Supplemental Material:
Mechanical Activation of a Multimeric Adhesive Protein through Domain Conformational Change

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I. SUPPLEMENTAL MATERIALS AND METHODS

A. Purification of VWF multimers and human glycocalicin

Plasma VWF was purified from human cryoprecipitate by glycine and NaCl precipitation and chromatography on a Sepharose 4B column (2.5 × 50 cm a bed volume of 3000 ml; Pharmacia, Inc., Piscataway, NJ) as described previously [1]. ULVWF multimers were produced from HUVECs as described previously [1, 2]. The multimeric composition of purified plasma VWF and ULVWF was evaluated by SDS-1% agarose gel electrophoresis and chemiluminescence.

Human glycocalicin was purified from platelets as previously described [3]. Briefly, 10 liters of outdated PRP was centrifuged at 150 × g for 15 min. Platelet pellet was suspended in 500 ml of buffer B (10 mM Tris/HCl, 150 mM NaCl, and 2 mM $CaCl_2$, pH 7.4) and sonicated. The resultant suspension was incubated at 37°C for 30 min to allow calpain released from platelets to cleave glycocalicin from GPIbα . After removal of cell debris, glycocalicin in the supernatant was first absorbed to a wheat germ Sepharose 4B column and eluted with 2.5% N-acetyl-D-glucosamine and 20 mM Tris/HCl, pH 7.4. The elute was then applied to an ion exchange column (Q-Sepharose Fast-flow column; Pharmacia) and eluted with a linear salt gradient of 0–0.7 M NaCl in 20 mM Tris/HCl, pH 7.4.

B. Production analysis of ULVWF multimers

Endothelial cell-derived ULVWF multimers - VWF enriched in ULVWF forms was obtained from cultured human umbilical vein endothelial cells (HUVECs) as described previously [1, 2, 4]. Briefly, confluent HUVECs were washed with phosphate-buffered saline (PBS) and incubated with a serum-free M199 medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10 μg/ml of insulin, 5 μg/ml of transferrin, and 1% glutamine for 48–72 hours. The cultured HUVECs were then exposed to 100 μM histamine (Sigma Chemicals, St. Louis, MO) for 30 min at 37°C, in order to stimulate the release of VWF enriched in ULVWF multimers. After incubation, the serum-free medium was collected and centrifuged at 150g for 10 min to remove cell debris, and the supernatant was used as the source of ULVWF multimers. The multimeric composition of purified ULVWF was evaluated by SDS-1% agarose gel electrophoresis and chemiluminescence using a goat polyclonal

anti-VWF antibody (Bethyl Laboratories, Conroe, TX).

C. Cone-and-plate viscometry

pVWF multimers (500 μl at 10 μg/ml) were exposed to 100 dynes/cm^2 of shear stress for 3 min at 37°C on a cone-and-plate viscometer (RS1, HAAKE Instrument Inc., Paramus, NJ) as previously described [5]. The surface of cone and plate was coated with 5% of bovine serum albumin (BSA) overnight at room temperature and rinsed gently before experiments. Shear stress was calculated based on a constant shear rate of 10,000s^{-1} and a viscosity of 1 cp for VWF multimers in suspension. VWF was subjected to AFM experiments within 1 hr after being exposed to shear stress.

D. Immunostaining of VWF aggregation

pVWF multimers (10 μg/ml) were first exposed to 100 dynes/cm^2 of shear stress for 3 min at 37°C and then incubated with glass coverslips that were previous coated with glycocalicin (5 μg/ml, overnight at 4°C) for 10 min. The coverslips were then washed with PBS to remove unbound VWF and fixed with 5% paraformaldehyde for 20 min at room temperature. VWF captured to coverslips was incubated with a polyclonal VWF antibody (DAKO, Carpinteria, CA) for 60 min at room temperature. After washing with PBS, they were incubated with a HRP-conjugated mouse anti-rabbit IgG for 30 min at room temperature. The coverslips were then incubated with DAB substrate (DAKO) for 8 min to develop for color detection.

E. Generation of dimeric VWF

Recombinant dimeric human VWF was generated in Human Embryonic Kidney (HEK) 293 cells. cDNA for human VWF without the sequence for propeptide was transfected into 293 cells using lipids as carrier. Transiently transfected cells were grown first in DMEM medium (Invitrogen, Grand Island, NY) with 5% of fetal bovine serum for 48-72 hrs and then in serum-free DMEM for 24 hrs. The conditioned medium was collected and dimeric VWF was purified by affinity chromatography with a polyclonal VWF antibody (DAKO).

F. Shear-induced VWF binding to human platelets

Shear-induced activation of human VWF multimers was measured by VWF binding to washed platelets detected by flow cytometry as previously described [6]. The use of human blood was approved by the Institutional Review Board of Baylor College of Medicine. All donors signed consent forms before blood was drawn. Within 2 hrs after preparation, washed platelets were mixed with spVWF multimers for 10 min at room temperature. A FITC-conjugated polyclonal VWF antibody (DAKO) was then incubated with platelets for 20 min at room temperature. The antibody binding was detected on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). FITC conjugated mouse IgG was used to control for background fluorescence and pVWF before shear stress exposure to control for baseline VWF binding to platelets.

G. Single-molecule AFM experiments

An atomic force microscope was used to perform single-molecule manipulation of pVWF multimers, ULVWF multimers and spVWF multimers. Multimeric VWF molecules were equilibrated at 37°C prior to being deposited onto a fresh gold surface at room temperature for 10 min then rinsed with phosphate-buffered saline (PBS, pH 7.4) to remove unabsorbed molecules. The AFM tip (MLCT Veeco Probes) was brought in contact with the surface for 1–3 seconds in order to allow part of the protein to adsorb to the tip. To reduce the likelihood of attaching multiple molecules, sample concentration was reduced so that the rate of catching a molecule was low. Force measurements were taken in PBS buffer solution (137 mM NaCl, 11.9 mM phosphates, 2.7 mM KCl) with pulling velocities ranging from 100 nm/s to 5000 nm/s.

VWF molecules were immobilized through nonspecific binding. Single molecule pulling event was accomplished by creating low surface coverage of VWF molecules, which in term kept the success rate for pulling any molecule below 5%, making the probability of pulling more than one molecule at a time very low. In addition, single molecule pulling was verified by the observation of only one detachment peak in the force-extension curve. This method was used in studying single titin molecule with success.

II. SUPPLEMENTAL RESULTS

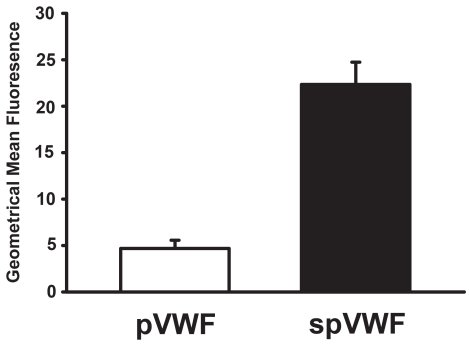

FIG. 1: Shear stress-induced VWF binding to platelets. Washed platelets were incubated with spVWF for 10 min at 37°C (pVWF as control). VWF binding was detected by a polyclonal FITC-VWF antibody on a flow cytometer. VWF binding was quantified by geometrical fluorescent intensity (n = 3, the paired t test, p = 0.002).

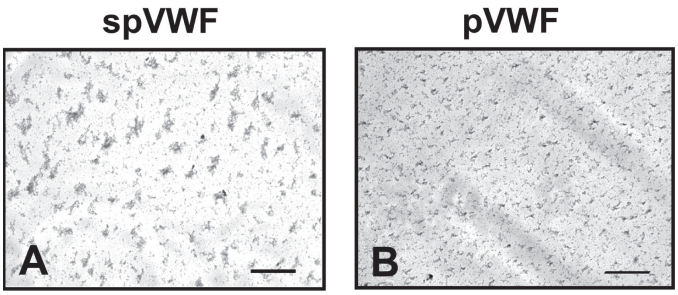

FIG. 2: Shear-induced VWF aggregation. VWF multimers purified from plasma samples of healthy subjects were exposed to 100 dynes/cm^2 of shear stress for 3 min at 37°C. spVWF captured by immobilized glycocalicin was detected by a polyclonal VWF antibody (A). pVWF before shear exposure was stained for negative control (B). Figures are representative of 3 separate experiments (bar = 200 μm).

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