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Lipid Binding Studies of Blood Coagulation Factor VIII C1 and C2 Domains

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Introduction

Factor VIII (fVIII) is an essential protein cofactor in the mammalian blood coagulation cascade. When fVIII is missing or defective, it causes Hemophilia A which is a X-linked bleeding disorder.[1] Hemophilia A affects 1 in 5,000 males worldwide and results in bleeding in joints, muscles, and soft tissues.[1] For treatment, 75% of patients receive infusions of concentrated or recombinant fVIII. Recombinant fVIII is produced in eukaryotic cell lines and is often genetically engineered to improve circulation half-life, secretion levels, stability, and decrease immunogenicity. However, approximately 30% of hemophilia A patients develop anti-fVIII pathogenic antibodies (inhibitors) that reduce treatment efficacy.[1] Not only that, but hemophilia A treatment is very expensive at a cost of over $50,000 a year.[3]

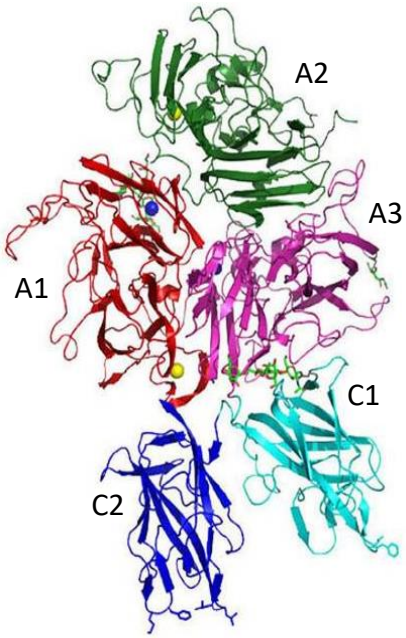


Figure 1. fVIII Crystal Structure.[2]

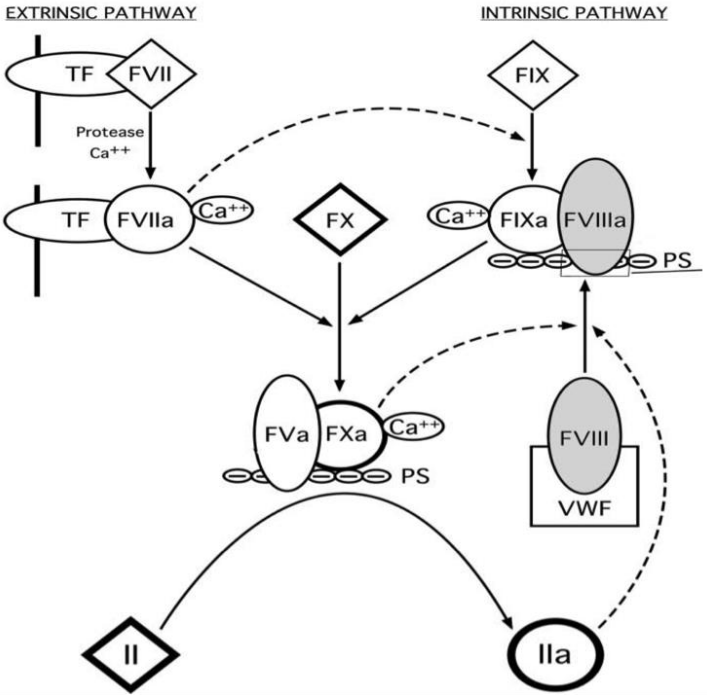


Figure 2. Blood coagulation cascade. Inactive fVIII circulates in complex with vWf. Following activation by fXa or thrombin, fVIII dissociates from vWf, associates with fIXa, and binds active platelet surfaces. This membrane complex then activates fX, which then activates thrombin. [2]

In plasma, fVIII circulates tightly bound to von Willebrand factor (vWf), which protects fVIII from rapid degradation. In order to function as a cofactor, fVIII must bind to the anionic

phosphatidylserine lipid head groups on the surface of platelets localized to the site of injury.[1] In order to do so, it must be proteolytically activated (cleaved) by thrombin or factor Xa and the now activated fVIII (fVIIIa) dissociates from vWf as a heterotrimer consisting of five domains (A1/A2/A3-C1-C2).[1] Two of these domains, C1 and C2, are known to be involved in lipid binding.[1] Along with binding the lipids, fVIIIa participates as a cofactor to factor IXa in the factor X activating (tenase) complex.[1]

The working model of how C1 and C2 bind to the platelet lipids is lacking. This work focuses on using mutational studies to identify the necessity of different amino acids in C1 and C2 in binding platelet membranes. Understanding the role of specific residues may further the model of fVIII lipid binding and provide the bases for the development of more effective therapeutics.

There are five specific mutations being studied currently on four different residues. On the C2 domain, there are R2320T, R2320S, and R2215A. On the C1 domain, there are R2163H and R2159H.

Methods

Over the course of two years, the methods used for each step have been altered as the most efficient protocols were determined. The methods explained here are what were used in the most recent round of each step, although they may not have been what was used every time.

Site-Directed Mutagenesis. An Agilent QuikChange Lightening Site-Directed Mutagenesis kit was used to introduce mutations into the WT hC1 (human C1) and WT hC2 (human C2) proteins. Primers were designed on Agilent online and ordered through Integrated DNA Technologies (IDT). Polymerase chain reaction (PCR) was performed and Dpn-1

restriction enzyme was added to digest the parental supercoiled DNA. The DNA was then transformed into XL10-Gold ultracompetent cells. After a heat shock and ice bath, the transformed cells were added to Luria-Burtani (LB) broth and incubated at 37°C. The cells were then plated on LB/ampicillin (50 μg/mL) (Amp-50) agar plates and grown in the 37°C incubator. After 16 hours, the colonies were counted and three were scraped into 10 mL of LB each and grown overnight at 37°C with shaking.

Plasmid Preparation. Glycerol cell stocks were made from the XL10-Gold overnight cell growths, and the rest of the cell growths were used to extract the plasmids from cells for sequencing. A QIAprep spin miniprep kit was used with the appended protocol for low copy number plasmids. The XL10-Gold cell growths went through the QIAprep spin miniprep buffer system and the DNA was eluted from the resulting supernatant. The DNA was quantified on a Nandodrop and sent to Nevada Genomics Center for sequencing to confirm that the correct mutant was made. DNA samples were stored at -20°C.

Chemical Transformation. Competent *E. coli* SHuffle B cells were transformed with five mutations in pET32a: hC2 R2320T, hC2 R2320S, hC2 R2215A, hC1 R2163H, hC1 R2159H. Previous rounds of transformation included transformation into SHuffle K12 cells and BL21(DE3) cells. The DNA for each mutant protein was added to SHuffle B cells and incubated. Following heat shock and an ice bath, LB broth was added. After a short period of growth, the cells were plated onto LB/Amp-50 agar plates and incubated overnight at 37°C. After about 15 hours of incubation, the colonies were counted and added to 10 mL LB/Amp-50 broth to grow at 30°C overnight with shaking. Glycerol cell stocks were made from each culture, and the rest of the 10 mL growth was used for further protein growth and overexpression. The cell stocks were frozen in liquid nitrogen and stored at -80°C.

Protein Overexpression. If a transformation was not done immediately previously, 10 mL overnight growths were done by adding 10 μL Amp-50 followed by 2 μL of the protein cell stock to 10 mL LB and growing it overnight at 30°C with shaking. Once the 10 mL overnight growths have been shaking for ~18 hours (min: 16 hours, max: 20 hours), they are added to 1 L of sterile LB/Amp-50 broth. Growths were typically 6 L total. The 6 x 1 L cultures were incubated at 30°C with shaking until an optical density, measured at 600nm (OD_{600}), of 0.5-0.6 (min: 0.4, max: 0.8) was reached. Once reached, the cultures were induced with IPTG for a final concentration of 0.4 mM isopropyl β-D-1-thiogalactopryanoside (IPTG). The 1 L cultures were then grown for ~18 hours at 16°C with shaking. The cells were harvested through centrifugation at 6000 rpm on a F12 rotor for 10 minutes at 4°C. The cell pellet was used for purification immediately or stored at -20°C.

Protein Purification and Dialysis. The cell pellets were defrosted if necessary and resuspended in 35 mL of Load/Lysis Buffer (300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM imidazole (pH 8.0), 10% (v/v) glycerol, 0.5% (v/v) Triton) on ice with each 2 L cell pellet being kept separate. The following steps were repeated for each resuspended 2 L cell pellet. Lysozyme and phenylmethane sulfonyl fluoride (PMSF) were added to the cell suspensions and they were incubated horizontally in ice with shaking for 30 minutes. The cell suspensions were sonicated for 3x30 seconds with 1 minute intervals. A 100 μL aliquot of the whole cell extract (crude cell lysate) was saved in a microcentrifuge tube. The crude cell lysates were then centrifuged at 17,500 rpm on a F20 rotor for 30 minutes at 4°C. The resulting supernatant was separated, an 100 μL aliquot of it was saved in microcentrifuge tube, and the insoluble pellets were discarded. The supernatant was first syringe filtered through a 5 μm filter, followed by a 0.45 μm filter. Each 2 L of growth is still being kept separate. Gravity-flow immobilized metal affinity

chromatography (IMAC) with a 50% slurry of TALON resin was used to purify the protein. 2 mL of 50% slurry (for 1 mL of resin) was added to each of three columns and cleaned. The resin was then added to the filtered supernatant with a spin bar in an Erlenmeyer flask and spun in the cold room for 1 hour. After incubation, the resin and protein mixtures were poured back onto the columns. An 100 μL aliquot of the flow through was saved in a microcentrifuge tube and the rest of the flow through went into waste. Load/Lysis buffer was used to rinse the Erlenmeyer flasks which were then added to each column and ~10-15 column volumes (cv) were poured over through the columns as wash I. An 100 μL aliquot of wash I was saved in a microcentrifuge tube. Next, the columns were rinsed with ~20 cv of wash II buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM imidazole (pH 8.0), 10% (v/v) glycerol) and an 100 μL aliquot of wash II was saved in a microcentrifuge tube. The protein was eluted with ~10-15 cv of elution buffer (150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 150 mM imidazole (pH 8.0), 10% (v/v) glycerol) and all the elution was collected. Dialysis tubing, with a molecular weight cut off (MWCO) of 12-14 kDa, was soaked in Nanopure water for 5 minutes. Each elution collection was added to one dialysis tube and clipped off with a dialysis clip at each end. The tubes were placed in 1 L of storage (dialysis) buffer (150 mM NaCl, 25 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol) overnight at 4°C.

Protein Quantitation. The dialyzed protein was concentrated using a 10,000 MWCO Millipore spin concentrator. At this point, all of the protein from the growth can be combined into one sample. Each round of centrifugation was at 4000 rpm, 4°C, acceleration of 9, deceleration of 9, for 5-12 minutes. Centrifugation was repeated until a volume under 1.5 mL was reached. Between each round, the spin concentrator was checked for aggregation. If significant aggregation was observed, the protein was aliquoted into microcentrifuge tubes with

1 mL fractions and spun down in cold room at 13,200 rpm for 4 minutes. The supernatant was then added back to the spin concentrator, leaving the pellets of protein aggregate behind, and concentration continued. Once under 1.5 mL, or at a high concentration, the protein was added to a microcentrifuge tube and centrifuged in cold room at 13,200 rpm for 4 minutes. The resulting supernatant was added to a new microcentrifuge tube and the concentration was tested from that sample on the Nanodrop. The dialysis storage buffer was used a blank. A sample was set aside for electrophoresis and stored at -20°C and the rest of the purified protein was frozen in liquid nitrogen and stored at -80°C.

Electrophoresis. A 15% discontinuous sodium dodecyl sulfate (SDS) gel was made and set up in an electrophoresis chamber for SDS polyacrylamide gel electrophoresis (SDS-PAGE). The resolving layer of the gel (15%) was 1.5 M Tris-HCl (pH 8.8), 40% (v/v) 1:19 bis-acrylamide mix, 10% SDS, 10% APS, TEMED, and Nanopure water. The stacking layer of the gel (1.5X) was 1.0 M Tris-HCl (pH 6.8), 40% (v/v) 1:19 bis-acrylamide mix, 10% SDS, 10% APS, TEMED, and Nanopure water. Tris-glycine running buffer was added to the electrophoresis chamber. The samples (100 µL samples collected at each purification step) were prepared by adding 15 µL of each sample to 5 µL of 4X load dye, centrifuging on pulse for 10 seconds, and heating the mixture in boiling water (100°C) for 5 minutes. The protein sample was prepared in the same way with 10 µg of the protein. The protein was brought to 15 µL with Nanopure water before 5 µL of 4X load dye was added. The samples were loaded onto the gel with 10 µL of the Spectra BR protein ladder. Electricity was applied to the gel at 90V through the stacking layer and 120V through the resolving layer (or at 100V throughout the whole gel) until the load tracking dye reached the bottom of the gel. Coomassie brilliant blue stain was used

to stain the gel overnight on the shaker table. The gel was then destained for 24-48 hours and imaged.

Further Protein Purification. IMAC did not purify some of the mutant proteins enough (need 90-95% purity), and further purification methods were required. The purity of the proteins was determined with SDS-PAGE. Fast protein liquid chromatography (FPLC) with size exclusion chromatography (SEC) columns and a cation exchange Mono S column were found to be the best methods for further purification. Using a 10 mL superloop, rather than a 1-2 mL loop, was found to improve purification as well because the proteins did not have to be placed in a buffer without salt or glycerol, that could be diluted instead which helped avoid aggregation. The eluted protein was concentration and run on SDS-PAGE to compare the purity of the samples.

LCMS-ESI. Liquid chromatography mass spectrometry (LCMS) with electrospray ionization (ESI) was used to confirm the identity of the purified protein. Samples of each mutant protein were prepared by diluting in buffer (150 mM NaCl, 25 mM Tris-HCl (pH 8.0)) and placing 200 μL into autosampler vials. The resulting spectra were analyzed to determine the mass of the protein present. The identity of the protein was confirmed based on the mass.

ELISA. In order to study the lipid binding of the C1 and C2 domains of factor VIII, enzyme-linked immunosorbent assays (ELISAs) were performed. The lipids used were 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (DOPS), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). The ratio plated on the 96-well Nunc Polysorp plate was 80% DOPC, 15% DOPS, and 5% DOPE because that is the natural ratio of these lipid head groups found in the exposed platelet membrane. The negative control was 100% DOPC to account for non-specific binding. The lipids were diluted in methanol immediately before being plated. After adding the lipids, the plate was left at room

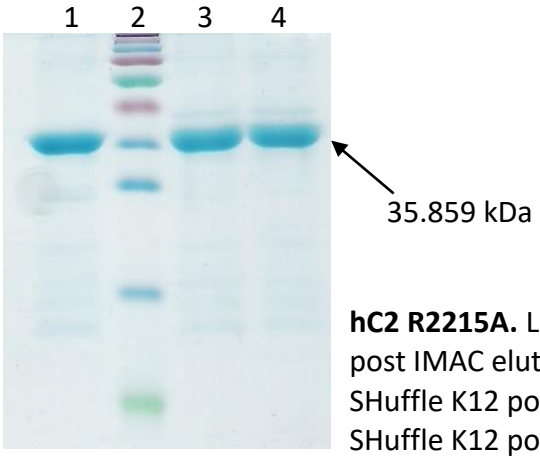
temperature to allow the methanol to evaporate overnight. After the lipids bound the well walls, the remaining areas of the walls that did not have bound lipids were blocked with 1% bovine serum albumin (BSA) in 1X Tris-buffered saline (TBS) with shaking at 37°C for 45 minutes. The protein was serially diluted in a 1:2.5 dilution ratio in 1% BSA/1X TBS. After blocking, the protein is plated and incubated at 37°C with shaking for 1.5 hours. The plate was washed 3x with 1X TBS using an ELx405 microplate washer. Ni-NTA•HRP (nickel nitrilotriacetic acid conjugated with horseradish peroxidase) diluted in 1% BSA/1X TBS was added to plate and incubated at 37°C with shaking for 30 minutes. The plate was washed 3x with 1X TBS again and the colorimetric reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), was added. The plate was incubated at 37°C with shaking until blue-green color developed and was the absorbance was read on a plate reader at 405nm at various time intervals. The resulting data was analyzed on GraphPad PRISM.

Recombinant Enterokinase Cleavage. Cleavage reactions for the proteins were set up and allowed to sit at 4°C overnight (min: 5-6 hours at room temperature). The recombinant enterokinase (rEK) cleaves the thioredoxin tag, S tag, and His tag from the fusion proteins that have been purified. Cleaving these tags is necessary for circular dichroism and protein crystallization experiments. Once cleaved, the rEK was bound to agarose and the rEk bound agarose was separated from the cleaved protein. The protein was then purified to remove the cleaved tags with TALON. The cleaved protein was then concentrated and a sample run on SDS-PAGE to confirm that cleavage was successful and analyze purity. There will a change in the molecular weight of the protein after cleavage because the tags have been removed (tags are ~14.5 kDa total).

Results

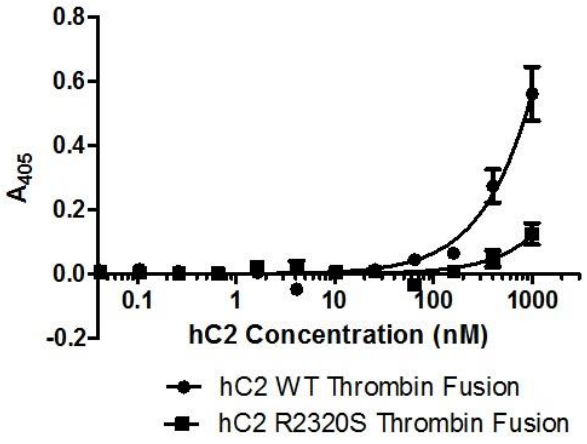
The results here are the most recent or best results related to the C1 and C2 domain lipid binding studies portion of the factor VIII project.

C2 Mutants. For C2 R2215A, a pure sample (~90-95% purity) has been achieved. The SDS-PAGE results are below.

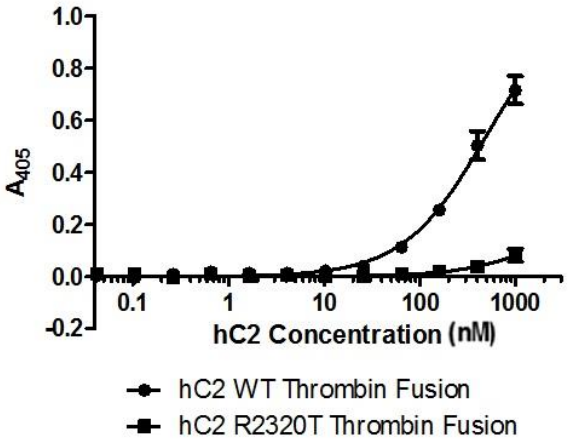


hC2 R2215A. Lanes: 1, hC2 R2215A SHuffle B post IMAC elution; 2, ladder; 3, hC2 R2215A SHuffle K12 post IMAC elution; 4, hC2 R2215A SHuffle K12 post IMAC elution

For C2 R2320S and C2 R2320T, ELISAs have been completed. Although we do not yet see saturated binding, we can see the relative binding of the WT and mutant to DOPS head groups. The ELISA results, analyzed on GraphPad PRISM, are below.

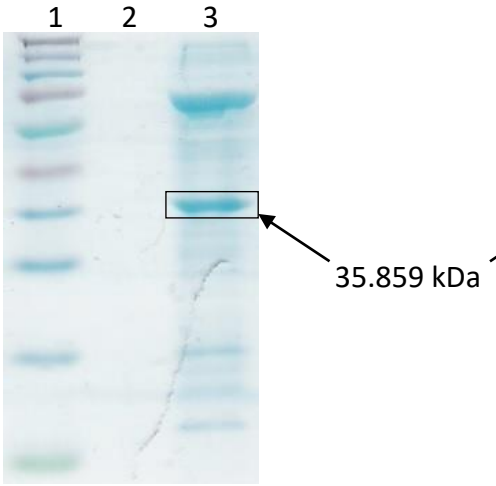


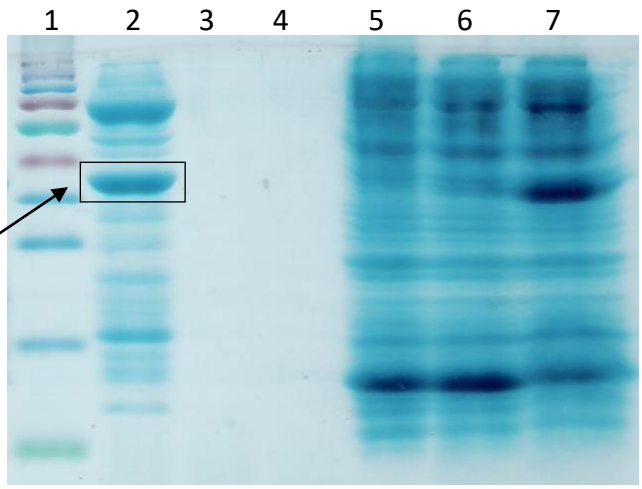
ELISA: hC2 WT v hC2 R2320S binding to PS. Reading taken after 1 hour.



ELISA: hC2 WT v hC2 R2320T binding to PS. Reading taken after 2 hours.

C1 Mutants. The C1 mutants have proven to be much harder to purify than the C2 mutants and C1 does not seem to be as stable in solution. When going through further purification steps, it is also more likely than C2 to aggregate. The SDS-PAGE results for C1 R2159H and C1 R2163H are below.





hC1 R2159H. Lanes: 1, ladder; 2, nothing; 3, hC1 R2163H post IMAC elution

hC1 R2163H. Lanes: 1, ladder; 2, hC1 R2163H post IMAC elution; 3, wash II; 4, wash I; 5, flow through; 6, high speed supernatant; 7, crude cell lysate

Discussion

When I initially started doing research, I was growing, overexpressing, and purifying WT C2 and three C2 mutants: R2320T, R2320M, R23020S. All of these mutations cause hemophilia A with differing levels of severity. We wanted to analyze if it is a difference in lipid binding that correlates to their inefficiency causing disease. We were able to purify all of these mutant proteins well, getting 95% purity, using IMAC with TALON resin. The only mutant that needed further purification on the FPLC using SEC was the R2320M mutant. Once our proteins were pure, we used LCMS-ESI to confirm their identity. Unfortunately, after getting our spectra back, we found that masses we got did not match the masses we expected. The table below explains these results.

Protein	Mass calculated from mass ladder by Vmware software (kDa)	Mass Difference (WT-mutant) (kDa)	What protein we thought it was
hC2 WT	20191	0	WT
hC2 E2322D	20176	14	R2320T
hC2 E2322D	20177	15	R2320M
hC2 V2321F	Signal to noise ratio is too high for Vmware to reconstruct	n/a	R2320S

The sequences were rechecked, and it was confirmed that the mutants were made incorrectly. We then started over from scratch, and redesigned and order primers to make the desired mutants. Site-directed mutagenesis was completed and the plasmid DNA was isolated and sent to Nevada Genomics center to confirm the correct mutants were made. The results were double checked to make sure that the primers made the correct mutants.

While that process was going on, we decided to work with another mutant that had been made previously: hC2 D2187A. This residue was interesting because it was adjacent to 2320 in the hC2 domain, and therefore we thought it may bind activated platelet surfaces. If it did bind lipids, we predicted that it bound with a higher affinity than the WT because it is changing from a negatively charged to a neutral residue and the lipids it would bind (DOPS) is negatively charged. This mutant was not naturally occurring in hemophilia A patients. I grew, overexpressed, and purified this mutant. Both the mutant and WT were pure enough to perform ELISAs to compare the binding affinity of these proteins to PS (the head group of DOPS) which simulates the binding affinity to platelet membranes. The ELISA protocol was created based off Stephen Mullen's and Michelle Weurth's notebooks as well as previous knowledge of ELISAs. The initial concentration of the ELISAs was increased from 1000 nM to 4000 nM to see if there

would be an increase in binding saturation, but I saw none. Preliminary results did show that D2187A was binding PS more than WT, which is what we expected.

After the correct R2320T, R2320S, and R2320M mutants were made and the sequences double checked, the mutants were grown and purified with IMAC. These growths resulted in low yields with most of the protein going into the insoluble fraction. Throughout last spring, we worked to improve the purification steps (including adding Triton to the Load/Lysis buffer and switching from 2.5% glycerol to 10% glycerol in the buffers). Over the summer, Serena Wo, a graduate student, was able to find a better expression plasmid and cell line that increased the yields and solubility of the different mutants. However, we could not get R2320M to express as well as the other mutants, and we dropped it from the project to focus on other mutants instead.

In the fall, I continued to work on the D2187A ELISAs as well as start R2320S and R2320T ELISAs now that we had sufficiently purified protein. For the 2320 mutants, we expected a decrease in binding compared to the WT because the residue was changing from positive to neutral and the PS they are binding is negatively charged. The results confirmed our hypothesis and can be found in the results section of this paper. After performing FPLC using SEC on IMAC purified D2187A and WT, the ELISAs from the year before were repeated and I got conflicting results. Some of the ELISAs showed WT binding slightly more than D2187A, but overall they were inconsistent and inconclusive. After discussion with Dr. Spiegel and Serena Wo, we decided to drop D2187A from the project as well and choose some new mutants to focus on. After looking at the structure of C2 in more detail, we also concluded that it was possible that 2187 was not as involved in membrane binding as we originally thought.

Now with only two mutants being looked at, R2320T and R2320S, we decided to design and order primers to make three new mutants, one on the C2 domain, R2215A, and two on the

C1 domain, R2163H and R2159H. The C2 domain mutant R2215A we chose because it is on one of the loops that interact with the lipid membranes, but it is unclear how involved it is in binding the DOPS head groups. We predicted that the mutant would have an increase in binding affinity because the location of the residue means it could be interacting with the tails of the lipids, rather than the head groups. Therefore, going from a positive charge to a neutral charge when interacting with something positive would be favorable. The C1 domain mutants were chosen because the residue 2163 is analogous to 2320 on the C2 domain and 2159 is another similar residue and both mutations chosen cause hemophilia A. We hypothesized that both mutants would decrease the binding affinity because they are going from being positively charged to neutral while interacting with something negative, and they cause hemophilia A indicating that there is an issue with the normal function of fVIII. The site-directed mutagenesis and plasmid preparation for these new mutants was performed by Serena Wo over winter break.

After winter break, I did a growth, overexpression, and purification of C2 R2320S and then moved on to focus on growing, overexpressing, and purifying the C1 R2163H mutant. It was during the process of purifying C1 R2163H that I started doing 3 2L purifications side-by-side in order to improve purity. C1 mutants are not as pure post-IMAC as C2 mutants are and need further purification before being used in any further experiments. SEC, Q columns, and S columns on FPLC were tried. The S column with a superloop was the most promising with the C2 mutants and it was done of the C1 R2163H mutant, but it was not as effective as for the C2 mutants.

At the same time, I continued trying to improve the ELISA protocol to get a better binding curve. Working with the C2 R2320S, I went through a process of troubleshooting the ELISAs and remade everything and ordered new lipids. Serena Wo got a pure sample of C2

R2215A and I was able to perform an ELISA on that mutant as well. It was the opposite results as expected. There was a decrease in binding, indicating that 2215 is likely interacting with the DOPS head groups, not the tails, because it is changing from a positive to a neutral charge and the positively charged arginine has a more favorable interaction with the negatively-charged PS than the neutral alanine. However, in the process of troubleshooting, I found that the R2215A ELISA performed actually had 5% DOPS present, not 15% (done with 80% DOPC, 5% DOPS, and 15% DOPE), which is why the results are not present in the results section of this paper.

During this last quarter of research, I have continued trouble shooting the ELISA protocol with the C2 R2320T and R2320S mutants. We now have a functioning plate washer which helps with the inconsistencies of the washing step when using a squirt bottle. Additionally, we have switched to using Polysorp (completely hydrophobic) Nunc plates rather than Maxisorp (partially hydrophobic) Nunc plates, which will hopefully improve the binding curves. More ELISAs were repeated, and thanks to some non-lipid binding ELISAs done by Mikko Sayre, it was determined that the lipids were what was causing the ELISAs to not work (rather than the 1% BSA, TBS, Ni-NTA•HRP, or ABTS as he used all of those things and his ELISA worked well). New lipids have been ordered, and I am hopeful that ELISAs will be able to be done with better results as soon as they arrive.

This quarter, I also learned how to perform rEK cleavages and did them for one of my C1 R2163H growths and one of Lilly Konek's C2 R2320T growths. It can be beneficial to cleave the proteins before further purification on the FPLC (Serena Wo tested this for the different mutants), and it is necessary to cleave the tags before circular dichroism (CD), fluorescence, and crystallization studies, which Serena Wo was been performing this quarter. I know that the

proteins I'm studying are folding correctly thanks for Serena's work with CD, fluorescence, and pull down assays.

Future Research for lipid binding studies include improving upon the C2 R2320S, C2 R2320T and C2 R2215A ELISAs. Once the purity of C1 R2163H and C1 R2159H are improved, ELISAs can be performed on them as well. Lastly, the ELISA results can be confirmed through liposome sedimentation assays which I have written up a protocol for.

Reflection

The last two years of research have been one of the best learning experiences in my undergraduate career. I have learned a lot in terms of lab techniques, but I have also learned a lot about myself, my work ethic, and how I deal with frustration and mistakes in the process. There have been a few hurdles, some my own fault and some completely out of my control, but I have learned how to deal with the mistakes and more forward in research which is incredibly important to be able to do in this field. My experience with undergraduate research in general have helped shape my career plans and given me an opportunity to understand the material I learn in classes at a deeper level than I otherwise would be able to. Not only have a learned more than I can articulate from Dr. Spiegel, but my peers and lab mates have taught me more than I ever expected.

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