

MINI-REVIEW

A biophysical view on von Willebrand factor activation

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The process of hemostatic plug formation at sites of vascular injury crucially relies on the large multimeric plasma glycoprotein von Willebrand factor (VWF) and its ability to recruit platelets to the damaged vessel wall via interaction of its A1 domain with platelet GPIIb₃. Under normal blood flow conditions, VWF multimers exhibit a very low binding affinity for platelets. Only when subjected to increased hydrodynamic forces, which primarily occur in connection with vascular injury, VWF can efficiently bind to platelets. This force-regulation of VWF's hemostatic activity is not only highly intriguing from a biophysical perspective, but also of eminent physiological importance. On the one hand, it prevents undesired activity of VWF in intact vessels that could lead to thromboembolic complications and on the other hand, it enables efficient VWF-mediated platelet aggregation exactly where needed. Here, we review recent studies that mainly employed biophysical approaches in order to elucidate the molecular mechanisms underlying the complex mechano-regulation of the VWF-GPIIb₃ interaction. Their results led to two main hypotheses: first, intramolecular shielding of the A1 domain is lifted upon force-induced elongation of VWF; second, force-induced conformational changes of A1 convert it from a low-affinity to a high-affinity state. We critically discuss these hypotheses and aim at bridging the gap between the large-scale behavior of VWF as a linear polymer in hydrodynamic flow and the detailed properties of the A1-GPIIb₃ bond at the single-molecule level.

KEYWORDS

biophysics, cell adhesion, GPIIb₃, hemostasis, platelets

1 | INTRODUCTION

Platelet adhesion to subendothelial matrix proteins and platelet aggregation are crucial initial steps for the formation of hemostatic plugs at sites of vascular injury. These processes are highly complex and dynamic and involve a variety of specific adhesion receptors and ligands, as reviewed for example by (Jackson, 2008; Ruggeri & Mendolicchio, 2007). A key player in platelet adhesion and aggregation is the large multi-domain plasma glycoprotein von Willebrand factor (VWF). Each mature VWF monomer possesses a number of domains with distinct structures and functions. As recently re-annotated by Zhou et al. (2012), these domains are D'D3, A1, A2, A3, D4, C1 to C6, and CK (Figure 1a). Via its domains A1 and A3, VWF can bind to constituents of the extracellular matrix of the subendothelium, for instance to different types of collagen (reviewed in [Lenting, Casari, Christophe, & Denis, 2012]). VWF can further directly interact with platelets, as it exhibits binding sites for the N-terminal domain of

GPIIb₃ (Vicente, Kostel, & Ruggeri, 1988), which is part of the platelet membrane receptor complex GPIIb-IX-V, and for the platelet integrin $\alpha_{IIb}\beta_3$, located in its A1 (Fujimura et al., 1986) and C4 domain (Plow, Pierschbacher, Ruoslahti, Marguerie, & Ginsberg, 1985), respectively.

After secretion from megakaryocytes, platelets, and vascular endothelial cells, either constitutively or via a stimulated pathway (Lopes da Silva & Cutler, 2016), VWF circulates in the plasma in the form of multimers that comprise a varying number of linearly connected monomeric subunits (Figure 1b) (Wagner, 1990). VWF multimers can reach an immense length, in the order of several micrometers, and adopt a loosely collapsed, globular conformation under normal blood flow conditions (Schneider et al., 2007). In this form, VWF can bind coagulation factor VIII (Owen & Wagner, 1972), while exhibiting only a very low affinity to platelets (Jackson, 2008; Ruggeri and Mendolicchio, 2007).

The prerequisite for an increase in the affinity, and thus, binding of the VWF A1 domain to platelet GPIIb₃, is that VWF

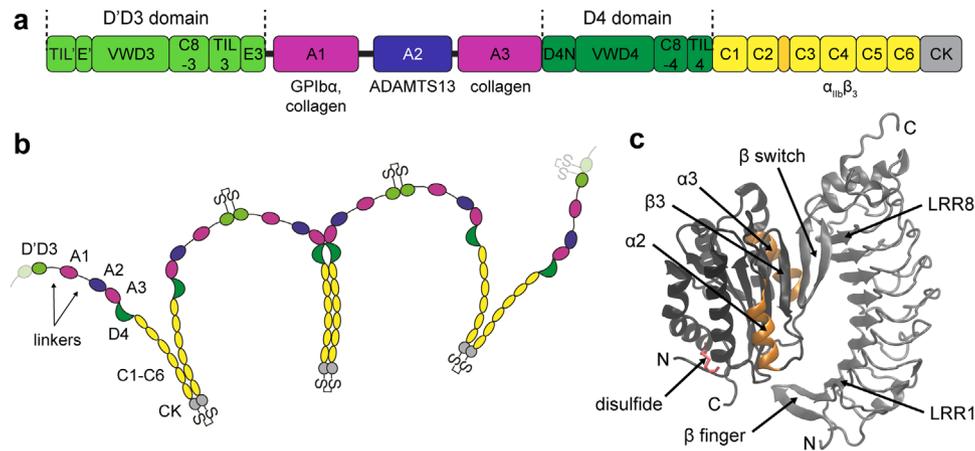


FIGURE 1 Architecture of VWF and structure of the A1-GPIIb/IIIa complex. (a) Domain annotation of a mature VWF monomer, according to (Zhou et al., 2012). The locations of binding sites for different interaction partners are indicated. (b) Schematic architecture of a VWF multimer. Multimers are built from monomers that are linearly linked head-to-head and tail-to-tail via disulfide bonds. (c) Crystal structure of the complex of the wtA1 domain with the N-terminal domain of GPIIb/IIIa, shown in cartoon representation (PDB: 1SQ0, [Dumas et al., 2004]). A1 and GPIIb/IIIa are depicted in black and gray, respectively. Helices $\alpha 2$ and $\alpha 3$ of A1 (orange) and the long-range disulfide bond between A1's N- and C-termini (red) are highlighted. The image was created using VMD, v.1.9.3 ([Humphrey, Dalke, & Schulten, 1996], <http://www.ks.uiuc.edu/Research/vmd/>)

experiences increased hydrodynamic forces (Savage, Saldívar, & Ruggeri, 1996) due to alterations from the normal blood flow profile, in particular as a result of increased elongational flow (Sing & Alexander-Katz, 2010; Springer, 2014; Zhang, Halvorsen, Zhang, Wong, & Springer, 2009).

Such conditions can for instance be found at sites of vascular injury where vasoconstriction reduces the vessel diameter, thereby increasing hydrodynamic forces enough for VWF multimers to be elongated, resulting in efficient binding of VWF to subendothelial collagen (Schneider et al., 2007). When immobilized, a VWF multimer experiences further increased tensile force along its contour, which appears to promote activation of binding to platelet GPIIb/IIIa. The latter also holds for VWF multimers still bound to the surface of endothelial cells after stimulated secretion (Dong et al., 2002; Mourik et al., 2013; Wang et al., 2012). Furthermore, pathologically high shear rates can occur in stenosed vessels or at prosthesis. In this case, the A1-GPIIb/IIIa interaction can even mediate adhesion and aggregation of non-activated platelets without involvement of integrins (Ruggeri, Orje, Habermann, Federici, & Reininger, 2006).

The need for activation of VWF for GPIIb/IIIa binding by hydrodynamic forces is not only highly intriguing from a biophysical perspective, but also of eminent physiological importance. While VWF-mediated platelet aggregation is necessary at sites of vascular injury, it would rapidly induce thrombotic occlusions if activated under normal blood flow conditions.

Especially in the last decade, tremendous efforts have been made to apprehend the molecular basis of this complex mechano-regulation of the VWF A1-GPIIb/IIIa interaction. The insights gained led to the proposal of different mechanisms that may be involved in VWF's force-induced activation. These suggested regulatory mechanisms can roughly be grouped into two main pictures. First, the A1 domain may initially be "shielded" by neighboring VWF peptide

sequences and only be accessible for GPIIb/IIIa binding upon force-induced opening of these intramolecular interactions under elevated flow conditions. Second, the A1-GPIIb/IIIa bond per se may possess an intrinsic force dependence and exhibit an enhanced affinity upon loading with force, resulting from force-induced conformational changes within A1 and/or GPIIb/IIIa.

Here, we review studies that promoted advances in understanding the molecular mechanisms underlying the regulation of the VWF A1-GPIIb/IIIa interaction, often by applying biophysical approaches. We conclude that the above pictures of VWF's activation do not exclude each other, but rather may well be two sides of the same coin that are both essential for VWF's intricate mechano-regulation.

2 | VWF A1 DOMAIN SHIELDING BY INTRAMOLECULAR INTERACTIONS

2.1 | The globule-stretch transition of VWF

It has been shown that VWF multimers adopt a loosely collapsed, globular conformation in solution (Schneider et al., 2007). The precise nature of the interactions that promote this conformation is not yet well understood, but a recently identified strong intradimer interaction mediated by VWF's D4 domain can be assumed to contribute by promoting a compact conformation of the individual dimers within a multimer (Müller, Löf, et al., 2016, Müller, Mielke, et al., 2016). Very likely, also additional interactions between the different dimers of a multimer are involved. Such inter-dimer interactions might be of a more unspecific nature, for example, based on electrostatic or van der Waals interactions or on hydrophobic effects.

When subjected to hydrodynamic forces above a certain threshold, globular multimers undergo an abrupt transition into an

elongated, “stretched” form (Alexander-Katz, Schneider, Schneider, Wixforth, & Netz, 2006; Schneider et al., 2007). In the vasculature, this conformational change likely occurs primarily at sites of vascular injury or in stenosed vessels, where alterations from the normal blood flow profile can be expected to result in increased elongational forces acting on VWF multimers (Sing & Alexander-Katz, 2010; Springer, 2014; Zhang et al., 2009). A key aspect to comprehend the abruptness of VWF’s elongation is the positive feedback between the effective length of a multimer and the hydrodynamic force it experiences in flow (Müller, Mielke, et al., 2016; Springer, 2014; Zhang et al., 2009). In other words, initial partial stretching of a multimer leads to higher forces acting on the multimer, which in turn promote further elongation. Here, elongational flow is particularly effective, as the relaxation of a multimer is suppressed compared to simple shear flow (Perkins, Smith, & Chu, 1997; Sing & Alexander-Katz, 2010; Zhang et al., 2009). Also due to the relation between multimer length and force, larger multimers—comprising more monomeric subunits—will experience higher forces in the blood flow than smaller ones. Indeed, the latter have been reported to be the less hemostatically active multimers, since in von Willebrand disease (VWD) patients the reduction of high-molecular-weight multimers leads to bleeding symptoms (reviewed in [Sadler, 2005; Sadler et al., 2006]). Although trivial, it is important to note that force acting along the contour of a multimer also impacts each domain within the multimer, unless the domains are shielded by intramolecular interactions within VWF (Müller, Löf, et al., 2016; Müller, Mielke, et al., 2016).

In microfluidic experiments, Schneider et al. (2007) showed that elongation of VWF above a certain threshold shear rate directly correlated with immobilization of VWF onto a collagen-coated surface. Presumably, elongation of VWF leads to increased exposure of interaction sites that facilitate multivalent binding events. It is easily conceivable that, similarly, also the accessibility of A1 domains, containing the binding site for GPIIb, increases upon elongation of VWF. However, such a simple correlation between elongation of VWF and binding to GPIIb has, to the best of our knowledge, not been shown experimentally. In addition, a markedly higher shear rate threshold has been observed for VWF-mediated aggregation of non-activated platelets than for activation of VWF for collagen binding (Ruggeri et al., 2006). Especially the latter observation suggests that at least one force-dependent regulatory mechanism is present within VWF that tunes the affinity of the A1 domain for the platelet receptor GPIIb-IX-V. This hypothesis is supported by the fact that the A1-GPIIb interaction can be induced in the absence of force by certain modulators that interact with VWF’s A1 domain, for example, the bacterial glycoprotein ristocetin (De Luca et al., 2000; Dong et al., 2001; Howard & Firkin, 1971) or the snake venom protein botrocetin (Fukuda, Doggett, Laurenzi, Liddington, & Diacovo, 2005; Read, Shermer, & Brinkhous, 1978).

Motivated by the above considerations, a series of studies were performed to elucidate whether interdomain interactions within VWF inhibit its binding activity. The studies summarized below have identified such interactions between the A1 domain (aa1260–1479) and its neighboring peptide sequences.

2.2 | Regulation of the A1-GPIIb interaction by D’D3 and the N-terminal flanking peptide of A1

First indication that A1-domain-neighboring amino acid sequences are involved in the regulation of the A1-GPIIb-interaction was provided in 1988 by Mohri et al. (1988), who showed that addition of isolated peptides comprising the sequence N-terminal of the A1 domain (aa1232–1261) inhibited binding of purified VWF to platelets. Five years later, Sugimoto, Dent, McClintock, Ware, & Ruggeri, 1993 found that stepwise deletion of the amino acid segment aa1204–1270 increased binding of the remaining A1 peptide to GPIIb.

Intriguing further information on A1 activation was gained by investigation of certain VWF mutants. Dysfunction of VWF induced by mutations in the *vwf* gene causes the most common hereditary bleeding disorder, VWD (reviewed by [Sadler, 2005]). Depending on which domain function within VWF is disrupted by genetic alterations, different types of VWD can be distinguished that influence platelet binding (Sadler, 2005; Sadler et al., 2006). Certain mutations primarily localized in the A1 flanking regions result in VWD type 2B, which is characterized by an increased affinity of VWF for GPIIb even without prior activation. In type 2M, mutations are localized in the A1 domain and cause a decreased affinity for platelet GPIIb.

Alanine-scanning mutagenesis indicated that several mutations in regions flanking the A1 domain result in a gain-of-function phenotype for GPIIb binding that is similar to type 2B VWD (Matsushita and Sadler, 1995). Based on these mutagenesis studies and the amino acid position of naturally occurring type 2B VWD mutations, three segments (aa1260–1274, aa1450–1461, and aa1303–1341) were identified as potential inhibitors of GPIIb binding. Since the first two segments were acidic and the proposed GPIIb binding site basic, it was proposed that intramolecular electrostatic interactions among these sites regulate VWF binding to GPIIb.

Building on the latter study, Nakayama et al. (2002) further characterized the role of these regions in regulation of GPIIb binding by measuring binding of mutants with deletions in the flanking regions. They identified Arg1308 as an important interacting amino acid, as its mutation to alanine increased GPIIb binding. In this context, additional deletions of aa1260–1271 or aa1459–1472 affected the p.Arg1308Ala phenotype and caused marked spontaneous binding. These observations suggested that conformational integrity between Arg1308 and the A1 flanking regions is important for regulating VWF-GPIIb binding. Co-crystallization of gain-of-function mutants of both VWF A1 and the N-terminal domain of GPIIb was in line with these findings (Huizinga et al., 2002).

Auton, Sowa, Behymer, and Cruz (2012) further provided evidence that regulation by the N-terminal flanking region of A1 extends to aa1238, by showing that a truncated A1A2A3 domain construct comprising aa1261–1874 exhibited an apparent binding activity for GPIIb higher than that of a longer construct (aa1238–1874). Thermal unfolding, monitored by circular dichroism spectroscopy and differential scanning calorimetry, indicated that this N-terminal peptide provides structural stability to the A domain complex and plays a role in modulating the A1-GPIIb interaction.

It should be noted that most of the above-described studies relied on assays performed under static conditions and often required the

use of modulators such as ristocetin to induce binding of A1 domain variants to GPIIb. Thus, the implications of the described observations for the physiologically relevant case—binding of A1 to GPIIb under flow and in the absence of modulators—are not always entirely clear. However, several further experiments performed under shear flow yielded results that were in line with the abovementioned studies. In particular, A domain constructs lacking the A1 N-terminal flanking peptide exhibited enhanced binding to GPIIb peptides as well as to platelets under flow (Auton et al., 2012; Ju, Dong, Cruz, & Zhu, 2013; Madabhushi, Zhang, Kelkar, Dayananda, & Neelamegham, 2014).

Ulrichs et al. (2006) further postulated that also the D'D3 domain serves as a masking environment that inhibits binding to the GPIIb-IX-V receptor. This idea was based on the finding that an antibody that recognizes an epitope in the D'D3 domain (aa764–1035) increased VWF-platelet binding (Tornai, Arnout, Deckmyn, Peerlinck, & Vermylen, 1993). They produced dimeric VWF constructs either with (plusD'D3) or without (Δ D'D3) the D'D3 domain and found that the deletion increased ristocetin-induced binding to recombinant GPIIb. It is worth mentioning that the Δ D'D3 construct contained domains A1-CK and was therefore, C-terminally dimerized. In contrast, the plusD'D3 construct lacked domains D4-CK and was thus N-terminally dimerized by intra-dimer disulfides between the D'D3 domains. It could be argued that this difference partially contributed to the observed shielding effect of D'D3, but later on Madabhushi et al. (2014) showed the same effect also with C-terminally linked dimers lacking D'D3. Additional evidence for a direct inhibition of the A1-GPIIb interaction through D'D3 was provided by showing that the presence of isolated D'D3 domain peptides directly inhibited ristocetin-stimulated platelet agglutination mediated by the C-terminally dimerized Δ D'D3 construct (Ulrichs et al., 2006). Cross-blocking experiments further showed that the D'D3 domain was more accessible when A1 was occupied by antibodies. These data support a model of D'D3 translocation from A1 that allows interaction with GPIIb (Ulrichs et al., 2006). Counterintuitively and contrarily to the above-described finding by Tornai et al. (1993), Madabhushi et al. (2014) reported that an anti-D'D3 antibody blocked shear-induced platelet agglutination in whole blood. However, in apparent contrast to this latter observation, they also showed that a Δ D'D3 construct exhibited increased platelet adhesion under flow conditions.

Taking into account the main results of all of the above studies, it appears plausible that intramolecular, most likely electrostatic, interactions of the A1 domain with its N- and C-terminal flanking peptides as well as with D'D3 occur that shield the GPIIb binding site, at least as long as VWF adopts a globular conformation. It can be assumed that such an inhibitory effect could be lifted upon elongation of VWF multimers by increased hydrodynamic forces.

2.3 | Interaction of A2 with an “active” conformation of A1

Nishio, Anderson, Zheng, and Sadler (2004) showed that deletion of the A1 domain dramatically increased the cleavage of A2 by the VWF-specific protease ADAMTS13. Lankhof et al. (1997) further reported that a VWF construct with an A2 domain deletion exhibited a slightly increased

ristocetin-induced binding to GPIIb. As these data indicated an inhibitory interaction between these two domains, Martin, Morales, and Cruz (2007) aimed to investigate if the A1 domain is shielded by the C-terminal A2 domain. They detected binding of the purified A2 domain to the isolated A1 domain in pull-down experiments and further showed that the isolated A2 domain binds to full-length VWF. The observation that the latter interaction only occurred when VWF was immobilized or activated by ristocetin, suggested that soluble A2 recognized only a putative activated form of A1. Taken together, these data suggest an autoinhibitory effect between A1 and A2 that could regulate the sequential activation of platelet binding and subsequent cleavage by ADAMTS13. Aponte-Santamaría et al. (2015) performed molecular dynamics (MD) simulations and—at least in silico—found strong indication that the two domains can directly interact in the context of the full-length protein and that stretching of the two connected domains would indeed first uncover the GPIIb binding site, and secondly lead to exposure of the cleavage site. In line with the MD simulations, microfluidic measurements indicated that deletion of the A2 domain yields a gain-of-function VWF variant with enhanced binding to GPIIb under flow.

Summarizing, these data suggest that some kind of, potentially electrostatic, intramolecular interaction exists between the domains A1 and A2. If such an interaction—in context of the full-length protein—has an inhibitory effect on A1-GPIIb binding and/or plays a role in regulation of A2 cleavage by ADAMTS13 requires further experimental investigation.

2.4 | The role of glycans in the VWF-GPIIb interaction

VWF is a highly glycosylated and sialylated protein and its N- and O-glycans have been implicated to affect many of VWF's properties and functions, such as processing and secretion (Galnack, Williams, & Rick, 1983; McKinnon et al., 2010; Wagner, Mayadas, & Marder, 1986), as well as its half-life in blood (Sodetz, Pizzo, & McKee, 1977). Already in 1985 it has been reported that desialylation of VWF enhances its binding to platelets via GPIIb (De Marco, Girolami, Russell, & Ruggeri, 1985).

Recently, Fallah et al. (2013) showed that overall N-deglycosylation of VWF increased platelet adhesion both to immobilized and to soluble VWF. Madabhushi et al. found that the influence of the N-terminal peptide of the A1 domain depends on its glycosylation status. In particular, removal of the O-glycans by exchange of the sugar-carrying amino acids to alanine enhanced platelet binding (Madabhushi et al., 2014).

Thus, the above studies indicate that VWF's glycans may have a crucial impact on the A1-GPIIb interaction and its regulation. However, extensive further research in this direction is required to fully understand the molecular basis of this potential regulatory mechanism.

3 | INTRINSIC FORCE-REGULATION OF THE A1-GPIIb INTERACTION

The above-described potential mechanisms for regulating VWF's hemostatic activity are primarily based on initial shielding of the A1 domain by intramolecular interactions and an increased exposure of A1 domains under elevated flow conditions. However, already two

decades ago it has been proposed that A1 also possesses an intrinsic regulatory mechanism, as studies on a series of fragments of the A1 domain had suggested the existence of two different conformations of A1 exhibiting markedly distinct kinetics of binding to GPIIb α (Miyata & Ruggeri, 1999; Miyata, Goto, Federici, Ware, & Ruggeri, 1996). Force acting on A1 may induce the transition from its low-affinity state to a distinct high-affinity conformation. Especially in the last few years, strong experimental evidence for such a scenario has been put forward, and effort has been made to find mechanistic explanations.

3.1 | Force-dependent binding kinetics

To elucidate the long-known force-enhancement of binding of A1 to GPIIb α (Savage et al., 1996), Yago et al. (2008) proposed that the A1-GPIIb α interaction exhibits “catch-bond” characteristics, that is, that within a certain force range bond lifetimes increase with increasing force. To test this hypothesis, they performed flow experiments with isolated A1 domain peptides immobilized on the surface of a parallel-plate flow chamber. A1-mediated rolling of platelets or GPIIb α -coated microspheres exhibited biphasic velocity curves as a function of the prevailing wall shear stress. The rolling velocity decreased with increasing shear stress up to an optimal value before increasing again at higher shear stress. The elevated adherence of platelets to A1 with increasing force thus suggested catch-bond behavior of the A1-GPIIb α interaction. However, since many A1-GPIIb α bonds can be expected to be present at the same time on a rolling platelet, it is difficult to draw conclusions at the single-bond level.

Furthermore, the above observations by Yago et al. were in line with AFM force measurements of the bond lifetimes performed in the same study. However, considering the relatively simple experimental setup chosen for these AFM measurements, it appears possible that multiple A1-GPIIb α bonds have been probed rather than single bonds. In addition, it should be noted that, both in the AFM and in the flow experiments, A1 domains were immobilized on a surface and not subjected to significant forces prior to binding, in contrast to the situation *in vivo*, where A1 domains within elongated VWF multimers in flow can be expected to experience forces that are large enough to potentially induce relevant conformational changes (Kim, Hudson, & Springer, 2015; Zhang et al., 2009).

In two further AFM-based studies, Wijeratne et al. (2013, 2016) performed force measurements on VWF multimers and dimers after exposure to high shear stress. They reported that peaks in the obtained force-extension traces, which were interpreted as domain unfolding events, occurred at higher forces for sheared than for non-sheared multimers. These results are in contradiction to the expectation that all domains of VWF except A2 are protected from unfolding by long-range disulfide bonds (Zhou et al., 2012). Indeed, only unfolding of A2 has been observed in other studies, and at markedly lower forces than reported by Wijeratne et al. (Müller, Löf, et al., 2016; Müller, Mielke, et al., 2016; Zhang et al., 2009). Taking into account that in their approach multimers were unspecifically adsorbed to a gold surface and not pulled via specific handles, it appears likely that also unspecific protein-surface or protein-protein interactions have been probed.

Detailed insights at the single-molecule level into how the application of force modulates the interaction between A1 and GPIIb α have been gained recently by two force spectroscopic studies by Kim et al. employing optical tweezers (Kim, Zhang, Zhang, & Springer, 2010; Kim et al., 2015). They engineered a protein construct comprising both A1 and the N-terminal domain of GPIIb α , connected by a flexible polypeptide linker, and used this construct to investigate the force dependence of the binding and unbinding kinetics of the A1-GPIIb α bond. Force-induced switching of the bond to a distinct second state was observed at forces above ~ 10 pN, that is, in the physiologically relevant force range (Zhang et al., 2009). This second bond state exhibited a greater force resistance, a markedly longer lifetime due to a lower off-rate (Kim et al., 2010), and a higher on-rate (Kim et al., 2015). Thus, the observed mechanism of force-induced switching to a second high-affinity bond state provides an explanation for the enhanced binding of A1 to GPIIb α under elevated flow conditions. Importantly, the existence of two distinct bond states—which were observed not only for unbinding, but also for rebinding—again suggests the existence of two different conformational states of A1 already before binding to GPIIb α . This idea is in line with the observation that the modulator ristocetin, which is thought to activate A1 in a manner closely resembling force-dependent activation (Dong et al., 2001), selectively stabilizes the second bond state (Kim et al., 2010). In contrast, for the modulator botrocetin, which induces activation by a different mechanism not resembling force-induced activation (De Luca et al., 2000; Dong et al., 2001; Fukuda et al., 2005), only a single bond state was observed (Kim et al., 2010). Presumably, binding of ristocetin to the A1 domain induces or stabilizes a configuration of A1 corresponding to its force-induced active conformation. One should however, bear in mind that due to the artificial connection between the two proteins in this experimental setup not only A1 but also GPIIb α might undergo relevant force-induced conformational changes before binding, whereas in the situation *in vivo* only A1 is expected to be subjected to significant forces prior to bond formation (Kim et al., 2015).

Kim et al. (2015) further investigated the effect of two gain-of-function mutations in A1 (p.Arg1306Gln) or in GPIIb α (p.Met239Val) that lead to a higher affinity of A1 and GPIIb α for each other and cause type 2B VWD and platelet-type VWD, respectively. Force-induced switching between two different bond association/dissociation pathways was still observed for both of these mutants, at forces similar to the wildtype (wt). In the first bond state at low force, binding kinetics exhibited only modest changes compared with the wt—counterintuitive to the fact that VWF mutants associated with type 2B VWD exhibit enhanced binding to platelets also under static conditions (Sadler et al., 2006). The second bond state at higher forces however, showed markedly faster bond association and slower bond dissociation than observed for the wt, indicating that type 2B VWD mutations enhance the impact of force on the modulation of the A1-GPIIb α interaction.

For two gain-of-function A1 domain mutants (p.Arg1306Gln and p.Arg1450Glu), Yago et al. did not observe biphasic velocity curves for platelet rolling. In contrast to wtA1, low rolling velocities were observed already at low shear stress that increased monotonically with

increasing shear stress (Yago et al., 2008). These data are in apparent contradiction to the study of Kim et al., as they do not show force-enhancement of A1-GPIIb binding for type 2B mutants. The reasons for this discrepancy are unclear, but might be related to the fact that the flow experiments have not probed the behavior of single bonds. Interestingly, in a later study employing very similar flow experiments Coburn et al. (2011) did observe biphasic velocity curves not only for platelets rolling on wtA1, but also on a gain-of-function VWD type 2B mutant as well as on a loss-of-function, type 2M mutant (p.Arg1450Glu and p.Gly1324Ser, respectively). The range over which rolling velocities decreased with increasing shear stress was observed to be shifted to lower (gain-of-function A1) and higher (loss-of-function A1) shear stress values, respectively. Intuitively, the latter finding would suggest that for gain-of-function A1 mutants force-induced switching to a high-affinity bond state occurs at lower forces than for wtA1. However, this was not observed in the optical tweezers study by Kim et al. (2015). Ju et al. (2013) noted that discrepancies between studies might originate from the fact that different A1 domain constructs were used and only some of them comprised the N-terminal flanking region (aa1238–1260). This idea was substantiated by their observation that platelet rolling velocities observed in flow experiments as well as A1-GPIIb bond lifetimes measured by using a biomembrane force probe showed differences when using A1 constructs with and without the N-terminal flanking region. In particular, the presence of this flanking region appeared to stabilize platelet attachment under high shear stress and to increase bond lifetimes at higher forces.

Taken together, there is strong evidence that the A1-GPIIb interaction indeed possesses an intrinsic mechanism for force-regulation that may rely on the existence of two distinct conformations of A1 and/or GPIIb associated with different affinities.

3.2 | Distinct conformational states of A1

Gain-of-function mutations in A1, associated with type 2B VWD, are primarily located in regions close to the N- and C-termini of A1, which are connected by a long-range disulfide bond (Emsley, Cruz, Handin, & Liddington, 1998). This is intriguing not only because these peptide sequences are distal to the main binding surface for GPIIb observed in crystal structures of A1-GPIIb complexes (Dumas et al., 2004; Huizinga et al., 2002) (Figure 1c), but also because they are the sites where force is applied to the A1 domain in elongated VWF multimers. Consequently, it has been proposed that force acting on A1's termini induces conformational changes that result in an activated conformation of A1 (Emsley et al., 1998; Huizinga et al., 2002).

Crystal structures of complexes of A1 with the N-terminal domain of GPIIb (Figure 1c) have revealed a discontinuous binding interface between the globular A1 domain and the curved, concave face of GPIIb, which is formed by eight leucine-rich repeats (LRRs) (Dumas et al., 2004; Huizinga et al., 2002). The main interaction occurs near the "top" face of A1—distal from A1's termini—and involves the central $\beta 3$ strand, the $\alpha 2$ helix, and the $\alpha 3$ - $\beta 4$ loop of A1, as well as LRRs 5–8 and the C-terminal flanking region of GPIIb. A part of this flanking region, the so-called β switch, forms a β sheet with A1's $\beta 3$ strand upon

binding. A less extensive contact was observed at A1's "bottom" face between the N-terminal, so-called β -finger region of GPIIb and the $\alpha 1$ - $\beta 2$ loop of A1, involving also LRR1 of GPIIb and loops $\beta 3$ - $\alpha 2$ and $\alpha 3$ - $\beta 4$ of A1.

Based on the crystal structure of a complex between gain-of-function mutants of both GPIIb (p.Met239Val) and A1 (p.Arg1306Gln), it has been hypothesized that force-induced displacement of A1's termini might lead to increased accessibility of the contact area between the $\alpha 1$ - $\beta 2$ loop of A1 and the β -finger region of GPIIb (Huizinga et al., 2002). Indeed, a crystal structure obtained for the wtA1-GPIIb complex revealed structural deviations in this region from the gain-of-function mutant complex, while the overall complex structure was very similar (Dumas et al., 2004). In a recent study, however, Blenner, Dong, and Springer (2014) argued that this contact between A1's $\alpha 1$ - $\beta 2$ loop and the β -finger region of GPIIb may represent a clash rather than a favorable binding interaction. Based on several newly solved crystal structures of A1-GPIIb complexes of both wt and gain-of-function mutants, these authors instead proposed that LRRs 2–5 of GPIIb play an essential role for the high-affinity A1-GPIIb binding induced by force. Such a scenario is in line with earlier reports that this region is crucial for GPIIb-mediated binding of VWF to platelets under flow, as was inferred from investigation of canine-human chimeras of GPIIb (Shen et al., 2000, 2006). Indeed, in crystal structures of certain gain-of-function mutant A1-GPIIb complexes, Blenner et al. (2014) identified a previously undescribed interaction between LRRs 4 and 5 with residue Lys1371 at the C-terminus of A1's $\alpha 3$ helix. This interaction was enabled by straightening of the concave surface of GPIIb, which allows for a closer approach of A1 and increases electrostatic interactions between the electrostatically complementary interfaces of A1 and GPIIb. Interestingly, strain appeared to occur both in wt and mutant complexes in regions close to the locations of gain-of-function mutations. The authors therefore, hypothesized that the currently known crystal structures of A1-GPIIb complexes may not yet represent the putative high-affinity conformation between A1 and GPIIb induced by force. The high-affinity conformation may be a result of relatively large force-induced conformational changes of A1 that dissipate strain and allow A1 to form a binding interface complementary to the concave surface of GPIIb (Blenner et al., 2014).

Large-scale conformational changes of A1 in connection with its activation have also been proposed by Auton, Cruz, and Moake (2007) who monitored the urea-induced unfolding of VWF's A domains by circular dichroism spectroscopy in order to investigate their conformational stability. The A1 domain was observed to unfold via a structured intermediate state with high stability, in contrast to the structurally very similar domains A2 and A3, which both exhibited simple two-state unfolding. The authors hypothesized that hydrodynamic forces in the blood flow could partially unfold A1 to the observed structured intermediate state, which may be capable to bind efficiently to GPIIb. This hypothesis was later supported by the observation that certain gain-of-function mutations associated with type 2B VWD decreased the thermodynamic stability of A1's native state and thus favored the intermediate state, whereas certain loss-of-function mutations associated with type 2M VWD stabilized the

native conformation of A1 (Auton et al., 2009). Furthermore, disruption of A1's long-range disulfide bond was observed to result in a structural transition of A1 to a "molten globule" state with a severe loss of tertiary structure (Tischer, Madde, Blancas-Mejia, & Auton, 2014), but strikingly at the same time to enhance binding of platelets in flow at low shear rates (Miyata & Ruggeri, 1999; Tischer, Madde, Blancas-Mejia, et al., 2014). This observation again suggests the existence of constraints in the globular state of A1 that confine it to a low-affinity conformation and that might be released by force acting on A1.

A more rigorous analysis of a series of both gain- and loss-of-function A1 domain mutants—combining thermal and chemical denaturation with circular dichroism and fluorescence spectroscopic approaches—revealed however a more complex picture of the impact of mutations on the overall structure of A1 (Tischer, Madde, Moon-Tasson, & Auton, 2014). Remarkably, a variety of different conformations was observed for both kinds of mutations, ranging from natively structured to molten globule conformations. This finding indicates that, ultimately, local effects on certain regions of A1 rather than on its overall structure underlie the changes in affinity for GPIIb α , although these local effects may be accompanied by further large-scale conformational changes.

Based on a comparison of the locations and structural effects of different mutations, Tischer, Madde, Moon-Tasson, et al. (2014) proposed that the α 2 helix of A1 (highlighted in Figure 1c) inhibits binding to GPIIb α , and that destabilization or unfolding of this helix result in an enhanced binding affinity. Local unfolding of the α 2 helix induced by force acting on A1's termini therefore appears to be a likely mechanism for A1's force-enhanced binding to GPIIb α . Notably, the α 2 helix can interact with the N-terminal arm of A1 via the salt bridge Arg1341-Glu1264 (Emsley et al., 1998). Conversely, the α 3 helix (highlighted in Figure 1c) was suggested to be essentially involved in binding to GPIIb α , as its destabilization was a common feature observed in loss-of-function mutants (Tischer, Madde, Moon-Tasson, et al., 2014). This hypothesis is in agreement with the abovementioned interaction between a region of A1 in close proximity to the α 3 helix and GPIIb α (Blenner et al., 2014).

These proposed roles of helices α 2 and α 3 are in line with several other experimental and computational studies. An impact of the local dynamics of the α 2 helix on A1's affinity for GPIIb α had been suggested before by all-atom molecular dynamics simulations on the wt A1 domain and two gain-of-function A1 mutants (p.Arg1306Gln and p.Ile1309Val) (Liu, Fang, & Wu, 2013). Involvement of the α 3 helix in the A1-GPIIb α interaction was at least hinted at by the crystal structure of GPIIb α in complex with a short α -helical peptide that very efficiently inhibits binding of GPIIb α to the A1 domain (McEwan, Andrews, & Emsley, 2009). Superimposition of this structure on the one of the A1-GPIIb α complex (Huizinga et al., 2002) revealed an overlap of the inhibiting peptide with A1's α 3 helix. This observation should however, not be overinterpreted since the peptide also interacts with the β switch region of GPIIb α that is essential for β sheet formation with the central β 3 strand of A1. In another computational approach, Zimmermann, Tischer, Whitten, and Auton (2015) investigated which segments of A1 are most likely to locally unfold, utilizing a recently

developed algorithm that aims at identifying structural segments in a protein that fold/unfold cooperatively, based on computationally determined values for free energy differences between folded and unfolded states (Porter & Rose, 2012). This analysis indicated that a region comprising helices α 2 and α 3 and the connecting loop possesses low structural cooperativity, implying that α 2 and α 3 may be prone to local unfolding (Zimmermann et al., 2015). This was further backed up by the observation that α 2 and α 3 are more susceptible to proteolysis by trypsin than other structural elements of A1 (Tischer et al., 2016). These results suggest that the local dynamics of helices α 2 and α 3 and their inclination toward local unfolding may be crucial for the force-regulation of the affinity of A1 for GPIIb α . The importance of the local dynamics of the binding interface region was further underlined by two rare loss-of-function type 2M VWD mutations (p. Gly1324Ser/Ala, located close to the N-terminus of A1's β 3 strand) that were shown to restrain the flexibility of the binding interface region and to reduce the possibility of local unfolding (Tischer et al., 2016). Taken together, it appears plausible that the local flexibility of the binding interface region and the stabilities of competing helices α 2 and α 3 govern A1's affinity to GPIIb α .

Overall, force acting on A1 very likely regulates its affinity for GPIIb α by inducing conformational changes within A1, thus, switching A1 to a high-affinity state distinct from the low-affinity state it adopts in unstretched VWF. This likely includes local conformational changes in the main binding interface region—for example local unfolding of α 2—but possibly also further large-scale structural rearrangements that allow for enlarging the contact area with GPIIb α .

4 | CONCLUSION

Despite of considerable effort and major advances, a comprehensive picture of the force-induced activation of the VWF-GPIIb α interaction is still difficult to define. Full apprehension of VWF's intricate mechano-regulation is aggravated by the sheer complexity of the processes involved. One sees himself confronted with the task to connect the behavior of the whole VWF multimer as a polymer in hydrodynamic flow with intramolecular interactions within the multimer and finally with detailed properties of the A1-GPIIb α bond at the single-molecule level.

Therefore, it is not surprising that essentially all studies on VWF's activation for binding to GPIIb α have to deal with methodical difficulties one way or the other. For instance, most of the studies reviewed here have been performed using isolated A1 domain constructs that might not reflect the natural structure of the domain in the context of the full-length protein. On the other hand, simplification of the investigated system by reducing it to its essential components is indispensable for gaining mechanistic insights into the molecular basis of VWF's activation. Here, meticulous design of the experimental setup is of utmost importance. We especially would like to stress that in order to gain reliable insights at the single-molecule level, one has to assure that measurements are indeed performed on single molecules or single bonds. Another example is the widespread use of static assays for

assessing the binding of A1-containing VWF constructs to GPIIb, often in connection with the use of modulators such as ristocetin. Despite of their undisputed usefulness, the results obtained by such assays are generally hard to interpret with respect to the physiologically relevant case of force-induced binding.

Still, combining the insights gained from a variety of different approaches spanning different levels of complexity, and thus, different levels of detail, allows to propose a rather conclusive picture of the regulation of VWF's hemostatic activity and to point out issues that are still unresolved.

As a first important step of VWF's force-induced activation for GPIIb binding, loosely collapsed, globular VWF (Figure 2a, top), becomes elongated by increased hydrodynamic forces that occur in connection with vascular injury (Figure 2b, top) (Schneider et al., 2007; Springer, 2014). This process is thought to lead to the exposure of collagen binding sites (mainly in domain A3), thus, facilitating immobilization of soluble VWF to the subendothelium (Figure 2b, top), but can be expected to also result in higher accessibility of A1 domains. Notably, the precise nature of the interactions promoting the globular conformation of VWF multimers under normal blood flow conditions is still incompletely understood.

Due to the positive feedback between the effective length of a multimer and the hydrodynamic force it experiences in flow (Springer, 2014; Zhang et al., 2009), the largest multimers, that is, the ones comprising the most monomeric subunits, can be expected to be the

first to respond to irregular blood flow conditions. Furthermore, it is important to note that for the same reason markedly higher forces will act on a multimer after elongation compared with its globular conformation. Consequently, also the individual domains within the multimer will experience higher forces upon elongation. Even further increased forces can be expected for multimers that are tethered to the subendothelium. It can therefore, be assumed that an opening of intramolecular interactions between the A1 domain and neighboring peptide sequences within a multimer occurs upon elongation of VWF, thus, lifting the initial shielding of A1. Both the D'D3 domain and the N- and C-terminal flanking regions of A1 can likely mediate such shielding interactions (Figure 2a, bottom). The possible physiological role of another intramolecular interaction between A1 and the A2 domain is however, less clear, as it was reported to only occur between A2 and a putative activated conformation of A1 (Figure 2b, bottom) (Martin et al., 2007). In principle it is conceivable that such an interaction could also play an autoinhibitory role, as suggested by MD simulations (Aponte-Santamaria et al., 2015), either by suppressing binding of A1 to GPIIb or by protecting A2 from cleavage by the protease ADAMTS13, or both. It should however, be stressed that to date no study has been published that showed direct interaction of A1 and A2 within a multimer, nor has such an interaction been comprehensively characterized experimentally at the single-molecule level. When investigating intramolecular interactions within VWF dimers by performing single-molecule AFM force measurements,

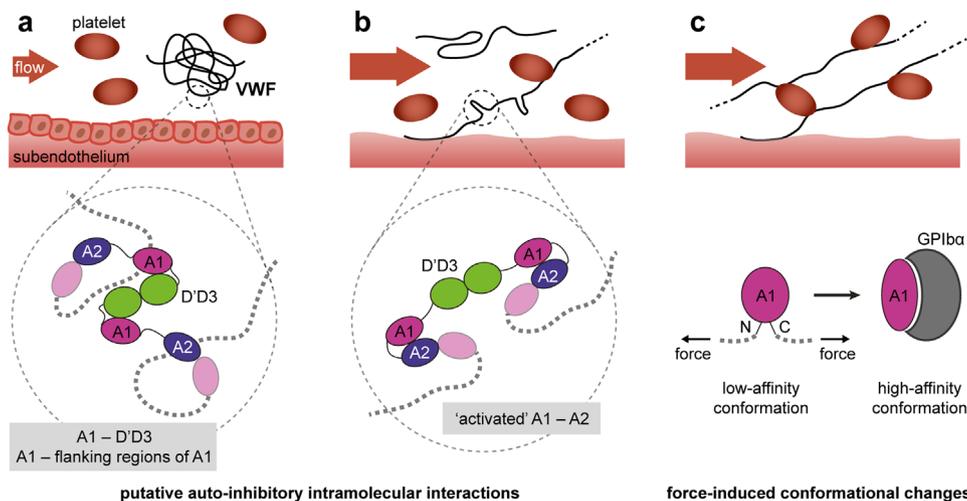


FIGURE 2 Possible regulatory mechanisms of the force-induced activation of VWF binding to platelet GPIIb. (a) Under normal blood flow conditions, VWF multimers adopt a loosely collapsed, globular conformation, and do not exhibit significant binding to platelets (top). Autoinhibitory intramolecular interactions between the A1 domain and D'D3 and/or the N- and C-terminal flanking regions of A1 (bottom) likely contribute to preventing platelet binding by inhibiting the A1-GPIIb interaction. (b) When subjected to elevated hydrodynamic forces above a certain threshold, as for instance occurring at sites of vascular injury, VWF multimers undergo an abrupt transition from the globular to an elongated conformation, which correlates with immobilization of VWF to the damaged vessel wall via binding to subendothelial collagen (top). This elongation of VWF likely increases exposure of A1 domains and, due to the higher hydrodynamic forces acting on an elongated multimer, can be expected to go along with opening of abovementioned autoinhibitory intramolecular interactions. An additional interaction between VWF's A2 domain and an "activated" form of A1 has been proposed, which in principle might also have an autoinhibitory effect on the A1-GPIIb interaction (bottom). (b) Due to a positive feedback between the effective length of a multimer and the hydrodynamic force it experiences in flow, markedly higher forces will act on a multimer after elongation. Further increased force will act on the multimer after immobilization. These relatively high forces exerted on an A1 domain within an elongated multimer very likely induce conformational changes that result in a conformational state of A1 with distinctly higher affinity for GPIIb than its state in globular VWF (bottom), allowing for efficient platelet binding upon increased force exposure (top)

Müller, Löf, et al. (2016); Müller, Mielke, et al. (2016) did not detect any strong interaction between these domains. Also in optical tweezers experiments by Ying, Ling, Westfield, Sadler, and Shao (2010) on a construct containing three repeats of domains A1, A2, and A3, no such interaction could be found.

An auto-inhibitory role in regulating the A1-GPIIb α interaction might further be played by VWF's glycans, as suggested by several studies that showed that (partial) deglycosylation of VWF enhances binding to GPIIb α (De Marco et al., 1985; Fallah et al., 2013; Madabhushi et al., 2014). Unfortunately however, the possible influence of glycans has only rarely been taken into consideration in studies investigating VWF's activation, so that insights into this subject are very limited. Moreover, it should be noted that recombinant VWF constructs expressed in different cell lines can markedly differ in their glycosylation status, which might complicate interpretation of results with respect to their physiological relevance as well as comparison of different studies.

Strong evidence exists that, in addition to the abovementioned autoinhibitory intramolecular interactions within VWF multimers, the A1 domain also possesses an intrinsic force-dependent regulatory mechanism. In this—well-substantiated—picture, the relatively high force that acts on an A1 domain within an elongated VWF multimer can induce conformational changes of A1 that result in enhanced affinity for GPIIb α (Figure 2c, bottom), thus, enabling efficient platelet binding to VWF (Figure 2c, top). However, the exact nature of these conformational changes remains to be elucidated. An obvious obstacle on the way to obtaining high-resolution structural information on an “activated” conformation of A1 is the impossibility to produce crystals of a protein subjected to an external force, leaving crystallographic approaches only with the option to investigate gain-of-function A1 domain mutants or A1 in combination with modulators. Nonetheless, combining insights from crystal structures of certain gain-of-function A1-GPIIb α mutant complexes with findings from other—thermodynamic, biophysical, microfluidic, and computational—approaches allows postulation of some features of the putative activated configuration of A1. It appears likely that primarily the local flexibility of A1's main binding interface for GPIIb α around its β 3 strand determines the affinity for GPIIb α , and that this flexibility can be increased by force-induced conformational changes within A1, for example, local unfolding of its α 2 helix. Also large-scale structural rearrangements of A1, which could markedly increase its contact area with GPIIb α , are conceivable.

Finally, it should be noted that in principle also GPIIb α might undergo relevant force-induced conformational changes that tune its interaction with A1. Indeed, force-induced unfolding of a “mechanosensitive domain” in GPIIb α has recently been observed (Zhang et al., 2015). However, this domain is not located in the N-terminal VWF-binding domain of GPIIb α , but between GPIIb α 's macroglycopeptide region and its transmembrane helix, and its unfolding is thought to play a role for platelet activation and platelet clearance rather than for regulating the binding of GPIIb α to the VWF A1 domain (Deng et al., 2016; Zhang et al., 2015). It seems reasonable to assume that the force-regulation of the affinity of A1 and GPIIb α for each other primarily traces back to conformational changes of A1, since in the physiological case of platelet binding to VWF only A1, but

not GPIIb α , is expected to experience significant forces prior to binding (Kim et al., 2015; Zhang et al., 2009).

Taken together, it is likely that the mechano-regulation of the VWF-GPIIb α binding is based on two central aspects: First, autoinhibitory intramolecular interactions within VWF shield the A1 domain under normal blood flow conditions and keep VWF in a low-affinity state. This inhibition can be lifted by increased hydrodynamic forces acting on VWF, as occurring primarily at sites of vascular injury. Second, force-induced conformational changes within the A1 domain convert A1 to a high-affinity state, which exhibits markedly increased binding to GPIIb α . In combination, these two aspects represent a sophisticated mechanism for ensuring that VWF remains inactive under normal blood flow conditions, thus, preventing thrombotic complications, and is reliably and quickly activated to efficiently perform its hemostatic function precisely when and where needed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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