulation model in which thromboplastin, now known as tissue factor (TF), was released by damaged vessels to convert prothrombin into thrombin in the presence of calcium (246). Thrombin then converted fibrinogen into fibrin resulting in the formation of a blood clot. However, this four-clotting factor model could not fully explain the complex process of coagulation. Around the 1950s, many of the remaining factors had been characterized, such as von Willebrand factor (VWF) and factors V, VII, VIII, IX, and XI (FV, FVII, FVIII, FIX, FXI). Deficiency in some of these factors was linked to bleeding diseases, such as FVIII deficiency in hemophilia A and FIX deficiency in hemophilia B (72). In the 1960s, two independent groups constructed a model for coagulation that resembled a waterfall or cascade. Therefore, this model was aptly named the coagulation cascade model (67, 168). Herein, each clotting factor consists of a proenzyme that is converted to an active enzyme by the upstream activated clotting factor **(FIGURE 1)**. It was also suggested that two different cascades exist that converge in FX activation. These are named the intrinsic pathway, so called because all the components are present in the blood, and the extrinsic pathway requiring an external factor (TF from the extravascular tissue). The intrinsic pathway becomes activated in vitro once blood comes into contact with hydrophilic surfaces. This is triggered by autoactivated FXII cleaving prekallikrein into kallikrein, which

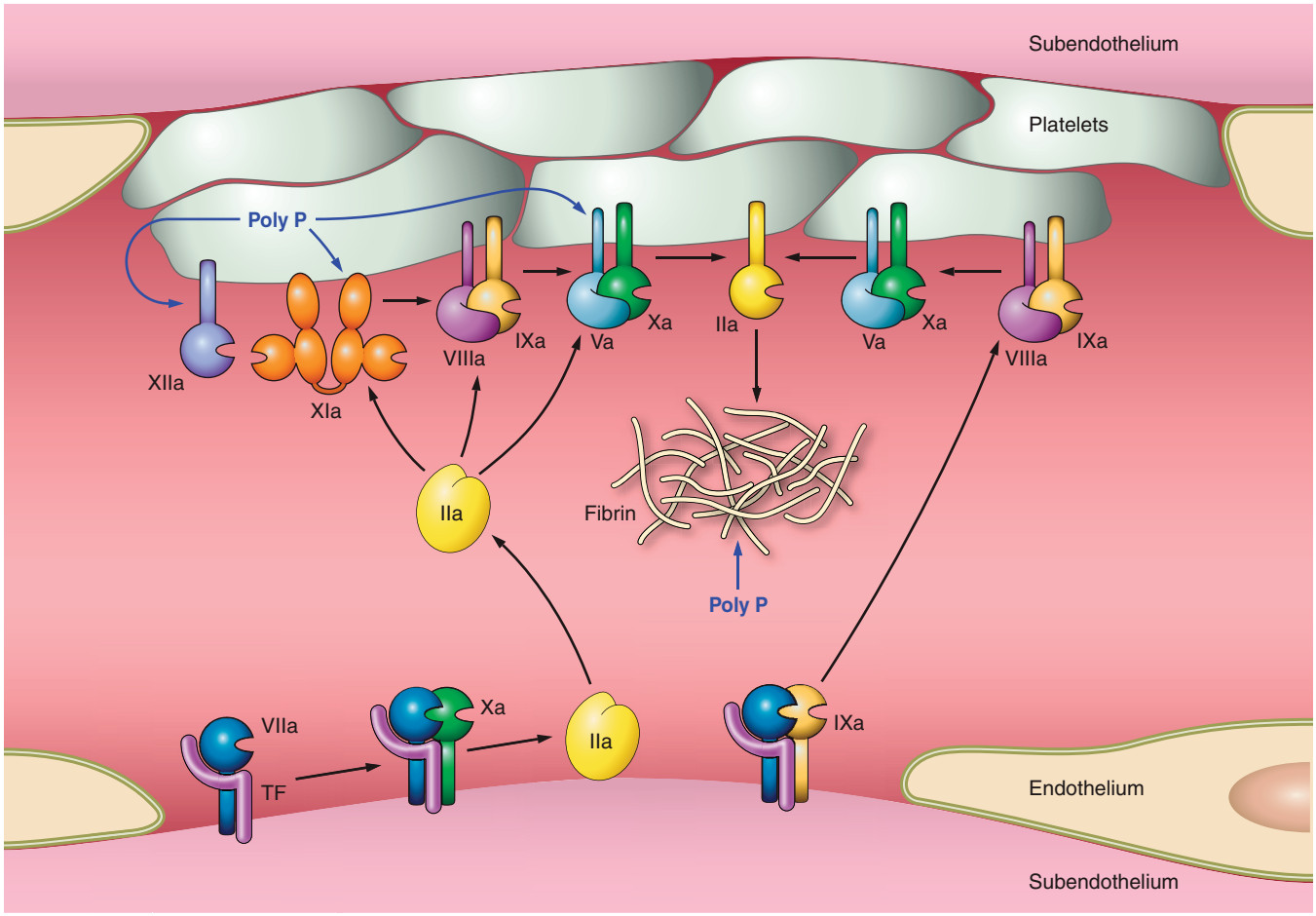

FIGURE 1. The coagulation cascade. Upon endothelial damage, tissue factor (TF) is exposed to the bloodstream and binds factor VII, which is activated to factor VIIa. The TF:VIIa complex enables subsequent activation of factor X and prothrombin, after which small amounts of thrombin activate the factor XI-IX feedback loop on the platelet surface. Factor IXa will then activate additional factor X. Simultaneously, the trace amounts of thrombin will then activate factors VIII (cofactor to factor IX) and V (cofactor to factor X), which dramatically enhances catalytic activity of factors IX and X. Finally, thrombin (factor IIa) activation leads to fibrin deposition. In parallel, local polyphosphate (polyP) release by activated platelets may additionally stimulate activation of factor XII, factor V, and FXI and inhibit clot lysis.

leads to a subsequent activation pathway of FXI, FIX, FX, and prothrombin. The extrinsic pathway starts with TF and activated FVII, which directly induces sequential activation of FX and prothrombin.

The current concept of hemostasis, outlined in detail below, is as follows. Upon vessel damage, platelets adhere to the damaged site and aggregate through interactions of platelet receptors with extracellular ligands and soluble proteins. Vascular damage-induced exposure of subendothelial TF generates trace amounts of thrombin with multiple effects on other coagulation factors and platelets. Via multiple enforcement loops in the coagulation system and in platelet activation, large amounts of fibrin are formed stabilizing earlier formed platelet thrombi. In this review we will first discuss the latest insights in function and regulation of the coagulation cascade, and then discuss the complex interaction of platelets with the endothelium and the extracellular matrix. As hemostasis is now considered to include wound healing and endothelial barrier protection, we will also discuss the role of coagulation factors in these important processes. Of note, we will not extensively discuss the fibrinolytic pathway.

II. NEW FUNDAMENTALS IN COAGULATION

A. Various Phases of Coagulation

According to a widely used current model (179), coagulation can be divided into three separate phases: *1*) an initiation phase, in which low amounts of active coagulant factors are generated; *2*) an amplification phase, in which the level of active coagulation factors is boosted; and *3*) a propagation phase, in which coagulation factors bind to highly procoagulant membranes of activated platelets and fibrin clots are

formed. While this cell biological model of coagulation is gaining attention, the more classical division between intrinsic and extrinsic pathway is still widely used (170).

1. Initiation phase

The initiation phase, classically referred to as the extrinsic pathway of coagulation, starts when the vasculature is disrupted, and subendothelial cells like smooth muscle cells and fibroblasts become exposed to the bloodstream **(FIGURE 1)** (179). These cells expose a key initiator of the coagulation cascade, TF, which binds coagulation FVII. By acting as a cofactor for FVII, TF promotes proteolysis and activation to FVIIa. TF largely resides on the cell surface in an inactive (cryptic) configuration, but under certain conditions it is readily decrypted as described below. It is not precisely understood how FVII is cleaved into FVIIa, but a proteolytic role is suggested for either the minute amounts of FVIIa that circulate in the blood (100 pM) (198) or the factor VII-activating protein (FSAP), but recent data argue against the latter (276). At physiological concentrations, FVIIa without TF shows little activity, because of a unique sequence characteristic that retains the FVIIa in a zymogen-like conformation (230). Thus FVIIa activity at physiological levels is entirely TF dependent.

The TF/FVIIa complex proteolytically cleaves traces of FIX and FX into FIXa and FXa, respectively. This allows FXa to associate with cofactor FVa to form a prothrombinase complex on TF-expressing cells (179), which serves to convert prothrombin (FII) into thrombin. FXa may dissociate from these TF-expressing cells to form prothrombinase complexes on distant cell membranes. However, the presence of protease inhibitors in plasma such as the Kunitz-type protease inhibitor tissue factor pathway inhibitor (TFPI), and the serine protease inhibitor antithrombin (AT), will limit such diffusion (40, 135). FIXa is not targeted by TFPI and hence can diffuse more easily to other cell surfaces to participate in the propagation phase.

2. Amplification phase

The slowly accumulating amounts of thrombin will further activate platelets that have adhered to a site of injury, as discussed in section IV. In parallel, thrombin will convert (platelet-derived) FV into FVa, thus amplifying prothrombinase activity, and convert FVIII into FVIIIa, which acts as a cofactor to FIXa on the surface of activated platelets to support FXa generation. In addition, thrombin converts FXI into FXIa **(FIGURE 1)**.

3. Propagation phase

Whereas in current models the initiation phase takes place on TF-expressing surfaces, the propagation phase occurs away on surfaces containing procoagulant phospholipids, such as activated platelets. Activated FXI converts FIX into FIXa, which then associates with thrombin-cleaved FVIII **(FIGURE 1)**. Absence or near-absence of FVIII or FIX leads to severe bleeding complications (hemophilia A and B, respectively), thus underlining the importance of these coagulation factors for normal hemostasis. On phosphatidylserine-exposing cell membranes, the tenase complex of FIXa/FVIIIa catalyzes the conversion of FX to FXa, after which the FXa/FVa complex produces sufficient amounts of thrombin to massively form fibrin fibers. As a final step, the thrombin-activated plasma transglutaminase FXIIIa catalyzes the formation of covalent crosslinks between adjacent fibrin chains to yield an elastic, polymerized fibrin clot (8).

B. Reappraisal of the Intrinsic Coagulation Pathway

According to the cell biological model of coagulation, the intrinsic FXI-FXII pathway only serves as an amplification loop initiated by the extrinsic TF pathway. Several pieces of evidence indicate that this understates the role of the intrinsic pathway. Recent studies indicate that in mice, the intrinsic pathway is activated more or less in parallel with the extrinsic pathway. Three physiological triggers of the intrinsic pathway have been discovered, namely, collagen (302), linear phosphate polymers termed polyphosphates (245), and neutrophil extracellular traps (NETs) (308). Cell- and platelet-derived polyphosphates bind to and activate FXII, thereby leading to the subsequent activation of plasma kallikrein, FIX, and further downstream coagulation factors (184). In particular, a role has been proposed for platelet-derived polyphosphates, as platelets are abundantly present at the sites of vascular injury. Polyphosphate-dependent FXII activation does not appear to lead to a faster clot formation, but rather to increased fibrin clot stability (225, 240). This could also explain why high levels of FXII associate with thrombosis, while FXII deficiency may render the clot unstable and lead to embolization. Recent findings further indicate that polyphosphates can act as a cofactor for thrombin-mediated activation of FV and FXI (50), and that it inhibits clot fibrinolysis, presumably through activation of TAFI (275). Markedly, the shorter chain polyphosphates secreted by platelets appear to be more active in FV conversion, while longer chain polyphosphates more efficiently contribute to FXII activation, thus suggesting that different polyphosphate pools act on these distinct pathways (275). Another phosphate source, extracellular RNA, was recently also shown to stimulate activation of the intrinsic pathway components such as FXII and FXI, but this has been associated with thrombosis, rather than with hemostasis (140). NETs also lead to the activation of FXII to FXIIa, and this influences clot formation. Whether FXIa is formed remains unclear (308). Taken together, one can speculate that polyphosphate- and NET-dependent activation of FV and FXI plays a role in hemo-

stasis, while the polyphosphate- and RNA-dependent activation of FXII plays a role in thrombosis.

C. Coagulation Proteases

The coagulation factors are usually divided into two biochemical classes: the serine proteases [i.e., (pro)thrombin, FVII(a), FIX(a), FX(a), and FXI(a)] and serine protease cofactors [i.e., thrombomodulin, TF, FV(a), and FVIII(a)]. The natural anticoagulant protein C also belongs to the group of serine proteases. These proteolytic enzymes are the active components of the clotting machinery and share many structural features. Full-length FVII, FIX, FX, and protein C consist of an NH_2-terminal γ-carboxylated glutamic acid (Gla) domain, two epidermal growth factor-like domains (EGF1 and EGF2), and a serine protease domain. Prothrombin has a similar structure but contains two kringle domains instead of the EGF domains, while FXI is a homodimer with four apple domains in each subunit. Sequence analysis of these serine proteases in different organisms including primates, rodents, and fish suggests that these serine proteases all originate from a common ancestral gene and are the result of gene reduplications (66). The prothrombin gene may originate from the same ancestral gene, but appears to have undergone an EGF to kringle domain substitution.

Posttranslational modification is of key importance to the function of coagulation serine proteases. Glutamate residues in the NH_2-terminal domain are converted into γ-carboxylated glutamic acids by the enzyme γ-glutamyl carboxylase (GGCX), which is an endoplasmic reticulum resident protein, mostly expressed in the liver. After hepatic secretion into the circulation, two or three of the Gla residues of the serine protease bind a Ca^{2+}, which promotes a conformational change (287). This conformational change confers to the Gla domain-containing protease the ability to bind to procoagulant phospholipid surfaces, which is a requirement for efficient hemostasis, as discussed above.

D. Role and Activation of TF

TF, also known as thromboplastin or CD142, is a 47-kDa transmembrane glycoprotein that is expressed in extravascular tissue, particularly in fibroblasts and smooth muscle cells, serving as a hemostatic "envelope," poised to activate coagulation upon vascular injury (78, 318). Generally, active TF is not exposed to the bloodstream, but endothelial cells and adhered leukocytes may express active TF as a response to injury or to inflammatory stimuli such as endotoxin, chemokines, or cytokines (84, 311). As discussed later, this may cause severe deregulation of hemostasis.

The mature TF protein comprises a 219-amino acid extracellular region, a hydrophobic transmembrane region, and a short intracellular tail of 21 amino acids. The external part consists of two fibronectin-type III domains, each with an extracellular disulfide bond (Cys^{49}-Cys^{57} and Cys^{186}-Cys^{209}). Only breaking of the second bond distorts the coagulant function of TF. Intracellularly, TF may be anchored in the cell membrane via acylation of palmitic and stearic acids, likely serving to target TF to glycosphingolipid- and cholesterol-rich microdomains (76). TF-like DNA sequences have also been identified in fish and in insects like *Anopheles gambiae* and *Drosophila*. Structurally, TF shares a high degree of homology with the class II interferon receptors (20). On cells, TF can be regarded as a true receptor for FVIIa, also given the fact that two cytoplasmic serines (Ser^{253} and Ser^{258}) become phosphorylated in the presence of FVIIa as a (co-)ligand (1).

E. TF Encryption/Decryption

Although extravascular cells amply express TF, TF procoagulant activity often remains surprisingly low. On the other hand, stimulating these cells with agonists such as Ca^{2+} ionophore, hydrogen peroxide, or proteases, but also disruption of cells, can lead to a dramatic increase, often up to a 100-fold, in TF-dependent procoagulant activity (12, 176). Therefore, it has been suggested that procoagulant activity is controlled by cellular mechanisms, that keep TF in an inactive or "encrypted" state and regulate decryption after an appropriate stimulus. Although substantial evidence for this hypothesis is available, the exact nature of the underlying mechanism remains controversial. Below we will discuss earlier and recent models that have been put forward to understand the regulation of TF decryption.

Classically, it has been assumed that TF encryption-decryption depends on the phospholipid environment (13), i.e., under cryptic conditions, TF is located in a noncoagulant membrane. Upon cell activation and subsequent increased cytosolic Ca^{2+}, the inner plasma membrane leaflet-residing phospholipid phosphatidylserine is transported to the outer leaflet, a process modulated by flippase, floppase, and scramblase-type lipid transporters (331) (**FIGURE 2, *A* AND *A′***). The identity of these proteins on the surface of TF-exposing cells remains largely unknown, but ABC-class transmembrane transporters and TMEM16F are candidate floppases and scramblase, respectively (64, 282). The exposed negatively charged phosphatidylserine accelerates coagulation reactions on TF-containing membrane surfaces by stimulating tenase and prothrombinase activities (14, 150). The direct association of TF and phosphatidylserine seems to restrict the orientation of the TF/FVIIa complex to align the active site with the scissile peptide bonds in membrane-bound FX/FIX (19). In support of this view, cells typically show more TF-dependent procoagulant activity upon phosphatidylserine exposure that occurs during apoptosis (108). Nevertheless, phosphatidylserine exposure does not fully explain TF encryption/decryption. For instance,

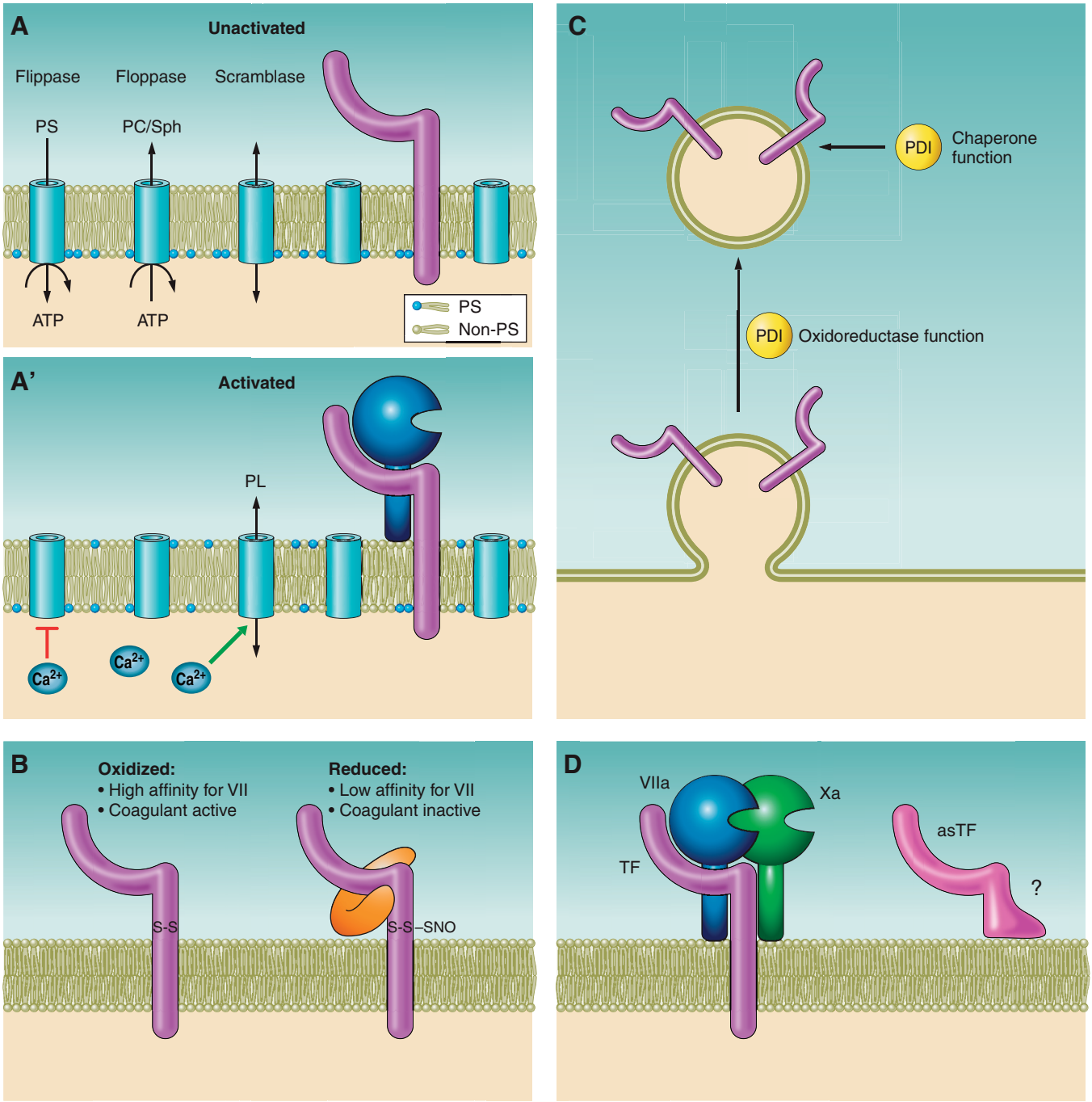

FIGURE 2. Regulation of TF activity. *A*: induced exposure of phosphatidylserine (PS). Under resting conditions, TF-exposing cells maintain a nonsymmetrical membrane composition, resulting in low PS contents in the outer membrane leaflet and high contents in the inner leaflet. This asymmetry is maintained through ATP-dependent inward transport of PS by flippases and outward transport of non-PS by floppases. Upon stimulation, calcium transients will inhibit flippase and stimulate the nonselective lipid transporter scramblase, resulting in PS exposure and the creation of a negatively charged surface that functions to bind coagulation factors. *B*: TF disulfide regulation. TF is kept on the cell surface in an inactive state through PDI- and/or nitric oxide (NO)-dependent reduction of the TF allosteric disulfide. Oxidation of the allosteric disulfide restores TF coagulant function. *C*: intravascular cells, such as activated monocytes, PDI-dependently shed TF-positive MPs. *D*: asTF on cellular surfaces or MPs may synergize with normally spliced TF to enhance TF procoagulant activity.

annexin A5, which binds to phosphatidylserine and thus competes with TF/FVIIa and FX for binding sites, does not appear to inhibit the TF/FVIIa-induced FX to FXa conversion (155), e.g., on ovarian carcinoma cells.

Another model explains TF-dependent procoagulant activity by oxidation and reduction of the TF COOH-terminal Cys^{186}-Cys^{209} bond. This disulfide bond between two antiparallel β-strands is less stable because of a strained bond geometry. Breaking of this disulfide bond may cause conformational changes that alter the affinity of TF for FVIIa. TF mutants lacking this disulfide bond show a much lower affinity for FVIIa and loss of coagulant function (242, 301), and pools of TF showing either high or low affinity for FVIIa may be found on the surface of cells that endogenously express TF (2, 155).

The Cys^{186}-Cys^{209} bond shows a typical right-handed staple allosteric disulfide conformation and may thus be prone to modulation by protein disulfide isomerases (PDI) (263) (**FIGURE 2*B***). Indeed, these isomerases have been proposed to break the allosteric disulfide bond to produce coagulant inactive TF, while PDI-dependent oxidation of this bond restores coagulant activity. PDI is a 64-kDa protein that is critically involved in protein folding and quality control in the endoplasmic reticulum, by using catalytical thiols that are used to enzymatically break or form disulfides, so-called oxidoreductase function, or reshuffle disulfides between cysteine residues, which is known as "isomerization" (319). However, PDI is also present on the surface of various cells, through electrostatic interaction with the membrane (3, 87, 328). In keratinocytes, PDI covalently complexes with TF and keeps TF in an inactivated state, but strong oxidants induce a PDI-dependent oxidative activation of TF function (2). Moreover, PDI activates TF on the surface of microparticles and stimulates thiol-dependent shedding of active TF on monocyte-derived microparticles (98). Finally, in murine models, PDI enhances coagulation by converting inactive TF into active disulfide bond-containing TF, a feature that is sensitive to TF free thiol-blockade (243), and promotes fibrin generation (49, 243). PDI-dependent covalent modification of TF cysteines with nitric oxide and/or glutathione, which also produces coagulant-inactive TF, has also been postulated and may form an additional level of regulation of TF. In this context, it is noteworthy that vascular-protective NO synthesis is frequently perturbed in atherosclerosis, diabetes, or inflammation (80), all conditions associated with thrombotic risk. Therefore, uncoupling of NO synthesis may shift cell-surface TF activity towards coagulation.

Although allosteric disulfide modulation forms an attractive explanation for TF decryption, this model has been extensively debated in the field. Some studies find that TF disulfide mutants are completely inactive and cannot be decrypted, whereas other studies attributed significant function and decryption potential to these mutants. Finally, TF redox switching does not appear to take place in pathological settings such as cancer, and this may account for the relatively high TF activity often observed on the surface of cancer cells (227).

A third model assumes that decryption relies on TF dimer formation. Like other members of the class II interferon receptors, TF indeed has the capability to dimerize in a manner determined by the redox environment and the exposure of phosphatidylserine (254). However, both monomeric and dimeric forms of TF appear to possess procoagulant activity, depending on the experimental conditions (13, 301).

A last model predicts that TF becomes encrypted after localization to lipid rafts, as lipid rafts are known to be poor in phosphatidylserine (38). In endothelial cells, assembly of the ternary TF/FVIIa/FXa complex indeed results in TF translocation to caveolae, where TF is then rendered inactive (269). In agreement with this, microparticles derived from cholesterol-rich monocyte rafts contain inactive TF. TF is activated upon microparticle fusion with platelets (70), presumably by platelet-dependent phosphatidylserine enrichment. In contrast, disruption of lipid rafts by cholesterol depletion appeared to decrease TF activity in fibroblasts, and it was suggested that raft-localized cholesterol functions as a positive regulator of TF function by maintaining TF receptors in a high-affinity state for FVIIa binding (171). Thus the importance of lipid rafts for TF function is still unclear. Based on these inconsistencies, one might speculate that cryptic TF is not a single entity and that encryption is differentially regulated in different cells. Alternatively, it is possible that TF needs to proceed through sequential steps, e.g., relocalization from rafts to phosphatidylserine-rich microdomains, followed by oxidation, to become active.

F. Blood-Borne TF

The traditional assumption is that expression of active TF is confined to extravascular cells. However, especially under pathological conditions, detectable amounts of TF are found in circulating blood. Recent insights predict that the coagulant activity of these TF pools may be tightly regulated by the environment and may also contribute to normal hemostasis under physiological conditions.

1. TF on intravascular cells

During sepsis, cell wall components from Gram-negative bacteria like lipopolysaccharide Toll receptor-dependently induce the expression of active TF on intravascular cells, such as circulating monocytes and endothelial cells in the microvascular system (77, 226). The consequence is widespread activation of coagulation, formation of fibrin, and

consumption of clotting factors. Whether intravascular cells other than monocytes and endothelial cells express TF has been the subject of debate for over 10 years. Expression of TF on neutrophils has been reported by some authors (102, 125), but denied by others (221). This controversy may be explained by the possibility that neutrophils can take up TF from other blood cells (113).

Expression of TF on platelets forms another controversial topic. Platelets appear to acquire TF through interaction with TF-bearing microparticles from monocytes (70, 88, 127), but platelets also contain TF pre-mRNA, which can be spliced into mature mRNA upon platelet activation, culminating in limited TF protein expression and procoagulant activity (267). Because platelets secrete large quantities of the TF antagonist tissue factor pathway inhibitor (TFPI), the physiological relevance of this low expression of TF to hemostasis is unclear.

2. TF on microparticles

Microparticles are 50- to 1,000-nm membrane vesicles that are released from various cells and may mediate intercellular signaling and regulate hemostasis (95). Especially disease states such as sepsis, inflammatory responses, and cancer are accompanied by shedding of TF-positive microparticles **(FIGURE 2*C*)**, and microparticles may also contribute to thrombotic risk. Thus enhanced TF activity on tumor cell-derived microparticles correlates with disease progression, and also associates with an increased risk of venous thrombosis (291). Here, we will only discuss the role of microparticle-associated TF pools in the regulation of normal hemostasis.

The available evidence suggests a prohemostatic effect of TF-positive microparticles. Although numbers in blood from healthy individuals may vary, microparticles can accelerate or increase formation of fibrin-rich thrombi (185). TF-positive vesicular structures are detected in thrombi formed under flow (102), and they appear to home to developing thrombi in vivo by interaction of microparticle P-selectin with PSGL-1-expressing platelets (89). This suggests that this pool of blood-borne TF stimulates fibrin formation after a thrombus has buried the extravascular source of TF. This view in which microparticle TF replenishes the TF pool at the site of clot formation has been criticized. First, in healthy subjects, the blood concentration and activity of microparticle-associated TF is relatively low (136, 291). Second, in static models of coagulation, this TF pool does not appear to mediate clot growth (215, 219). In a physiological context, however, blood flow and therefore continuous supply of microparticles may lead to local TF concentrations that can drive thrombus growth in vivo.

Cellular sources of coagulant active TF on microparticles include monocytes, platelets and endothelial cells (70, 185), but in vivo models predict that especially hematopoietic cells contribute to hemostatic TF-MP generation (224). Interestingly, TF on platelet and monocyte microparticles likely requires activation by certain changes in the intravascular environment. Notably, TF microparticles generated from platelets become procoagulant upon association with activated neutrophils (185), and TF on monocyte-derived microparticles shows activity after fusion with phosphatidylserine-expressing platelets, but not resting platelets (70). As discussed above, PDI-dependent decryption of microparticle TF may play a role here (307).

3. Alternatively spliced TF

The primary transcript encoding full-length TF (flTF) contains six exons, but an alternatively spliced form of TF (asTF) exists in which exon 5 is spliced out. Because of a 3′ frame shift mutation, the flTF transmembrane and cytoplasmic tail are replaced with a hydrophobic COOH-terminal domain, which renders asTF soluble. asTF is expressed in lung, pancreas, placenta, heart, endothelium, and monocytes (31, 285, 286). Although the level of asTF in human plasma may be substantial, amounting to 10–30% of total TF (107), the question of whether it contributes to coagulation is a matter of debate. Several observations suggest that asTF has a role in hemostasis: *1*) asTF localizes on platelet surfaces in experimental blood clots; *2*) the 165–166 lysine doublet involved in FVIIa binding is maintained in asTF, and theoretically, activity of the TF/FVIIa complex is maintained; *3*) when added to plasma in vitro, asTF shortens the clotting time (31); and *4*) while cell-expressed asTF alone does not appear to have procoagulant properties (32, 47), endothelial asTF expression in the presence of TF may be procoagulant, as depletion of asTF reduced TF-dependent coagulant activity (285). Thus asTF and flTF may cooperate in a synergistic fashion **(FIGURE 2*D*)**, but such a mechanism, as well as how asTF would enhance function of normally spliced TF, remains speculative.

In conclusion, on the basis of the above-described studies on blood-borne TF, a model is likely in which damaged vascular cells shed MP-TF that fuse with platelets and leukocytes to form a "second-generation" procoagulant platform that is in part dependent on asTF for optimal activity.

G. Inhibition of the Coagulation Process

Considerable efforts have been made in recent years to unravel the suppressor mechanisms of the coagulation process. Studies with patients showing deficiency in specific coagulation inhibitors and genetically modified mice have clearly shown that extensive negative control of coagulation is essential, to prevent uncontrolled, widespread clot formation. First, circulating protease inhibitors, such as antithrombin, heparin cofactor II, TFPI, and C1 inhibitor, eliminate activated coagulation factors by attacking their active sites. The second anticoagulant modality is provided

by the enzyme-based protein C/protein S pathway. Interestingly, the latter is implicated in endothelial-based pathways of coagulation inactivation.

1. The protein C/protein S pathway

Coagulant activity of tenase and prothrombinase complexes is dependent on the cofactors FVIIIa and FVa, respectively. Since the 1980s, it is known that activated protein C (APC) in complex with protein S establishes proteolytic inactivation of FVIIIa and FVa, thus suppressing tenase and prothrombinase actions **(FIGURE 3)**.

Protein C is a 419-amino acid anticoagulant factor with high homology to the vitamin K-dependent procoagulant factors (see above). For full anticoagulant control, it needs to be cleaved into APC and bind to its cofactor, protein S, also a vitamin K-dependent protein (635 amino acids). Protein C, aside from an NH_2-terminal phospholipid-binding Gla domain, contains a thrombin-sensitive region, four EGF-like domains that are required for protein S interaction, and two laminin G-type domains which synergistically with FV target FVIIIa (see below) (61).

As its concentrations gradually rise during coagulation, thrombin binds to thrombomodulin, a 60-kDa transmembrane protein that is expressed on endothelial cells (283). The extracellular domain of thrombomodulin consists of an NH_2-terminal lectin-like domain, six EGF-like repeats, and a short serine/threonine-rich domain, of which the EGF-like repeats 5 and 6 bind to exosite I of thrombin. Modification of the serine/threonine-rich region by chondroitin sulfate can induce binding to exosite II of thrombin (298).

Once bound to thrombomodulin, thrombin proteolytically cleaves and activates protein C that is bound to nearby endothelial protein C receptor (EPCR), an activity that is dependent on thrombomodulin's EGF-like repeats 4–6 (146, 190). Activation of protein C occurs after cleavage at Arg^{169}, thereby removing the activation peptide. Other thrombomodulin domains do not appear to play a role in anticoagulant activity but are important in inflammation, a link that will not be discussed here.

There is evidence that binding of thrombin to thrombomodulin is not strictly required for protein C activation, although its cleavage is extremely slow in the absence of thrombomodulin (86). Due to the large endothelial surface area in capillary beds, activation of protein C in these small vessels is relatively efficient. In larger vessels, where the endothelial surface area-blood volume ratio is low, addi-

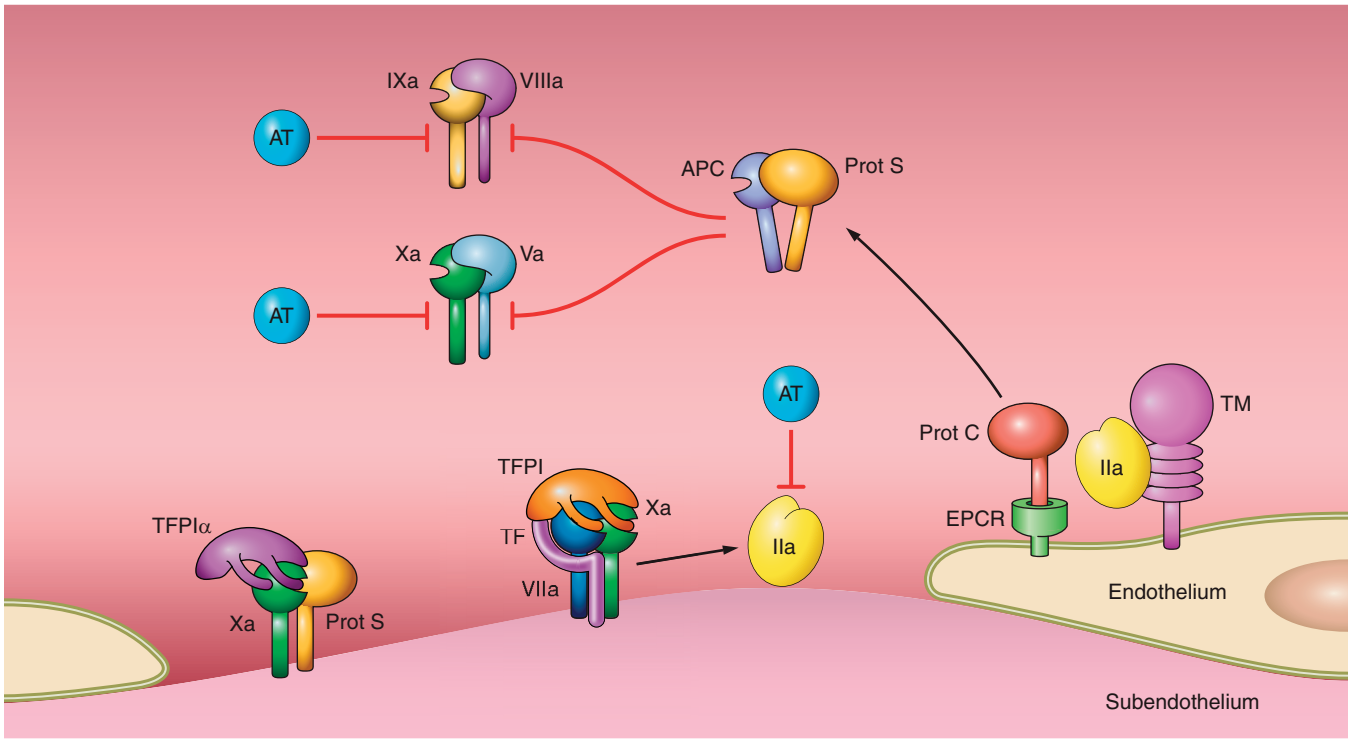

FIGURE 3. Negative regulation of the coagulation cascade. TFPI binds to FXa or the TF-FVIIa-FXa complex to restrict coagulation function. Protein S (prot S) may additionally bind to TFPIα to further inhibit FXa activity. Generated thrombin at sufficient amounts binds to thrombomodulin and is presented to protein C in complex with EPCR, after which protein C (prot C) is activated (APC). APC in complex with its cofactor protein S then inactivates FVa and FVIIIa. Antithrombin (AT) forms another level of control as it inhibits function of thrombin, FIXa, and FX.

tional presence of EPCR is required for protein C binding and presenting it to the thrombomodulin-thrombin complex (152).

Structurally, EPCR is a class I transmembrane glycoprotein, which shares homology with the histocompatibility class 1 family of receptors (MHC1) involved in immune and inflammation responses (96). Strikingly, both MHCI and EPCR contain a binding groove that tightly binds phospholipids and glycolipids, respectively. However, whereas glycolipid binding by MHC1 functions to stimulate the immune response to bacteria, the phospholipid binding by EPCR is necessary for tight protein C binding. (213). Studies with mice lacking EPCR indicate that EPCR not only suppresses thrombosis, but is also essential for normal embryonic development (110). Embryos deficient in EPCR die around day 10 due to a dramatic increase in TF-dependent fibrin formation around the trophoblast giant cells and in the primitive placenta.

Depending on the experimental system, the presence of EPCR enhances APC formation by 6-fold (cell cultures) to 20-fold (in vivo) (277, 289). This agrees with the presumption that the binding and activation of protein C are endothelial cell membrane-restricted processes. On the membrane, APC associates with its cofactor protein S to form a complex that proteolytically attacks FVa and FVIIIa, which are mostly membrane-bound as well. The structural requirements for this cofactor inactivation have been studied in detail (62). Both FV and FVIII are characterized by an A1-A2-B-A3-C1-C2 domain structure. The A domains shape into a globular structure with the B domain protruding from it. The latter is removed during activation of the cofactors by thrombin or FXa (61). While the A domains interact with FXa and FIXa, respectively, the C domains establish binding to the phospholipid membrane surface (92, 138, 220, 228).

The inactivation complex of APC with protein S cleaves FVa at Arg^{306}, Arg^{506} and Arg^{679} leading to complete downregulation of prothrombinase activity (139, 172). Interestingly, in the presence of protein S, intact FV functions as an additional cofactor to APC in the cleavage of FVa and FVIIIa (271). For FV to function as an APC cofactor, integrity of the FV B domain as well as APC-dependent FV cleavage at Arg^{506} are required (200).

The majority of protein S in plasma circulates in complex with C4b-binding protein (C4BP). Recent evidence indicates that C4BP-bound protein S also facilitates APC-dependent FVa cleavage, but at a reduced rate (174). Protein S also displays anticoagulant activity in the absence of APC by various mechanisms, i.e., by competing with prothrombin for direct binding to FVa, by inhibiting FXa, or by promoting the FXa-TFPI interaction (111, 119, 120).

Until recently, the APC pathway was seen as a means to terminate coagulation by cleaving FVIIIa and FVa. New insights have shed a different light on the role of APC. A key observation was that APC only inactivates FVa when the thrombin-generating surface is provided by endothelial cells, but not if it comes from platelets (214). In agreement with this, other authors report that platelets offer protection against FVa cleavage by APC (43). Taking into consideration the claim that activation of protein C is restricted to endothelium of vascular beds where thrombomodulin expression is high, it is now assumed that APC does not so much function to switch-off coagulation, but rather to prevent clotting reactions on healthy, uninjured vessels and in capillary beds.

Factor V^{Leiden} is a common gene defect that is detected in about one-third of the (Caucasian) patients suffering from venous thromboembolism (253). Because of an Arg to Gln mutation at Arg^{506}, this form of FV cannot be cleaved by APC and cannot support the APC-driven inactivation of FVIIIa (28). As a result, individuals who are heterozygous or homozygous for FV^{Leiden} have a 5- or 50-fold increased risk of venous thrombosis, respectively (61). Several other mutations or polymorphisms of coagulation factor genes are known to associate with an altered thrombotic risk (253), but due to space limitations, these cannot be mentioned.

Apart from controlling the protein C pathway, thrombomodulin and EPCR can also affect coagulation in other ways. Thrombomodulin appears to impair clot lysis by suppressing fibrinolytic activity (18). Via its EGF-like domains 3–6, it binds the plasma zymogen thrombin-activable fibrinolysis inhibitor (TAF+I) (144). The latter is activated by thrombin into TAFIa. TAFIa removes COOH-terminal lysine residues from partially degraded fibrin, and because these residues are important for further stimulation of the fibrinolytic pathway, their removal leads to impaired fibrinolysis. However, TAFIa has other functions. It inhibits binding of plasminogen to a fibrin clot, decreases the tissue-type plasminogen activator (tPA) cofactor activity of partially degraded fibrin, and reduces the capacity of fibrinogen to protect plasmin from inactivation by α2-antiplasmin (258, 265, 310). The activation of TAFI by thrombin appears to be much more efficient in the presence of thrombomodulin (18). Therefore, the thrombin-thrombomodulin complex is considered to be the main physiological activator of TAFI.

Recent findings provide evidence that EPCR also functions as a scavenging receptor for FVII/FVIIa. Structurally, the Gla domains of protein C and FVIIa are highly homologous, and both proteins hence bind to EPCR with similar affinity (100). The relevance of this interaction in terms of coagulation remains a matter of speculation, but a dampening effect is likely. Binding to EPCR also results in inter-

nalization of FVIIa by endothelial cells, thereby clearing FVIIa from the circulation. In addition, EPCR binding reduces TF/FVIIa-dependent FX activation, which provides an alternative explanation for the inhibitory role of EPCR on coagulation initiation (165). Another proposed mechanism is that EPCR inhibits FVII activation by FXa on endothelial cells, which may suggest that EPCR inhibits coagulation by sequestering FVII(a) from the procoagulant phospholipid environment (236). Some controversy exists with regard to the exact mechanism underlying EPCR-dependent inhibition of FXa generation. While some researchers primarily attribute this effect to inhibitory effects of EPCR on the FVIIa Gla domain, others find that the FX/FXa Gla domain is the primary EPCR target (74). Despite the lack of experiments demonstrating the relevance of these findings in vivo, EPCR appears to be a bona fide inhibitor of coagulation initiation.

Similar to other receptors involved in hemostasis, both thrombomodulin and EPCR can be cleaved from the cell surface. Thrombomodulin cleavage is induced by neutrophil-associated proteases, metalloproteinases, and rhomboids, whereas EPCR cleavage is accomplished by metalloproteinases and thrombin (164, 323). In healthy persons, the cleaved soluble thrombomodulin and EPCR fragments circulate in plasma at appreciable concentrations of 50 and 100 ng/ml, respectively, leaving open the possibility that these soluble proteins have a biological function. This is confirmed by the observation that soluble EPCR retains its affinity for protein C and APC. Interestingly, soluble thrombomodulin concentrations in plasma correlate with a decreased risk of coronary heart disease (322), whereas soluble EPCR levels may correlate with an increased risk of deep venous thrombosis (192, 260), implying that soluble EPCR but not soluble thrombomodulin, functions as a negative regulator of protein C activation. However, in mice expressing increased levels of soluble EPCR, a role for this fragment in APC formation could not be established (329). Hence, the main function of the cleavage products of these endothelial receptors still remains unclear.

2. Coagulation protease inhibitors

Out of the many plasma proteins that exert negative regulatory control on the coagulation process, TFPI and antithrombin are the most studied and best understood inhibitors. For this reason, we will primarily focus on these two proteins, each representing different classes of protease inhibitors: TFPI is a Kunitz-type protease inhibitor that limits coagulation initiation, while antithrombin is a serpin (acronym for serine protease inhibitor) type of inhibitor.

The Kunitz-type domains in TFPI act on coagulation proteases by mimicking their substrates. Upon binding of the enzyme, cleavage of the pseudosubstrate occurs at slow rates, or not at all. TFPI is present in platelets and on the microvascular endothelium, where it remains associated with the cell surface in a yet poorly characterized manner (39). Eighty percent of the circulating pool of TFPI is associated with lipoproteins, while the remaining 20% is lipid-free (114). In human plasma, three TFPI isoforms, resulting from alternative splicing, have been identified. Best characterized is TFPIα, which contains all three Kunitz domains. It circulates at a concentration of ~0.2 nM (39), but levels increase after heparin infusion (206), or by secretion from platelets at sites of vascular injury (207). In the second isoform, TFPIβ, the third Kunitz domain and COOH terminus are replaced by a neo COOH terminus containing a glycophosphatidylinositol anchor. This isoform is expressed in endothelial cells, albeit at lower levels than TFPIα, and was identified as the major endothelial cell surface-associated form of TFPI (104). Given that it is easily cleaved from the phospholipid anchor, TFPIβ may well be the predominant TFPI pool available in the body (104). A third TFPI isoform containing only Kunitz domains 1 and 2, TFPIδ, has only been identified at the mRNA level.

TFPI inhibits coagulation in two distinct manners, namely, by direct inhibition of free FXa and by interaction with the transient TF/FVIIa/FXa complex **(FIGURE 3)** (105). Kunitz domain 2 in TFPI serves to block FXa activity, while inactivation of TF/FVIIa is mediated via the Kunitz domain 1 (105). Recently, protein S has been identified as an important cofactor for TFPI-dependent inhibition of FXa, but not TF/FVIIa, at low procoagulant stimuli **(FIGURE 3)** (111, 194). Protein S potently increases the affinity of TFPI for FXa in a procoagulant phospholipid-dependent manner, bringing TFPI concentrations needed to block FXa function well within the range of free TFPIα concentrations. TFPI-protein S complexes exist in plasma, and protein S deficiency results in lower TFPI levels as well, suggesting that protein S improves TFPI stability in plasma (45). Although the function of TFPI Kunitz domain 3 remains to be firmly established, it may be that this domain and the COOH terminus serve to dock TFPI onto phospholipid-bound protein S that is in close proximity to phospholipid-bound FXa (195), and it is then likely that protein S only serves as a cofactor for full-length TFPIα.

It is currently unclear why different pools of TFPI (truncated TFPI and TFPIα) exist. Nonetheless, it should be noted that the TFPIα/protein S does not inhibit thrombin generation in the presence of high TF concentrations, unless sufficient APC at later points in coagulation is generated to slow down thrombin generation (229). Thus it may be that TFPIα and carboxy-truncated TFPI fulfill different anticoagulant actions during different phases of coagulation.

In conclusion, given the fact that *1*) TFPI function is partially dependent on protein S, *2*) both are contained in platelets at high concentration, and *3*) protein S deficiency lowers TFPI levels, the field may witness a dramatic reevaluation of TFPI function in hemostasis.

Antithrombin (previously antithrombin III) is considered one of the most important inhibitors of thrombin generation and function. This is exemplified by its high affinity towards three key coagulation proteases, i.e., FIXa, FXa, and thrombin **(FIGURE 3)**. Clinically, heterozygous deficiency in antithrombin confers a 10-fold higher risk of venous thrombosis, while true homozygous deficiency has never been observed in humans, probably because it is lethal (71). Antithrombin-deficient mice also die in utero, supporting the notion that complete AT deficiency is incompatible with life (131).

Serpins (serine protease inhibitor) like antithrombin belong to a class of protease inhibitors distinct from Kunitz-type inhibitors. They display a typical protruding reactive center loop (RCL), which is subject to protease attack. After cleavage, the protease covalently links to the cleaved RCL, and the RCL incorporates into the main body of the serpin (154). The direct effects of these events are threefold: *1*) the serpin adopts a hyperstable conformation, *2*) the protease catalytic domain is distorted, and *3*) the protease structure is disordered, adopting a zymogen-like state (129).

The natural inactivation of coagulation proteases by antithrombin is strongly enhanced by heparin. Long-chain heparins function to bind antithrombin as well as protease FIXa, FXa, or thrombin, thereby bringing these components together in close proximity (217, 218). Additionally, heparin pentasaccharide binding to antithrombin results in a conformational change that promotes antithrombin recognition by FIXa and FXa, but not thrombin (160, 216).

H. Roles of Protease-Activated Receptors in Coagulation, Wound Repair, and Endothelial Barrier Function

Key proteases of the coagulation cascade cleave and activate a particular class of intravascular receptors, the family of protease-activated receptors (PARs). PARs are seven-transmembrane domain G protein-coupled receptors that are activated by proteolytic removal of their NH_2-terminal exodomains (59). Receptor cleavage results in the liberation of a neo-NH_2 terminus, which serves as a tethered ligand that folds back into the ligand-binding pocket of the receptor. This results in the activation of a set of signal-transmitting GTP-binding proteins, namely, G_i, G_{12}/G_{13}, and G_q (212). There are four isoforms of PAR, PAR1–4, each with different specificities towards coagulation proteases. PAR1 is primarily activated by thrombin, but is also subject to cleavage by other proteases such as APC, FXa, the ternary TF/FVIIa/FXa complex, high concentrations of plasmin, and matrix metalloproteinase-1 (33, 149, 247–249, 309). Thrombin most effectively activates PAR1, since its exosite binds a hirudin-like sequence in PAR1 thus promoting efficient thrombin-PAR1 assembly (163). PAR2 is not activated by thrombin, but rather by FXa, TF/FVIIa, trypsin, and mast cell tryptase (42, 209, 249). On endothelial cells, FXa is a main trigger of PAR2-induced signaling (65). PAR3 and PAR4 are mainly thrombin receptors, but marked interspecies differences in activation of these receptors exist. The isoform PAR3, when present on mouse platelets but not on human platelets, is not cleaved by thrombin, but serves as a coreceptor for PAR4 (191). Thrombin can bind to a hirudin-like sequence of PAR3 while directly targeting the PAR4 NH_2 terminus. On human vascular smooth muscle cells, PAR3 appears to be directly targeted by thrombin (37, 222). PAR4 is cleaved and activated by thrombin in a similar way as PAR1. Members of the PAR family have a variety of functions in hemostasis. The roles of PAR1/PAR4 in human platelets and PAR3/PAR4 in mouse platelets are described below in section IV.

PAR1, PAR2, PAR4, and possibly also PAR3 are expressed on the endothelium (210, 264). In endothelial cells, thrombin stimulates the release of procoagulant factors, such as VWF and platelet-activating factor, and mediates the surface exposure of TF and P-selectin, especially via PAR1 (58, 151, 280). Endothelial cell activation and secretion induced via PAR2 have been demonstrated as well (65, 143).

PAR2 is expressed on endothelial cells, smooth muscle cells and fibroblasts, but is absent from platelets. Activation of PAR2 by TF/FVIIa or FXa is not necessarily directly relevant for coagulation, but it is currently speculated that this event is more important for wound healing, angiogenesis and tissue remodeling, now regarded as a downstream effects of coagulation (126). Especially PAR2-dependent angiogenesis appears to be critically regulated by TF, as phosphorylation of the TF cytoplasmic tail enhances PAR2 activation (23). PAR2 signaling via TF/FVIIa also promotes the proliferation and migration of epithelial cells into a wound (126). As squamous epithelial cells express high levels of PAR2, it is likely that TF/FVIIa signaling via PAR2 also contributes to cutaneous wound healing (259). In support of this notion, TF/FVIIa signaling in keratinocytes upregulates expression of "wound-healing" genes such as hbEGF, CTGF, FGF-5, IL-8, the PGE_2 receptor, as well as MMP-1 and -13 (42). In keratinocytes, TF phosphorylation as a consequence of PAR2 signaling also regulates $\alpha 3\beta 1$ integrin function and cell migration on extracellular matrix components (75). Other involvement of the coagulation system in wound healing occurs via thrombin-induced PAR1 signaling regulating angiogenesis, and via a scaffold function of fibrin networks for reepithelialization (153). As these processes are key in embryonic development and cancer, it is tempting to speculate that the roles of coagulation factors in wound healing mimic those in development and disease states. Indeed, overexpression of many coagulation factors is observed in tumors, leading to an exaggerated "wound-healing" and angiogenic response (300).

Vascular integrity is maintained by endothelial cells, providing a well-controlled barrier for blood components. Loss of the barrier function is accompanied by exposure of the subendothelial matrix, which as indicated triggers hemostatic responses. In a pathological context, thrombin-induced endothelial activation, e.g., upon sepsis, may impair vascular integrity. Recent findings indicate that thrombin at high (>100 pM), but not at low (<50 pM) concentrations can disrupt the endothelial barrier via activation of PAR1 (15, 93). Several downstream signaling events may be implicated, among which caveolin-1-dependent weakening of endothelial tight junctions (147), and G_{12}/G_{13}-dependent RhoA activation and concomitant actin stress fiber formation (303).

In contrast to thrombin, the anticoagulant protease APC appears to promote endothelial barrier function via signaling pathways dependent on Rac activation and cortical actin rearrangements (94). Intriguingly, also the APC-dependent barrier effects are elicited via PAR1 activation. The proposed mechanism is that APC-activated PAR1 generates the bioactive lipid sphingosine 1-phosphate, which in turn activates G protein-coupled EDG receptors to improve barrier function (93). This raises the question how PAR1 stimulation by thrombin or APC can have opposing effects on cellular responses, especially considering that thrombin is a more potent activator of PAR1 than APC (167). A reasonable explanation is that PAR1 cleaved and activated by high thrombin concentrations is rapidly internalized and broken down, whereas PAR1 activated by APC remains on the endothelial cell surface, even in the presence of thrombin (266). Another explanation is that binding of APC to EPCR results in relocalization of PAR1 out of raft domains, rendering PAR1 more sensitive to APC-mediated cleavage (16). Thus EPCR may be implicated in the activation of PAR1 by thrombin or APC. Jointly, this points to a complex interplay between PAR1 and EPCR in regulating endothelial activation, secretion, and barrier function. Given the multifunctionality of these receptors with, in part, different physiological consequences, we speculate that still unknown adjunct receptors or receptor-associated proteins are involved in the fine-tuning of the effects of thrombin and APC.

At this point, it is not clear why thrombin would disturb vascular integrity. However, it is known that coagulation further amplifies the inflammatory response, and it may be speculated that sepsis-induced coagulation, both as a result of TF upregulation and increased exposure of extracellular matrix, functions as a positive feedback loop to help clear invading pathogens.

III. NEW FUNDAMENTALS IN PLATELET-VESSEL WALL INTERACTION

In the adult human body, 1×10^{12} blood platelets continuously flow over 1,000 m^2 of vascular surface with normally minimal adhesion or aggregation. Upon disruption of the vessel wall or at sites of activated or damaged endothelium, swift and complex interactions occur between vascular cells, extracellular matrix components, platelets, and the coagulation system. The traditional concept of "sealing" a damaged vessel wall assumes that platelets first aggregate to form a primary plug, after which a fibrin clot forms as a consequence of activation of the coagulation system, phases that are termed primary and secondary hemostasis. As will be pointed out, current insights point to a more dynamic and intricate interplay between platelet responses, coagulation proteins, and components of the vessel wall where the relative contribution of many of the numerous molecules in thrombus formation still is a matter of debate.

Resting platelets are kept in a discoid and nonadherent state by the activity of endothelial cells, which on the one hand produce substances that strongly inhibit platelets like prostaglandin I_2 and nitric oxide, and on the other hand metabolize platelet agonists like ADP and thrombin to inactive products (54). Platelets become activated upon endothelial dysfunction or disruption: they change in shape, increase in adhesiveness, and acquire a prohemostatic surface. Studies with genetically modified mice and with patients showing gene defects (208) have convincingly demonstrated that several classes of surface glycoproteins, which are expressed in large numbers, are essential for these primary platelet responses. Increased platelet adhesiveness is achieved by a variety of mechanisms: assembly and clustering of receptors, formation of neoepitopes on receptors due to conformational changes, increased expression of receptors by pseudopod formation and secretion, and sudden availability of receptor agonists. Interestingly, the subsequent activation changes in platelets can be well compared with the fast intercellular communication pathways in nerve terminal synapses, in terms of regulation by ion channel activities and release of soluble autocrine and paracrine agents. In the following, we discuss current insights into the relative importance of platelet glycoproteins (leucine-rich repeat, immunoglobulin, and integrin receptors), their adhesive ligands, and platelet receptors for soluble (autocrine) agonists. It is aimed to link the consequences of receptor ligandation to common signaling pathways and physiological responses of platelets, with emphasis on hemostasis and thrombosis.

A. Platelet Leucine-Rich Repeat and Immunoglobulin Family Receptors

Both in vivo studies with animals (usually mice), investigating platelet adhesion upon vascular damage (79), and flow chamber studies, monitoring platelet adhesion during perfusion of isolated blood over a vessel wall substrate (261), have yielded important new information about the platelet activation processes, the various sets of receptors, and the complex intracellular signaling pathways that are involved

in the buildup of a multiplatelet thrombus under physiological flow conditions. Although details may differ, various researchers have described quite similar models of thrombus formation (36, 101, 186, 261, 279, 313). This is exemplified in **FIGURE 4,** which also visualizes key interactions between platelet and coagulation processes. In brief, discoid platelets interact via adhesive receptors with the extracellular matrix components VWF and collagen, resulting in unstable and later stable adhesion. The adhered platelets become activated, change their shape to become rounded and form pseudopods, express activated integrins, and secrete autocrine agents. Multiple flowing platelets then aggregate via fibrinogen bridges, produce fibrin clots through the action of thrombin, and finally contract to form a tightly packed thrombus. Patches of procoagulant platelets generate a phosphatidylserine-exposing membrane surface (188), which dramatically increases the formation of tenase and prothrombinase complexes of activated coagulation factors, leading to massive thrombin generation. Phosphatidylserine exposure thus firmly links the processes of platelet activation and thrombin generation (332). Thrombin in turns plays a central role in activating coagulation factors as well as platelets (284). Recent studies point to a gradient in platelet activation, with discoid, loosely aggregated platelets at the surface of a thrombus (197), and a gradually increased activation level deeper in the thrombus where tight platelet-platelet contacts are formed (11, 36). Pathological thrombus formation, which we will not discuss in detail, additionally involves other factors like high shear stress and dysfunctions of endothelial cells (255). The functions and activation mechanisms of major platelet adhesive receptors are described below.

Interaction of the GPIb-V-IX receptor with VWF is one of the first steps in platelet adhesion and tethering. VWF is stored inside Weibel-Palade bodies and α-granules in endothelial cells and platelets, respectively, but it also circulates in blood plasma. Once released from the endothelial cells, it

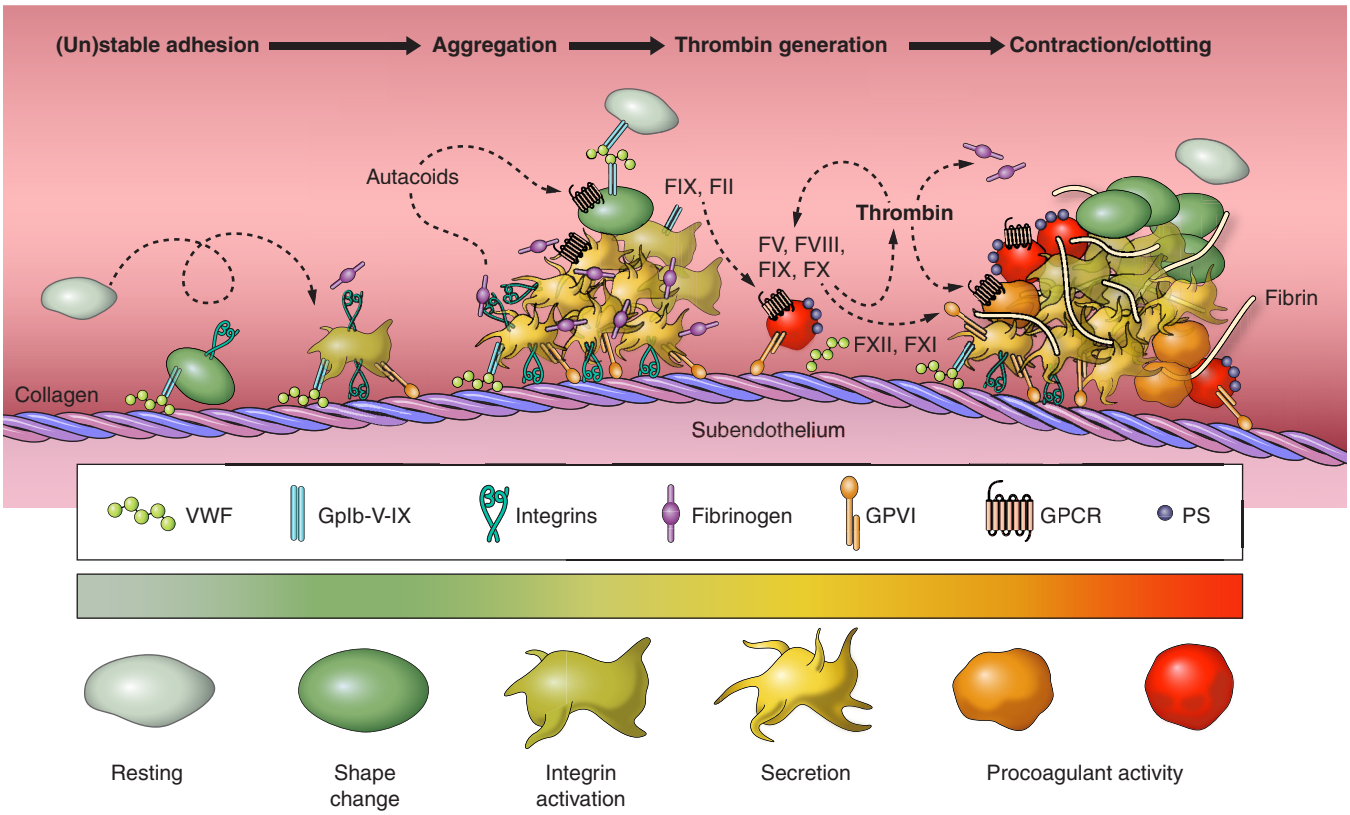

FIGURE 4. Stages of platelet activation and thrombus formation. Platelets adhere to a von Willebrand factor (VWF)/collagen matrix, get activated, secrete granular contents, aggregate via integrins, produce thrombin after developing a procoagulant surface, and form a contracted thrombus with fibrin. Heat map with color codes from green (low Ca^{2+} signal) to red (high Ca^{2+} signal). Interactions of platelets with coagulation factor are indicated, as described. Note that procoagulant platelets provide a phosphatidylserine (PS)-exposing surface for the tenase complex (activated FVIII and FIX) and the prothrombinase complex (activated FV and FX). Formed thrombin provides positive-feedback reactions to activate platelets via GPCR, to activate coagulation factors, and to convert fibrinogen into fibrin.

adheres to the cell surface membrane as ultra-large multimers that form characteristic strings before cleavage by ADAMTS-13. In addition, VWF binds to subendothelial matrix proteins, in particular collagen types I and III. Platelet adhesion to immobilized VWF via GPIb-V-IX is considerably accelerated by the high shear forces present in the arterial circulation, as a consequence of conformational changes in the immobilized VWF (133).

The transmembrane complex GPIb-V-IX is generally considered to consist of two pairs of the subunits GPIbα, GPIbβ and GPIX, which are all glycoproteins with leucine-rich repeats, next to one GPV subunit (6). About 25,000 copies of the complex are present on the cell membrane of a platelet. Tight connections between GPIb-V-IX and the membrane cytoskeleton, via the actin-binding protein 14–3-3ζ, are important in platelet production. Mutations in the various leucine-rich subunits give rise to a bleeding disorder, Bernard-Soulier syndrome, which is characterized by the presence of low numbers of giant platelets (macrothrombocytopenia) (208). A similar phenotype is seen in mice lacking the GPIbα β chains. Other dysfunctional mutations are present in subtypes of von Willebrand disease (208). Platelets also contain other classes of leucine-rich repeat motif receptors, such as Toll-like receptors, but the role of these is still under investigation (53).

A direct effect of engagement of GPIb-V-IX by VWF is restructuring of the actin cytoskeleton (132). The ligand-bound GPIbα also transmits weak intracellular signals by activating Src-related protein kinases, phosphoinositide 3-kinases (PI3K) and small GTPases, which cause Ca^{2+} fluxes and mediate integrin $\alpha_{IIb}\beta_3$ activation and platelet spreading (44, 133). GPIb-dependent platelet activation engages further lipid signaling, in that phospholipase D1 activation produces phosphatidic acid, which may contribute to integrin activation (82).

The current understanding is that GPIb-V-IX has a much broader role than only serving as a VWF receptor, as the complex also interacts with several plasma proteins and with counterreceptors on other cells. For instance, it contributes to platelet rolling on the endothelium via P-selectin (252), and it binds to the neutrophil receptor integrin $\alpha_M\beta_2$ (Mac-1), thus controlling platelet-neutrophil interactions (274). Furthermore, GPIb-V-IX binds with high affinity to thrombin, which can support thrombin-induced activation of the receptors PAR1 and PAR4, although the physiological importance of the GPIb pathway is debated (68). In addition, GPIbα has been reported to bind the coagulation factors FXI, FXII, and high-molecular-weight kininogen (7) and may also function as a coreceptor for FVIIa (314) and FXI(a) (17). On the other hand, recent findings suggest that FXI binds to platelets via the receptor LRP8 (ApoeER2) (317). LRP8 may also serve as a binding site for APC (326). Interestingly, β_2 glycoprotein-1, a plasma protein implicated in lupus anticoagulant, has been shown to interfere with the binding of VWF to GPIbα (156). One can speculate that it is because of this combination of properties that GPIb-V-IX is so important in normal hemostasis, such as apparent from the severe bleeding phenotype in patients with type 3 von Willebrand disease, lacking this glycoprotein complex (162).

By a mechanism that is not fully understood, but is considered to involve binding of VWF and coagulation factors, the GPIb-V-IX complex enhances platelet procoagulant activity (surface exposure of phosphatidylserine) and thereby the formation of thrombin (22, 316) and fibrin (56). Several glycoprotein chains of the complex can be inactivated by ectodomain cleavage. Under conditions that are not well clarified, the extracellular protease ADAM-17 cleaves both GPIbα and GPV in activated platelets (7).

GPVI, the major signaling collagen receptor on platelets, is a member of the immunoglobulin superfamily, which also includes Fc receptors and the T- and B-cell receptors (52, 203). Deficiency in GPVI leads to impaired collagen-induced platelet adhesion and aggregation and is associated with a mild bleeding disorder (182). GPVI expression is dependent on association with the FcR γ-chain, which expresses as a homodimer containing two so-called ITAM motifs (two adjacent YxxL sequences), requiring tyrosine phosphorylation for signal transmission (313). Collagen binding and dimerization of GPVI mediates activation of Lyn tyrosine kinase and other Src-family kinases (SFK) leading to phosphorylation of the ITAM motifs (237, 262). This in turn leads to binding of protein tyrosine kinase Syk and generation of a large signaling complex (signalosome). A major effector protein in this complex is phospholipase Cγ2 (PLCγ2), which becomes activated with support of the scaffold membrane proteins LAT and SLP-76, the GTP-exchange factors Vav1 and Vav3, the small GTPase Rac1, several isoforms of PI3K and Tec family tyrosine kinases, and other proteins (312). Initial phosphorylation events in GPVI-induced activation are under control of the membrane tyrosine phosphatase CD148 (268).

Both in vitro and in vivo thrombosis studies have established that GPVI is a crucial receptor in collagen-dependent thrombus formation (118, 148, 201). GPVI is a low-affinity receptor for collagen, and the interaction is enhanced by three mechanisms: *1*) coadhesion via integrin $\alpha_2\beta_1$ which complements and extends GPVI-dependent signaling (unifying two-site two-step model) (10, 48); *2*) GPIb-V-IX binding to collagen-bound VWF, which also enhances GPVI activation (130, 273); and *3*) receptor dimerization. GPVI is furthermore implicated in platelet adhesion to laminin, likely with integrin $\alpha_6\beta_1$ as coreceptor and dependent on GPIb-V-IX under flow conditions (130).

Recent findings point to a subtle regulation of GPVI expression on platelets. Once activated, GPVI is easily shed from the platelet surface by the extracellular proteases ADAM-10 and -17, which abolishes GPVI-induced responses (24). Interestingly, under clotting conditions this proteolytic cleavage appears to be regulated by FXa, but not by thrombin (4). This finding demonstrates a novel negative-feedback mechanism of the coagulation process on a platelet activation pathway. Unfortunately, patients with GPVI deficiency are extremely rare (208) so that the physiological role of this receptor in human hemostasis is still unclear.

The C-type lectin receptor CLEC-2, highly expressed on platelets relative to other hematopoietic cells, is characterized by the intracellular presence of a hem-ITAM motif (one YxxL sequence), which needs to become tyrosine-phosphorylated to transmit intracellular signals via the tyrosine kinase Syk. Clustering of CLEC-2 evokes a similar set of signaling events as seen with GPVI, including tyrosine phosphorylation by SFK, Syk, and Tec-family kinases and signal complex assembly around scaffold proteins (313). Platelet stimulation via CLEC-2 relies on autocrine effects via release of secondary mediators like ADP and thromboxane A_2 (233). There is still discussion on the possible existence of a physiological ligand for CLEC-2 receptors on platelets, which could potentially be CLEC-2 itself (281). An established ligand is the transmembrane protein podoplanin, which is widely expressed outside of the vasculature on tissues such as lymphatic endothelial cells and certain cancer cells, but is absent on platelets and endothelial cells. Depletion of CLEC-2 in mouse platelets impairs thrombus formation consistent with the presence of a physiological ligand in platelets or in plasma (175, 281).

The low-affinity IgG receptor FcγRIIa, which contains one ITAM, also evokes Syk-dependent signaling pathways in human platelets that are enforced by autocrine stimulation (159). Signaling via FcγRIIa is induced by clustering of two or more receptor chains, e.g., by autoimmune antibodies against the complex of platelet factor 4 and heparin, leading to heparin-induced thrombocytopenia.

B. Platelet Integrins and Other Adhesive Receptors

Integrins are required for the stable adhesion of platelets to the vessel wall or a growing thrombus. Integrins exist as noncovalent heterodimeric complexes of a transmembrane α and β chain, both of which are largely extracellular and contain short cytoplasmic tails (124). It is currently accepted that integrins need to be in an active conformation, achieved by declasping of the α and β membrane domains and bending out of the extracellular domains, to develop high-affinity ligand binding (231, 270). Integrins on platelets, once activated, interact with several adhesive proteins of the extracellular matrix (collagens, elastin, laminin, vitronectin) or in the blood plasma (VWF, fibrinogen, fibronectin) (116).

Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is the most abundant glycoprotein with more than 80,000 copies expressed per platelet and additional pools of up to 40,000 copies held in the open canicular system and in α-granules (231). In the active, "open" conformation, $\alpha_{IIb}\beta_3$ binds to several bivalent ligands, particularly fibrinogen, VWF, fibronectin, vitronectin, and CD40L. Integrin antagonists and knockout mice have established that $\alpha_{IIb}\beta_3$ is the most important adhesive receptor for platelet aggregation. Activation of $\alpha_{IIb}\beta_3$ is evoked by the vast majority of platelet agonists and is the result of a complex chain of intracellular signal transduction events, referred to as inside-out signaling. Integrin-activating agents include the soluble agonists ADP, epinephrine, thromboxane A_2, and thrombin, as well as the ligands of adhesive receptors like GPVI (collagen) and CLEC-2 (podoplanin). At least in response to ADP and low concentrations of most agonists, integrin activation is a reversible process, requiring continuous signaling to maintain its active conformation (55). Congenital absence of $\alpha_{IIb}\beta_3$, as in Glanzmann's thrombasthenia, results in a severe bleeding phenotype (208). In agreement with its crucial role, integrin $\alpha_{IIb}\beta_3$ is a widely used target of antiplatelet medication (drugs: abciximab, eptifibatide, and tirofiban).

Activated integrin $\alpha_{IIb}\beta_3$ also contributes to the platelet activation process (41). This so-called outside-in signaling by the integrin relies on the clustering of ligand-occupied integrins. In spite of the short cytoplasmic integrin domains, they appear to assemble large signaling complexes consisting of adaptor proteins, cytoskeletal proteins (talin-1, skelemin), and multiple protein phosphatases and kinases (231, 270). The integrin-associated tyrosine kinases Src and Syk in particular regulate further downstream signaling. Integrin outside-in signaling regulates platelet spreading over a fibrinogen surface and platelet-dependent clot retraction, suggesting that this pathway is particularly relevant for platelets adhered to a surface.

Integrin $\alpha_2\beta_1$ (GPIa/IIa) is a less abundant platelet integrin, expressed at 1,500–4,000 copies per platelet (53). In the active, extended conformation, it binds with increased affinity to collagens (83, 183). One way to achieve the conformational change of $\alpha_2\beta_1$ is by $\alpha_{IIb}\beta_3$-dependent signaling (299). At least in mouse blood, both the α_2 and β_1 chains are redundant for collagen-dependent platelet adhesion and thrombus formation under flow (148, 201). However, $\alpha_2\beta_1$ integrin does sustain and enforce human platelet adhesion via GPIb-V-IX and GPVI (273). As platelets on immobilized $\alpha_2\beta_1$ substrates show limited signaling, it appears that this integrin mainly serves to support platelet interaction via the other collagen receptor, GPVI (187, 203).

Integrin $\alpha_v\beta_3$ (binding vitronectin and fibrinogen), integrin $\alpha_5\beta_1$ (binding fibronectin), and integrin $\alpha_6\beta_1$ (binding laminin) are moderately expressed on platelets at ~1,000 copies per cell (53). The relative importance of these adhesive integrins likely is also moderate, as their contribution in thrombus formation is only demonstrated in the absence of other functional integrins ($\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$) (109).

The glycoprotein CD36 (GPIV, GPIIIb) is abundantly expressed on platelets (10,000–25,000 copies) as well as on mononuclear cells, macrophages, and endothelial cells. While recognized as a fatty acid binding/transporting protein in some cells, on platelets and other cells of the vascular system it acts as a receptor for thrombospondin-1, oxidized low-density lipoprotein, and oxidized lipids (scavenger receptor function) (196, 232). Platelet adhesion to (immobilized) thrombospondin causes significant signaling events, enforced by autocrine agents. These involve several protein kinases, including SFK and Syk, and result in Ca^{2+} rises. Another reported pathway is inhibition of the cAMP-dependent protein kinase A (PKA) (250). In the past, CD36 has been proposed as a collagen receptor. However, it later appeared that CD36 deficiency did not alter platelet interaction with collagen (141). Its role in the thrombus may be confined to conditions of dyslipidemia and oxidant stress (232).

In addition to the key suppressors of platelet activation, prostaglandin I_2 and nitric oxide, platelet inhibition may also be achieved via adhesive receptors. Platelet-endothelial cell adhesion molecule-1 (PECAM-1), present at 5,000–9,000 copies per cell, acts as a negative regulator of platelet activation (101, 205). Homophilic PECAM-1-PECAM-1 interactions can occur, but may not be involved in platelet PECAM-1 function. However, it is shown that cross-linking of two PECAM-1 molecules results in phosphorylation of their inhibitory ITIM motifs, and causes recruitment of the tyrosine phosphatases SHP-1 and SHP-2 (51, 205). In vivo experiments with mice have demonstrated that platelet PECAM-1 is a negative regulator of collagen-dependent thrombus formation (89). Two other ITIM-containing membrane proteins, also recruiting SHP-1 and also negatively regulating platelet signaling by collagen, are the weakly effective glycoprotein CEACAM-1 (320) and the more active immunoglobulin receptor G6b-B (181).

C. Platelet Receptors for Soluble Agonists

Platelet activation by extracellular matrix proteins results in the production of a range of autacoids or soluble secondary mediators, which can activate and trap nearby platelets. Most or all of these autacoids are only transiently present, and most become degraded or inactivated within minutes (133). Examples are the nucleotides ADP and ATP released from platelet-dense granules (degraded by the endothelial exonucleotidase, CD39), serotonin from the same granules (taken up by platelets), thromboxane A_2 produced by the platelet cyclooxygenase/thromboxane synthase complex (unstable eicosanoid, spontaneously decaying), and thrombin formed at the platelet surface (inactivated by antithrombin and other plasma components). Below we discuss current insights in platelet activation via receptors for these soluble agonists.

Platelets respond to soluble agonists mostly via G protein-coupled receptors (GPCR) **(FIGURE 6)**. Once ligand-bound, these receptors regulate various heterotrimeric G proteins, the activities of which depend on whether the G protein α-subunits have bound GTP instead of GDP. Regulator of G protein signaling (RGS) proteins help to hydrolyze GTP, and thereby shorten the time period of G protein activation and thus of signaling (35). Various prominent GPCR on platelets signal to inhibit cAMP formation via adenylate cyclase, i.e., the adrenergic α_{2A}-receptor for epinephrine (coupled to G_z, also to G_i), the EP3 receptor for PGE_2 (coupled to G_i), and the $P2Y_{12}$ receptor for ADP (also coupled to G_i) (211). Current insight is that PAR isoforms couple but indirectly to G_i via PAR-mediated ADP secretion and $P2Y_{12}$ signaling. The classical concept is that G_i activation and adenylate cyclase inhibition lead to diminished activation of PKA, which in turn functions as an overall inhibitor of platelet responses. However, current perception is that there is a more important role for G_i in stimulating PI3K activation (46). On the other hand, adenylate cyclase and hence PKA are stimulated by endothelial-derived prostaglandin I_2 (prostacyclin) binding to the platelet IP receptor (coupled to the G_s protein). The consequence of treatment with prostaglandin I_2 and PKA activity is overall platelet inhibition. The alpha2 type of adenosine receptors also suppress platelet aggregation by coupling to G_s (325).

The ADP receptor $P2Y_{12}$ is a well-established target to suppress platelet function in cardiovascular disease and to prevent secondary thrombosis, e.g., after stent placement (46). The successful clinical use of (pro)drugs directed against this receptor, i.e., clopidogrel, prasugrel, and ticagrelor, demonstrate that autocrine secretion of ADP and ensuing integrin activation via the $P2Y_{12}$ is a main driving force of thrombus formation. It is speculated but not completely sure that the clinical efficacy of $P2Y_{12}$ inhibition relies on signal transmission to G_i and PI3K (regulating integrin activation), rather than on $P2Y_{12}$-mediated suppression of adenylate cyclase.

Platelets contain two GPCR for thrombin, PAR1 and PAR4 in human and PAR3 and PAR4 in mouse. Stimulation of these thrombin receptors results in robust platelet activation, which is predominantly mediated by the coupling of PAR1/4 to the G_q protein and activation of PLC-β isoforms. The high potency of thrombin to activate human platelets is primarily dependent on PAR1 but enforced by PAR4, which is believed to be cleaved at higher thrombin concen-

trations (5). Markedly, recent clinical trials aiming to reduce platelet function with PAR1 antagonists are of miscellaneous success and even show increased (intercranial) bleeding (294). Whether platelet inhibition is the reason for this undesired side effect needs to be established.

As indicated above, there is evidence for PAR1 cleavage and activation by other proteases (295). The physiological importance of cleavage by non-thrombin proteases however is unclear. The PAR isoforms couple not only to G_q, but also to G_{13} family proteins, which mediate activation of the small G protein RhoA (178). RhoA contributes to platelet secretion but, in particular, controls the activity of RhoA kinase (ROCK). The latter protein kinase inhibits myosin light-chain phosphatase and, hence, augments phosphorylation of the myosin light chain (MLC), which is a requirement for platelet shape change.

The second platelet ADP receptor, $P2Y_1$, coupling to G_q, has a prominent role in initial platelet responses, in contrast to $P2Y_{12}$ which is more active in later stages (117). The TP receptor for autocrine produced thromboxane A_2 (suppressed by the drug aspirin) and the 5-HT_{2A} receptor for serotonin also couple to G_q, the latter of which has limited signaling capacity (211). Platelets contain several cation and anion channels, but their function in hemostasis is rather unexplored. Only in the case of the $P2X_1$ Ca^{2+} channel has a significant enhancing role been established in collagen-induced platelet activation and thrombus formation (97, 223). Jointly, for the receptors of soluble platelet agonists, only the $P2Y_{12}$ (ADP) and TP (thromboxane A_2) receptors have been shown to be successful targets for platelet inhibition, suggesting that especially these two play major roles in pathological platelet activation.

D. Platelet Common Signaling Responses

Stimulation of most platelet receptors leads to integrin $\alpha_{IIb}\beta_3$ activation and platelet aggregation. By implication, integrin activation is an easily inducible platelet response that even occurs at low cytosolic Ca^{2+} levels. On the other hand, only combinations of strong agonists (collagen, thrombin) induce the platelet procoagulant response, which is highly dependent on elevated cytosolic Ca^{2+}. Important recent progress has been made in deciphering the signaling pathways leading to both integrin activation and Ca^{2+} signal generation.

Studies using knockdown strategies in mice have helped to unravel the molecular pathways of integrin $\alpha_{IIb}\beta_3$ activation. Key signaling pathways involve activation of PI3K (through 3-phosphorylated phosphoinositides and possibly the Akt protein kinases) and, in parallel, activation of the small G protein regulator CalDAG-GEF1. Both pathways establish stimulation of the G protein Rap1b, which binds to and switches on its molecular effector RIAM (26, 321). The latter, in a still unsolved way, prompts cytoskeleton-bound talin-1 together with kindlin-3 to unclasp the integrin α and β chains (202, 270). Patients with a deleterious kindlin-3 mutation (leukocyte adhesion deficiency 3) thus have platelets that are defective in $\alpha_{IIb}\beta_3$ activation (26, 270). The precise mechanism of kindlin-3 action is still discussed (327). In agreement with the early evidence that direct stimulation of protein kinase C (PKC) results in platelet aggregation (272), the PLC/PKC pathway also triggers integrin activation, possibly also involving Rap1b, and secretion of ADP. Full-size thrombus formation most likely requires the participation of the various signaling proteins, including CalDAGGEF1 and Rap1b as well as isoforms of PI3K and PKC (134).

Elevation of cytosolic Ca^{2+} is a key signaling event, in that short-term or prolonged Ca^{2+} rises differentially control the type of platelet responses (**FIGURES 5 AND 6**). Several new proteins regulating Ca^{2+} signal generation have recently been discovered. The common stimulus for Ca^{2+} elevation is activation of PLC isoforms, either via the tyrosine kinase Syk (PLC-γ2) or via the G_q protein (PLC-β). Both PLC isoforms produce the second messengers inositol 1,4,5-trisphosphase ($InsP_3$) and 1,2-diacylglycerol (121). The former releases Ca^{2+} through $InsP_3$ receptors in the endoplasmic reticulum membrane, while the latter activates conventional and novel isoforms of PKC (see below). Initial Ca^{2+} release from endoplasmic reticulum storage sites acts as a trigger for further Ca^{2+} rises through the process of store-regulated Ca^{2+} entry. Herein, the reticular membrane protein STIM1 plays a crucial role in sensing Ca^{2+} store depletion and interacting with the plasma membrane Ca^{2+} channel Orai1 (304). Thus mice deficient in either platelet STIM1 or Orai1 are greatly impaired in thrombus formation and platelet procoagulant activity (103, 304). The precise role of other proposed Ca^{2+} channels in platelets is still unclear.

Platelet responses induced by short-term rises in Ca^{2+} are shape change and granular secretion, the latter of which also requires PKC-dependent phosphorylation events. Weak platelet agonists (epinephrine, PGE_2, serotonin, ATP) induce minimal Ca^{2+} responses without secretion, unless in combination with other agonists (272). Moderate agonists like ADP and thromboxane A_2 cause repetitive spiking Ca^{2+} transients, and limited secretion (123). Dependent on a prolonged Ca^{2+} rise is the platelet procoagulant response (phosphatidylserine exposure), which is only induced by combinations of strong agonists, like thrombin and collagen (121). For a long time, investigators have been searching for the Ca^{2+}-dependent phospholipid scramblase, which is responsible for phosphatidylserine externalization and platelet-dependent thrombin generation (332). On the basis of genetic analysis of patients with Scott syndrome, whose platelets are deficient in phosphatidylserine exposure, the membrane protein TMEM16F has now been recognized as a good candidate protein implicated in phospholipid scrambling (282). In secretory cells like platelets, Ca^{2+}

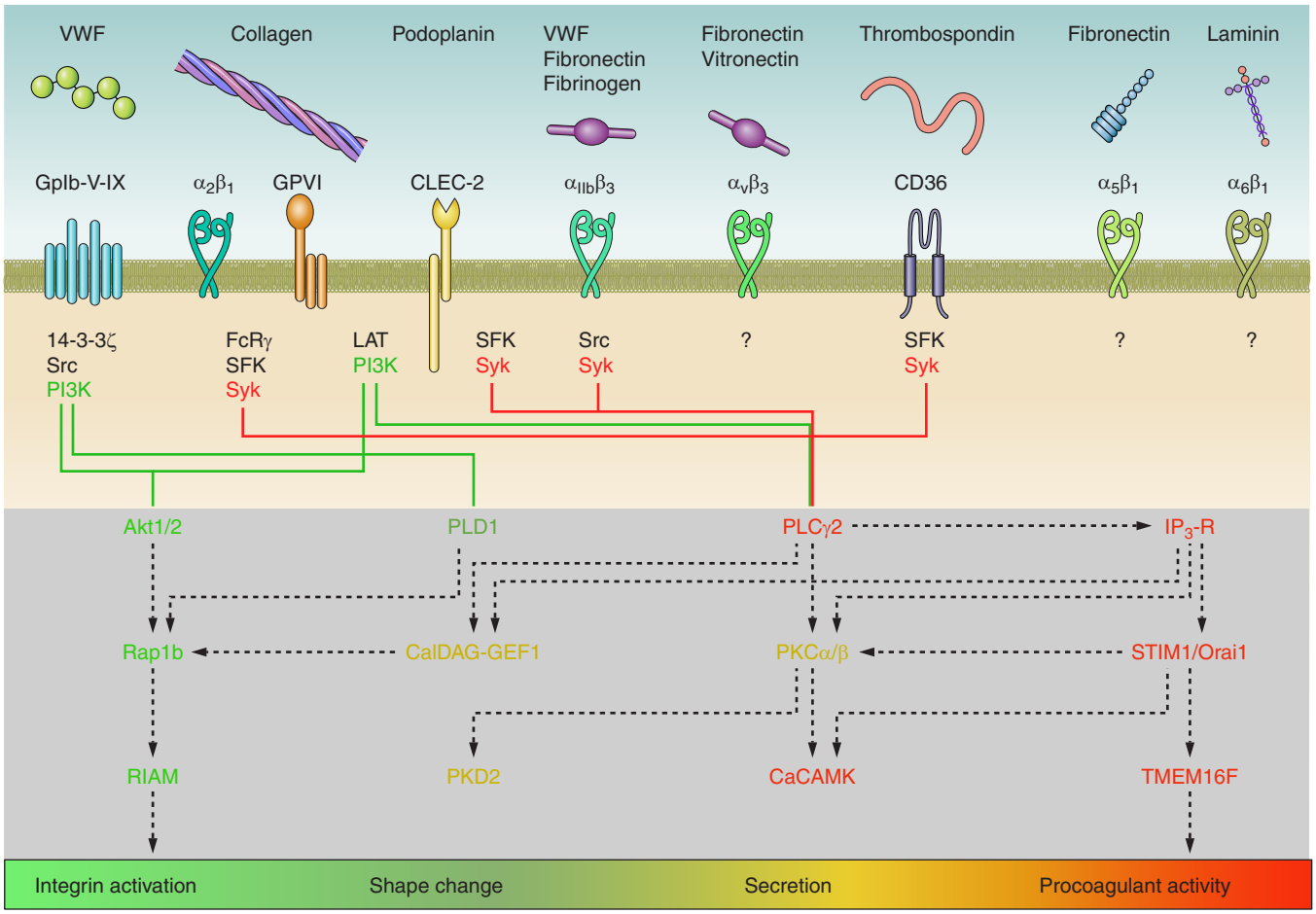

FIGURE 5. Ligands and key signaling events of major adhesive receptors of platelets. Signaling proteins that are associated with the various receptors are placed close to the receptor symbols. Gray box shows networks of signal transmitting proteins that act independently of the type of receptor. Only key network interactions are indicated. Heat map is shown with color codes from green (low Ca^{2+} signal) to red (high Ca^{2+} signal). Platelet activation pathways may also be graded on other axes than the amount of Ca^{2+} signal.

acts to trigger many effector molecules, among which actin cytoskeleton proteins, PKC, Ca^{2+}-calmodulin kinase (Ca-CAMK), and the Ca^{2+}-regulated protease calpain. The role of PKC, however, is more complex than earlier anticipated. The two conventional isoforms, PKCα and -β, stimulated by diacylglycerol, not only evoke integrin activation and secretion, but also support Ca^{2+} rises in part by promoting Ca^{2+} extrusion pumping (122). A novel downstream mediator of PKC is the protein kinase PKD2 (145). The precise phosphorylation targets of these protein kinases are for the most part unclear. On the other hand, the novel PKC isoforms, PKC-δ and -θ, seem to have different functions. Interestingly, the novel isoform PKC-θ even suppresses collagen-dependent platelet activation and procoagulant activity in a not fully clarified way (115). Downregulation of platelet Ca^{2+} signaling is provided by back-pumping via Ca^{2+}-ATPases in the endoplasmic reticulum (SERCA) and plasma membrane (PMCA) (241).

An increasing number of soluble and membrane-bound proteins, stored in the platelet α-granules, are found to play a role in platelet aggregation and thrombus formation. These secreted proteins trigger additional feed-forward loops that appear to be required for stable and tight platelet-platelet contacts. For instance, activated platelets express on their surface members of the TAM family of Gas6 receptors which, by interacting with plasma Gas6, support integrin activation and thrombus formation (57). Tight intercellular contacts of platelets in a thrombus are established by a number of signaling interactions: *1*) the EphA4 receptor kinase with ephrinB1, *2*) the receptor semaphorin 4D (CD100) with plexin (36), *3*) P-selectin (CD62P, in α-granules) with its ligand PSGL-1 (292), and *4*) the TNF-family receptor CD40 with CD40L (161). Thrombus formation is further supported by other secretion products from the platelet α-granules (thrombospondin-1, VWF, fibrinogen, FV) and from the dense granules (ATP, ADP, polyphosphates) (36). On the other hand, some receptor-ligands pairs provide a negative feedback in platelet activation and thrombus formation. These include the CTX family receptor members, ESAM (in α-granules) and JAM-A, likely functioning by forming homophilic interactions (36);

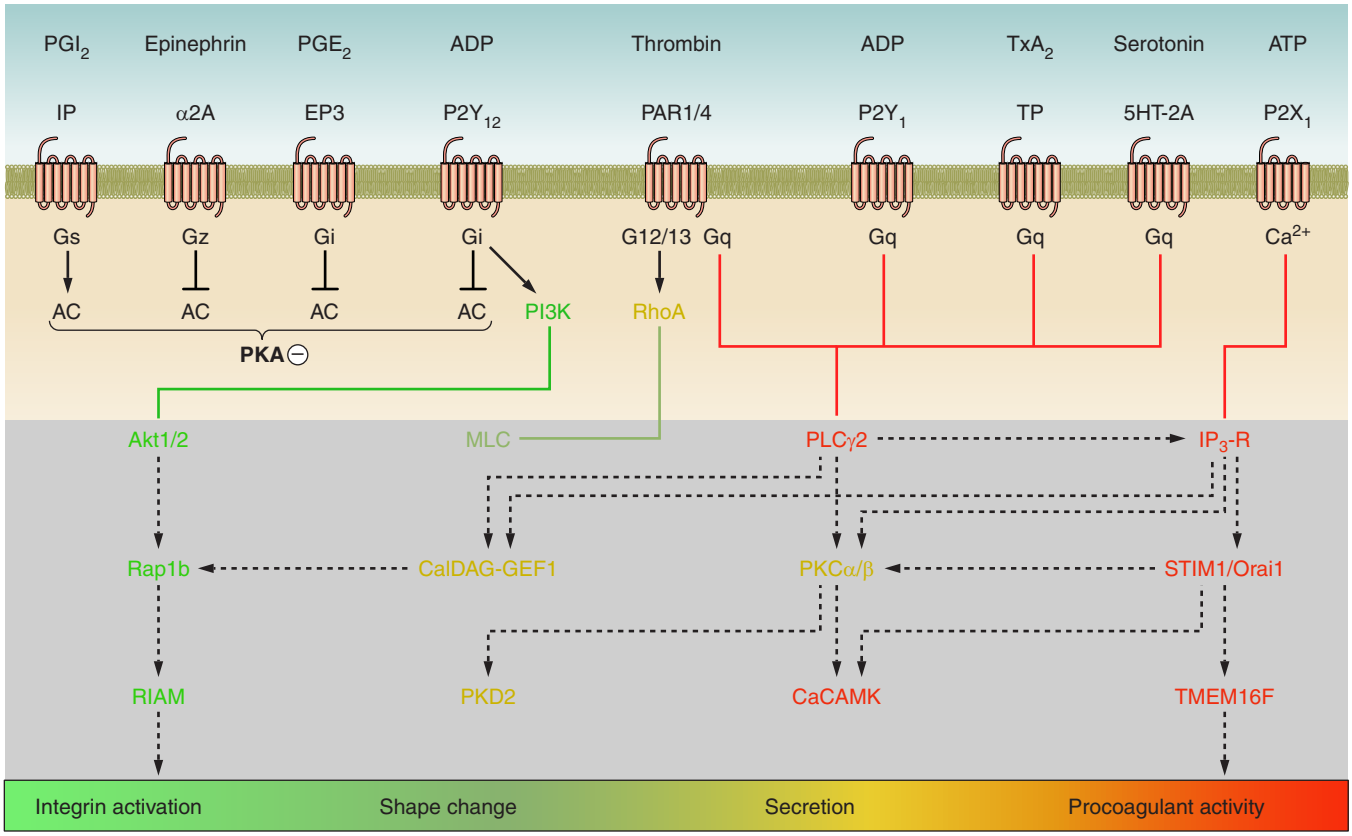

FIGURE 6. Ligands and key signaling events of major G protein-coupled receptors of platelets. Signaling proteins that are associated with the various receptors are placed close to the receptor symbols. Gray box shows networks of signal transmitting proteins that act independently of the type of receptor. Only key network interactions are indicated. Heat map is shown with color codes from green (low Ca^{2+} signal) to red (high Ca^{2+} signal). Platelet activation pathways may also be graded on other axes than the amount of Ca^{2+} signal.

and Wnt3a interaction with the Wnt frizzled-6 receptor (278). Some of the above-mentioned receptors are known to be shed from the membrane of activated platelets, yielding soluble cleavage products, for example, semaphorin 4D, P-selectin, and CD40L (7). This cleavage can provide another negative feedback mechanism limiting thrombus formation.

Given the many signaling proteins that play a role in platelet adhesion, activation, and thrombus formation and given the multiple interactions that exist between these signaling proteins, it is more and more difficult to construct a simple, arrow-based signaling scheme explaining cellular responses. Furthermore, platelets in an "activating" environment will be exposed to multiple agonists at the same time, implying that signaling pathways triggered by receptors for various agonists will operate in synergy. Efforts are now directed at the development of multiedge networks to incorporate all known weak and strong interactions between signaling proteins. **FIGURES 5 AND 6** provide a framework for the linking of platelet receptors, the signaling pathways, and networks controlled by these receptors with the ultimate platelet responses, assuming the extent of intracellular Ca^{2+} rise as a main parameter of platelet activation. Similar frameworks can clearly be constructed for other platelet activation parameters. The current challenge is to extend such frameworks for (physiologically relevant combinations of) all ligands, receptors, intracellular signaling proteins, and secretory mediators, to get a complete understanding of how the interaction with a specific vessel wall segment determines platelet activation. A public accessible database, in which the molecular events of platelet signaling are linked together, as well as the biochemical reactions of coagulation and clot dissolution, is provided by the Reactome (http://www.reactome.org). The reaction lists of such databases may be integrated into frameworks as described above, e.g., by providing a weighted contribution of each of the reactions to the overall process of platelet activation. Such systems biology approaches may finally explain why dysfunction of some platelet proteins, but not of others, associate with a bleeding phenotype not only in mouse models, but also in patients with certain gene defects (208, 315). In addition, this may give insight into the conditions and reactions of platelets that give rise to hyperfunction and thrombosis.

E. Adhesive Proteins and Platelet Function

Thrombus formation to stop bleeding or in thrombosis often is started by platelet interaction with a vulnerable or

damaged blood vessel. The prevailing concept is that platelet stimulation via adhesive receptors and by soluble agonists (autocrine substances and coagulation products) is needed to build a stable thrombus, where coagulation factors can be activated and thrombin and fibrin can be formed **(FIGURE 4)**. There is increasing knowledge on how several "sticky" proteins, present in blood plasma and the (damaged) vessel wall, act as cement for platelet adhesion, aggregation, procoagulant activity, and fibrin clot formation.

VWF consists of larger and smaller multimers of disulfide-linked subunits, each comprising 2,050 amino acid residues and up to 22 carbohydrate chains (180, 256). The molecular mass of the multimers ranges from ~500 to >10,000 kDa. At high shear rate, multimeric VWF unrolls from a globular to a filamental conformation, up to several microns long (i.e., as large as a platelet), which becomes a high-affinity surface for the platelet GPIb-V-IX complex. The large VWF multimers are hemostatically more active than smaller molecules (9). The biochemical basis for this is that large multimers contain multiple domains that support the interactions between platelets, endothelial cells, and subendothelial collagen. VWF binds to matrix collagens via its A1 and A3 domains. The A1 domain also mediates binding to platelet GPIbα, the interaction of which is required for the fast capturing of platelets (128). Platelet adhesion to VWF is greatly further supported by VWF immobilization to a surface (collagen, other platelets) and by high shear stress. Binding of GPIb-V-IX to VWF primes for secondary interaction of VWF with activated integrin $\alpha_{IIb}\beta_3$, resulting in two-phased intracellular Ca^{2+} rises (133). Qualitative or quantitative deficiencies in VWF cause von Willebrand disease, a mild to severe bleeding disorder (162).

Large VWF multimers are cleaved by the plasma protease ADAMTS-13, which is mainly produced by the liver (99). This cleavage produces the smaller size VWF multimers that are circulating in plasma. Although other proteases, such as cathepsin G and plasmin, can also cleave VWF, their role in vivo is limited, because they are rapidly neutralized (72). It is suggested that the A1 domain of VWF inhibits the cleavage by ADAMTS-13, but this inhibition is weakened upon A1 domain binding to platelet GPIbα (204). The consequence of this is increased ADAMTS-13-mediated cleavage of VWF multimers adhering to platelets. Decreased ADAMTS-13 activity is linked to various microangiopathies with increased platelet activity, as indicated below.

Fibrillar collagens type I and IV are among the most potent platelet-activating constituents in the vessel wall. Many animal studies indicate that collagen exposure to the bloodstream is a major trigger for thrombus formation (202). With the use of novel technology of collagen peptide synthesis, it has become possible to identify separate motifs in the (triple-helical) structure of fibrillar collagens that recognize GPVI, integrin $\alpha_2\beta_1$, and the VWF A3 domain, thus establishing collagen as an efficient multireceptor substrate for platelet adhesion (90). Indeed, coimmobilization of collagen-based peptides recognizing GPVI, $\alpha_2\beta_1$, and VWF can fully mimic the role of intact collagen fibers in thrombus formation (235). For the presence of collagen receptors other than GPVI and $\alpha_2\beta_1$ on platelets is only limited evidence (203).

The adhesive molecule fibronectin, an ~500 kDa glycoprotein dimer, is abundantly present in blood plasma, megakaryocytes, and α-granules of platelets (173). Similarly to fibrinogen, fibronectin mediates platelet aggregation through integrin $\alpha_{IIb}\beta_3$, but it also binds to the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Data from men or mice lacking fibronectin suggest it has only a limited role in normal hemostasis. However, fibronectin levels do affect thrombus formation and thrombus stability, suggesting a role of this protein in pathological thrombus formation (173, 199). Vitronectin also binds to platelet $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins (293). This multimer-forming glycoprotein, abundantly present in plasma and the extracellular matrix, coordinates migration and signaling of blood cells and vascular cells, but its role in platelet activation is unclear (234). Other vitronectin ligands are PAI-1 and high-molecular-weight kininogen. Markedly, vitronectin binding stabilizes PAI-1 as a fibrinolytic inhibitor, which renders fibrin clots less susceptible for lysis (330). Of the specific platelet integrin ligands, in humans only severe fibrinogen lowering is known to be linked to impaired hemostasis and bleeding, particularly due to inability of fibrin clot formation.

Two other adhesive proteins with an established role in platelet-vessel wall interaction are thrombospondin-1 and laminin. Thrombospondin-1 is released from α-granules and binds to CD36, resulting in platelet activation, although it has also been proposed to bind to CD47 and GPIb (137, 196). In addition to a role in hemostasis by controlling the multimer size of VWF, thrombospondin furthermore functions in other vascular processes (would healing, angiogenesis) (34). Laminin is a large glycoprotein (920 kDa) of the extracellular matrix and the basement membrane, synthesized by endothelial cells, which becomes exposed after mild vascular injury (116). Laminin binding to platelets via integrin $\alpha_6\beta_1$ does not seem to result in platelet activation, but it can also serve as a ligand for GPVI (130).

Increased platelet stickiness has also been detected by the formation of so-called coated platelets, e.g., by stimulation with collagen/thrombin. This is a population of highly activated procoagulant platelets, where the surface is covered with fibrin(ogen), thrombospondin-1, VWF, and FVa, likely in a transglutaminase-dependent manner (63). In thrombi, the coated platelets at least in part overlap with phosphatidylserine-exposing platelets that are active in thrombin generation (186). Given the multitude of adhesive proteins interacting with platelets as discussed above, it is

not a surprise that, with the exception of VWF and fibrinogen which also act in platelet aggregation, deficiencies of single adhesive proteins do not appear to impair hemostasis, at least not in mouse models. This may point to functional redundancy.

IV. NEW FUNDAMENTALS IN COAGULATION-ASSOCIATED PATHOLOGY

A. Venous Thromboembolism

Our understanding of venous thromboembolism (VTE), the collective term used for venous thrombosis and pulmonary embolism, is based on the triad of Virchow which postulates that thrombosis is caused by changes in *1*) blood flow, *2*) the state of the vessel wall, and/or *3*) the composition of blood (189). In the most current view, stasis of blood and the accompanying low oxygen tension (in particular downstream of a venous valve), activation of the endothelium, activation of innate (involving monocytes and neutrophils and platelets) immunity, activation of blood platelets, concentration and nature of microparticles (MPs), and the individual concentrations and function of pro- and anticoagulant proteins all claim a role (see **FIGURE 7**) (169, 244). The complex interaction between these players leads to activation of intrinsic and extrinsic coagulation pathways and thus to fibrin formation and an intravascular blood clot.

Until quite recently, the notion was that in particular the concentration and function of hemostatic proteins were the main determinants of venous thrombotic risk. More recent data, largely from small-animal studies, indicate that cells and cellular components associated with (acute) inflammation and platelets are also rate-limiting in thrombus formation and thus codetermine thrombotic risk (308). This interpretation of small-animal data finds support in our current understanding of many clinical risk factors for venous thrombosis such as acute and chronic infection, obesity, smoking, and surgery (244).

From the perspective of treatment and prevention of VTE, this shift in view may become important. The emphasis on the role of coagulation factors forms the basis of the current treatment of VTE with heparins and vitamin K antagonists or direct thrombin and factor Xa inhibitors. If platelets and inflammation are also determinants of thrombotic risk, their inhibition could become an alternative, in particular in preventive strategies. The main advantage of such an ap-

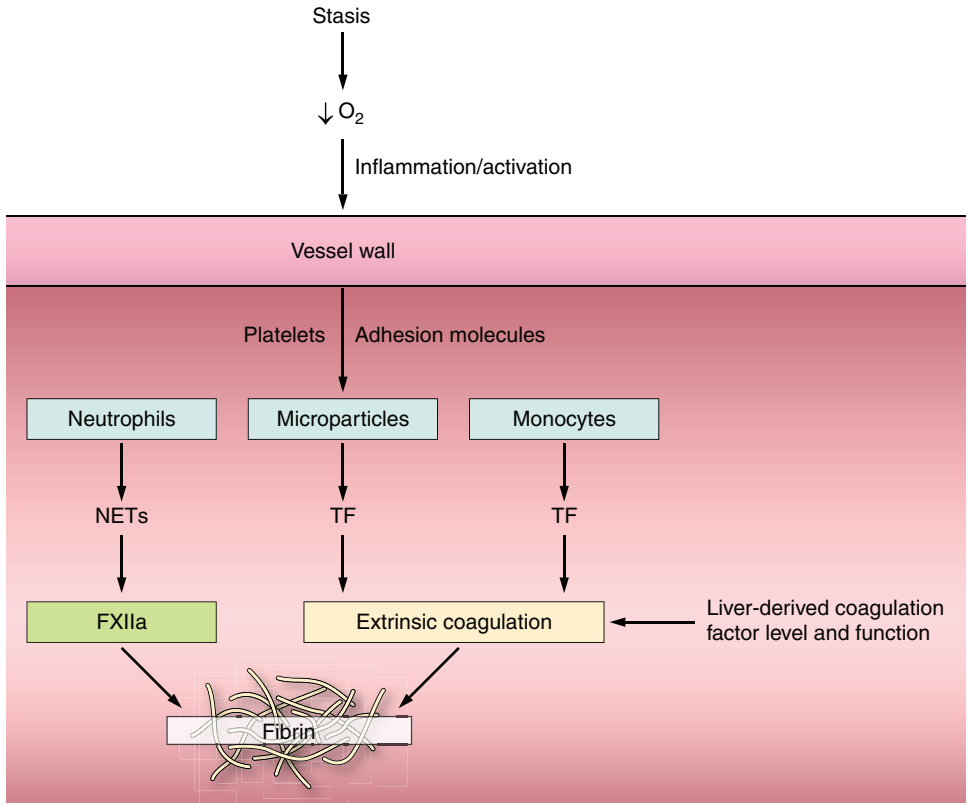

FIGURE 7. Simplified mechanistic schematic showing the role of key players in venous thrombosis. Obstruction of blood flow or stasis leads to hypoxic activation of the endothelium with the concomitant expression of adhesion proteins such as P-selectin. Circulating monocytes, neutrophils, platelets, and microparticles bind to the activated endothelium and locally provide ingredients, such as tissue factor (TF) and neutrophils extracellular traps (NETs), for initiation of extrinsic and intrinsic coagulation, thus triggering local thrombosis.

proach would be that there is less or no increased risk of bleeding with such therapies. This goes against the current dogma that effective prevention of VTE always increases risk of bleeding.

There is indeed evidence that the dogma may not hold. First, the platelet collagen receptor GPVI was identified in a genome-wide association study that searched for novel risk factors for VTE (29). Second, a recent clinical trial showed that treatment with aspirin strongly reduced the risk of recurrent VTE without increasing thrombotic risk (21).

Recent epidemiological studies have also provided intriguing evidence that treatment with statins may reduce the risk of (recurrent) VTE by as much as 30–50% both during and after anticoagulant treatment (106, 238, 251). Again, bleeding risk is not increased by statin treatment. The mechanism of VTE prevention by statins is unknown. A direct effect of statins on the liver production of coagulation factors is an unlikely mechanism. Unpublished data from the authors show that an 80 mg dose of simvastatin given for 6 months to individuals with familial hypercholesterolemia induces statistically significant changes in coagulation factor levels, but the absolute size of the effect is small and might not be biologically meaningful. These small effects are also in agreement with the notion that statins do not increase bleeding risk. Another candidate mechanism is inhibition of monocyte TF expression in vitro and in vivo (166, 169). Simvastatin reduced peripheral blood mononuclear cell TF expression and TF-positive MPs in hyperlipidemic monkeys (166, 169). Finally, simvastatin had anti-inflammatory properties in that it counteracted the effect of TNF-α on thrombomodulin, tPA, and PAI-1 (25).

B. The APC System and Disseminated Intravascular Coagulation

The APC system plays a central role in the pathogenesis of disseminated intravascular coagulation (DIC) and associated organ dysfunction (85, 157, 158). In patients with severe systemic inflammation, the protein C system is malfunctioning at virtually all levels. First, plasma levels of the zymogen protein C are low or very low, due to impaired synthesis, consumption, and degradation by proteolytic enzymes, such as neutrophil elastase (81, 305). Furthermore, a significant downregulation of thrombomodulin, caused by proinflammatory cytokines such as TNF-α and IL-1, has been demonstrated, resulting in diminished protein C activation (91, 193). Low levels of free protein S may further compromise an adequate function of the protein C system. In plasma, 60% of the cofactor protein S is complexed to a complement regulatory protein, C4b binding protein (C4bBP). Increased plasma levels of C4bBP as a consequence of the acute phase reaction in inflammatory diseases may result in a relative protein S deficiency. Support for this hypothesis comes from studies showing that the infusion of C4bBP in combination with a sublethal dose of *Escherichia coli* into baboons resulted in a lethal response with severe organ damage due to DIC (288). Finally, but importantly, in sepsis the EPCR has been shown to be downregulated, which may further negatively affect the function of the protein C system (290). Apart from these effects, a resistance toward activated protein C by other mechanisms may occur in DIC, partly dependent on a sharp increase in factor VIII levels (released from endothelial cells), but partly by yet unidentified mechanisms (69).

The hypothesis that restoration of the defective activated protein C pathways could be beneficial in the management of DIC has been extensively explored, in particular in the setting of severe sepsis. In murine models, a beneficial effect of APC in sepsis has been attributed to EPCR- and PAR1-dependent anticoagulant, cytoprotective, and endothelial barrier effects (142). However, a more recent view is that sepsis induces death by stimulating release of cytotoxic histones, while APC cleaves histones, reduces their cytotoxicity, and prevents lethality (324). In a human setting, a beneficial effect of rhAPC was demonstrated in a phase 3 trial (PROWESS) in patients with sepsis, that was prematurely stopped because of efficacy in reducing mortality in these patients (27). All-cause mortality at 28 days after inclusion was 24.7% in the rhAPC group versus 30.8% in the control group (19.4% relative risk reduction). The administration of rhAPC was demonstrated to cause an amelioration of coagulation abnormalities and a post hoc analysis of this trial demonstrated that patients with a diagnosis of DIC had a relatively greater benefit of rhAPC treatment than patients that did not have overt DIC (73). However, subsequent trials could not confirm the beneficial effect of rhAPC on mortality in sepsis. Meta-analyses of published literature conclude that the basis for treatment with APC, even in patients with a high disease severity, is not very strong or even insufficient. A recently completed placebo-controlled trial in patients with severe sepsis and septic shock was prematurely stopped due to the lack of any significant benefit of rhAPC (239). Subsequently, the manufacturer of rhAPC has decided to withdraw the product from the market.

A promising intervention for the management of DIC aimed at the APC system is represented by recombinant human soluble thrombomodulin. Several preclinical studies in experimental DIC models have shown that soluble thrombomodulin is capable of improving the derangement of coagulation and may restore organ dysfunction (158). Also, administration of recombinant soluble thrombomodulin caused significant anti-inflammatory effects (112). In a phase 3 randomized double-blind clinical trial in patients with DIC, administration of soluble thrombomodulin had a significantly better effect on bleeding manifestations and coagulation parameters than heparin, but the mortality rate at 28 days was similar in the two study groups (257). Cur-

rently, soluble thrombomodulin is being evaluated in a large phase 2/3 clinical study in patients with sepsis and DIC.

C. Platelets and Platelet-Adhesive Proteins in Thrombotic Angiopathies

The new insights in platelet-vessel wall interaction, as described above, have resulted in a better understanding of the pathogenesis of thrombotic vascular disorders (angiopathies) and may support attempts to come to a better clinical management of patients with these diseases.

Thrombotic microangiopathic syndromes are a group of disorders, comprising thrombocytopenic thrombotic purpura (TTP) and hemolytic uremic syndrome (HUS). These are characterized by thrombocytopenia as well as systemic and microvascular thrombotic occlusions, leading to ischemia and dysfunction of various organs, often in association with defective ADAMTS-13 (177). In HUS, microvascular thrombi predominantly occur in the kidney (leading to kidney failure), whereas in TTP often the brain is affected (causing neurological abnormalities) next to the kidney and other organs. A common pathogenetic feature of TTP and HUS is endothelial dysfunction, giving rise to ongoing platelet adhesion and aggregation, with secondary thrombin formation and an impaired fibrinolysis. The consequence is thrombocytopenia and mechanical injury of erythrocytes (hemolysis). The pathogenesis of these diseases is often linked to high concentrations of ultralarge VWF multimers and relative insufficiency of ADAMTS-13 to degrade these large multimers (99, 177). Under normal conditions, ADAMTS-13 activity cleaves large VWF multimers and prevents their binding and stretching at the endothelial surface. Deficiency in ADAMTS-13 impairs this downregulation and causes accumulation of ultralarge, highly hemostatically overactive VWF multimers at the endothelial cell surface, at which platelets spontaneously adhere via GPIb-V-IX and become activated by engagement of multiple adhesive receptors, as described above.

In most, if not all, cases of TTP, deficiency of ADAMTS-13 has also been identified as the underlying cause (99, 296). Genetic mutations in the ADAMTS-13 gene associated with lower activity of the protease have been linked to the occurrence of juvenile and familial TTP. Acquired deficiency of ADAMTS13, due to an autoantibody, has been demonstrated to occur in patients with sporadic TTP. Interestingly, the rationale for the treatment of TTP by infusion of large volumes of donor plasma or plasmapheresis, for a long time known to positively affect the clinical course of patients with this disease, has now become clear, since with this strategy the deficiency of ADAMTS-13 will be corrected.

Another acquired mechanism, though again centered around VWF multimers, may account for forms of HUS, caused by infections of shiga toxin-producing strains of *E. coli, Shigella dysenteriae,* and other microorganisms. Shiga toxins bind with high affinity to epithelial and microvascular endothelial cells, causing perturbation and the release of ultralarge VWF multimers. It is not completely clear why these ultralarge multimers are not cleaved by ADAMTS-13, since in patients with this acquired form of HUS levels of this protease are normal. It might well be that the massive release of ultralarge VWF may not keep pace with its cleavage, in combination with shiga toxin-induced activation of platelets.

Microangiopathic diseases may therefore be classified and treated according to the deficiency state of ADAMTS-13 rather than by the old classification, which was based on clinical criteria and known to be highly confusing since so many overlap situations occurred (297).

Several macrovascular alterations such as atherosclerosis and diabetes mellitus correlate with increased platelet activation (60, 306). Since in these cases there is no strong evidence for a gain in VWF function, it is likely that platelets themselves contribute to these diseases. The literature indeed contains evidence for this speculation, but this topic deserves much wider and more thorough investigation.

V. CONCLUSIONS

The research during the last decade has provided insight into the unexpectedly complex interplay between hemostatic processes in the vessel wall and the regulation of platelet and coagulation activation. The biochemical processes described above allow the construction of a general model of interactions between vessel wall, coagulation system, and platelet function. It is especially at the vulnerable or damaged vessel wall where the extrinsic and intrinsic coagulation mechanisms are initiated (e.g., by expression of tissue factor and collagen, respectively) and anticoagulation mechanisms are modulated (EPCR and PAR-type receptors). These are also the sites where circulating platelets adhere, form a plug, and immediately support thrombin generation by forming a procoagulant surface (exposed phosphatidylserine) and releasing procoagulant substances (polyphosphates). These platelets furthermore are the scavenging sites for fibrin clot formation and, at a later stage, they regulate fibrin clot contraction. This scheme is speculative with respect to the relative importance of these complex interactions in cases of hemostasis, hemostatic and prothrombotic disorders, and thrombosis in various vessels.

Answering these questions may allow new options for prevention and treatment of thrombotic disease.

Some of the important open questions that still need an answer are the following: Why do some adhered platelet cluster into aggregates, while others form a procoagulant surface? How is the anticoagulation system regulated during plug formation and particularly which are the roles of the vessel wall (endothelium and subendothelium), monocytes and neutrophils, and activated platelets therein? How does the adjacent vessel wall respond to the plug and clot formation? And finally, what is the regulatory potential of the adapted blood flow after plug and clot formation and secondarily vascular contraction?

ACKNOWLEDGMENTS

Inge Kos is acknowledged for constructing figures. Dr. Steve P. Watson (Univ. of Birmingham) is acknowledged for critically reading the manuscript and providing useful comments.

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GRANTS

H. H. Versteeg is supported by Netherlands Organization for Scientific Research (NWO) Grant nr 17.106.329. J. W. M. Heemskerk and P. H. Reitsma acknowledge financial support from the Center for Translational Molecular Medicine (Innovative Coagulation Diagnostics).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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