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

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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 phosphatidylserine 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).

Heterotrimeric FVIIIa binds to negatively charged phospholipids, and participates as a cofactor to factor IXa in the factor X activating (tenase) complex.

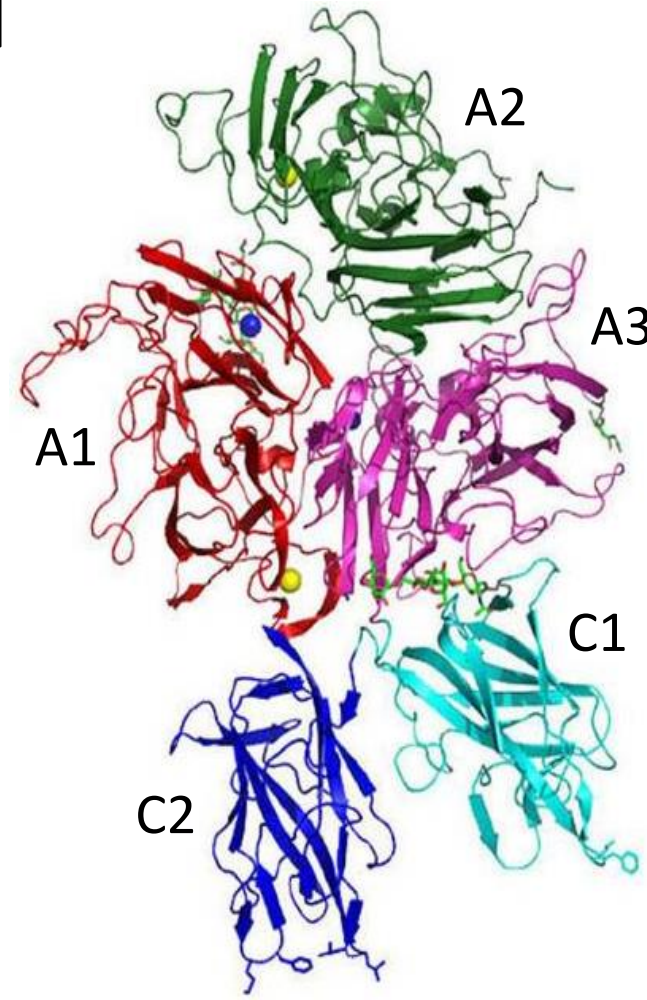


Figure 1. FVIII Crystal Structure.¹

Blood Clotting Cascade

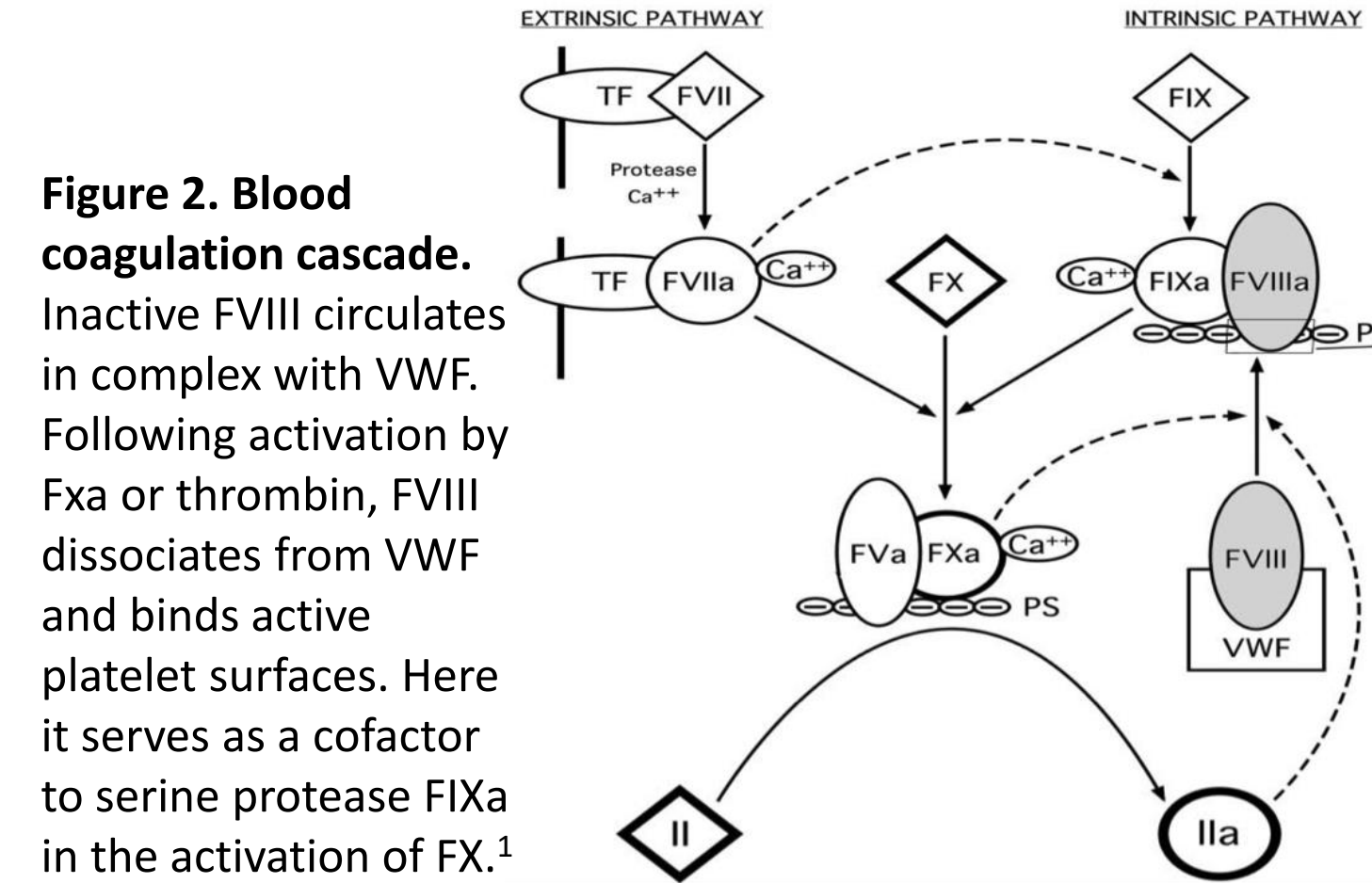


Figure 2. Blood coagulation cascade. Inactive FVIII circulates in complex with vWf. Following activation by Fxa or thrombin, FVIII dissociates from vWf and binds active platelet surfaces. Here it serves as a cofactor to serine protease FIXa in the activation of FX.¹

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 phosphatidyl serine layer of activated platelet membranes.

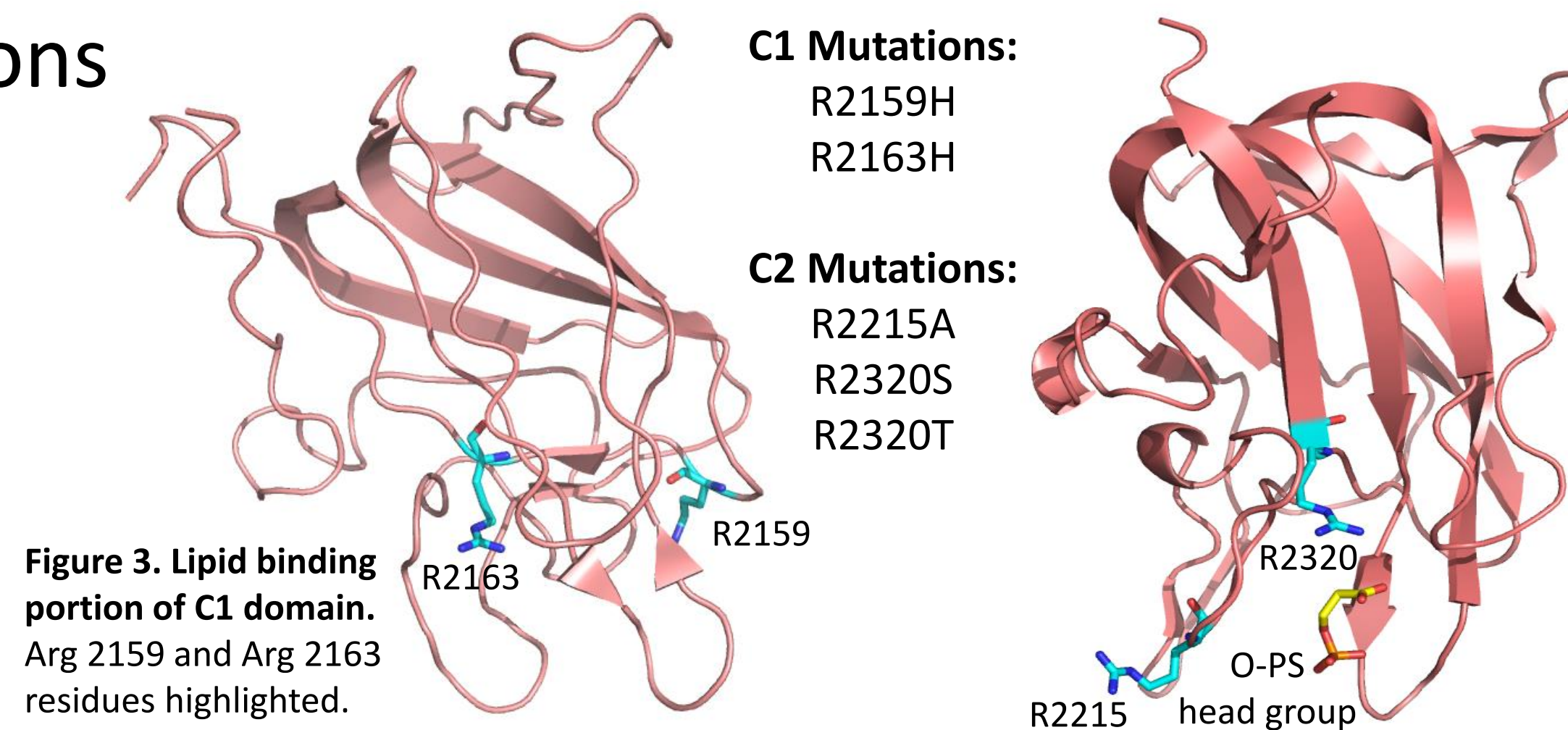


Figure 3. Lipid binding portion of C1 domain. Arg 2159 and Arg 2163 residues highlighted.

C1 Mutations:
R2159H
R2163H

C2 Mutations:
R2215A
R2320S
R2320T

	R2320T	R2320S	R2159H	R2163H
Hemophilia Severity	Moderate	Mild	Mild	Moderate
Factor VIII Clotting Percentages	5%	6%	~25%	2-6%

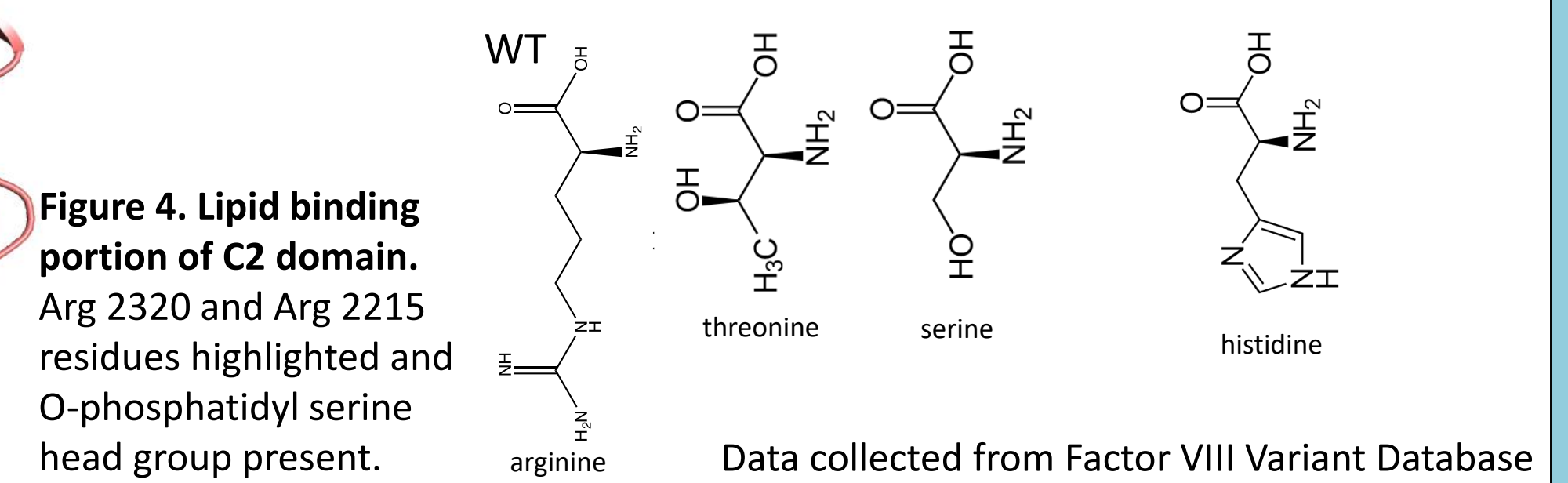


Figure 4. Lipid binding portion of C2 domain. Arg 2320 and Arg 2215 residues highlighted and O-phosphatidyl serine head group present.

Data collected from Factor VIII Variant Database

Methods

Site Directed Mutagenesis

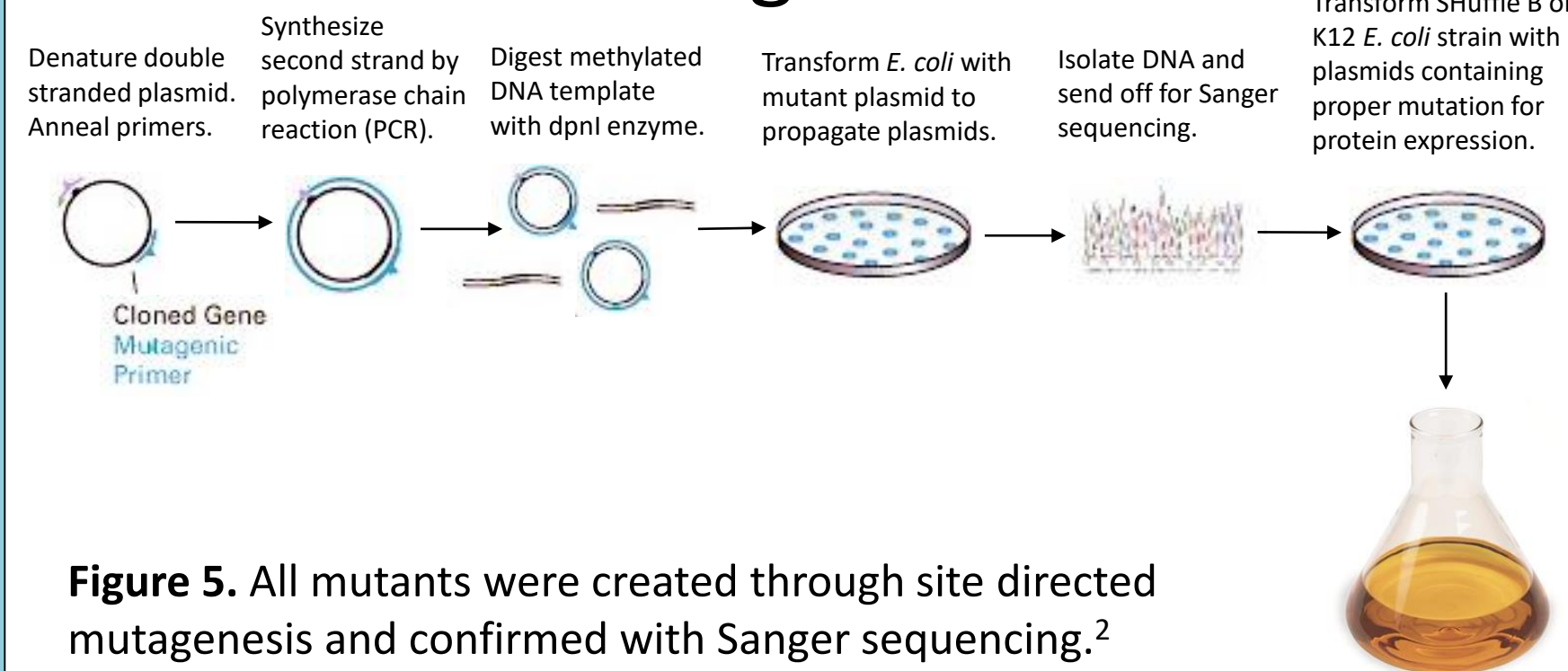


Figure 5. All mutants were created through site directed mutagenesis and confirmed with Sanger sequencing.²

Purification

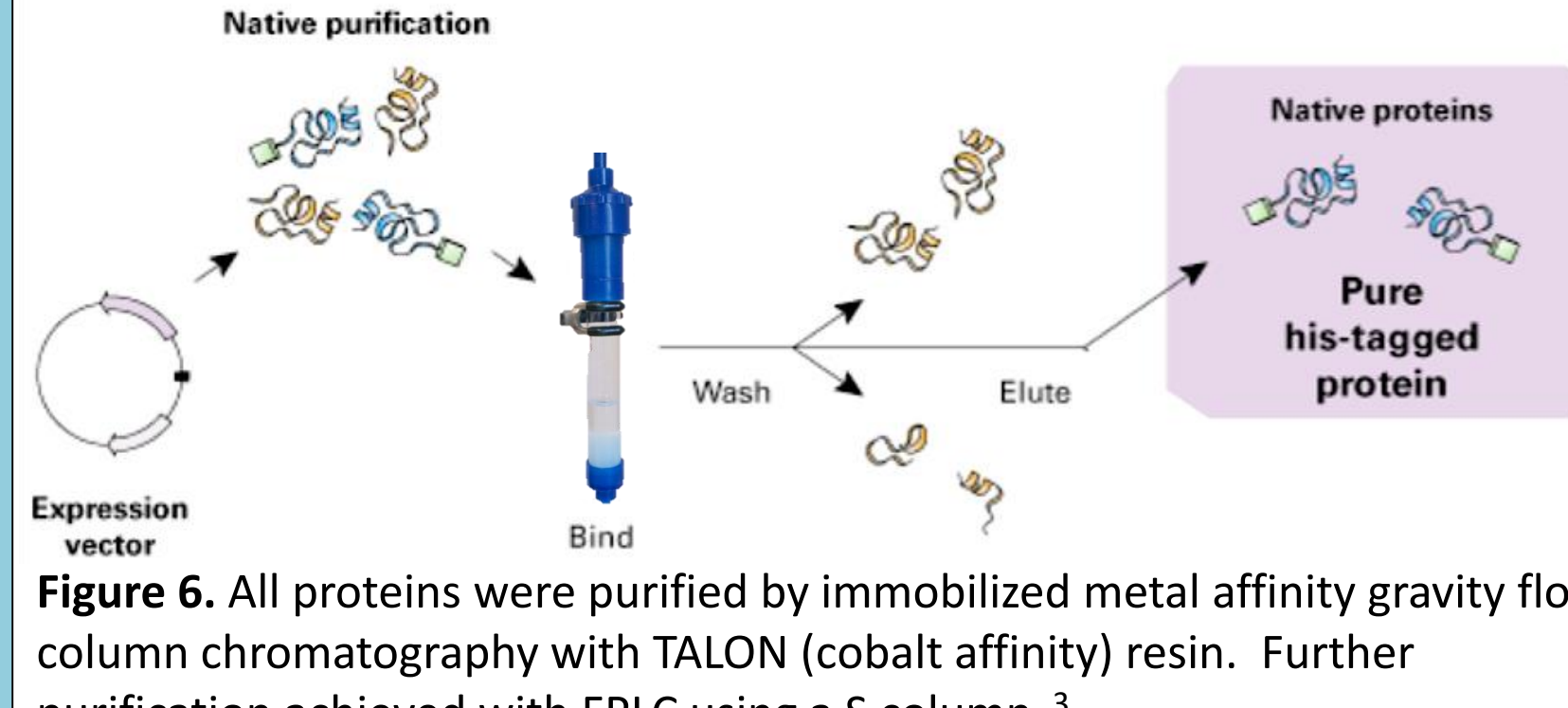


Figure 6. All proteins were purified by immobilized metal affinity gravity flow column chromatography with TALON (cobalt affinity) resin. Further purification achieved with FPLC using a S column.³

ELISA

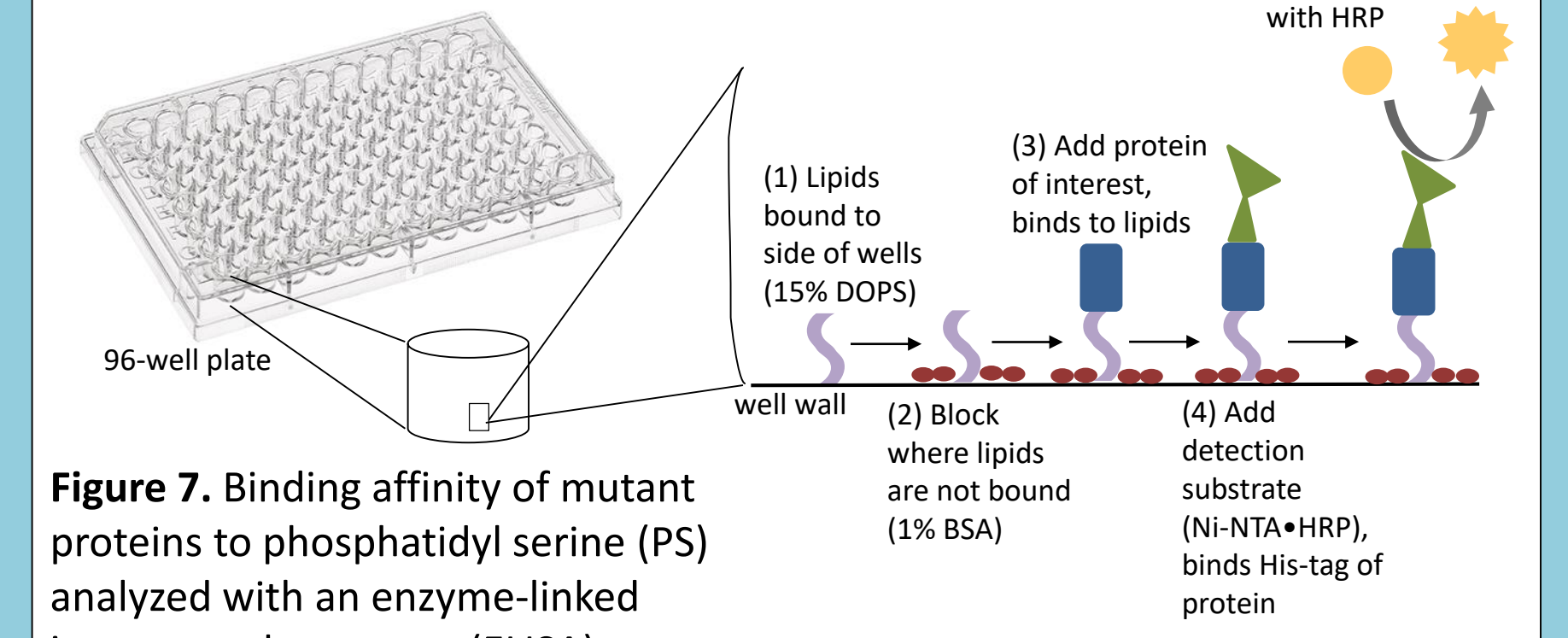
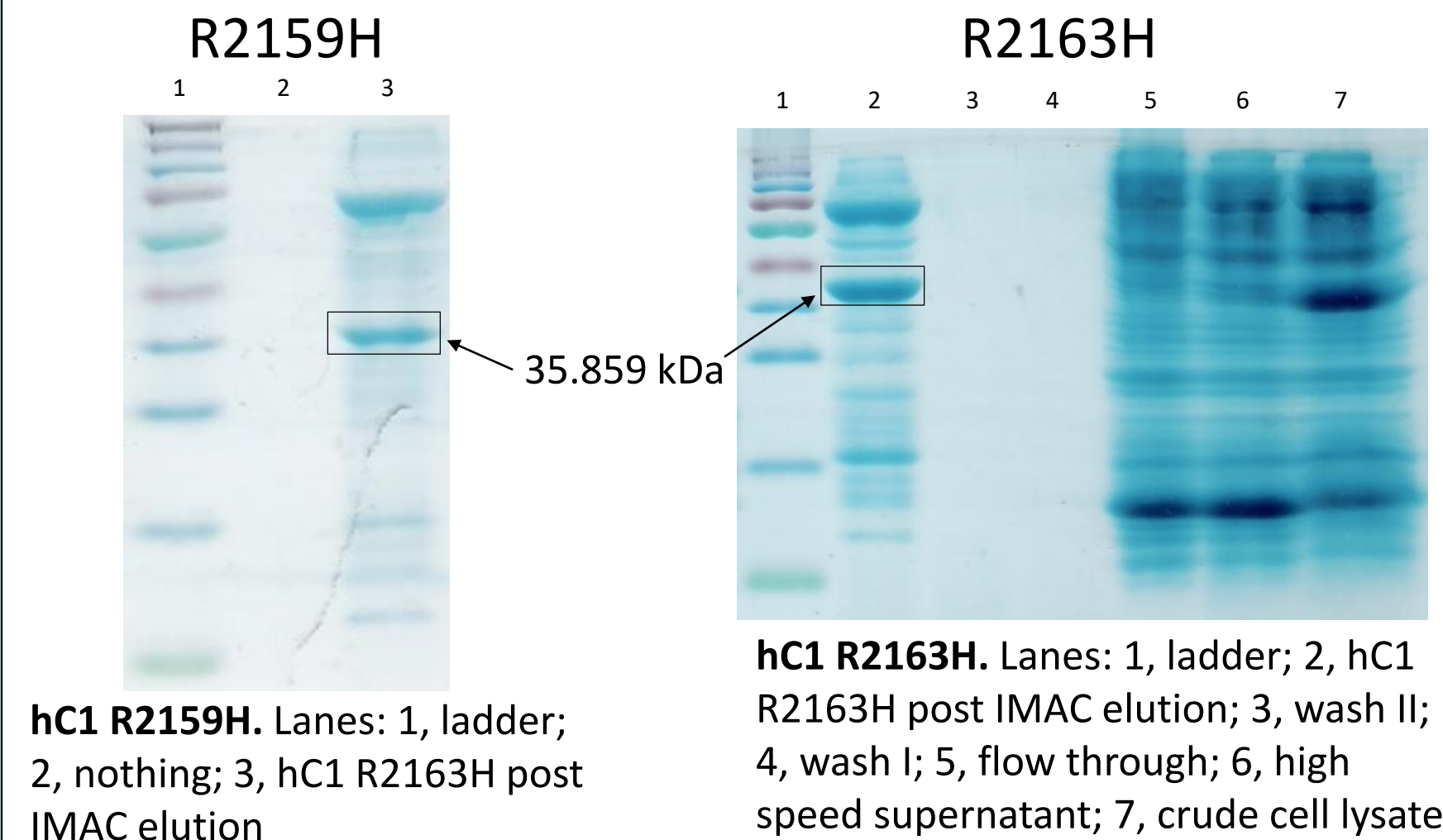


Figure 7. Binding affinity of mutant proteins to phosphatidyl serine (PS) analyzed with an enzyme-linked immunosorbent assay (ELISA).

Results

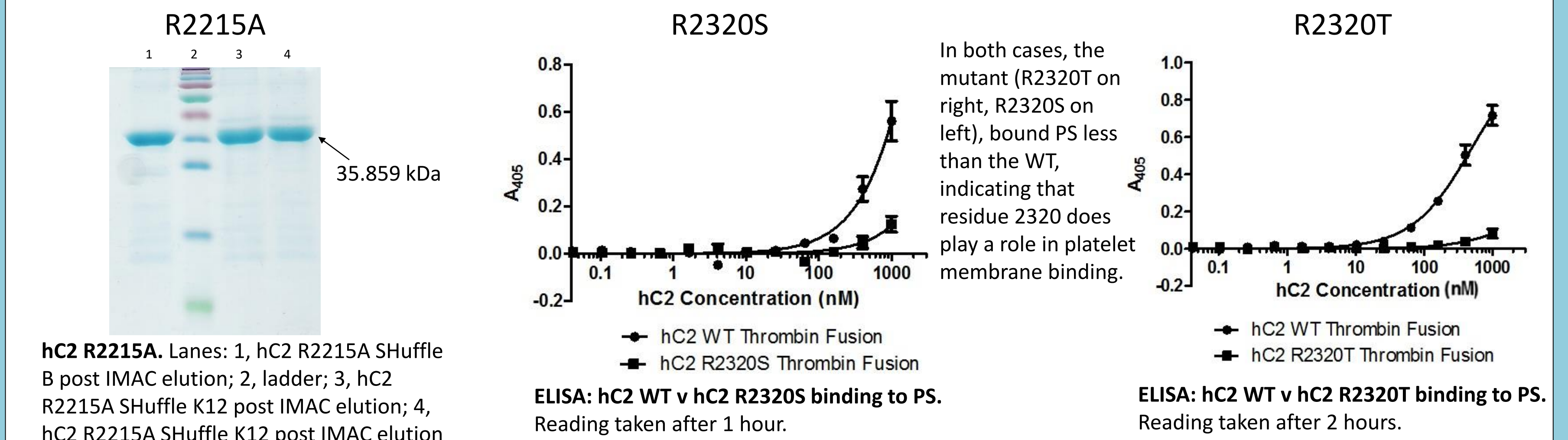
C1 Domain Mutations



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

C2 Domain Mutations



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

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.

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

- Shen, Spiegel, et al. *Blood* (2008), 111: 1240-1247.
- Original image from Takara Bio Inc.
- Original image from Takara Bio Inc.

Acknowledgements

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