Von Willebrand Factor propeptide (VWFpp) binding to VWF D'D3 domain attenuates platelet activation and adhesion

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Running head: VWFpp interacts with VWF in circulation

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METHODS

***VWF domain constructs**:* Standard molecular biology methods and primers listed in Supplemental Table I were used to create lentiviral vectors for the expression of individual domains (VWFpp, D′D3, A1, A2 and A3). In the first step, green fluorescent protein (GFP) present in the vector pCS-CG (plasmid # 12154, Addgene, Cambridge, MA) was replaced by the Kozak sequence followed by the VWF signal peptide, VWF propeptide (VWFpp) sequence, TEV (Tobacco Etch Virus) cleavage site and poly(6X)-His tag. To this end, the 1.5 Kb *Bam*HI to *Kpn*I fragment of pCS-CG was cloned into the pUC19 vector. Inverse PCR with phosphorylated primers (primers (1)-(2)) was performed on this template to simultaneously delete GFP and to introduce the restriction sites *Hpa*I and *Bst*BI, followed by TEV cleavage site, His-tag and stop codon. *Nhe*I just upstream of *Hpa*I and *Bst*BI was preserved in the parent template. The resulting product was re-ligated and the 0.9 Kb *Bam*HI to *Kpn*I fragment was transferred back into the parent pCS-CG vector. Next, the VWF signal peptide along with VWFpp (VWF aa 1-745) was PCR amplified using full length VWF cDNA in pCDNA3.1 as template [2]. *Nhe*I site and Kozak sequence were incorporated at the 5'-end while a *Hpa*I site was inserted at the 3'-end (primers (3)-(4)) of the PCR product. This product was ligated into pCR-Blunt II TOPO (Invitrogen, Carlsbad, CA). The *Nhe*I to *Hpa*I fragment was also introduced into the above His-tag modified pCS-CG vector generated in the previous step. This final vector, where the CMV promoter drives the production of His-tagged VWFpp is called "pCS-CG-VWFpp-His".

In the second step, the VWFpp sequence in "pCS-CG-VWFpp-His" was replaced with the 22 amino acid VWF signal peptide. To accomplish this, the above TOPO vector containing the VWFpp insert was used as template and the VWF signal peptide was amplified with primers (primers (5)-(6)) containing *Age*I and *Sac*II sites. A phosphorylated-ACC sequence was present at the 5' end of the antisense primer since this provides a *Hpa*I compatible blunt end. After digestion with *Nhe*I, the PCR product was ligated into "pCS-CG-VWFpp-His" cut with *Nhe*I and *Hpa*I. This second vector is called "pCSCG-KZK-SS-His".

In the final step, the VWF A1-domain (aa 1243-1480) was introduced into pCSCG-KZK-SS-His (primers (7)-(8)). During this process, the FLAG epitope followed by *Bst*BI was incorporated at the C-terminal of A1 domain PCR product. This was digested with *Age*I and *Bst*BI and ligated into pCSCG-KZK-SS-His. This back bone was designated 'pCSCG-KZK-SS-A1-FLAG-His'. The VWF-A1 domain was replaced in this vector with the D′D3- (aa764-1243), A2- (aa 1481-1668) and A3- (aa 1671-1875) domains using additional PCR primers having *Age*I and *Hpa*I compatible restriction site overhangs (primers (9)-(14)).

Stable CHO cells expressing individual VWF domains: In order to establish stable Chinese Hamster Ovary (CHO) cell lines that secrete individual VWF domains (VWFpp, D′D3, A1, A2 or A3), lentivirus was generated in human embryonic kidney HEK 293T cells. Viral particles thus generated were applied to transduce CHO cells. Briefly, 293T cells were grown to 50% confluence in DMEM containing 10% FBS. Culture medium was then supplemented with 25μM chloroquine. Transfection was performed using calcium phosphate method and a DNA mix that contained 15μg of one of the above pCS-CG vectors, helper plasmids 10μg pMD2.G (Addgene, Cambridge, MA) and 10μg psPAX2 (Addgene). After overnight culture, medium was changed to DMEM containing 10% FBS with 10mM sodium butyrate for additional 24-30h. The medium with lentiviral particles was then collected, filtered through 0.45μM syringe filter and concentrated 100-fold by centrifugation at 50,000g for 2h at 4°C. The viral pellet was dissolved in serum free IMDM, and used either immediately or stored at -80°C. During the transduction step, high-titer virus corresponding to each of the constructs was added to CHO cells grown to 40% confluence in 500μl DMEM media containing 10% FBS and 8μg/mL polybrene. Viral transduction using this method is efficient and typically >98% of CHO cells stably express the transgene. After 48h, CHO cells were transferred into larger tissue-culture flasks for scale-up. DMEM with 10% FBS was the media during scale-up. Once ~90% cell confluence was achieved, media was changed to protein free ProCHO-AT (Lonza, Walkersville, MD), and cell culture supernatant was collected 3 and 6 days later. Typical

scale up involves culturing cells in 20 T-150 flasks to produce 1L of cell culture supernatant. VWF domains were secreted into cell culture media at 4-12 μg/mL.

Affinity and ion-exchange purification of individual domains: Individual VWF domains were purified using a combination of poly-Histidine based affinity- and ion exchange- chromatography. In all cases, 1L of cell culture media was pooled and passed through a HisTrap HP column (GE Healthcare, Piscataway, NJ) equilibrated with 20mM HEPES salt (pH 7.4) containing 300mM NaCl (binding buffer). In case of the A1 domain, non-specifically bound protein was washed with 20mM HEPES (pH 7.4), 300mM NaCl and 66mM imidazole using an AKTAprime plus system (GE Healthcare). A1 domain was then eluted with binding buffer containing 200mM imidazole. For the rest of the proteins (VWFpp, D′D3, A2 and A3), the individual domains were directly eluted with 20mM HEPES salt (pH 7.4), 300mM NaCl and 250mM imidazole in the absence of the low-imidazole wash step. Fractions containing the proteins of interest were then pooled, diluted 10-fold in 20mM HEPES (pH 7.4) to reduce salt concentration and subjected to anion-exchange chromatography using a Capto-Q column (GE Healthcare). In this step, after passing the pooled fractions, the column was washed/equilibrated with 5ml of 20mM HEPES, and the proteins of interest were eluted using a salt gradient where the second buffer was 20mM HEPES containing 1M NaCl. Buffer exchange was performed as necessary using a Zeba spin desalting column (7K MWCO, Thermo, Rockford, IL).

Silver stain, western blot, dot blot: SDS-PAGE under standard Laemmli reducing conditions was performed using 4-20% gradient gels (Thermo-Fisher, Rockford, IL). In some cases, β–mercaptoethanol was withheld from the sample buffer and this condition is termed 'non-reducing'. Protein purity was assessed using silver staining kit from Thermo-Pierce. Western blotting was performed by transferring proteins onto nitrocellulose membrane and detecting them using HRP conjugated goat anti-His polyclonal Ab (Bethyl Laboratories, Montgomery, TX).

In some instances, dot blot assays were performed by spotting 50ng of either D'D3 or VWFpp onto nitrocellulose membrane, either in native form or upon denaturation by boiling in the presence of 1.4M β–mercaptoethanol. The membrane was then dried at 37ºC, blocked with TBST (Tris-buffer saline with 0.1% Tween-20) containing 5% milk, and the protein spot was detected using 2μg/mL of anti-VWFpp/D'D3 mAb and HRP-conjugated goat-anti-mouse Ab (Jackson Immuno., West Grove, PA).

Epitope Mapping with bovine and porcine VWF: The binding of anti-D'D3 mAbs to VWF from different species (human, bovine, porcine) was performed using sandwich ELISA. 1μg/mL rabbit-anti-human VWF polyclonal Ab (Dako, Carpinteria, CA) was bound onto 96-well MaxiSorb plates (Thermo-Fisher) overnight at 4^0C. The wells were then blocked with HEPES buffer containing 3% BSA for 2h at room temperature. 1:4 human and porcine plasma or 1:2 bovine plasma (Sigma) was then added to anti-VWF wells for 1h at room temperature. After washing with TBST, mAbs DD3.1 and DD3.2 (~0.2μg/mL) were added for 1h at room temperature. Bound mAb was detected using HRP conjugated goat-anti-mouse Ab and o-phenylenediamine substrate (OPD, Sigma). VWF immobilization was quantified in under identical conditions as above, only using a HRP conjugated polyclonal anti-VWF antibody.

Blocking VWFpp-VWF interaction using mAbs against VWFpp and D'D3: MaxiSorb plates were incubated with 1μg/mL VWFpp overnight at 4^0C and the surface was then blocked with HEPES buffer containing 3% BSA (Sigma) for 1h at room temperature. While 10μg/mL of each of the anti-VWFpp mAbs were incubated with the wells for 15min in some cases, 1μg/mL anti-D′D3 mAbs were added to multimeric VWF in other cases. Following this, 2.5μg/mL VWF, with or without anti-D′D3 mAb, was added to VWFpp wells for 1h in one of two buffers: (i) MES buffer (pH 6.2) with 10mM Ca^{2+} and 1% BSA, or (ii) HEPES buffer (pH 7.4) with 2.5mM Ca^{2+} and 1% BSA. After three TBST washes, 1μg/mL rabbit polyclonal anti-VWF Ab (Dako) was added along with 1μg/mL HRP conjugated donkey anti-rabbit

Ab (Jackson Immuno.) for 1h in either MES or HEPES buffers. After several more washes, the binding of VWF to wells was measured based on HRP signal quantified when OPD was substrate.

Studies that measured VWFpp binding to ΔPro-VWF and ΔD'D3-VWF were performed similar to above. In this case, 2µg/mL of VWFpp was immobilized overnight on MaxiSorb plates and the binding of 10µg/mL of the VWF-variants was measured in MES buffer (pH 6.2) containing 10mM Ca^{2+}, 1% BSA and 0.01% Tween-20.

Factor VIII binding assay: 2µg/mL of anti-His antibody or AVW-1 was immobilized on MaxiSorb plates overnight at 4^0C. The wells were then blocked with HEPES containing 3% BSA for 2h at room temperature. 2µg/mL of D'D3 or VWF in HEPES containing 1% BSA was added to the anti-His/AVW-1 immobilized wells for 1h at room temperature. After several washes with TBST, 1µg/mL Factor VIII (Advate, Baxter, Deerfield, IL) was added in the presence or absence of 20µg/mL ESH-4 (anti-Factor-VIII blocking mAb, American Diagnostica, Stamford, CT). Following this, 1µg/mL HRP conjugated sheep anti-Factor VIII antibody (Enzyme Research, South Bend, IN) was added to the wells, and the signal was developed using OPD as substrate.

The above protocol was slightly modified to assay the effect of VWFpp on VWF-Factor VIII binding. In this case, 2.5µg/mL human VWF from plasma cryoprecipitate was immobilized on MaxiSorb wells. Different concentrations of VWFpp (1.25-40µg/mL) or 20µg/mL mAb DD3.1 in HEPES (pH 7.4) containing 2.5mM $CaCl_2$ and 1% BSA were added to the VWF immobilized wells. After 15 minutes of incubation, Factor VIII was added to these wells.

Ristocetin induced binding of A1 domain to platelets: Platelets in platelet rich plasma (PRP) obtained from human blood was labeled with 1µl of 1:10 diluted CD31 PerCP-efluor710 (ebiosciences, San Diego, CA) [3]. 10µg/mL of the A1 domain was added to ~300,000 labeled platelets/mL in the presence of 1.5mg/mL ristocetin (MP Biosciences, Solon, OH) at room temperature. A1 domain binding to platelets was measured using Alexa-488 labeled anti-tetra His Ab (Qiagen, Valencia, CA) in the flow cytometer. 10µg/mL anti-Gp1bα blocking antibody AK2 was added to platelets in some runs to inhibit this molecular interaction.

A2-domain cleavage measured using ADAMTS-13: A2 domain (25µg/mL) was incubated with varying concentrations of urea in the presence or absence of concentrated, recombinant, human ADAMTS13 [1] for 20h at RT in 50mM Tris buffer containing 12.5mM $CaCl_2$ and 0.1% BSA. Western blot using anti-His antibody was performed as described above to observe cleavage of this domain.

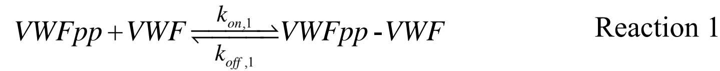
VWFpp-plasma VWF binding in blood plasma: Two types of assays were performed:

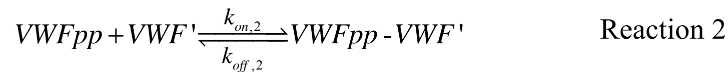
In some runs, 30µg/mL anti-VWF Ab or control (anti-myosin) was added to 1mL Platelet Poor Plasma (PPP) obtained from heparinized human blood in the presence or absence of 30µg/mL DD3.1 overnight at 4ºC. Protein A/G beads (Calbiochem-Merck) was added to immunoprecipitate VWF for 4h at 4ºC. Beads were then washed with MES buffer (pH 6.2) containing 10mM Ca^{2+} and 1% BSA. Following this, VWFpp was released using HEPES buffer (pH 7.4) with 5mM EDTA and 1% BSA. After 30 min, released VWFpp was detected using a sandwich ELISA where 5µg/mL 242.2 mAb was immobilized on a MaxiSorb plate to capture VWFpp and 5µg/mL HRP conjugated 239.3 was used for detection.

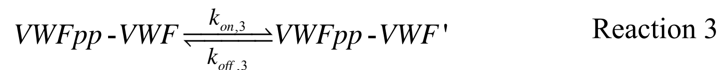
In other runs, MaxiSorb plates were immobilized with anti-FLAG antibody (clone M2, Sigma) for 3h at room temperature followed by blocking with HEPES buffer containing 3% BSA. Platelet poor plasma (PPP) was isolated from freshly drawn human blood in 20U/mL heparin [4]. 35µl PPP was incubated with different concentrations of VWFpp (0-20µg/mL), a FLAG-tagged recombinant protein, for 10 min. In some cases (shown in the inset), 50 µg/mL of DD3.1 or control antibody DD3.3 was incubated with PPP for 10 minutes prior to incubation with 10µg/mL of VWFpp. This PPP with VWFpp was then added to the anti-FLAG bearing wells for 1h at room temperature. The plates were washed five times using HEPES buffer with 2.5mM Ca^{2+}. Plasma VWF bound to the wells was detected using 1µg/mL HRP conjugated polyclonal anti-VWF Ab diluted in MES buffer (pH6.2) containing 10mM Ca^{2+} and 1% BSA.

Following additional washes in MES buffer, HRP signal was detected using OPD as substrate. Several controls were performed including runs where MaxiSorb plates were blocked without immobilized anti-FLAG mAb.

Clamp XP model fits: SPR data for VWFpp binding to multimeric VWF under low pH and high calcium conditions were fit using Clamp XP [5]. In this model, VWFpp clusters/aggregates formed at low pH can interact with one D'D3 site alone (reaction 1). They can also interact with adjacent D'D3 units located at the N-terminal of multimeric VWF simultaneously (reaction 2). VWFpp clusters binding to single D'D3 from reaction 1 may also transition to multivalent binding to two adjacent D'D3 sites (reaction 3).

 Reaction 1

 Reaction 2

 Reaction 3

To fit this model to experimental data, the rate constants for the first reaction was set based on measurements of VWFpp binding to single D'D3 at low pH (i.e. $k_{on,1}$=0.55 x 10^5 $M^{-1}s^{-1}$; $k_{off,1}$=110x10^{-5} s^{-1} Figure 6B). The rate constants for the second and third reactions were fitted using ClampXP. These fitted reaction constants were $k_{on,2}$ = 4.52 x 10^5 $M^{-1}s^{-1}$; $k_{off,2}$= 0 s^{-1} ; $k_{on,3}$ = 3.923 x 10^{-4} s^{-1}; $k_{off,3}$= 0 s^{-1}.

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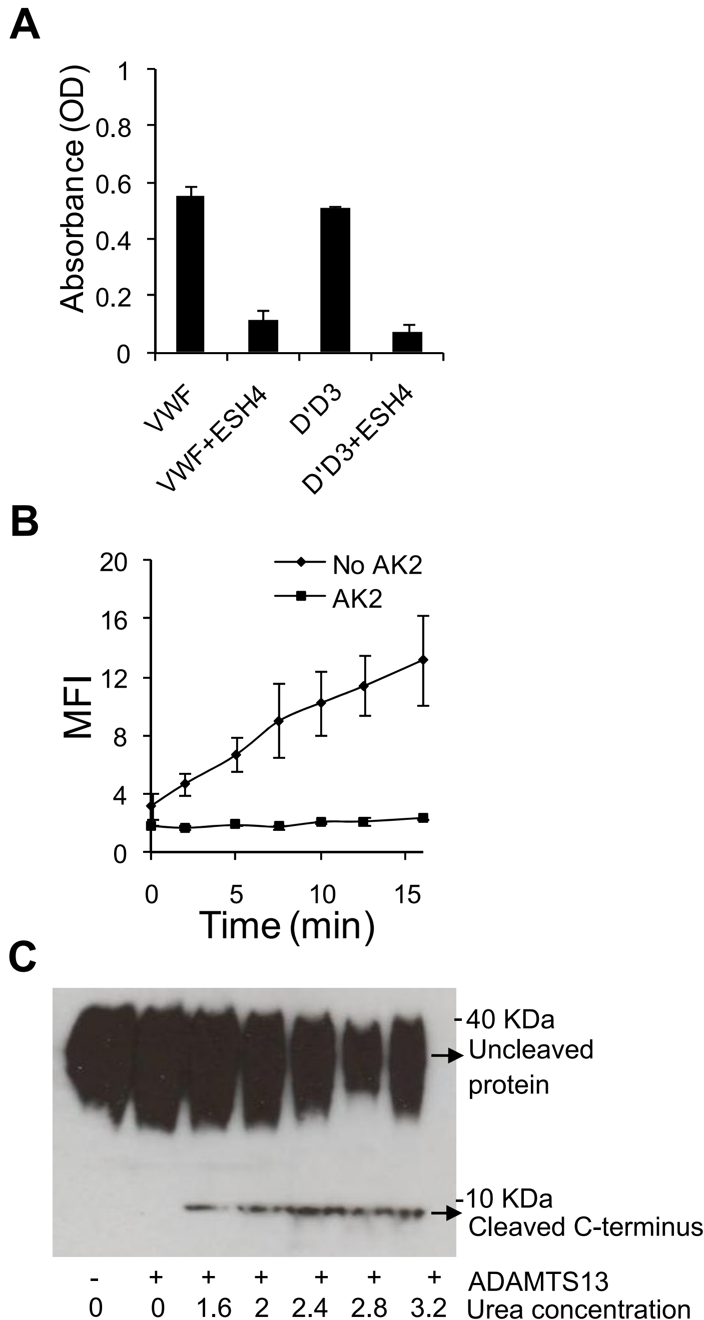
Supplemental Table 1. List of PCR Primers

Primer (number) name	Sequence (5′-3′)
(1) pCSCG_His_inv_sense	[Phos]GGC*CATCACCATCACCATCAC***TGA**CTCGAGACCTAGAAAA ACATGGAG
(2) pCSCG_His_inv_asense	CTGGAAGTACAGGTTTTCGAAGTTAACACCGGTAGCGCTAGCGG ATCTGAC
(3) VWF_D1_NheI_Kozak	GCTAGC*GCCGCCACC*ATGATTCCTGCCAGATTTGCCGGGGTG
(4) VWFD2_HpaI_antisense	GTTAACTCCGGGGACTCCACTCATGGTACAG
(5) pCR2bluntSense	AGTGTGCTGGAATTCGCCCTT
(6) Sigpep-AgeI-asense	[Phos]AACCCGCGGACCGGTACAAAGGGTCCCTGGCAAAATG
(7) VWF_A1_AgeIF	CGCGCCGACCGGTCTGGTGGTGCCTCCCACAGATGCC
(8) VWF_A1_BstBI_FLAG_R	CTCCTCCTTCGAACTTATCGTCATCGTCCTTGTAGTCGTTAACCG GGCCCACAGTGACTTGTGCCATG
(9) VWF_D′D3_AgeI for	CGCGCGGACCGGTAGCCTATCCTGTCGGCCCCCCATG
(10) VWF_D′D3_HpaI rev	[Phos]AACGCCTCCCGGCTCCTGGCAGGCTTC
(11) VWF_A2_AgeI for	CGCGCGGACCGGTGGGCTCTTGGGGGTTTCGACCCTG
(12) VWF_A2_HpaI rev	[Phos]AACCCTCTGCAGCACCAGGTCAGGAGC
(13) VWF_A3_AgeI	GCGCGCGACCGGTTCCGGAGAGGGGCTGCAGATCCCC
(14) VWF_A3_HpaI rev	[Phos]AACAAATCCAGAGCACAGTTTGTGGAGG

Enyzme sites: *Hpa*I: GTTAAC; *BstB*I: TTCGAA; *Nhe*I: GCTAGC; *Age*I: ACCGGT; *Sac*II: CCGCGG



Supplemental Figure S1. Characterization of recombinant proteins. A) ***D'D3 binding to Factor VIII:*** Anti-His antibody or AVW-1 was immobilized on microtiter plates. 2µg/mL D'D3 domain or VWF was captured on the substrates. ~ 1 µg/mL Factor VIII was then added in the presence or absence of 20 µg/mL ESH-4. B) ***Ristocetin induced A1 domain binding to platelets***: 10µg/mL of A1 domain was incubated with CD-31 PerCP labeled platelets in the presence of 1.5mg/mL ristocetin. Amount of A1 domain bound to platelets was measured using Alexa-488 conjugated anti-His antibody using flow cytometry. Binding of the A1-domain was blocked by anti-GpIbα mAb AK2. C) ***Cleavage of VWF-A2 domain***: A2 domain (25µg/mL) was incubated with varying concentrations of urea in the presence or absence of recombinant, human ADAMTS13 for 20h at room temperature in 50mM Tris buffer containing 12.5mM $CaCl_2$ and 0.1% BSA [1]. Western blot using HRP conjugated anti-His antibody shows the cleaved C-terminal A2-domain fragment at urea >2M in the presence of ADAMTS13.

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Human       SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCVA
Murine      SLSCRPPMVKLVCPADNPRAQGLECAKTCQNYDLECMSLGCVSGCLCPPGMVRHENKCVA
Porcine     SLSCRPPMVKLVCPADNPRAEGLECAKTCQNYDLECVSTGCVSGCLCPPGMVRHENRCVA
Bovine      SLSCRPPMVKVVCPADNPRAEGLECTKTCQNYDLECMSTGCVSGCLCPPGMVRHENRCVA

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Human       LERCPCFHQGKEYAPGETVKIGCNTCVCRDRKWNCTDHVCDATCSTIGMAHYLTFDGLKY
Murine      LERCPCFHQGAEYAPGDTVKIGCNTCVCRERKWNCTNHVCDATCSAIGMAHYLTFDGLKY
Porcine     LQRCPCFHQGREYAPGETVKVDCNTCVCRDRKWSCTDHVCDASCSALGLAHYLTFDGLKY
Bovine      LERCPCFHQGREYAPGDRVKVDCNSCVCQDRKWNCTDHVCDASCSALGLAHYFTFDGLKY

                                        *   *     *
Human       LFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKRVTILVEGGEIELFDGEVNV
Murine      LFPGECQYVLVQDYCGSNPGTFQILVGNEGCSYPSVKCRKRVTILVDGGELELFDGEVNV
Porcine     LFPGECQYVLVQDYCGSNPGTFRILLGNEGCGYPSLKCRKRVTILVDGGEIELFDGEVMV
Bovine      LFPGECQYVLVQDHCGSNPGTFRVLVGNEGCSVPSLKCRKRITILVEGGEIELFDGEVNV

                                                           *
Human       KRPMKDETHFEVVESGRYIILLLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQ
Murine      KRPLRDESHFEVVESGRYVILLLGQALSVVWDHHLSISVVLKHTYQEQVCGLCGNFDGIQ
Porcine     KKPLKDETHFEVVESGRFITVLLGSGLSVVWDRHLGISVFLKQTYQEQVCGLCGNFDGVQ
Bovine      KTPMKDETHFEVVESGRYITVLLGKALSVVWDGHLAVSVFLKRTYQERVCGLCGNFDGVQ

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Human       NNDLTSSNLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
Murine      NNDFTTSSLQVEEDPVNFGNSWKVSSQCADTRKLSLDVSPATCHNNIMKQTMVDSACRIL
Porcine     NNDLTGSSLQVEEDPVDFGNSWKVSPQCADTRKVPLDTSPATCHNNVMKQTMVDSSCRIL
Bovine      NNDLTSSSLQVEENPVDFGNSWKVSPQCADTQKVRLDSAPAICHDNVMKQTMVDSSCRVL

Human       TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKVVTWRTA
Murine      TSDVFQGCNRLVDPEPYLDICIYDTCSCESIGDCACFCDTIAAYAHVCAQHGQVVAWRTP
Porcine     TSDIFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYARVCAQHGKVVTWRTA
Bovine      TSDVFRECNRLVNPEPYLDVCIYDSCSCESIGDCSCFCDTIAAYAHECAQHGQVVTWRTA

                                          *
Human       TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
Murine      TLCPQSCEEKNVRENGYECEWRYNSCAPACPVTCQHPEPLACPVQCVEGCHAHCPPGRIL
Porcine     TLCPQNCEERNLREDGYQCEWRYNSCAPACPVTCQHPEPLACPVSCVEGCHAHCPPGKIL
Bovine      TLCPQNCEERNLKESGYQCEWRYNSCAPACPVTCQHPEPLACPVQCVEGCHAHCPPGKIL

                                  *          *           *
Human       DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVVNLTCEACQEPGG
Murine      DELLQTCVDPQDCPVCEVAGRRLAPGKKITLSPDDPAHCQNCHCDGVNLTCEACQEPGG
Porcine     DELLQTCVSPEDCPVCEAAGRRLAPGKKIILNPRDPAHCQICHCDGVNLTCEACAEPVP
Bovine      DELLQTCVNPEDCPVCQVEGRRLASGKKVTLNPGDPEHCQICHCDGVSLTCEACKEPGG
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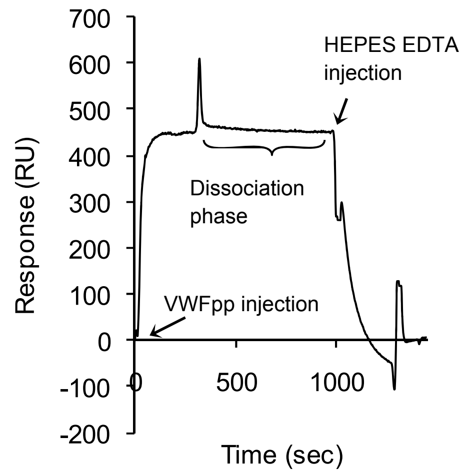
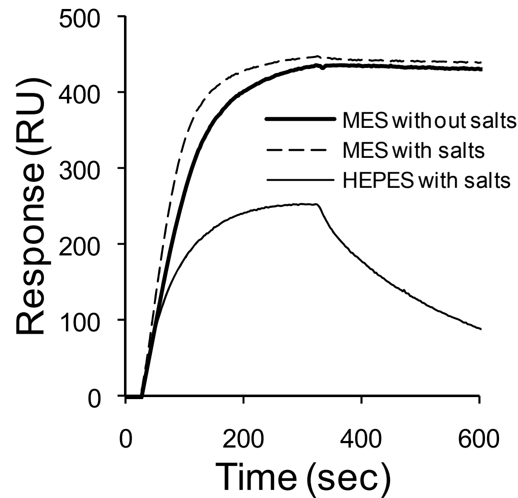
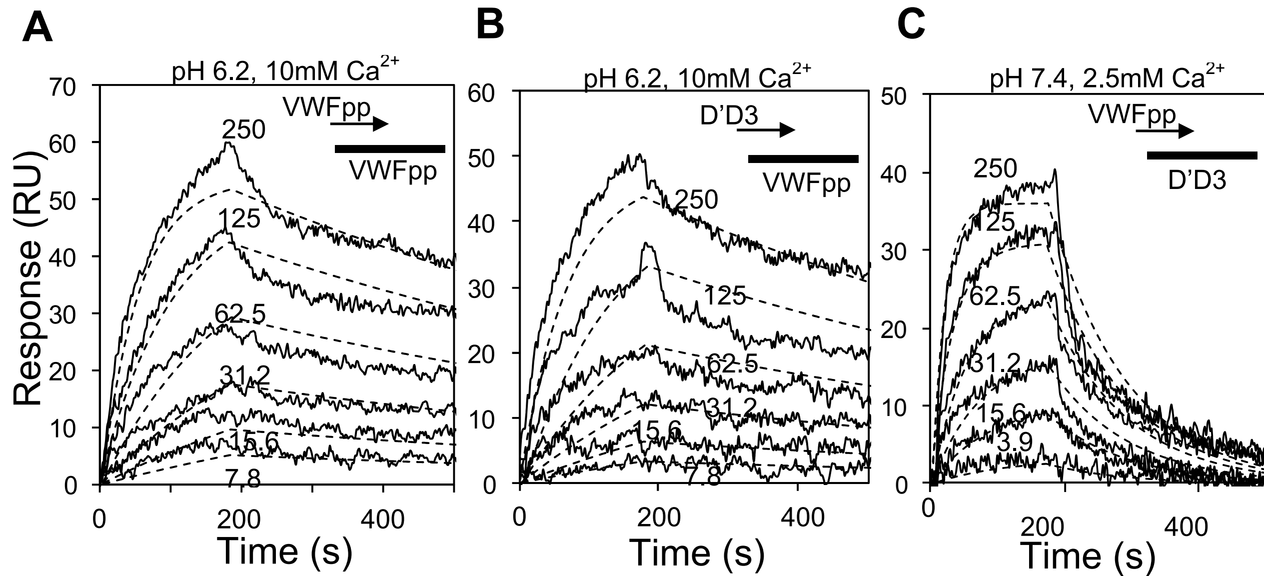
Supplemental Figure S2. Sequence alignment of D'D3 domain of human, murine, porcine and bovine VWF: The amino acid residues highlighted in gray are different between human and mouse species. Those indicated by '*'are unique to humans and they represent potential amino acids that bind mAbs DD3.1 and DD3.2.



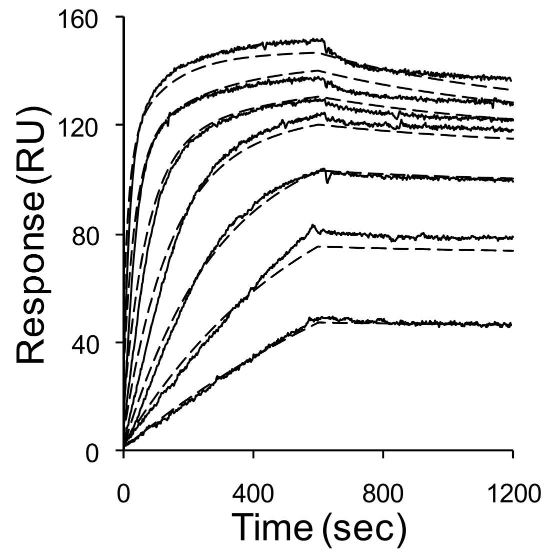
Supplemental Figure S3. SPR regeneration conditions: 1200 RU multimeric VWF was captured onto biosensor surface. 125nM VWFpp was then injected for 300s in MES buffer (pH 6.2), 10mM Ca^{2+}. Dissociation of VWFpp from substrate was measured from 300-900s in MES buffer containing 10mM Ca^{2+}. Very little dissociation is detected. Following this, dissociation buffer was changed to HEPES (pH 7.4) containing 1mM EDTA. HEPES-EDTA successfully regenerates the biosensor substrate.



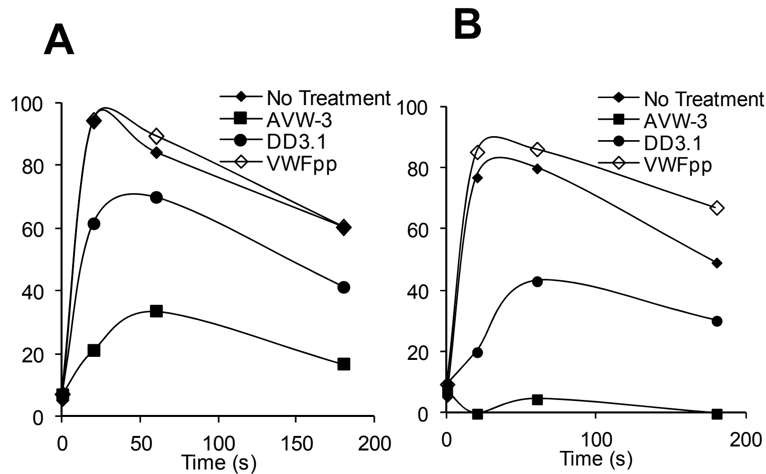
Supplemental Figure S4. Effect of salt: ~1500 RU multimeric VWF was captured onto biosensor surface via mAb AVW-1. 62.5nM VWFpp was then injected for 300s in MES buffer (pH 6.2, 10mM Ca^{2+}) either without or with salts: 110mM NaCl, 10mM KCl and 1mM $MgCl_2$. Dissociation of VWFpp from substrate was measured after 300s. Salt does not affect binding at low pH. When 62.5nM VWFpp in HEPES buffer (pH 7.4) containing 2.5mM Ca^{2+} was injected on the same surface, response was ~60% that of MES buffer runs.



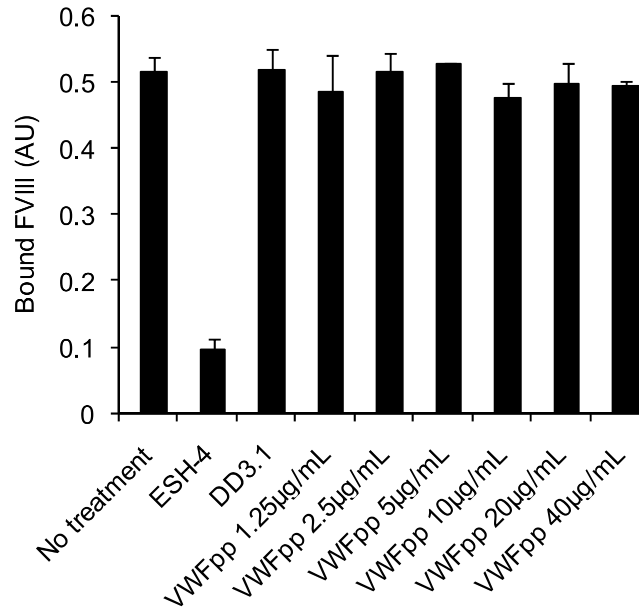
Supplemental Figure S5. VWF domain-domain interactions. A) 500 RU VWFpp was immobilized on the sensor surface and different concentrations of VWFpp (indicated in nM units) were injected in MES (pH 6.2) buffer with 10mM Ca^{2+}. B) Different concentrations of D′D3 were injected onto VWFpp (500RU) bearing substrate in MES (pH 6.2) buffer with 10mM Ca^{2+}. C) 800 RU of D′D3 was immobilized and different concentrations of VWFpp were injected in HEPES (pH 7.4) containing 2.5mM Ca^{2+}. Kinetic data were fit to a simple 1:1 interaction mode (dashed lines). Binding constants are summarized in Table 1.



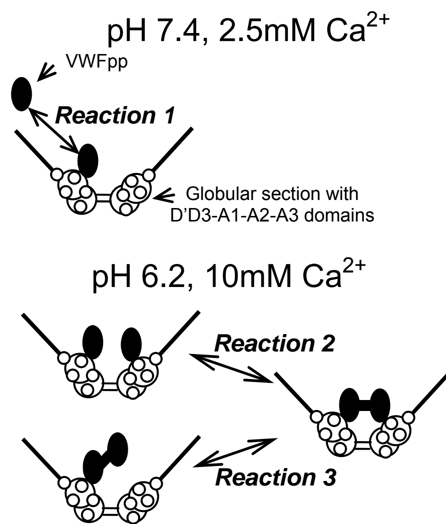
Supplemental Figure S6. Multivalent interaction model for low pH binding data MES binding data from Figure 5A fit using the ClampXP model that considers multivalent VWFpp binding to single and dual D'D3 epitopes. Model fit agrees well with experimental data.



Supplemental Figure S7. DD3.1 partially inhibits agonist mediated platelet aggregation. PRP isolated from blood was diluted in PPP to obtain a platelet concentration of 10^8/mL. This solution was incubated with AVW-3 (20 µg/mL) or VWFpp (100 µg/mL) or DD3.1 (100 µg/mL) for 10 minutes at room temperature. These were then sheared in a cone-plate viscometer at $9600s^{-1}$ in the presence of A) ADP (0.5µM) or B) TRAP (5µM). % platelet aggregation was quantified using flow cytometer by measuring depletion of single platelets. Data are representative of three independent experiments.



Supplemental Figure S8. VWFpp does not affect Factor VIII-VWF interaction The ability of VWF bearing microtiter wells to capture FVIII, in the presence or absence of different concentrations of VWFpp (or 20μg/mL mAb DD3.1), was measured. MAb ESH-4 blocks VWF binding to FVIII. Neither VWFpp not DD3.1 altered VWF binding to FVIII.



Supplemental Figure S9. Model for VWFpp-VWF interaction under different conditions. At pH 7.4, VWFpp does not aggregate. Here, VWF-VWFpp binding is described by a 1:1 interaction model. At pH 6.2, clusters of VWFpp interact with VWF. Thus, a complex set of interactions regulate overall binding kinetics at low pH. Reactions 1-3 are the three types of molecular interactions considered in the ClampXP model.