Design, expression, and initial characterization of von Willebrand Factor A2 domain and its mutants

Design, expression, and initial characterization of von Willebrand Factor A2 domain and its mutants

by

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Date

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Chairperson of Department：Dr. Anand Jagota

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Acknowledgments

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ABSTRACT

The von Willebrand Factor (VWF) is an essential clotting factor in hemostasis. It is secreted into blood plasma as an ultralarge multimer to mediate platelet adhesion at vascular injured sites. The VWF can be cleaved to smaller multimers by the metalloprotease ADAMTS13, which is the feedback control to maintain the proper thrombogenic potential of the VWF. The A2 domain of the VWF contains the only cleavage site for ADAMTS13. Mutations in the A2 domain may lead to type-2M von Willebrand Disease (VWD), which is characterized by a bleeding phenotype and a lack of large VWF multimers in blood plasma.

This project focuses on the design, expression, and characterization of three A2 mutants: Q1541R, N1515Q, and N1574Q. Q1541R is a newly identified, type-2M VWD mutation that causes a bleeding phenotype. N1515Q and N1574Q are artificial mutants with N-linked glycans removed at sites N1515 and N1574, respectively. Genes encoding wild-type and mutant A2 domains were PCR-amplified and constructed in the pHLsec vector. N-terminal SpyTag and C-terminal Avi- and His(6)-tags were added to the constructs. Wild-type and mutant A2 proteins were expressed in HEK 293T cells, and purified from a culture supernatant using Ni-NTA chromatography, followed by size exclusion chromatography. The proteins were biotinylated using an Avi-tag biotinylation kit. The purified A2 and mutants were characterized by the ADAMTS13 cleavage assay and optical-tweezer pulling assay. Our data suggest that the Q1541R and N1574Q mutants are more susceptible to forced unfolding and ADAMTS13 cleavage compared to the wild-type A2.

1. INTRODUCTION

Hemostasis is the process that stops bleeding and prevents blood loss following blood vessel injury. It contains two steps, primary hemostasis and the secondary hemostasis. The von Willebrand factor (VWF) plays a vital role in primary hemostasis, especially for arterial bleeding due to the high shear force. [1] The VWF is a kind of large multimeric glycoprotein, [2-3] which is stored as an ultra large (UL) form (ULVWF) in the Weibel-Palade bodies of endothelial cells. To generate varying sizes of VWF, the ULVWF is cleaved by the metalloprotease "a disintegrin and metalloproteinase with thrombospondin type 13" (ADAMTS13) after its release into the bloodstream by endothelial cells or the precursor of the platelets megakaryocytes. [4] During vessel injury, the VWF first acts as a signal to attract platelets to the injured site. Subsequently, the VWF helps the platelets bind with the subendothelial matrix proteins (e.g., collagen), and to bind with each other (platelet aggregation), finally forming a platelet plug.

The protein sequence of the wild-type VWF contains a signal peptide comprising 22 amino acids, one pro-peptide with 741 amino acids, and 2050 amino acid mature structures. A monomer VWF contains four types of domains in the following order: NH_2-D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK-COOH.[5] The ADAMTS13 cleavage site is located at the Y1605-M1606 peptide bond in the A2. The cleavage is highly regulated by the hydrodynamic force, which is capable of reducing the multimer's size and downregulating the VWF's potency in mediating adhesion between platelets and the subendothelial matrix protein.

The primary structure of the A2 domain contains 199 residues. With the glycans attached to the two N-link glycosylation sites N1515 and N1574, the molecular weight of A2 reaches about 30 kDa. The tertiary structure of A2 can be distinguished as a two-winged structure (shown in Figure 1[2]).

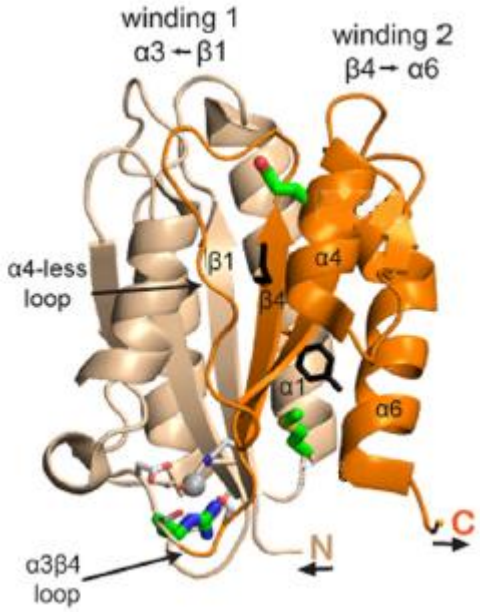

Figure 1. The tertiary structure of the A2 domain. [2]

Recently, Xu and Springer (2011) proposed a hypothesis that the initial A2 unfolding takes place in the winding 2. More precisely, they suggested it would happen close to the C-terminal around the α6 helix ends. The opposite site (N-terminal) in winding 1 contains three β-strands which protect the central part of the domain to be extended. Another possibility, also proposed by Xu and Springer (2013), is that A2 is more sensitive to force due to its lack of long-ranged disulfide bonds. They also proposed that the concatemer length, regulated by A2 unfolding, would have a huge effect on the VWF function. [5-6] Furthermore, N-linked glycosylation on sites N1515, N1574 may affect A2's sensitivity to the ADAMTS13 cleavage. [3] Comparing the cleavage sensitivity of the wild type (WT) A2 and mutants lacking glycans or causing VWD is one of the main goals of this project.

In this thesis, the unfolding of A2 is detected by optical tweezers. The optical tweezer is an increasingly important tool in nanometer-scale research. [7] Optical tweezers have the ability to generate extremely small forces by using a highly focused

laser beam. Usually, the beams focus with the help of a microscope's objective lens. Due to the conservation of momentum, when the photons pass through the beam, the particles will generate a force that attracts the particles along the gradient to the center of the beam. This is also called optical capture. The dual-laser optical tweezer that I used for this experiment is more stable because it can balance the two forces generated by the light reflection, which is one of the main noises from this machine.

This project can be divided into two parts. The first is about A2 expression and purification. I managed to express and purify the A2 domain proteins through the full-length VWF gene. I used SDS-PAGE gel electrophoresis and a western blot to test the expression result. The second part is called the "Initial characterization of A2 and its mutants," which aims to find the unfolding force difference between the WT and the Q1541R mutations by using an optical tweezer system. In addition, I used a cleavage assay to detect the difference between the WT A2 and the mutations of when subjected to cleavage by ADAMTS13.

2. EXPRESSION OF A2 AND ITS MUTANTS

2.1 Preparation of A2 DNA

Our lab already has the plasmids encoding the full length VWF. The first step of the project is to construct a plasmid containing the cDNA of the human VWF A2 domain (Pro1480 to Pro1678 with prepro-VWF numbering). To achieve this, I used a polymerase chain reaction (PCR) method to amplify the WT gene and the respective mutants of the A2 domain with a pre-designed PCR primer. After the PCR amplification, a DNA agarose gel was used with Ethidium Bromide (0.5 g agarose dilute with 50ml 1X TAE buffer, and 3µl Ethidium Bromide) to check the PCR purification result (shown in Figure 2).

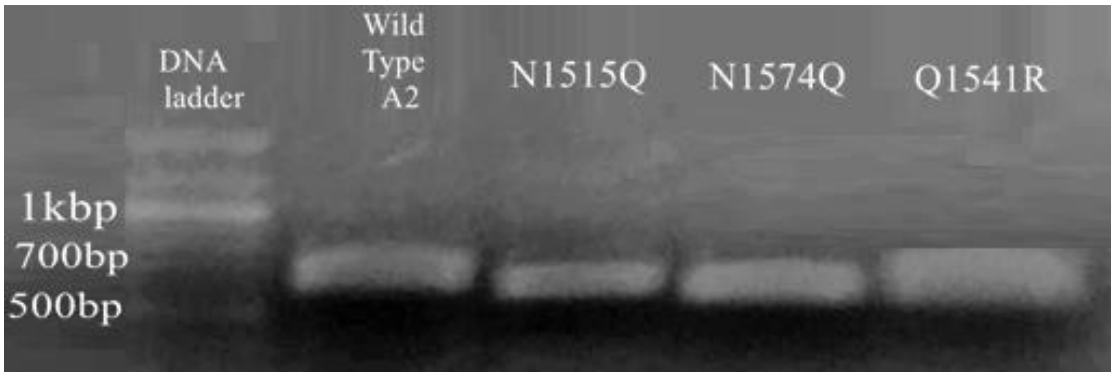

Figure 2. The DNA agarose gel result of the PCR products. The second lane (from left) is the WT A2 DNA. The N1515Q, N1574Q, and Q1541R are the three mutations of the A2 domain.

The DNA fragment size of the A2 domain should be 600 bp (for both the WT and mutations). This was proved by the DNA-gel result (Figure 2).

After purification, the PCR products were digested with restriction endonucleases PacI and PmeI at 37 degrees for one hour. The digested products were purified by gel extraction (using a QIAquick Gel Extraction kit) (shown in Figure 3).

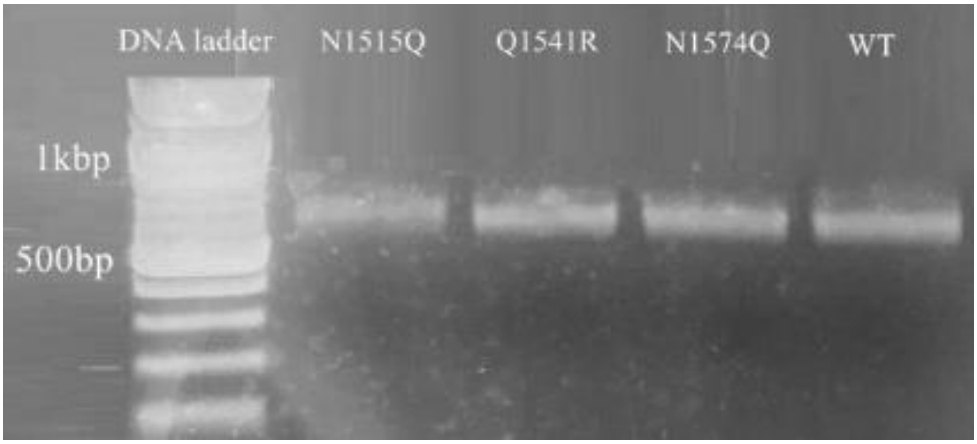

Figure 3. DNA gel for the DNA samples after treatment with restriction endonucleases.

2.2 Preparation of A2 in pHLsec vector

Our lab is an existing plasmid, X3 (containing genes for expressing human MAdCAM-1(D42A) fused with human integrin β7(1-703D)), which was built on a pHLsec vector backbone. The vector includes genes encoding an N-terminal SpyTag and a C-terminal AviTag for the optical tweezer pulling experiment, and a C-terminal His(6) tag for protein purification. In order to build the A2 constructs into the pHlsec vector, plasmid X3 was treated with Pac I and Pme I at 37 degrees for one hour to create the same sticky ends for insertion (shown in Figure 4). As such, the digested insert and vector can be ligated to generate new A2 plasmids.

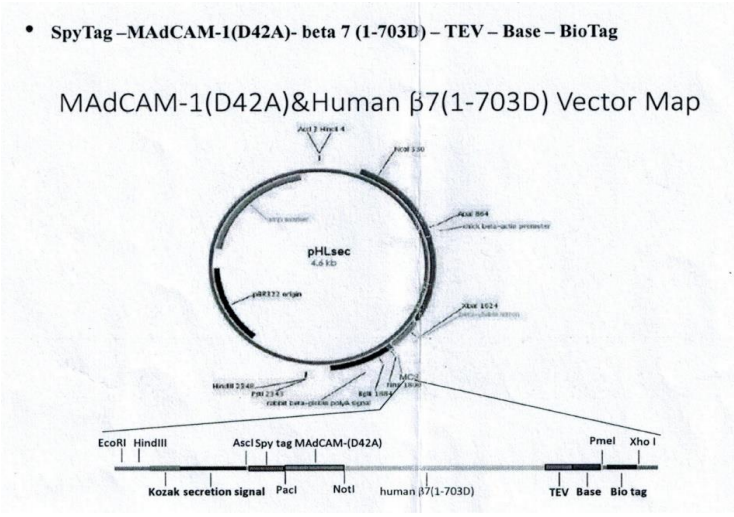

Figure 4. Vector map for the X3 plasmid.

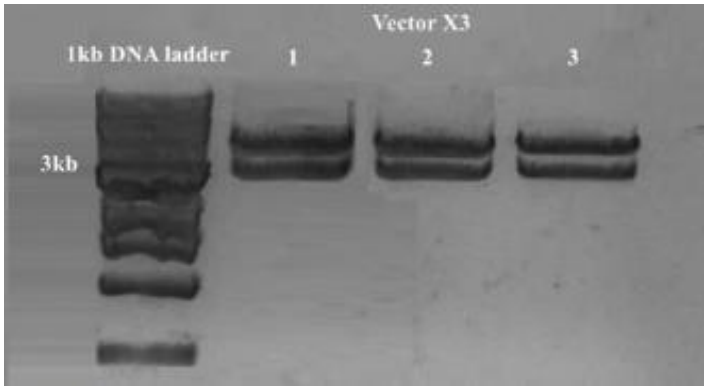

Figure 5. DNA agarose gel result for the X3 plasmid after treatment with restriction endonucleases Pac I and Pme I. (The upper band (4.6kb) is the pHLSec vector, which was used as the vector for constructing new A2 expression plasmids. The lower band (3.2 kb) is the original insert for the X3 plasmid.

Ligation was done by mixing the digested pHLsec vector and the insert (PCR products containing the A2 gene) with the ligation buffer and enzyme at 16 degrees overnight. This was one of the key steps throughout the whole process, as the ligation buffer contains ATP (Adenosine Triphosphate), which is temperature sensitive. After ligation, the ligated product was transformed into the NEB® 5-alpha Competent *E. coli* (High Efficiency) cells. According to the heat shock protocol provided at the NEB's website (https://www.neb.com/products/c2992-neb-5-alpha-fiq-competent-e-coli-high-efficiency), 1 ng of the ligated product was transformed to 50 µl of cells, for 42 seconds of heat shock time. The transformed *E. coli* was plated on LB-agar plates supplemented with 100 µg/ml ampicillin. After incubating at 37 degrees overnight, the clones were collected from the plates and cultured in 5 ml LB liquid medium (with100 µg/ml ampicillin) in the 37-degree shakers (220 RPM) overnight. Following incubation, a miniprep kit (QIAquick miniprep kit) was used to extract the plasmids. The concentration of the plasmids was measured with the OD 260/280 ratio using a Nanodrop 2000. The typical yield of our experiments was 50 µl of plasmid with a concentration of 300-400ng/µl. The OD 260/280 ratio was around 1.9, thus proving the

high purity of DNA solution. [8] The plasmid samples were also sent to the Genescript company for DNA sequencing. The sequencing results proved that plasmids with WT A2 or mutants were successfully made.

2.3 Protein expression

The next step in the protein expression was to transfect the plasmid to the mammalian cell line HEK 293T. The cells were cultured in a Dulbecco's Modified Eagle's medium (Gibco 1X DMEM) supplemented with 10% FBS (fetal bovine serum), 2% L-glutamine, and 1% penicillin/streptomycin. Typically, 24 hours prior to transfection, the HEK293T cells were divided into three T75 flasks. I chose the Orgigene Megtran 1.0 as the transfection reagent with the ratio 3µl: 1µg DNA. After transfection, the cells were cultured in a serum DMEM medium for 4-6 hours and subsequently changed to a serum-free medium (EX-CELL serum-free medium, Sigma) for 6 days. The culture media were harvested and centrifuged to remove the precipitation. The culture supernatant was stored at 4 degrees until the next protein purification steps.

2.4 Protein purification

The protein purification steps contain two main methods based on chromatography. The first is the purification of proteins from the harvest cell culture supernatant through Ni-NTA chromatography. This is followed by the second purification: size-exclusion chromatography. Purified proteins were stored in the PBS solution (155 mM NaCl, 3 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4).

Figure 6 shows the SDS-PAGE analysis of the samples purified from Ni-NTA chromatography. As the Ni-NTA column did not provide a satisfactory purification result, a further purification process by gel filtration chromatography was necessary. The principle of this method is based on the diffusion speeds of proteins with different

sizes (shown in Figure 7). When the smaller proteins diffuse further into the pores of the beads in the gel filtration column, due to their size it is easier for them to move through the beads more slowly. Larger proteins are retained less and thus eluted from the beds more quickly. [9]

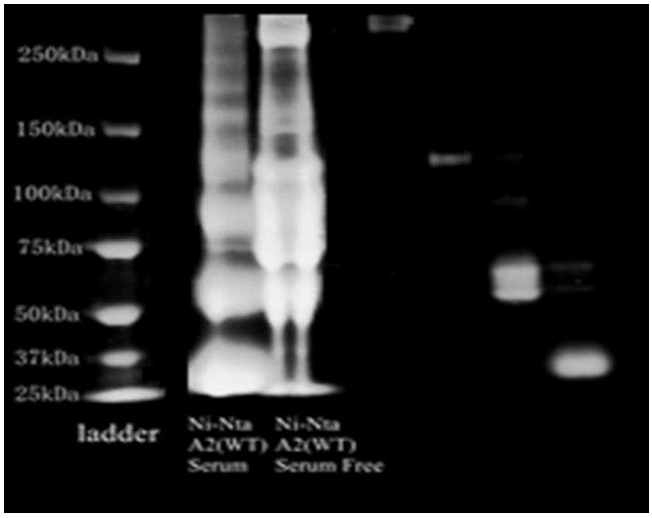

Figure 6. SDS-PAGE gel result for the protein samples purified from Ni-NTA affinity chromatography. Samples were run in a 15-well Genscript ExpressPlus™ precast gradient (4-20%) PAGE Gel, and stained with the Sigma-Aldrich EZ blue staining reagent. The protein ladder is the BIO-RAD Precision Plus Protein™ All Blue Prestained Protein Standards #1610373.

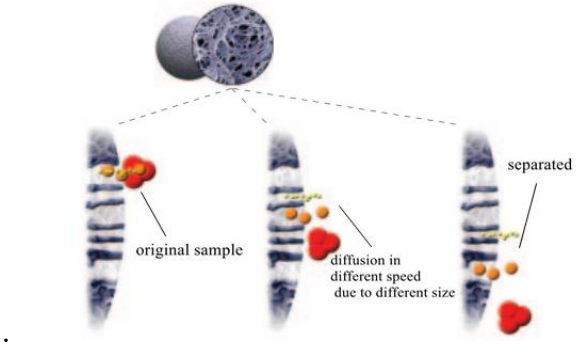

Figure 7. The principle of size exclusion chromatography (gel filtration).

The advantage of this method is that the separation accuracy is enhanced by the simultaneous UV detection of the protein concentration for each eluted fraction, as

shown in Figure 8.

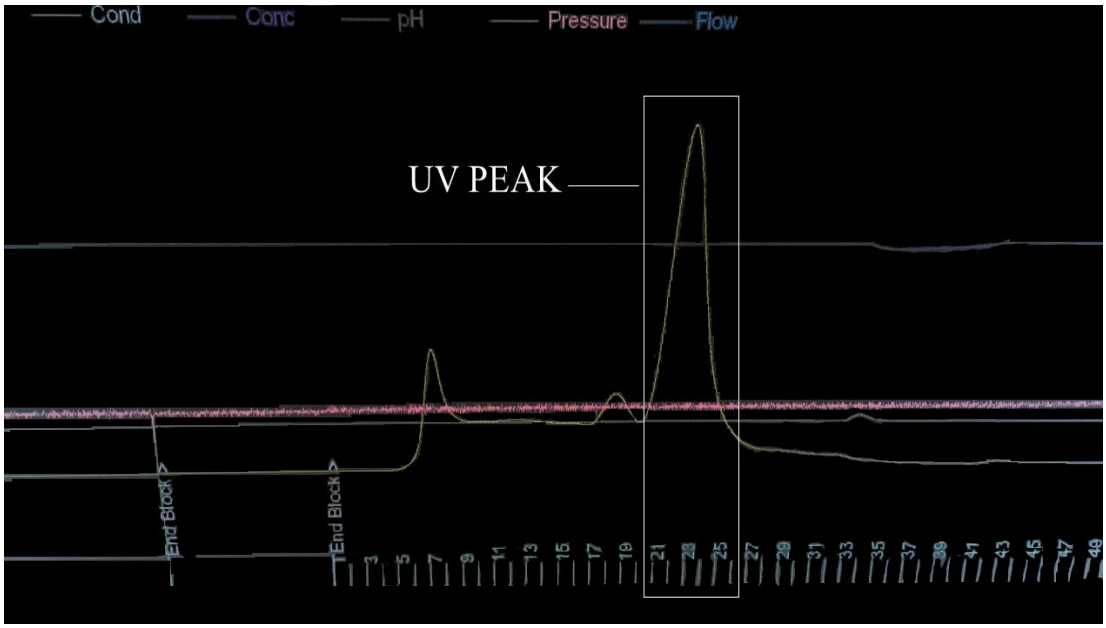

Figure 8. Typical gel-filtration result (showing the WT A2 protein). The numbers along the bottom demonstrate the order of fragment collection (0.5 ml each tube). The UV value peak highlighted in the rectangle shows the A2 domain sample. The sample buffer was a degassed Tris buffer containing 10mM Tris (hydroxymethylaminomethane, $((HOCH_2)_3CNH)$, 150mM NaCl, PH 7.5).

Figure 9 shows the SDS-PAGE of the protein samples after purification by gel-filtration chromatography. The gel clearly demonstrated that the size of the 21-26 fraction is around 30 kDa, with no other impurities.

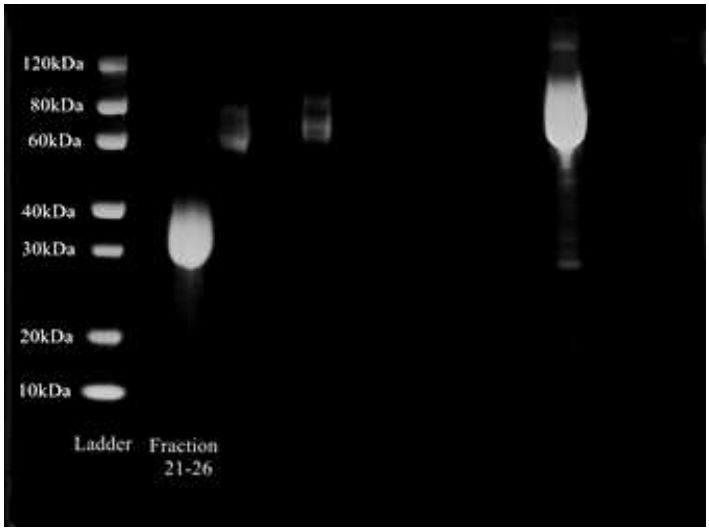

Figure 9. SDS-PAGE gel for the protein samples after further purification by gel-filtration chromatography. Samples were run in a 15-well Genscript ExpressPlus™ precast gradient (4-20%) PAGE Gel, and stained with the Sigma-Aldrich EZ blue staining reagent.

To further validate the result, I performed a Western blot test. The two bands in Figure 10 demonstrate that the protein purified from the gel-filtration column was successful. The samples could be used for a further cleavage assay and single molecular experiment.

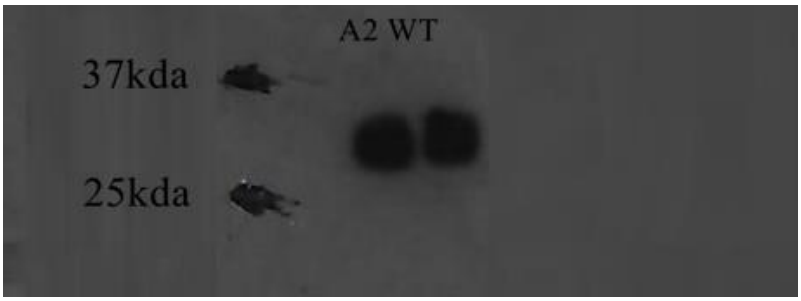

Figure 10. Western blot result for the WT A2 protein. The samples were run in a 15-well Genscript ExpressPlus™ PAGE Gel (4-20%) and transferred to a PVDF membrane with a Tris-Glycine Transfer Buffer for 1.5 hours under at 100 volts. The membrane was washed and blocked using PBST (1x PBS with 0.5% Tween20) with 5% non-fat milk for 1 hour. After washing 4 times with PBST, the membrane was incubated with 10 µl of anti-hVWF A2 primary antibody in 10 ml PBST milk at 4 degrees overnight, and subsequently incubated with a Peroxidase conjugated affinity purified goat anti-mouse lgG as a secondary antibody (10µl in 10 ml PBST milk) for 1 hour. Finally, the membrane was treated with a Thermo SuperSignal West Femto Maximum Sensitivity substrate and developed with an X-ray film in a darkroom.

2.5 Sample preparation

2.5.1 Couple protein with DNA handle

In order to conduct a single molecular experiment with an optical tweezer, the protein needs to couple with a DNA handle. The two predesigned 802-bp DNA handles were generated using a PCR method with a pGEMEX plasmid DNA template and New England Biolabs' DeepVent DNA polymerase with 20 mM DTT. The primers used were 5'thiolmodifier C6-SS- CGA-CGA-TAA-ACG-TAA-GGA-CAT-C and either 5'biotin- or 5'digoxigenin-CAA-AAA-ACC-CCT-CAA-GAC-CC primers (1 μM). After amplification by PCR, I followed the Qiagen HiSpeed Plasmid Maxi Kit's procedures to purify the handle.

The DNA handles were activated by 2,2′-Dithiodipyridine according to reference [1], and coupled with A2 proteins via a disulfide bond. To validate the coupling result, the coupled samples were analyzed in a Lonza PAGEr Gold Precast 4-20% TBE gel after being stained with an Invitrogen SYBR-green DNA gel stain. The result is shown in Figure 11. According to the ladder information and lanes 3 and 4, we were convinced that the coupling between the DNA handle and the A2 protein was successful.

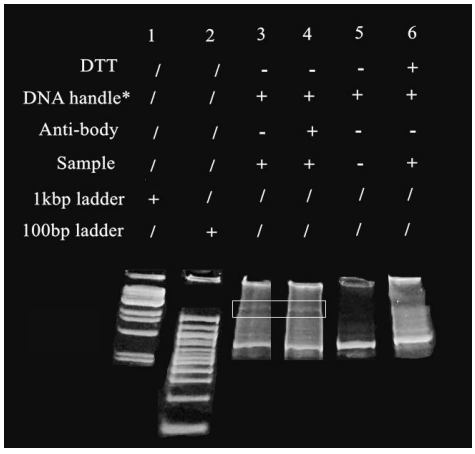

Figure 11. Tris-Borate-EDTA gel showing the DNA handle coupling result. Lane 1 is

the NEB 1kbp DNA ladder. Lane 2 is the NEC 100bp DNA ladder. Lane 3 is the WT A2 coupled with the handle. Lane 4 has an A2 antibody to increase the signal. Lane 5 is the negative control, containing only the DNA handle. Lane 6 contains DTT to reduce the protein sample. Boxed region indicates the A2-DNA coupled construct.

2.5.2 Biotin ligase biotinylation

According to the single molecular experiment design, the A2 domain must be labeled with biotin, so that it can be attached to the streptavidin beads. The bond between biotin and streptavidin (avidin) is the strongest noncovalent biological bond and has many applications in biology. [11] In order to biotinylate the A2 protein, I chose the AVIDITY BirA-500 BirA biotin-protein ligase reaction kit and performed the biotinylation with a 40 μM sample concentration with a 1-hour incubation of the sample and BirA ligase at 30 degrees.

After the biotinylation, a biotin-blot was performed to test the biotinylation result. This method is very close to the western blot. The main difference here was that the biotin acted as a primary antibody. Since the biotin had already been added to bind with the protein, it only needed to incubate the membrane with the Avidin-HRP Conjugate as the second antibody prior to treatment with the chemiluminescent substrate.

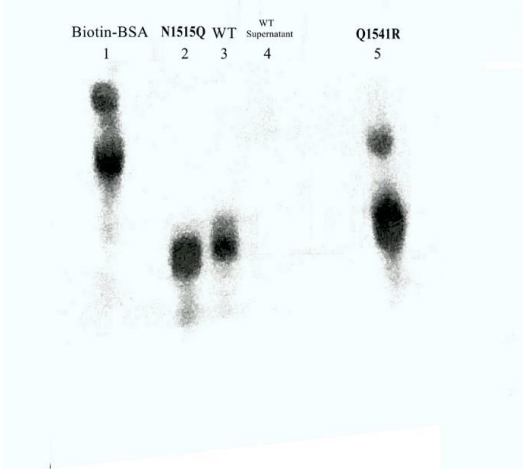

Figure 12. Biotinylation detection using a biotin-blot. Lane 1 is the Biotin-BSA as a

positive control. Lane 2 is the A2 mutation N1515Q after biotinylation. Lane 3 is the WT A2 after biotinylation. Lane 4 is the supernatant of the WT A2 cell culture supernatant. Lane 5 is the A2 mutation Q1541R after biotinylation.

Figure 12 shows the experiment's results [3]. The A2 domain is around 30 kDa, and the WT A2 is a little heavier than the N1515Q mutation, which proves a successful glycosylation at N1515. The final step of the protein biotinylation is passing the sample solution through a Thermo Zeba spin desalting column (0.5ml) twice to remove the ATP/ADP and biotin remains.

3. INITIAL CHARACTERIZATION OF A2 AND THE MUTANTS

3.1 Instrument description

A dual-beam optical trap was used to characterize the mechanical unfolding of WT A2 and mutants. A schematic of the machine is shown in Figure 13.

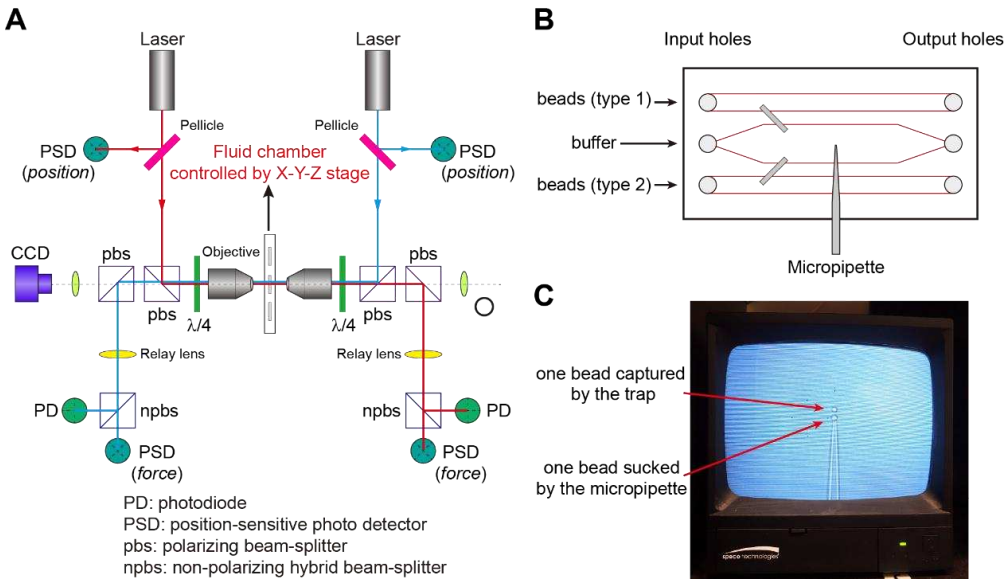

Figure 13. Schematic of the dual-beam optical tweezer system. [11]

Optical tweezers have the ability to control dielectric particles from nano- to micrometer size by using extremely small forces with a highly focused laser beam. [12] The basic principle of this system is that two lasers will symmetrically trap the beams at the focus point. Due to the conservation of momentum, the laser generates force, in both lateral and axial directions, through the refraction of the light, which causes photon momentum changes and finally generates the optical force. The equipment I used also has the ability to measure location and force by using a position sensitive photodetector (PSD) (Figure 13). Figure 13B shows the chamber structure for this equipment. The main part of the chamber contains three channels. The beads were injected from the input whole into the upper and lower channels using a 1 ml syringe. Usually it is first injected into lower channel, which is shown on a CCD camera

(Figure 13C). The beads first captured by the dual-trap are brought to the middle of the middle channel and are sucked up using a micropipette. The other beads are trapped in the upper channel in a similar way and move closer to the micropipette's position. Finally, these pairs of beads are allowed to move in retract-approach cycles to detect the A2 domain unfolding.

3.2 Single molecule experiments

All the experiments were based on a dual-beam optical tweezer system, as shown in Figure 13. The experiment's design is shown in Figure 14.

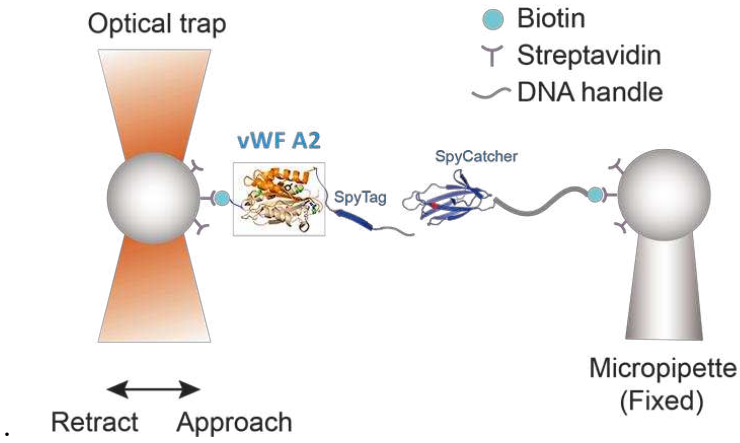

Figure 14. Principle of the single molecular experiment on the A2 domain.

The buffer used in the experiment was a TBS buffer (10 mM Tris, 150 mM NaCl, pH 7.5). All experiments were done at room temperature. The protein contains biotin in the N-terminal of the protein, which gives it the ability to bind the streptavidin (SA)-coated polystyrene beads (the right SA bead in Figure 14). The other beads were coupled with the DNA handle and spy catcher, which has the ability to catch the spy tag in the A2 protein. Prior to the pulling assay, the SA bead shown on the right (i.e., the fixed bead) was coupled with DNA catcher and protein. The SA bead held by the optical trap (i.e., the trapped bead) was coupled with the A2 protein. The dual-beam optical tweezer system was used to take the trapped bead into feedback-controlled

contact with the fixed bead. To further detect the unfolding force, different constant speeds were used (50, 100, 200, 400, 500 units: nm/s). With the help of the PSD, the force and extension curve were recorded. Figure 15 shows one example.

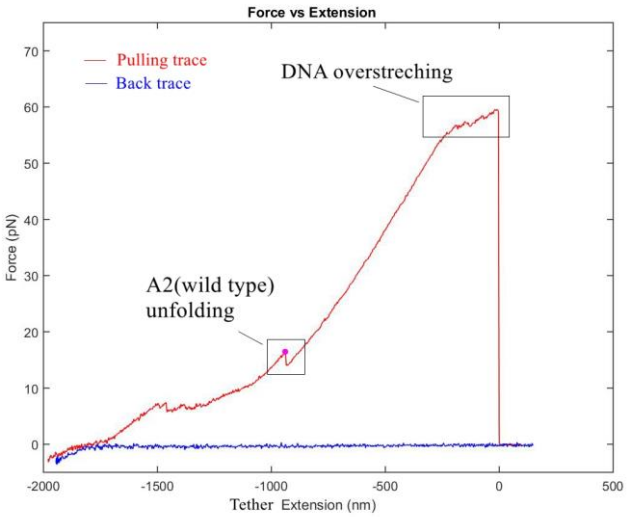

Figure 15. Force vs tether extension curve showing pulling and unfolding the WT A2. DNA overstretching is an important internal control to signal pulling a single molecule. The 65-pN overstretching force was also used to calibrate the force of the optical trap. [13]

3.3 Data analysis

3.3.1 Worm-like chain fit

The worm-like chain (WLC) model (or the so-called Kratky-Porod model) was designed to demonstrate the behavior of continuously flexible polymers. [14] This model assumes that all the polymers were considered to be an idealized, macroscopic, and circularly-symmetric beam element with flexural rigidity. The successive monomers in this model were forced to point in nearly the same direction, and the isotropic polymer chain was continuously flexible. Due to this feature, it has a wide range of applications to describe biological molecules, especially the single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), and polypeptides. Mathematically, WLC can be described as:

$$\frac{F(x)\cdot L_p}{k_B T} = \frac{1}{4}\left(1-\frac{x}{L_c}\right)^{-2} - \frac{1}{4} + \frac{x}{L_c}$$

In this equation, F is the force applied on the polymer, x is the end-to-end distance, k_B is the Boltzmann constant, L_c is the contour length of the polymer (the maximum length of the polymer when fully extended), L_p is the persistence length of the polymer, and T is the absolute temperature. All these parameter designs are based on an inextensible polymer and ignore a possible twisting effect. The persistence length L_p, which quantified the rigidity of the material, is recognized as the length over which correlations in the direction of the tangent are lost.

3.3.2 Force vs. extension

Using the optical tweezer system mentioned previously, I detected the unfolding force and divided the data according to different loading rates and different force strengths. Most probable unfolding forces are plotted versus averaged unfolding extension (Figure 16). WLC fit was performed to both WT A2 (Figure 16A) and Q1541R mutant (Figure 16B). The contour length for unfolding A2 is about 65 nm and consistent with the reported value [1]. Compared with the WT, the Q1541R mutant exhibit a shorter contour length and persistence length. However, more data is required to improve the accuracy of the fit.

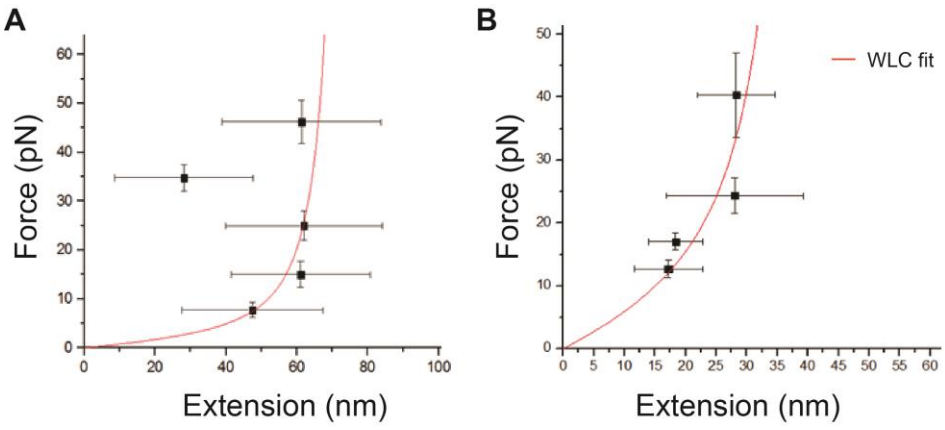

Figure 16. Worm-like chain fit for unfolding data of (A) WT A2 and (B) Q1541R mutant.

3.4 ADAMTS13 cleavage assay

As mentioned previously, ADAMTS13 cleavage is one of the most significant features of the VWF. According to the reference [1], the cleavage needs scissile bonds in the A2 domain, which are exposed upon forced unfolding. An enzymatic cleavage assay was performed to understand the effects of A2 mutations (Q1541R, N1515Q, and N1574Q) on the ADAMTS13 cleavage.

The ADAMTS13 was bought from R&D Systems (Recombinant Human ADAMTS13 Protein, CF 4245-AD). An assay buffer (50 mM Tris, 2 mM CaCl2, 0.01% (w/v) Brij-35, pH 8.5) was used to dilute the A2 protein (WT or mutants) to around 100 µg/mL. Meanwhile, the same buffer was used to dilute rhADAMTS13 to 10 µg/ml. The enzyme and the sample were mixed and incubated at 37 degrees for 2 hours. Control groups contain the protein but no enzyme. The reaction was stopped by adding 5 µl GenScript 5x sample buffer (MB01015); the sizes of the proteins were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining.

3.5 Silver staining

Silver staining is a conventional protein staining method invented by the Camillo Golgi. [15] It can detect very small amount of protein samples (ng range) on the protein gels. I used a Thermo Pierce Silver Stain kit to test the cleavage result (shown in Figure 18). I separated the Q1541R and the N1515Q/N1574Q samples on two sides of the gel. When cleavage occurs, the 30-kD A2 will be cleaved into two fragments, one about 20 kD and the other 10 kD.

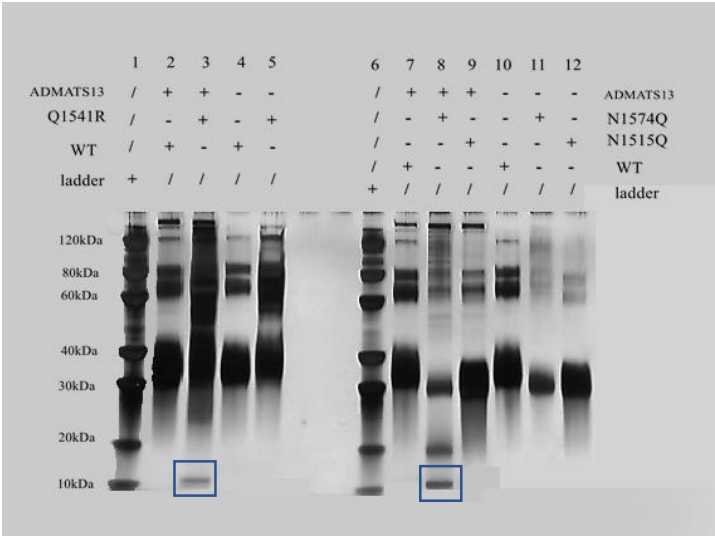

Figure 17. Silver staining result for the ADAMTS13 cleavage assay. Boxed regions indicate cleavage products.

As predicted, the WT A2 did not show a cleavage signal, consistent with the previous reported data that mechanical force or chemical denaturants are required to achieve efficient cleavage. N1515Q mutant did not show cleavage as well, suggesting that glycans on N1515 may not contribute to the structural stability of A2. Interestingly, both mutants Q1541R and N1574Q showed clear significant 10-kD digested fragments. The digested fractions were approximately 10% and 50% for Q1541R and N1574Q, respectively. The results indicate that both Q1541R mutation and removal of glycans on N1574 alter the structure or weaken the mechanical strength of A2. The easier cleavage in Q1541R can explain why this mutant causes a type 2M bleeding VWD phenotype.

4. CONCLUSION

In summary, as shown in Figures 9 and 10, I have successfully expressed and purified VWF A2 WT protein and mutants (Q1541R, N1515Q, and N1574Q). The ADAMTS13 cleavage assay indicates that Q1541R and N1574Q are more susceptible to ADAMTS13 cleavage than WT. Excessive cleavage of the VWF shifts the circulating multimeric VWF towards smaller multimers. This shift causes less hemostatic potential, which can lead to excessive bleeding disorders. The initial characterization of A2 and the mutation based on optical tweezer experiment suggested type-2M VWD mutant Q1541R may fold differently than the WT, causing an easier exposure of the scissile bond for cleavage. In addition, out data suggests the N-linked glycosylation on N1574 may play an important role in regulating the mechanical stability of A2.

5. DISCUSSION AND FUTURE REMARK

According to the results, the first goal of this project, expressing and purification of A2 and mutants, has been achieved. The key to success in this part was to avoid contamination throughout the experiments. Another key step is the ligation process, as the solution contains temperature sensitive ATP. I performed the ligation in 16 °C to minimize ATP hydrolysis.

In the single molecule experiment for WT A2, the unfolding data points, especially at 30-40 pN, did not fit the WLC model well. The main reason is that the data set was not large enough, especially compared with the Q1541R, which may have led to statistical errors. Moreover, the WLC model has its own limitations. It assumes the polymers are idealized, macroscopic, and have a circular-symmetrical beam element with flexural rigidity. The A2 domain may not be such a perfect polymer. The difference between the idealized polymers with the real A2 domain may cause the discrepancy.

In future experiments, more single molecular unfolding experiments will be collected on the WT A2. Also, I will perform unfolding experiments on the N1574Q and N1515Q mutants. In addition, according to the literature [6], Ca^{2+} may stabilize the A2 structure. Thus, detecting the unfolding of WT A2 and mutants with calcium would be an interesting extension. Finally, repeating the WLC fit for all the A2 samples using a larger data set would definitely make the conclusion more reliable.

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