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Mutual A domain interactions in the force sensing protein von Willebrand factor

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ABSTRACT

The von Willebrand factor (VWF) is a glycoprotein in the blood that plays a central role in hemostasis. Among other functions, VWF is responsible for platelet adhesion at sites of injury via its A1 domain. Its adjacent VWF domain A2 exposes a cleavage site under shear to degrade long VWF fibers in order to prevent thrombosis. Recently, it has been shown that VWF A1/A2 interactions inhibit the binding of platelets to VWF domain A1 in a force-dependent manner prior to A2 cleavage. However, whether and how this interaction also takes place in longer VWF fragments as well as the strength of this interaction in the light of typical elongation forces imposed by the shear flow of blood remained elusive. Here, we addressed these questions by using single molecule force spectroscopy (SMFS), Brownian dynamics (BD), and molecular dynamics (MD) simulations. Our SMFS measurements demonstrate that the A2 domain has the ability to bind not only to single A1 domains but also to VWF A1A2 fragments. SMFS experiments of a mutant [A2] domain, containing a disulfide bond which stabilizes the domain against unfolding, enhanced A1 binding. This observation suggests that the mutant adopts a more stable conformation for binding to A1. We found intermolecular A1/A2 interactions to be preferred over intramolecular A1/A2 interactions. Our data are also consistent with the existence of two cooperatively acting binding sites for A2 in the A1 domain. Our SMFS measurements revealed a slip-bond behavior for the A1/A2 interaction and their lifetimes were estimated for forces acting on VWF multimers at physiological shear rates using BD simulations. Complementary fitting of AFM rupture forces in the MD simulation range adequately reproduced the force response of the A1/A2 complex spanning a wide range of loading rates. In conclusion, we here characterized the auto-inhibitory mechanism of the intramolecular A1/A2 bond as a shear dependent safeguard of VWF, which prevents the interaction of VWF with platelets.

1. Introduction

von Willebrand factor (VWF) is a huge multimeric protein that plays a key role in hemostasis. It triggers platelet adhesion in areas of vascular damage by binding to exposed sub-endothelial collagen to assist wound closure. In this process, VWF domain A1 is responsible for mediating platelet adhesion under flow in areas of vessel injury through the platelet glycoprotein Ibα (GPIbα) (Huizinga et al., 2002; Dumas et al., 2004; Lou and Zhu, 2008; Kim et al., 2010; Blenner et al., 2014). Domain A2 is unfolded under shear, whereupon it exposes a proteolytic site cleaved by the metalloprotease ADAMTS13 (Sadler, 2002; Baldauf et al., 2009; Chen et al., 2009; Zhang et al., 2009; Wu et al., 2010; Ying et al., 2010; Lippok et al., 2015). In the resting state, i.e. under low shear-stress conditions, VWF is incapable of binding platelets. This behavior has been associated with shielding of the GPIbα binding site by the adjacent units to A1, namely, the D′D3 domain

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(Ulrichts et al., 2006), the linker connecting D′D3 with A1 (Auton et al., 2012), and the A2 domain (Martin et al., 2007; Aponte-Santamaría et al., 2015). We recently found that the GPIbα-binding site in A1 becomes accessible by force-induced dissociation of the A1–A2 complex, whereas A2 mostly withstands unfolding under these forces and thereby remains protected against cleavage (Aponte-Santamaría et al., 2015). These insights have put forward new scenarios how VWF, in a shear-flow dependent manner, dynamically balances intra versus intermolecular A1A2 interactions, thereby guiding VWF self-assembly and activation. However, the inter- and intra-molecular forces and energies underlying dissociation and unfolding have remained elusive and the lifetime of these interactions have not been quantified nor compared to physiological shear regimes.

We addressed these issues by using single molecule force spectroscopy (SMFS), Brownian dynamics (BD) and molecular dynamics (MD) simulations. SMFS provided evidence for specific interactions between the important VWF domains A1 and A2. Binding activities, forces and bond stability were determined and put into context of physiological shear rates. Overall, our results underpin the strategy of a safeguarding mechanism in the competition between clot formation and VWF cleavage, depicted by the hierarchy of A1–A2 dissociation versus A2 unfolding forces in primary hemostasis.

2. Materials and methods

2.1. SMFS investigations

SMFS measurements were performed using a Pico SPM Plus setup (Agilent Technologies, Chandler, AZ, USA) under physiological conditions. Single VWF A-domains or VWF A domain constructs were either coupled to the AFM tip or to the sample surface. For SMFS experiments non-conductive Silicon Nitride MSCT tips (Brucker Corporation, MA, USA) with small spring constants (k = 0.03 N/m) were utilized. The actual spring constant was determined using the thermal noise method (Hutter and Bechhoefer, 1993).

2.2. Materials

All chemicals were used in the highest available purity. 3-Aminopropyl-triethoxy silane (APTES; Sigma Aldrich, Vienna, Austria) was distilled at low pressure and stored under argon in sealed crimp vials over silica gel (to avoid polymerization) at −20 °C. MilliQ (Millipore, Massachusetts, USA) purified water was used for all aqueous solutions. Triethylamine (TEA, Sigma Aldrich, Vienna, Austria) was stored under argon and in the dark to avoid amine oxidation. Chloroform was purchased from J.T. Baker (Griesheim, Germany), Argon and N_2 from Linde Gas GmbH (Stadl-Paura, Austria). HCl was purchased from Sigma Aldrich (Vienna, Austria). Ethylenediaminetetraacetic acid (EDTA) and Tris base were purchased from VWR International (Vienna, Austria), Hepes and $NiCl_2$ were obtained from Merck (Darmstadt Germany) and TCEP (tris(2-carboxyethyl)phosphine) hydrochloride from Molecular Probes, Invitrogen (Vienna, Austria). Disulfide-tris-NTA was generously provided by the Tampé lab, Biocenter, Frankfurt am Main, Germany. The heterobifunctional crosslinker maleimide-PEG_{27}-NHS was purchased from Polypure (Oslo, Norway). The cDNAs' coding for recombinant human VWF constructs containing the A1A2 (aa 1230–1672) construct, the single A1 (aa 1230–1463) and the single A2 (aa 1494–1672) were cloned into the mammalian expression vector pIRES neo2 (Schneppenheim et al., 2010). All VWF constructs are labeled with a His_6-tag. Mutations were inserted by site-directed mutagenesis employing the QuickChange kit (Stratagene). The vectors were used to transform Top10 super competent cells (Invitrogen) and sequenced. Plasmid purification was performed using the Endofree Plasmid Maxi Kit (QIAGEN). HEK293 cells were cultured in Dulbecco Modified Eagle Medium (DMEM, Invitrogen) with 10% [v/v] fetal bovine serum (Invitrogen) and 1% penicillin/streptavidin at 37 °C and 5% CO_2. These cells were transfected with the VWF vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and recombinant expression of VWF variants was performed as previously described (Schneppenheim et al., 2001). His-tagged VWF domain constructs were purified employing the His-Pur Ni-NTA Resin (Thermo Fisher Scientific) according to the manufacturer's instruction for purification of His-tagged proteins using a gravity-flow column. Mica sheets were bought from Christine Groepl, Electron Microscopy (Tulln, Austria). For aqueous solutions, TBS buffer (50 mM Tris, 150 mM NaCl) and Hepes buffer (prepared from a 1 M solution of Hepes acid by adjustment of pH 7.5 or pH 9.6 – as stated in the text – with 20% NaOH) were used.

2.3. Tip chemistry for coupling VWF constructs

Proteins carrying a His_6-tag were coupled to the AFM tip (Verbelen et al., 2007) using the connector molecule tris-NTA and in the presence of $NiCl_2$. The protocols for the different steps of tip functionalization were optimized in our lab with respect to reproducibility, stability, and to the probability for binding on average one single ligand to the outer tip apex. They are available from the internet at http://www.jku.at/biophysics/content/e257042.

The first step was amino-functionalization of the tip surface: Commercial silicon-nitride cantilevers were washed with chloroform (3 × 5 min incubation) and dried in a gentle nitrogen gas stream directly before further treatment. The APTES functionalization was performed as described previously (Ebner et al., 2007): A desiccator (5 l) was flooded with argon gas to remove air and moisture. Then two small plastic trays (e.g. the lids of Eppendorf reaction vials) were placed inside the desiccator, 30 µl of APTES and 10 µl of triethylamine were separately pipetted into two trays, the AFM tips were placed nearby on a clean inert surface (e.g. Teflon) and the desiccator was closed. After 120 min of incubation, APTES and triethylamine were removed, the desiccator was again flooded with argon gas for 5 min, and the tips were left inside for two days in order to cure the APTES coating.

In a next step the coupling of the maleimide-PEG_{27}-NHS linker was performed: APTES-functionalized AFM tips were incubated in 0.5 ml of a 1 mg/ml solution of maleimide-PEG27-NHS in chloroform containing 0.5% (v/v) of TEA as base for two hours. Subsequently, the tips were rinsed in chloroform (3×) und dried in a gentle stream of nitrogen gas.

In the third step a tris-NTA function was coupled to the maleimide group on the outer end of the PEG chain. The cantilevers were then placed on parafilm in a polystyrene Petri dish. 100 µl disulfide-tris-NTA (1 mM in MilliQ water), 2 µl EDTA (100 mM, pH 7.5 in MilliQ water), 5 µl Hepes (1 M, pH 7.5), 2 µl TCEP hydrochloride (100 mM in MilliQ water) and 2.5 µl Hepes (prepared from a 1 M stock solution of Hepes acid by adjusting pH 9.6 with 20%NaOH) were mixed (final pH ∼ 7.5), pipetted on the tips, and incubated for two hours. Subsequently the tips were washed in TBS buffer (3 × 5 min).

In the last step the tris-NTA group was loaded with Ni^{2+} and His_6-tagged VWF proteins were bound: The cantilevers were again placed on parafilm in a polystyrene Petri dish and pre-loaded with 50 µl $NiCl_2$ (200 µM in TBS buffer, pH 7.5) for 5 min. Subsequently, 100 µl of the His_6-tagged protein was mixed with 4 µl 5 mM NiCl2 and incubated for 2 h. Finally, the tips were washed 3 times for

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