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

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Introduction

The blood coagulation protein, factor VIII (FVIII), is a necessary cofactor for factor IXa in the mammalian blood coagulation cascade. To function as a cofactor, FVIII must bind to the anionic phosphatidyserine head groups on the surface of platelets localized to the site of injury. Two domains, C1 and C2, are known to be involved in lipid binding, however the working model for platelet binding needs to be bolstered by mutational studies to identify the necessary amino acid contacts. This work uses site directed mutagenesis, metal affinity column chromatography, and enzyme-linked immunosorbent assays to directly compare the lipid binding affinities of single residue mutants of isolated FVIII C1 domain and C2 domain relative to wild type C1 domain and C2 domain. Understanding the role of both residues may further the model of FVIII lipid binding and provide the basis for development of more effective therapeutics.

Background

Blood Coagulation Factor VIII

In plasma, FVIII circulates complexed to von Willebrand factor (vWF), which protects it from rapid degradation. On cleavage by thrombin, activated FVIII dissociates from vWF, as a heterotrimer (A1/A2/A3-C1-C2).

Heterotrimetric FVIII binds to negatively charged phospholipids, and participates as a cofactor to factor IXa for the tenase complex.

Blood Clotting Cascade

Figure 1. Blood clotting cascade. Inactive FVIII circulates in complex with vWF. Following activation by factor IXa or thrombin, FVIII dissociates from vWF and binds active platelet surfaces. Here it serves as a cofactor to serine protease FIXa in the activation of FX.

Hemophilia A

- Hemophilia A is an X-linked bleeding disorder caused by inactive coagulation protein, FVIII. This deficiency affects 1 in 5,000 males worldwide and results in bleeding in joints, muscles, and soft tissues.
- 75% of patients receive infusions of concentrated or recombinant FVIII product. Recombinant FVIII is produced in eukaryotic cell lines and is often genetically engineered to improve stability, secretion levels, circulation half life, and decrease immunogenicity.
- Approximately 20% of hemophilia A patient develop anti-FVIII pathogenic antibodies, known as inhibitors, that reduce treatment efficacy.

C1 & C2 Domain Mutations

Project Goal:

To assess the importance of the four highlighted residues for C1 and C2 domain stability and membrane binding capability.

These residues were chosen because of their possible interactions with the phosphatidyserine head group of activated platelet membranes.

Methods

Site Directed Mutagenesis

- Dimensional change, monomer (globular protein)
- DNA (40,000 monomer units, single stranded DNA)
- Synthesize DNA strand
  - PCR
  - Second strand by enzymatic synthesis
  - Melt DNA
  - Cool to 60°C
  - Transform E. coli
  - Digest methylated DNA
  - Isolate DNA
  - Transform plasmid to new host (SHuffle K12)

- Transform SHuffle B or C12
- Amplify mutant plasmid to large scale
- Verify mutants by sequencing

- Clone mutants to plasmids containing ISilon vector
- Mutagenesis and confirmed with Sanger sequencing

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Figure 2. Blood clotting cascade. Inactive FVIII circulates in complex with vWF. Following activation by Factor IXa or thrombin, FVIII dissociates from vWF and binds active platelet surfaces. Here it serves as a cofactor to serine protease FIXa in the activation of FX.

Figure 3. Lipid binding portion of C1 domain. Arg 2159 and Arg 2163 residues highlighted.

Figure 4. Lipid binding portion of C2 domain. Arg 2320 and Arg 2215 residues highlighted and O-phosphatidyserine head group present.

Figure 5. All mutants were created through site directed mutagenesis and confirmed with Sanger sequencing.6

Figure 6. All proteins were purified by immobilized metal affinity gravity flow column chromatography with TALON (cobalt affinity) resin. Further purification achieved with FPLC using a 5 column.8

Figure 7. Binding affinity of mutant proteins to phosphatidylserine (PS) analyzed with an enzyme-linked immunosorbent assay (ELISA).

Figure 8. ELISAs, R2159H R2215A R2320S R2163H

Figure 9. ELISAs, H2215A R2215A R2320S R2163H

Results

C1 Domain Mutations

- R2159H
- R2163H

- 35.859 kDa
- M.C 125 kDa

C2 Domain Mutations

- R2215A
- R2215A

- 28.879 kDa
- M.C 125 kDa

Conclusions and Future Work

- C2 Domain: R2320 does play a role in membrane binding.
- Thermodynamic stability and proper folding of human C1 and C2 mutants will be measured with circular dichroism and intrinsic tryptophan fluorescence. Proper folding will also be confirmed with pull down assays.
- Binding capacity of C1 and C2 mutants for activated platelet surfaces will be measured with enzyme-linked immunosorbent assays (ELISAs) and liposome sedimentation assays.
- Crystallize the C2 domain mutants to understand conformational changes caused by each mutation and elucidate a working model for membrane binding by the C2 domain.

References

2. Original image from Takara Bio Inc.
3. Original image from Takara Bio Inc.

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