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The von Willebrand Factor A1-Collagen III Interaction is Independent of Conformation and Type 2 von Willebrand Disease Phenotype

Venkata R. Machha¹, Alexander Tischer¹, Laurie Moon-Tasson¹, and Matthew Auton^{1,*}

¹Division of Hematology, Departments of Internal Medicine and Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Abstract

The blood von Willebrand factor (VWF) mediates platelet adhesion to injured vessels by sequestering platelets from blood flow and depositing them to collagen and other exposed subendothelial matrix proteins. This process of capturing platelets to facilitate formation of platelet plugs occurs through transient interactions with platelet glycoprotein Iba via the VWF A1 domain which also binds collagen. Using a conformationally diverse collection of natively folded and mutation-induced misfolded von Willebrand Disease (VWD) variants, we test a recently proposed affinity up-regulation hypothesis which states that collagen binding changes the conformation of the A1 domain to a high affinity GPIba binding competent state. With surface plasmon resonance (SPR), we present this diversified collection to collagen and quantify the kinetics of association and dissociation to ascertain the conformational selectivity of collagen. With analytical rheology, we quantify real-time platelet pause times and translocation velocities across a Cu²⁺ HisTag-chelated and collagen bound A1 single domain and A1A2A3 tridomain fragment of VWF under shear stress in an *ex vivo* shear flow microfluidic chamber. In contrast to expected hypothetical outcomes, collagen has limited conformational selectivity for binding A1, is independent of gain- or loss-of-function phenotype, and platelet translocation pause times on collagen-bound A1A2A3 are either normal or shorter depending on whether A1 is concertedly bound with the A3 domain to collagen. With respect to A1, collagen has an inhibitory role that provides an explanation for the lack of thrombosis in patients with gain-of-function VWD.

Graphical abstract

*To whom correspondence is addressed, auton.matthew@mayo.edu.

Author Contributions: VRM performed and analyzed the SPR experiments. VRM prepared the plasma-free blood and performed the shear flow assays in presence and absence of collagen. AT performed the CD spectroscopy and analyzed the platelet pause times. VRM and AT prepared the figures. VRM, AT, and LMT expressed and purified the proteins. LMT performed the mutagenesis and cloning. MA, VRM and AT wrote the paper. MA designed the research.

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Keywords

Collagen; GPIb α ; von Willebrand factor; surface plasmon resonance; analytical rheology

3 Introduction

During primary hemostasis, the von Willebrand factor (VWF) contributes to the arrest of bleeding by sequestering platelets from vascular blood flow and mediating their adhesion to exposed subendothelial connective tissue at sites of vascular injury. This process of initiating the platelet plugs necessary to stop bleeding is driven by the VWF A1 domain binding to the platelet receptor GPIb α and, in part, the cooperative adhesion of the A1 and A3 domains to subendothelial collagen. The rheological biophysics of platelet adhesion to the A1 domain under shear flow is highly dependent on the flexibility of local secondary structure within the native state [1]. However, this dynamic adaptation to blood flow becomes disadvantageous in von Willebrand disease (VWD) when mutations induce misfolding of A1 in both gain- (2B) and loss-of-function (2M) phenotypes [2]. Both platelet adhesion to VWF and VWF adhesion to collagen are critical to normal hemostasis, but how VWD mutations affect VWF collagen binding and whether platelet and collagen adhesion are coupled or mutually exclusive remains unknown. Resolving these issues is important because both activities of VWF are used as criteria for the diagnosis of VWD [3].

Recent studies propose that collagen binding via the A1 domain induces a conformational change in A1 that enables tighter binding to platelet GPIb α [4]. The linkage principle, referred to as "affinity up-regulation", is an example of positive coupling such that collagen binding preferentially induces a shift in equilibrium of the A1 domain to a platelet binding competent conformation with higher affinity for GPIb α . It is qualitatively similar to known effects of some gain-of-function type 2B VWD mutations which shift the unfolding equilibrium of A1 toward nonnative conformations [5], enhance platelet GPIb α affinity [6], and increase platelet pause times under shear flow [2]. Evidence for a collagen-induced conformational change of A1 stems from the observation that the reactivity of A1 to "conformationally specific" monoclonal antibodies was significantly reduced when bound to type III collagen [7]. A thermodynamically stable and conformationally rigid type 2M loss-of-function VWD variant (G1324S) [1], did not display a loss of antibody response upon

binding collagen [7], but the epitopes of the particular antibodies used that would yield structural insight into the proposed conformational changes are not known [7, 8]. Evidence against a collagen-induced enhancement of GPIIb/IIIa binding to VWF originates more than two decades ago from competition experiments which demonstrated that recombinant GPIIb/IIIa inhibits the binding of VWF to collagen [9].

In this study, we first address the issue of conformational change by assessing the association and dissociation kinetics of a collection of common VWD variants of the A1 domain to collagen III using surface plasmon resonance (SPR) spectroscopy. This collection represents a mix of both natively folded states and mutation-induced misfolded states of the A1 domain in both gain- and loss-of-function phenotypes of VWD. According to the linkage principle above, our criteria for collagen-induced conformational change are twofold. **1)** Within the natively-folded class, VWD variants of the A1 domain have a range of thermodynamic stabilities that depend on the type and structural location of the mutation and, therefore, have altered propensities for populating locally unfolded conformations within the native state ensemble [1,2,5]. If collagen binding induces conformational changes in A1, variants that are more susceptible to local unfolding should respond with increased collagen association rates and/or decreased collagen dissociation rates. In contrast, more stable variants (like G1324S) should have decreased association rates and/or increased dissociation rates. **2)** Within the misfolded class, VWD variants of the A1 domain populate nonnative conformations that are locally unfolded in the structural region around the mutation [10]. If collagen favors any of these non-native conformations of the A1 domain, locally unfolded variants should bind with increased association rates and/or decreased dissociation rates relative to the natively folded class of A1 domain VWD variants.

Second, we address the issue of platelet GPIIb/IIIa binding affinity in the presence and absence of collagen using comparative SPR spectroscopy and real-time analysis of platelet adhesion pause times under shear flow. According to the linkage principle above, our criteria for positive coupling in the binding are two-fold. **1)** If collagen binding induces the A1 domain into a higher affinity conformation for GPIIb/IIIa, collagen should preferentially bind gain-of-function over loss-of-function VWD variants resulting in a clear demarcation between the VWD phenotypes. **2)** The proposed collagen-enhanced GPIIb/IIIa binding affinity of the A1 domain should also manifest as enhanced pause times of platelets interacting with the complex under rheological shear flow relative to the intrinsic platelet pause times in the absence of collagen.

The results of these comparative studies demonstrate that the GPIIb/IIIa and collagen binding functions of the A1 domain are not linked through conformational change. The evidence for this is borne out by a rigorous test of the above criteria which illustrate that the kinetics of association to and dissociation from collagen assessed by SPR are similar for most VWD variants of the A1 domain regardless of their conformation and functional phenotype. In addition, analytical rheology does not support “affinity up-regulation” of the A1-GPIIb/IIIa-collagen ternary interaction as hypothesized by others [4, 7]. Under shear flow, platelets translocating on A1 bound to collagen have shorter pause times than in the absence of collagen indicating a slight inhibition of the A1-GPIIb/IIIa interaction consistent with early observations [9]. Two populations of platelet translocation pause times are observed on

collagen bound A1A2A3 tridomain fragment of VWF, which has collagen affinity in both A1 and A3, indicating that the A1 domain is either collagen bound with A3 or free in solution to interact with platelet GPIIb α . Pause times in the presence of collagen, however, are never observed to exceed those in the absence of collagen at any shear rate. The significance of these findings are further discussed in the context of von Willebrand Disease.

4 Results

4.1 Surface Plasmon Resonance Spectroscopy

Previously, we have characterized the structure and platelet adhesive function of 19 variants of the A1 domain [1,2,11]. In Figure 1A, these VWD variants of the A1 domain are grouped into two classes: Natively structured and Non-Native misfolded conformations according to the secondary structure content as reported from circular dichroism (CD) spectroscopy at 222nm. These classes of folded and misfolded variants are further ranked in Figure 1B according to the pause times of platelet translocation on the A1 domain chelated to the surface of a shear flow chamber via the N-terminal 6xHis Tag at 1500s⁻¹. Figure 1C illustrates that the structural locations of these mutations in the cocrystal structure of the A1:GPIIb α complex [2,12] occur within multiple regions of the A1 domain structure. Taken together, panels A and C of Figure 1 demonstrate that mutations significantly affect local secondary structures within A1 resulting in variable losses of helicity (CD at 222nm) depending on the structural location of the mutation. Mutation-induced local misfolding of the A1 domain occurs in secondary structures that are either necessary for binding resulting in loss of function (Type 2M VWD) or in secondary structures that are conformationally flexible such that local unfolding in these regions enhances function (Type 2B VWD) [2, 10]. Figure 1D illustrates that the pause times for platelet translocation are inversely proportional to reported blood platelet counts in VWD patients with these mutations. RCAM (Reduced and CarboxyAMidated) A1 is representative of cysteine mutations that break the disulfide bond. Frontroth *et al.* recently demonstrated transient thrombocytopenia following DDAVP desmopressin administration in two patients with C1272W and C1458Y indicating a strong gain-of-function phenotype [13]. The higher the A1:GPIIb α interaction pause time is, the greater the thrombocytopenic deficiency of platelets will be in blood [2]. These common VWD mutational variants of the A1 domain form the basis set for which to test the proposed linkage hypothesis that collagen binding changes the conformation of A1 to a higher GPIIb α affinity state [4].

Using surface plasmon resonance spectroscopy, collagen III was immobilized onto CM5 biochips by amine coupling and the A1 variants introduced in Figure 1 were injected at various μ M concentrations to assess the kinetics of A1 association to collagen and dissociation of A1 from collagen. Figure 2 illustrates the quality of the sensorgram responses obtained for the binding at the various injected concentrations (0.1 to 40 μ M) of the A1 domain. Sensorgrams are grouped by conformational class: natively structured (top panel) and non-native misfolded (bottom panel) and ranked according to the slowest apparent dissociation rate (k_{dB}); increasing from left to right.

Rate constants during the association and dissociation phases of the binding were derived from a triexponential fitting of each association and dissociation phase independently as

described in the methods [1]. This analysis yielded three sets of rate constants (fast, medium and slow) within the association and dissociation phases which are reported as a function of protein concentration for all A1 domain variants in Figures 3 and 4, respectively. Due to the heterogeneity of the collagen surface, the number of distinct binding sites for the A1 domain variants is not known, but the triexponential behavior of both association and dissociation phases indicates multiple sites (at least 3) on collagen for which there is some affinity for the A1 domain to bind.

All A1 variants had triexponential behavior in both the association and dissociation phases excepting RCAM A1 which had biexponential behavior in the association phase. The rate constants within each phase were independent of protein concentration. As with Figure 2, rate constants in Figures 3A and 4A are also grouped by conformational class and ranked according to the slowest rate constant, k_{dB} (right plot of Figure 4B). The average $\pm$ standard deviation of the fast, medium and slow association rates obtained for all protein concentrations reported in Figure 3B demonstrate that although rates in the association phase are quite variable within an order of magnitude, they are similar to the WT A1 domain within ± 2 standard deviations of WT. Similarly, the average $\pm$ standard deviation of the fast and medium rates in the dissociation phase are quite comparable to the WT A1 domain within an order of magnitude and ± 2 standard deviations (Figure 4B). The slowest rate constant within the dissociation phase, k_{dB} , demonstrated the greatest range of variability among the A1 variants. k_{dB} was therefore considered to be the parameter most sensitive to any specific binding interactions with collagen. This interpretation is justified since the A1 concentrations are significantly less than the equilibrium dissociation constant as indicated by the linear increase in total response with respect to A1 concentration (Figure 2 and Methods). Therefore, any affinity of the interaction manifests in the dissociation rate, k_{dB} , when tight binding between A1 and collagen occurs. Relative to WT A1, most of the variants have similar k_{dB} 's within the experimental error excepting RCAM A1, S1285F, V1314D, and V1316M which displayed significantly slower dissociation phases (Figure 2) that are reflected in the lower values of k_{dB} (right plot of Figure 4B). The k_{dB} 's of R1306Q and A1437T were statistically higher than the ± 2 standard deviation range for WT A1.

The kinetic results obtained from SPR are related to the experimentally determined secondary structure content observed by CD in Figure 5. Rate constants are grouped for these variants by the mean residue molar ellipticity obtained by CD as Native ($\Theta^{MRW} \approx -9$) and Non-Native ($-7.5 \leq \Theta^{MRW} \leq -5$). Our criterium for conformation dependent collagen binding is that rates during the association phase should increase when the native state is more flexible and when the A1 domain is locally misfolded to conformations that favor binding. Panel A illustrates that the rate constant, k_{a3} , is essentially independent of mean residue molar ellipticity with an average for all variants = $5.2074 \pm 1.6512 \times 10^{-3}$. Likewise, rates during the dissociation should decrease, but Panel B illustrates that k_{dB} for most of the A1 variants is identical within experimental error ($5.1241 \pm 2.2720 \times 10^{-4}$). Four exceptions do occur. Both the type 2B gain-of-function variants V1314D and V1316M and the type 2M loss-of-function variant S1285F have diminished dissociation rates. RCAM A1 essentially does not dissociate from collagen.

Our criterium for conformation dependent positive coupling between collagen binding and platelet adhesion is that collagen should preferentially bind gain-of-function over loss-of-function variants of the A1 domain. The results of Figure 5 demonstrate that collagen has little preference for VWD phenotype regardless of the conformational class because both phenotypes bind collagen in both conformational classes. In most cases, both type 2B and type 2M variants bind collagen equally well. This general observation is also evident in clinical assays of VWF collagen binding in patient samples reported as ratios of collagen binding activity relative to VWF antigen. Figure 6 compares k_{CB} obtained from SPR with (VWF:CB/VWF:Ag) ratios reported from the literature of patients with known VWD genotypes (see Table 1). Two features become apparent: **1)** The clinical data show a considerable overlap of both type 2B and type 2M phenotypes in the normal range of VWF:CB/VWF:Ag (0.7 – 1.2); **2)** The k_{CB} determined from SPR and the results of the collagen binding clinical assay with patient VWF are not correlated.

4.2 Platelet adhesion to A1 and A1A2A3 in presence/absence of collagen III

Figure 7A, B, & C illustrates a comparison of the collagen binding of A1, A3 and the A1A2A3 via SPR. Although the A3 domain has been referred to as the essential domain for VWF adhesion to collagen [25, 26], A1 also binds collagen in the same affinity range in agreement with others [7,25,27]. Like the variants of A1 described above, A3 and A1A2A3 rate constants in the association and dissociation phases are essentially concentration independent (Figure 7E). It might be expected for A1A2A3 to have significantly slower rates of dissociation from collagen since A1 and A3 both bind collagen and there are potentially some interactions from the A2 domain. Under equilibrium conditions, however, interactions between the A domains stabilize tridomain quaternary interactions between domains resulting in a globular associated complex [28]. This intrinsic domain mediated stabilization of the quaternary A1A2A3 complex limits the inhibitory capacity of GPIIb α to block binding of A1A2A3 to collagen. Although the equilibrium affinity of GPIIb α for A1A2A3 is weak, some inhibition of collagen binding is observed in the SPR as a reduction in the total SPR response units as GPIIb α concentration is increased relative to a constant A1A2A3 concentration (Figure 7D). In the presence of GPIIb α , the collagen association and dissociation rates are also constant as a function of GPIIb α because they are independent of concentration of A1A2A3 in the absence of GPIIb α (Figure 7F). The reduced SPR response in the presence of GPIIb α is consistent with a competitive inhibition mechanism, which does not account for the possibility of a ternary complex, under equilibrium conditions.

Under rheological shear, the A1A2A3 tridomain complex opens up to adhere more strongly to the collagen and noncompetitive inhibition of platelet adhesion (accounting for a ternary complex between VWF, GPIIb α and collagen) becomes apparent. In Figure 8, we have assessed the interactions of platelets obtained from plasma-free blood (see methods) with WT A1 and A1A2A3 in the presence and absence of collagen III under shear flow. Our criterium for enhanced affinity of A1 in the collagen bound state is that pause times of platelets interacting with the A1-collagen or A1A2A3-collagen complex should be enhanced relative to platelet adhesion in the absence of collagen. Figure 8A demonstrates that platelet pause times on A1 bound to collagen are actually decreased relative to A1 immobilized by the N-terminal HisTag over the full range of shear rates. The shorter pause times of platelets

on A1 bound to collagen are observed as faster translocations by comparing Supplemental Movies S1 and S2. Figure 8A also places this slight inhibition of platelet adhesion to A1 by collagen in context with a type 2M loss-of-function G1324S variant characterized by very short pause times ($\sim 0.1s$, Supplemental Movie S3) [1]. In contrast to A1, the effect of collagen on platelet adhesion to the A1A2A3 tridomain was more pronounced. Platelets initially translocated very fast resulting in short pause times ($\sim 0.3s$) and after perfusion, a second population of platelets was observed to interact more strongly with collagen bound A1A2A3 resulting in longer pause times (Figure 8B). The longer pause times occurred predominantly at higher shear rates and were equivalent to platelet pause times on A1A2A3 immobilized by the C-terminal HisTag on the A3 domain. However, the number of platelets adhering to collagen bound A1A2A3 with long pause times was significantly fewer than those with short pause times or those interacting with A1A2A3 in the absence of collagen (Supplemental Movies S4–S6). Pause times of platelets obtained from perfusion of whole blood or plasma-free blood over A1A2A3 chelated by the HisTag were identical over the full range of shear rates (Figure 8B). These observations are further confirmed from the perspective of platelet pause time and translocation velocity distributions. Figures 8C & D demonstrate that platelets translocating on A1 and A1A2A3 in the absence of collagen have statistically higher pause times and slower velocities excepting the fast translocation population of platelets on A1A2A3 in the presence of collagen with short pause times. When the velocities are slow, the pause time distributions skew to higher values increasing the mean pause times. When pause times are short, the velocity distributions skew to higher values.

Figure 9A illustrates our observations that A1 bound to collagen has shortened pause times and increased velocities, a slight inhibition. However, A1A2A3 bound to collagen has two populations of platelet pause times in the presence of collagen. The observation that long adhesion pause times to A1A2A3 in the presence of collagen are equivalent to those in the absence of collagen suggests that two A1A2A3 collagen bound states exist where A3 is bound to collagen and either A1 is collagen bound or completely free to interact with platelets in solution, Figure 9B.

Control experiments performed at a $1500s^{-1}$ shear rate illustrate that collagen is essentially inert to platelets obtained from plasma-free blood, but whole citrated blood which retains the plasma VWF forms large thrombi in the presence of collagen. These control experiments shown in Supplemental Movies S7 and S8 indicate that the observed interactions with the recombinant VWF A domains bound to collagen were specific.

Overall, the analytical rheology confirms a noncompetitive inhibition mechanism under shear stress in that while platelet adhesion to collagen bound VWF A1 domains is supported, the strength of adhesion is reduced. It is probable that this inhibition is due to sterically hindered binding interactions in the ternary complex.

5 Discussion

The present study provides a structural and functional test of a hypothesis proposed a decade ago that binding to collagen alters the conformation of the VWF A1 domain [7] and

evaluates the validity of the recent proposal that this interaction results in a high affinity GPIIb α binding competent state [4]. Here, we take advantage of a conformationally diverse set of VWD variants of the A1 domain in both gain-and loss-of-function phenotypes and use logical criteria to decipher the role of collagen in VWF mediated primary hemostasis. These criteria enumerated in the introduction establish expected outcomes, provided the hypothesis is true, to which our experimental outcomes are then compared.

It is important to recognize how the conformational change hypothesis was established. Weiss *et al.* initially demonstrated that platelet adhesion to an exposed vascular surface was dependent on both the shear rate and the presence of VWF [31], an observation that we have reproduced with respect to collagen in this study (Supplemental Movies S7–S8). Prior to knowledge that the A1 domain of VWF could simultaneously support platelet adhesion when bound to collagen [7], a key observation established that a recombinant fragment of the platelet GPIIb α receptor could inhibit the binding of ^{125}I radiolabeled VWF to a mixture of types I and III collagen [9]. Although the equilibrium solution affinity of A1A2A3 for GPIIb α is weak, our SPR observation that GPIIb α mixtures with A1A2A3 reduce the total collagen binding response confirms that a slight inhibition of collagen binding by GPIIb α does occur (Figure 7D). Early attempts to decipher conformational mechanisms of platelet adhesion to VWF involved raising a barrage of monoclonal antibodies against a disperse fragment of VWF [32] containing the A1 domain, some with known sequential epitopes and others in which the epitope is nonsequential and presumably dependent on local conformation [8]. In particular, 5D2 has an unidentified nonsequential epitope and is capable of blocking botrocetin-, ristocetin-, and shear-induced platelet aggregation [8,33], but it does not recognize the WT A1 domain bound to collagen despite its inability to block A1 binding to collagen [4, 7]. The only evidence for 5D2's conformational specificity is its ability to recognize a type 2M VWD variant, G1324S, when bound to collagen [7]. The implication is that the high thermodynamic stability of G1324S [5] precludes the collagen-induced change in A1 conformation [7]. However, polystyrene also reduces the avidity of 5D2 for the WT A1 domain [34], which brings into question the specificity the proposed conformational changes induced by collagen. In fact, 5D2 also has avidity for the disulfide reduced A1 domain which is known to have a molten globule structure [8,11]. The advantage of circular dichroism over monoclonal antibodies is that CD provides a direct measurement of the overall secondary structure content within the A1 domain conformational states. Our strategy was to present to collagen a diversified collection of A1 conformations found in VWD and use quantitative kinetics of binding/unbinding to ascertain the conformational selectivity of collagen.

Our recent studies have established that the native state of A1 domain is highly flexible. VWD mutations have a range of effects on thermodynamic and kinetic stability and cause localized misfolding within specific secondary structures of the A1 domain [1, 5, 6, 28, 29]. This results in significant effects on platelet adhesive capacity under shear stress [2, 10, 11]. If collagen binding were to alter the conformation of the A1 domain, natively folded variants with different thermodynamic stabilities and thus different probabilities for local unfolding would have altered association and/or dissociation rates and, therefore, altered affinities for collagen relative to the WT A1 domain. In contrast to this expectation, statistical differences in the association and dissociation rate constants are not observed among any of the natively

structured variants (Figure 5). Likewise, misfolded variants of the A1 domain populate non-native locally unfolded conformations and should have altered binding competencies for collagen. However, our observations demonstrate that rate constants within the association phase are not significantly different experimentally to that of the natively folded variants, nor are the dissociation rates excepting only a few (V1314D, V1316M, S1285F and RCAM A1) whose amino acid substitutions dramatically disrupt the hydrophobic core of the domain. Strong evidence against the probability of collagen-induced conformational change of A1 is provided by the fact that A1's collagen binding competency is not significantly altered for the majority of VWD variants in both native and non-native conformational classes.

If conformational changes in the A1 domain do occur upon binding collagen, it is more likely that they are relatively minor compared to the known conformational effects of VWD mutations [2]. In contrast to the proposed positive coupling in which collagen binding induces a conformational change to a higher affinity state, the SPR results demonstrate that there is little preference for the gain-of-function phenotype in either the native or misfolded conformational classes. This lack of preference for gain-of-function is also clearly evident in clinical VWF collagen binding assays because both type 2M and type 2B patient VWF can have VWF:CB/VWF:Ag ratios in the normal range (Table 1 and Figure 6). Given a remote possibility that the conformation of the collagen bound A1 domain is different than what is induced by gain-of-function VWD mutations, the expectation of enhanced platelet pause times under shear flow must be evident for the "affinity up-regulation" hypothesis to be upheld. In contrast to single molecule forced-unbinding bond lifetime studies [4], the flow dependent platelet adhesion studies shown here demonstrate that pause times on the A1 domain are actually shortened rather than prolonged and translocation velocities are statistically faster rather than slower. These observations exemplify the shear flow equivalent of the inhibition observed by static binding competition experiments showing GPIIb/IIIa inhibition of radiolabeled VWF binding to collagen [9]. Without debating a litany of differences between single molecule and flow chamber experiments, the advantage of analytical rheology is that the simultaneous measurement of hundreds of platelet-VWF interaction events enable a statistically robust quantification of the platelet adhesion dynamics in an *ex vivo* environment that closely mimics the vascular environment.

How are stable platelet plugs formed if collagen bound VWF has reduced platelet adhesive function to the A1 domain relative to free VWF? Analytical shear flow studies using the A1A2A3 tridomain reveal evidence for two populations of platelet translocations (Figure 9). Depending on whether the A1 domain is collagen bound or free, pause times are either short or long, respectively. These two bound states of the tridomain can only be inferred from the pause times on collagen, but the fact that the long pause times on collagen are experimentally identical to those where the tridomain is chelated to surface via the C-terminal HisTag lends a measure of confidence to the interpretation. In fact, both fast and slow translocations of platelets are also observed when whole blood is perfused over collagen (Supplemental Movie S8) indicating that the multimeric plasma VWF that associates to collagen has some A1 domains that are more exposed and can firmly adhere the platelets whereas other A1 domains are more occluded by the collagen interaction and do not adhere platelets efficiently resulting in fast translocations. Some platelets do not

translocate suggesting that other platelet receptors are involved, such as GPIIb/IIIa (α IIb β 3), which binds the C-domains of VWF and fibrinogen [35] and GPIa/IIa (integrin α 2 β 1) or GPVI which bind collagen [36]. Some evidence suggests that the A1-GPIb α interaction can even activate α IIb β 3 to bind fibrinogen [28] and α 2 β 1 to bind collagen under shear flow conditions [37]. In addition to plasma VWF, much of the work of capturing platelets from blood flow is initiated by the stimulated release of unusually large hyperactive VWF multimers (ULVWF) from vascular endothelial cells [38]. ULVWF is known to make high strength bonds with platelet GPIb α [39] that can be visualized as ULVWF-platelet strings under shear flow [40]. The observation that pause times of platelet adhesion to free A1 are greater than collagen bound A1 in the context of the A1A2A3 tridomain is consistent with a mechanism by which platelets strongly adhere to ULVWF prior to their deposition onto collagen, subendothelial VWF, and other exposed subendothelial matrix fibrillar proteins at a damaged vascular site.

Although evidence exists that the A1 domain has affinity for other types of collagen, such as types I, IV, and VI, clinical assays for VWF collagen binding typically use collagen III as a substrate [41,42]. It is possible that these other types may be more selective to various conformations of the A1 domain in VWF because some mutations in the α 4-helix of A1 have been reported to impair collagen IV and VI binding [43, 44]. However, the probability of highly specific conformationally regulated interactions between VWF and other collagen types is currently uncertain but likely very low because cohort studies demonstrate that healthy controls have statistically equivalent VWF:CB activities for types I, III, and VI, as do type 1 VWD patients with quantitatively deficient VWF levels [45]. Furthermore, the link between the extent of the loss of high molecular weight VWF multimers in type 2 VWD and VWF:CB activity is not clearly established.

In conclusion, what is clear from these studies is that the VWF A1-collagen III interaction is not conformationally dependent and the principle of "affinity up-regulation" does not apply to VWF-collagen binding activity. Based on our results, our view is that the A1 domain's purpose is simply to transiently capture and decelerate flowing platelets enough to enable stable adhesion by other receptors so that secondary hemostasis and coagulation can ensue. Putting the onus on the A1 domain alone to do all of the regulation of platelet adhesion to an exposed subendothelium, as implied by the tested hypothesis, would be inefficient. Furthermore, "affinity up-regulation" could pose a significant risk of thrombosis in patients with type 2B VWD, a complication that is not observed [46, 47].

7 Methods and Materials

7.1 Protein expression, purification and quantification

VWF A1 domain (amino acids $Q_{1238} - P_{1471}$) and all mutants were expressed in *E.coli* as fusion constructs containing an N-terminal 6 $\times$ His-Tag. Protein expression, inclusion body preparation, the protein refolding procedure and the purification via Ni²⁺-NTA and Heparin Sepharose affinity chromatography was performed as recently described [2,49]. VWF A3 (amino acids $S_{1671} - G_{1874}$) domain, also expressed as fusion construct containing an N-terminal 6 $\times$ His-Tag, was prepared as previously described [48]. A1A2A3 triple domains (amino acids $Q_{1238} - G_{1874}$) were expressed as fusion proteins containing a C-terminal 6 $\times$

His-Tag and purified as described previously [50]. Protein concentrations were determined on a Shimadzu UV2101PC spectrophotometer. Extinction coefficients at 280nm for A1 (15350L/mol/cm), A3 (10490L/mol/cm) and A1A2A3 (74497L/mol/cm) were calculated from the amino acid sequence.

7.2 Surface plasmon resonance

SPR kinetic experiments were performed on a Biacore T-100. Human placenta collagen III was purchased from Sigma-Aldrich and solubilized in 20mM sodium acetate pH 4.0 to a final concentration of 1 mg/ml. A portion of this stock solution was diluted 100 fold into 10mM HEPES, 150mM NaCl, 3mM EDTA, 0.05% (v/v) surfactant P20 buffer pH 7.0 to make a fibrillar collagen solution which was covalently coupled via primary amines to the active channel of a CM5 chip using amine coupling reagents (EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), NHS (N-hydroxysuccinimide) and ethanolamine). Kinetic studies of collagen fibril formation and atomic force imaging of collagen coated glass surfaces demonstrate that neutral pH is necessary for the formation of ordered collagen fibrils and VWF binding [51, 52]. The immobilization of collagen III resulted in approximately 3000 response units, which corresponds to approx. 30ng/mm² collagen. The reference channel (Fc1) was equally treated with same amine coupling reagents in the absence of collagen III. In order to correct for nonspecific interactions the response from the reference channel (Fc1) was subtracted from the response of active channel (Fc2). All kinetic experiments were performed in Tris buffered saline at 25 °C at a flow rate of 30μL/min. For each experiment increasing concentrations ranging from 1 to 40μM protein were injected over the biosensor for 500s. Dissociation of VWF A1 mutants from the collagen was initiated by injecting TBS buffer for 500s. After each injection, the biosensor was regenerated by injection of 1mMEDTA, 2MNaCl, 0.1M sodium citrate, pH 5 for 60s at a flow rate of 10μL/min followed by injection of 0.1M H₃PO₄ for 30s at a flow rate of 30μL/min.

7.3 Analysis of SPR sensorgrams

Due to the complex heterogeneity of the collagen surface, fit equations using 1:1 binding, bivalent analyte, or heterogeneous ligand models for the association and dissociation phases provided by the Biacore evaluation software were not able to yield a satisfying fit quality to adequately compare all variants of the A1 domain. Therefore, sensorgrams were exported to SigmaPlot 11 and the association and the dissociation phases were fit independently using triexponential functions by non-linear least squares fitting routines as described previously [1]. For the association phase of the kinetics of binding, Equation 1, was used to describe the association phase of the binding kinetics yielding three rate constants, fast k_{a1} , medium k_{a2} and slow k_{a3} .

$$RU_a[t] = a * (1 - e^{(-k_{a1} * t)}) + b * (1 - e^{(-k_{a2} * t)}) + c * (1 - e^{(-k_{a3} * t)}) \quad (1)$$

In the range of concentrations of the A1 domain attainable for this analysis, the total response units obtained at the end of the association phase as well as the predicted total

response units, sum of the pre-exponential factors (a+b+c) obtained from the fit, increase linearly with A1 concentration. This indicates that the equilibrium dissociation constant is much greater than the A1 concentration, *i.e.* $K_D \gg [A1]$. Therefore, the rates obtained from this analysis should not be interpreted as association rates, rather as rate constants during the association phase.

Similarly, a triexponential decay function, Equation 2, was used to describe the dissociation phase of the unbinding kinetics yielding three rate constants, fast k_{d1} , medium k_{d2} and slow k_{d3} .

$$RU_d[t] = a * e^{(-k_{d1}*t)} + b * e^{(-k_{d2}*t)} + c * e^{(-k_{d3}*t)} \quad (2)$$

In all cases the fit quality had a coefficient of determination $R^2 = 0.99$. Fast, medium and slow rate constants for both association and dissociation phases were directly compared among all A1 domain variants in Figures 4B and 3B after ranking the variant according to the slowest dissociation rate, k_{d3} , which provides the best approximation for collagen binding specificity (right panel of Figure 4B). A biexponential analysis of SPR sensorgrams yielded significant periodicities in the fit residuals, thereby justifying the need for triexponential functions. Furthermore, analysis of the pre-exponential factors a, b, and c, in both association and dissociation phases of the sensorgrams demonstrated that all A1 domain variants have proportionately similar magnitudes of a, b and c thereby eliminating the remote possibility of A1 switching between two or more conformational states for any given VWD variant.

7.4 Preparation of plasma-free blood

Washed platelets were prepared as described by [7,37]. Blood was collected from healthy donors into a syringe containing one part of acid-citrate-dextrose (ACD, pH 4.5) and supplemented with 2 U/mL apyrase, 10 μ M prostaglandin E1 and 5 mM EDTA to block platelet activation. Then the blood was centrifuged at 2100 g for 13 min at room temperature. The supernatant plasma was discarded and the cells were resuspended to their original volume with Hepes-Tyrode buffer (10 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4 , 10 mM NaHCO_3 , 5 mM dextrose, and 5 mM EDTA pH 6.5) containing 1 U/mL apyrase. Resuspended cells were centrifuged again and the supernatant was discarded. This process was repeated twice. In the third wash cycle the cells were resuspended into Hepes-Tyrode buffer (pH 6.5) without apyrase. After this the cells were resuspended in Hepes-Tyrode buffer, pH 7.4 containing 20 mg/mL BSA, 1 μ M of prostaglandin E1 and 5 mM EDTA.

7.5 Platelet binding under shear stress

Flow chamber studies were performed as previously described [1] on a Zeiss Axio Observer A1 microscope operated by Zen 2012 Software. Movies were recorded for using a PCO edge camera at 25 frames per second.

For the shear flow assay of WT A1 in absence of collagen, whole citrated blood was perfused over the protein that was immobilized via its N-terminal 6xHis tag on Cu^{2+} -treated Vena8 Chips (Cellix, Dublin, Ireland) prior to the experiment. The analogous experiment with WT A1A2A3 (immobilized on the Cu^{2+} -treated surface via a C-terminal 6xHis tag) was performed in the same manner but with plasma-free blood. In all cases, approximately $5\mu M$ protein was immobilized on the Cu^{2+} -treated surface.

For the shear flow experiments in presence of collagen III, NHS-treated Vena8 Chips were used and collagen III was immobilized using the same procedure as for the SPR experiments. After immobilization, the channels were flushed multiple times with WT A1 or WT A1A2A3. The experiments in presence of collagen III were performed using plasma-free blood.

The analysis and determination of mean platelet pause times was performed as previously described [29]. For the Cu^{2+} immobilized proteins multiple shear rates could be applied on a single channel that was initially loaded with blood at $800\ s^{-1}$. After removal of the majority of the red blood cells, the shear rate was increased at logarithmic intervals to $9000\ s^{-1}$. For WT A1 and WT A1A2A3 bound to collagen III one channel was used per shear rate due to faster clearance of the platelets from the channel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

SPR	surface plasmon resonance
CD	circular dichroism
VWF	von Willebrand factor
ULVWF	unusually large von Willebrand factor
VWD	von Willebrand disease
DDAVP	1-desamino-8-D-arginine vasopressin
RCAM	reduced and carboxyamidedated

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2 Research Highlights

1. The VWF A1-collagen-GPIIb α ternary interaction is not conformationally coupled.
2. VWF A1 binding to collagen is independent of Type 2 VWD phenotype.
3. VWF A1 affinity for platelet GPIIb α is down-regulated in presence of collagen.

Figure 1.

Structure and platelet adhesion function of the A1 domain [2]. A) CD ellipticity at 222nm of non-native (grey) and natively structured (black) A1 domain mutants. B) Platelet adhesion pause times of non-native (grey) and natively structured (black) A1 domain variants at 1500s⁻¹ shear rate ranked according to pause time. C) Structural locations of mutations in the A1 domain complex with GPIIb/IIIa. D) Platelet counts reported for VWD patients with known mutations as a function of the platelet pause times obtained at 1500s⁻¹ shear rate. The linearity has a correlation coefficient of determination ($R^2 = 0.90$). RCAM A1 has the disulfide bond C1272–C1458 reduced and carboxyamidated.

Figure 2.

Interaction of A1 domain VWD variants with Collagen III. Sensorgrams of the interaction with collagen III are shown for native (top) and non-natively (bottom) structured A1 domain mutants. A series of concentrations of A1 mutants (ranging from 0.1 to 40 μM) were injected through Fc1 (control) and Fc2 (active) channels for the measurements. Concentration ranges are indicated in each panel. All proteins are injected at a flow rate of 20 $\mu\text{L/s}$.

Figure 3.

Concentration dependency of the exponential rate constants within the association phase. Rate constants (k_{a1} , k_{a2} , and k_{a3}) for native (top) and non-natively (bottom) structured A1 mutants are plotted as function of protein concentration. In each plot the average rate $\pm$ 1SD is represented by black solid lines and black dashed lines respectively. The bar plots in the lower panel represent the association rate constants (k_{a1} , k_{a2} , and k_{a3}) for all native (black) and non-natively (grey) structured A1 mutants. Average rates for WT A1 are represented by black solid lines; the 1 SD and 2 SD range for WT A1 are shown by light grey and dark grey dashed lines respectively. All proteins are ranked according to increasing k_{a3} as shown in Figure 4.

Figure 4. Concentration dependency of the exponential rate constants within the dissociation phase. Rate constants. Apparent dissociation rate constants (k_{d1} , k_{d2} , and k_{d3}) for native (top) and non-natively (bottom) structured A1 mutants are plotted as function of protein concentration. In each plot the average rate ± 1 SD is represented by black solid lines and black dashed lines respectively. The bar plots in the lower panel represent the dissociation rate constants (k_{d1} , k_{d2} , and k_{d3}) for all native (black) and non-natively (grey) structured A1 mutants. Average rates for WT A1 are represented by black solid lines; the 1 SD and 2 SD range for WT A1 are shown by light grey and dark grey dashed lines respectively. All proteins are ranked according to increasing k_{d3} .

Figure 5.

Correlations between rate constants k_{a3} (panel A) and k_{d3} (panel B) with the secondary structure content monitored by CD at 222 nm. Average rates for all variants are represented by the black solid lines and the ± 1 SD range are shown by dashed lines.

Figure 6.

Correlation between the slowest rate of dissociation (k_{d3}) and the collagen binding capacity in VWD patients (ratio of VWF CB to VWF:Ag) reported in Table 1.

Figure 7. Sensorgrams of A1 (A), A3 (B) and A1A2A3 (C) tridomain binding to Collagen III. The small numbers indicate the used protein concentration in μM . The insets show typical fit residuals that lie within 0.1 – 1 % of the signal amplitude. D) Inhibition of A1A2A3 binding to collagen by increasing concentrations of GPIIb α (indicated). E & F) Apparent rate constants for collagen association and dissociation as a function of A1, A3 and A1A2A3 concentration in absence of GPIIb α (E) and presence of GPIIb α (F) Average rates are represented by horizontal lines.

Figure 8.

Platelet pause times for the interaction of WT A1 (A) and WT A1A2A3 (B) with platelets as function of shear stress in presence and absence of Collagen III. Pause times are reported as the mean $\pm$ standard deviation for a minimum of 3 separate experiments. C) Normalized platelet pause time distributions (log scale) on A1 ($1320s^{-1}$) and A1A2A3 ($1500s^{-1}$) in the presence and absence of collagen. n_{pt} equals the number of platelet pause time events analyzed in each cumulative distribution. D) Normalized platelet translocation velocity distributions (linear scale) on A1 ($1500s^{-1}$) and A1A2A3 ($3200s^{-1}$) in the presence and absence of collagen. n_v equals the number of instantaneous velocity events analyzed in each distribution. Instantaneous velocities in the direction of flow were calculated from platelet translocation trajectories using a Savitzky-Golay numerical derivative of the position versus time according to methods established in [29,30]. Velocity distributions were fit with a double gaussian function to account for the positive skew toward higher velocities.

Figure 9.

Model of platelet interactions with A1 and A1A2A3 tridomain in the presence and absence of collagen indicating long pause times and slow translocation velocities when the A1 domain is free in solution and short pause times and fast translocation velocities when A1 is interacting with collagen.

Table 1

Clinical data for the VWF:CB-III/VWF:Ag ratio.

Mutation	VWD Type	Conformation	VWF:CB/VWF:Ag ratio	Mean $\pm$ SD	Reference
F1369I	2M	Non-Native	like WT	1	[14]
E1359K	2M	Non-Native	Not Reported		
I1425F	2M	Non-Native	like WT	1	[14]
S1285F	2M	Non-Native	Not Reported		
R1374H	2M	Non-Native	0.83 ± 0.12	0.83 ± 0.12	[15]
G1324S	2M	Native	Not Reported		
G1324A	2M	Native	Not Reported		
A1437T	2M	Non-Native	Not Reported		
R1308L	2M	Native	0.87 ± 0.37	0.71 ± 0.37	[15]
			0.54 ± 0.05		[16]
WT A1	Normal	Native	1.06	0.99 ± 0.17	[43]
			1.03 ± 0.09		[41]
			0.91		[19]
R1341Q	2B	Non-Native	0.48	0.46 ± 0.04	[15]
			0.43		[41]
R1306Q	2B	Native	like WT	1	[20]
I1372S	2B	Native	0.84	0.96 ± 0.17	[21]
			1.08		[21]
H1268D	2B	Non-Native	Not Reported		
P1337L	2B	Non-Native	0.70	0.54 ± 0.23	[15]
			0.38 ± 0.11		[19]
I1309V	2B	Native	0.87 ± 0.09	0.87 ± 0.09	[15]
V1316M	2B	Non-Native	0.34 ± 0.22	0.22 ± 0.19	[15]
			0.05		[22]
			0.43		[23]
			0.06		[24]
V1314D	2B	Non-Native	Not Reported		
C1272W	2A/2B	Non-Native	0.17^*	0.33 ± 0.23	[13]

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<u>Mutation</u>	<u>VWD Type</u>	<u>Conformation</u>	<u>VWF:CB/VWF:Ag ratio</u>	<u>Mean ± SD</u>	<u>Reference</u>
C1458Y	2A/2B	Non-Native	0.49 *		[13]

* 30 minutes Post DDAVP treatment reported by Frontroth J.P. *et al.* at the 2015 ISTH Congress [13].