7-2014

Characterization of Lipid-Anchored Inhibitor Rulers as a Measure of Enzyme Topography in Coagulation Enzyme Factor Xa

Robin C. Conley
Philadelphia College of Osteopathic Medicine, conleyrc@acomedu.org

Recommended Citation

Characterization of Lipid-Anchored Inhibitor Rulers as a Measure of Enzyme Topography in Coagulation Enzyme *Factor X$_a$*.

By:

Robin C. Conley

Department of Basic Sciences

Philadelphia College of Osteopathic Medicine, Georgia Campus

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Biomedical Science

May 2013

PCOM Biomedical Sciences Degree Programs

Signatory Page for Master's Thesis

We approve the thesis of Robin Christina Conley.

7/17/2014

Date of Signature

Kimberly J. Baker, PhD

Assistant Professor of Biochemistry

Thesis Advisor

7/17/2014

Date of Signature

Francis E. Jenney, Jr., PhD

Associate Professor of Biochemistry

7/17/2014

Date of Signature

Harold Komiskey, PhD

Professor of Neuroscience, Physiology, and Pharmacology

7/17/2014

Date of Signature

Brian M. Matayoshi, PhD

Graduate Program Director and Professor of Physiology

Table of Contents

List of Figures

List of Tables

Abstract:

The blood coagulation cascade is activated under various circumstances such as an injury. This system involves a tightly regulated series of events. The enzymes involved assemble with their respective cofactors on lipid membranes to reach their full pro-coagulant complex potential. The coagulation cascade is divided into intrinsic and extrinsic portions, both of which converge into a common pathway with the activation of factor X (FX_a). FX_a is a very important part of the blood coagulation cascade because its activation is primarily responsible for thrombin generation. Activation of FX_a alone is, however, insufficient to produce a fully active thrombin. For this to occur, FX_a must form a complex with its protein cofactor Factor V_a and its protein substrate prothrombin on a phospholipid surface. This suggests that the topography of the enzyme on the lipid surface changes when in a fully pro-coagulant state. However, the topography of FX_a alone and in complex with these cofactors is poorly understood. Kunitz-type protein inhibitors, such as basic pancreatic trypsin inhibitor (BPTI), are globular proteins, which inhibit serine proteases such as FX_a. These small protein inhibitors fit just inside the active site of the enzyme, making them ideal candidates with which to study the active site of FX_a. The goal of this project is to gain understanding of the topography of fully active FX_a by using lipid-anchored BPTI with linkers that act as molecular rulers to measure the range of reactive heights of the active site of FX_a in its pro-coagulant complex on a phospholipid surface.

Introduction

Hemostasis

Hemostasis is the process responsible for initiating and terminating the blood clotting mechanism upon injury (1) . It is a tightly regulated and dynamic process by which bleeding as a result of vessel damage is stopped and, ultimately, returned to normal (1-3) . The hemostasis process works to maintain blood in a fluid, clot-free state under normal conditions, form a solid clot to block bleeding upon injury, and then return blood to a fluid state upon repair of that injury. This process involves a subset of processes including blood clotting, platelet activation, and vascular damage repair (3). Coagulation is the first integral part of this process as it is the formation of blood clots which assist with sealing the injury and returning blood flow to normal (2-4) . The coagulation cascade includes three major components some of which circulate in the blood in a deactivated state and become activated upon vascular injury. These components are platelets, vessel endothelium, and coagulation factors. Platelet activation is important in hemostasis for sealing vessel damage and accelerating the coagulation system overall. Vessel repair is a result of the hemostatic plug composed of fibrin and platelets that seals the damage as well as endothelial components which repair the tissue (3). To understand more about the blood coagulation cascade, it is important to investigate the blood components involved more closely.

Platelets

Platelets are specialized blood cells that play an important role in hemostasis (2, 5, 6). The structure of both the un-stimulated and stimulated platelet is important in its

functioning. In a deactivated state, they are small, disk-like, non-nucleated cell

fragments of megakaryocyte cytoplasm in the blood (6). When activated, platelets

structure morphs from smooth and discoid to rough cells with pseudopodia. As the term

suggests, these pseudopodia are feet-like projections which serve as puzzle-like sites of

attachment for other platelets and fibrin. The cytoplasm is surrounded by a plasma

membrane constructed in an open canalicular system (6). This system transports

nutrients in and out of the cell. The plasma membrane is composed of over 30%

phospholipids and nearly 60% proteins. The phospholipids are organized as a bilayer

with polar head groups on both the external and internal layers in addition to a

hydrophobic center. Neutral phospholipids like phosphatidylcholine are oriented on the

external layer while anionic phospholipids are oriented internally. These phospholipids

include phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine (7) .

Tasks assigned to the phospholipids include solubilizing cholesterol such that cholesterol

can maintain the fluidity of the phospholipid bilayer. A small percentage of

carbohydrates are present in the membrane in the form of glycoproteins and

glycolipids.

The outer layer of the bilayer contains a glycoprotein complex known as the GPI

complex. There are subgroups of glycoproteins involved in this complex: GPI_a, GPI_b, and

$GPII_b$/$GPIII_a$. These proteins work together to attach platelets to collagen and bind von

Willebrand factor. This step-wise process involves the platelet exposure to coagulation-

inducing proteins, adhesion, and cellular reorganization. A well-characterized

coagulation factor named von Willebrand factor (vWf) attaches to a receptor on the GPI

complex during platelet adhesion. vWF is a multimer produced by endothelial cells and megakaryocytes which is composed of over 200,000 subunits connected by disulfide bonds which functions with coagulation factor VIII to assist in platelet adhesion (5, 8, 9). This complex plays a necessary role in the conformational changes and overall activation (*i.e.* pseudopodia, increases in surface area) platelets undergo after stimulation (10). GPI$_a$ is a receptor protein. It attaches to extracellular collagen exposed at the site of injury. Next, GPI$_b$ then becomes exposed to vWF. Another structural shift in platelets occurs which allows for GPII$_b$ and GPIII$_a$ to bind with vWF and collagen. This process is depicted in Figure I.
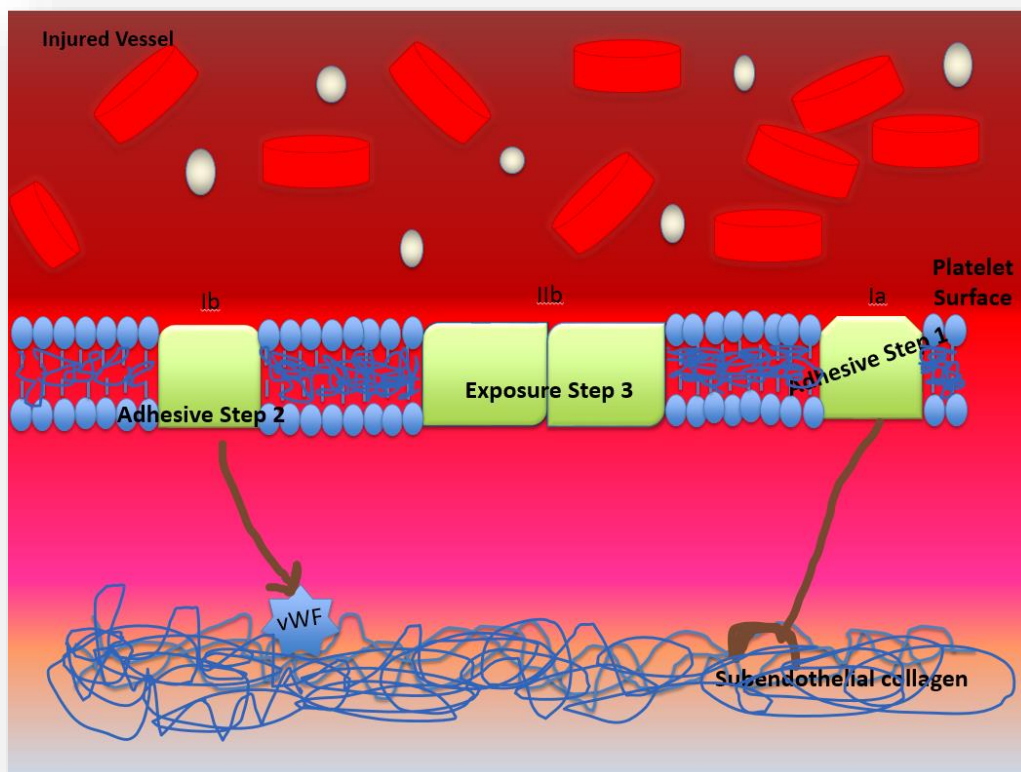
Figure I. GPI Complex. Depicted above is the phospholipid bilayer of a platelet cell containing GPIa, GPIb, GPIIb, and GPIIIa subunits and the step-wise platelet activation process. Adapted from (20)

The internal membrane of platelets has been likened to the sarcoplasmic reticulum of muscle cells because they contain dense tubing, mitochondrial membranes, and storage granules (11). These storage granules serve to release necessary components to platelet adhesion when needed and are divided into different categories including α, δ, and λ types. Enzymes including phospholipase A and cyclooxygenase assist in the transfer of Ca^{2+} from membrane stores within the platelet to the cytosol encouraging secretion from the storage granules (5). The α-granules contain fibrinogen, which upon vessel injury is later cleaved to become fibrin necessary for the hemostatic plug. Alpha granules also contain proteins like β-thromboglobulin. One of its functions is to assist in megakaryocyte maturation and therefore in platelet production. The δ granules also known as electron-dense storage granules contain metabolically-inactivated adenosine diphosphate or ADP (an agonist in platelet activation), serotonin (a common neurotransmitter), and calcium (metal ion involved in aggregation). Lambda granules contain lysosomal enzymes such as acid hydrolase important for the breakdown of waste materials (12).

Activated platelets undergo conformational changes as a result of contact with endothelial components. Under normal circumstances, the endothelial lining of the vessels is inert and platelets are not stimulated to adhere. When the blood vessel is damaged, platelets are exposed to subendothelial components including collagen, tissue factor, fibronectin, and glycosaminoglycans. Then, platelets undergo adhesion in which they attach to these macromolecules in subendothelial tissues at the point of injury. This collection of cells forms what is referred to as the 'adherent layer' (7) . In response

to the initial platelets' activation, other platelets aggregate to form a hemostatic plug (2, 6).

Aggregation is subcategorized into primary and secondary stages. Primary (1°) aggregation is a reversible process that arises from the stimulation (i.e. *signaling*) other platelets receive from the adherent layer. While platelets are aggregating with one another, they are also undergoing a process called secretion which stimulates the irreversible secondary (2°) aggregation. Like many biochemical processes, aggregation requires divalent cations such as Mg^{2+} or Ca^{2+} to function properly (6) . Platelets release several vasoactive amines and other important substances, including but not limited to ADP, β-thromboglobulin, epinephrine, and platelet-derived growth factor (6). Epinephrine, a hormone and neurotransmitter, binds to α-adrenergic receptors on the platelet surface thereby encouraging stimulation (13). The process is broken down into two major steps. First, storage granules cluster at the center of each platelet surrounded by microtubules. Then, the granules travel along the microtubules to the surface of the cell and fuse to the surface membrane and release their contents. The amount of substances released is dependent on the agonist involved as established by Kinlough *et al*. (14). Secretions such as these trigger other platelets in the area to adhere and continue forming the hemostatic plug.

Plug formation and pro-coagulant activity on platelet surfaces assists in the generation of the thrombin burst which leads to fibrin formation. Fibrin complexes with the platelets to form the fibrin clot in the final stages of coagulation. As wound healing takes place, both the platelet aggregate and fibrin clot are broken down over time in a

process called fibrin clot retraction (2, 6, 13, 15, 16). This process is also mediated by platelets. All of these components are part of an intricate stimulatory signaling pathway, which communicate messages to the platelets upon injury. There are also inhibitory components as well. One such pathway would be the adenylate cyclase enzyme complex. Adenylate cyclase and cyclic adenosine monophosphate (cAMP)-dependent phosphodiesterases work closely with one another to inhibit platelet function. These enzymes recruit the second messengers to attach to receptors on platelet surfaces sending signals to essentially de-activate each platelet cell (17).

Vasculature Walls

In addition to platelets, the vascular wall is an important component of blood coagulation. In general, blood vessels contain 3 layers: tunica intima, tunica media, and tunica adventitia (18). The tunica intima is the innermost layer composed of endothelial cells. The tunica media is the smooth muscle layer, which also contains elastic tissue as well. The tunica adventitia is composed of connective fibrous tissue. When the vessels are damaged, endothelial components such as tissue factor (TF) come in contact with platelets in the blood thereby activating them (2). Tissue factor, also known as factor III, is a protein that becomes exposed to intracellular blood components upon injury. It will be discussed further in subsequent sections. Coordinated interactions among these tissue components, plasma proteins, and platelet receptors lead to the primary hemostatic plug, which seals the damaged region of the vessel (2).

Ashford *et al* completed an electron microscopy study on particular endothelial components and the role they play in coagulation (19). The study was completed *in vivo*

in rats in which the vascular wall was manipulated and known endothelial components introduced, altered, or removed via purification.

Microscopic evidence indicates that platelets in proximity with fibrin were visibly altered and showed loss of granulation. Conversely, even with vascular injury, platelets that are not in close proximity to the fibrin remained intact. After a length of time neutrophils (known *inflammatory responders*) were recruited to the injured area. The study investigated activity around disrupted endothelium in the presence and absence of ADP, collagen, and thrombin. Data suggested that adding ADP induced platelet aggregation. Evidence indicated that collagen adheres rapidly to platelets after trauma. It also suggested that collagen encouraged the release of ADP. Another interesting finding was that cAMP exhibited inhibitory effects on platelets. Overall, the study surmised that the endothelium plays a passive barrier role separating circulating procoagulants in the blood from tissue-embedded activators necessary for the hemostatic plug.

Other endothelial components include vWf and fibrinogen. As mentioned, vWf is a large multimeric plasma protein, which mediates platelet adhesion by binding them to the collagen at injury sites on the damaged vessel (2, 6). Riddel *et al* (2) explored theories in blood coagulation by reviewing multiple studies that compared the accepted extrinsic/intrinsic model to the cell-based model theories proposed more recently. This publication describes vWF as an indispensable part of platelet-platelet interaction. Its primary role is to increase the efficiency of platelet attachment to one another. Its

secondary role is important for the survival of coagulation factor VIII described further in the <u>Non-Enzymatic Factors</u> section below.

Coagulation Factors

    <u>Serine Proteases</u>

The coagulation factors are proteins developed by hepatocytes and are another portion of the cascade necessary to form a clot (20). Early work by Orwen, Biggs, and MacFarlane in the 1940s demonstrated that many of the coagulation factors are the zymogen form of serine proteases important in clot formation that required activation to function properly.

Coagulation proteins are proteases activated by proteolytic cleavage. Many of these proteins are vitamin-K dependent. Vitamin K is necessary for γ-carboxylation of glutamate residues on the protease necessary to allow attachment to the membrane. The process of γ-carboxylation involves the reduction of vitamin K to vitamin KH_2 by the enzyme, quinone reductase (15). Vitamin KH_2 serves as a co-factor for carboxylase enzymes within hepatocytes that add a carboxyl group to the glutamate (Gla) domain on these serine proteases (15, 20-22) . Cleavage is a biochemical process by which one enzyme cleaves a portion of another enzyme adjusting its chemical composition converting it from an inactive state to an active one. Serine proteases are proteins which activate one another by this process. Because there are so many coagulation factors and more being discovered all the time, this publication will focus on discussing a select few in an effort to provide a general overview of their diverse characteristics.

Table I contains an abbreviated list of known serine proteases and their main functions

(20).

Coagulation Factors: Serine Proteases	Function
Factor II Prothrombin	When cleaved becomes thrombin Cleaves fibrinogen→fibrin
Factor VII Proconvertin	Forms complex with Tissue Factor to partially activate fX and FIX
Factor IX Christmas Factor	Partially activates fX
Factor X Stuart-Prower Factor	Cleaves prothrombin→thrombin
Factor XI Plasma Thromboplastin Antecedent	Partially activates fIX

Table I. Serine Proteases and their Functions

The activation of these factors leads to the initiation of thrombin cleavage.

Thrombin, a serine protease, is the activated form of coagulation factor II. Its main

responsibility is to cleave soluble fibrinogen, also known as factor I, converting it to

insoluble strands of fibrin. Fibrin sticks together with blood components including

platelets to seal the injured site. Previous work has established that hemostatic

constituents among vertebrates, including prothrombin, are conserved suggesting that

any vertebrate with a circulatory system contains these factors within that system (3,

23).

Non-enzymatic Factors

Factor III, often called Tissue Factor, was mentioned earlier to complex with fVII

in order to partially activate both fIX and fX. It is produced by endothelial cells and is,

therefore, categorized as a member of the extrinsic pathway. Two distinct coagulation

pathways–extrinsic and intrinsic—work together to maintain hemostasis upon injury.

After each participant of pathway has been activated and performed its task, the

cascades enters a third pathway called the Common Pathway in which the components

necessary to generate thrombin are fully activated. Factor VII complexes with non-

enzyme participant TF and performs its duties on the surface of TF-bearing cells (20).
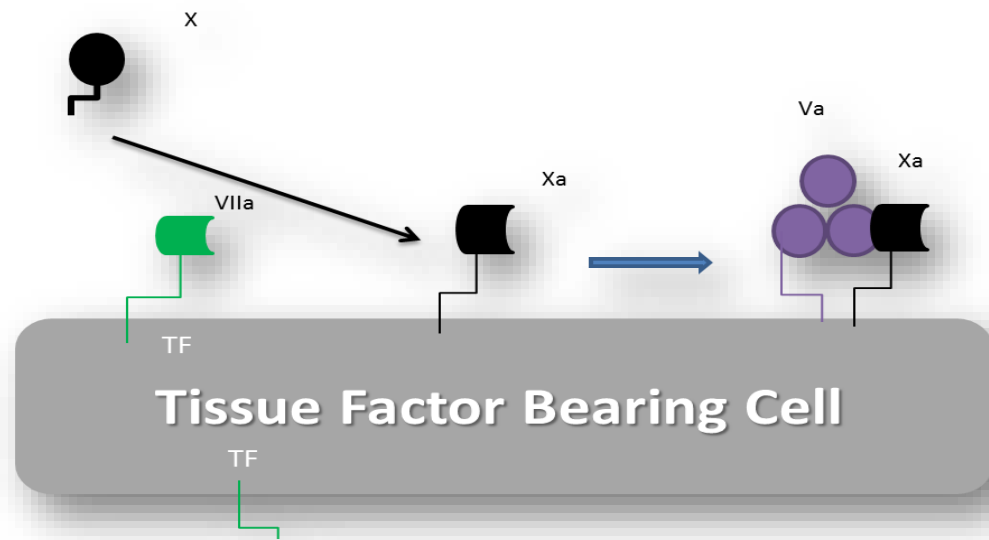
Figure II. TF Interaction on the Cell Surface during Coagulation. Depicted above is the interaction of tissue factor, factors Va, VIIa, and Xa with the endothelial cell surface. Adapted from (20)

This complex serves as a partial activator of factors IX and X respectively.

Factor VIII is another factor necessary for the activation of fX. Factor VII complexes with fIX such that fX is cleaved to a fully active state. vWF is critical for fVIII survival. FVIII is a labile co-factor meaning that it is easily degraded. vWF complexes with $FVIII_a$ prolonging its presence in the blood so that it may attach to fIX_a for fX activation. All three factors work together to cleave fII for a burst of thrombin generation.

Pro-accelerin or factor V is a member of the prothrombinase complex necessary for fX to cleave fibrinogen. The interaction between fV_a and fX_a can be seen in Figure III. It is activated by trace levels of thrombin to catalyze its attachment to fX on cell membranes (20). Further information on the integral role of fV is discussed below.
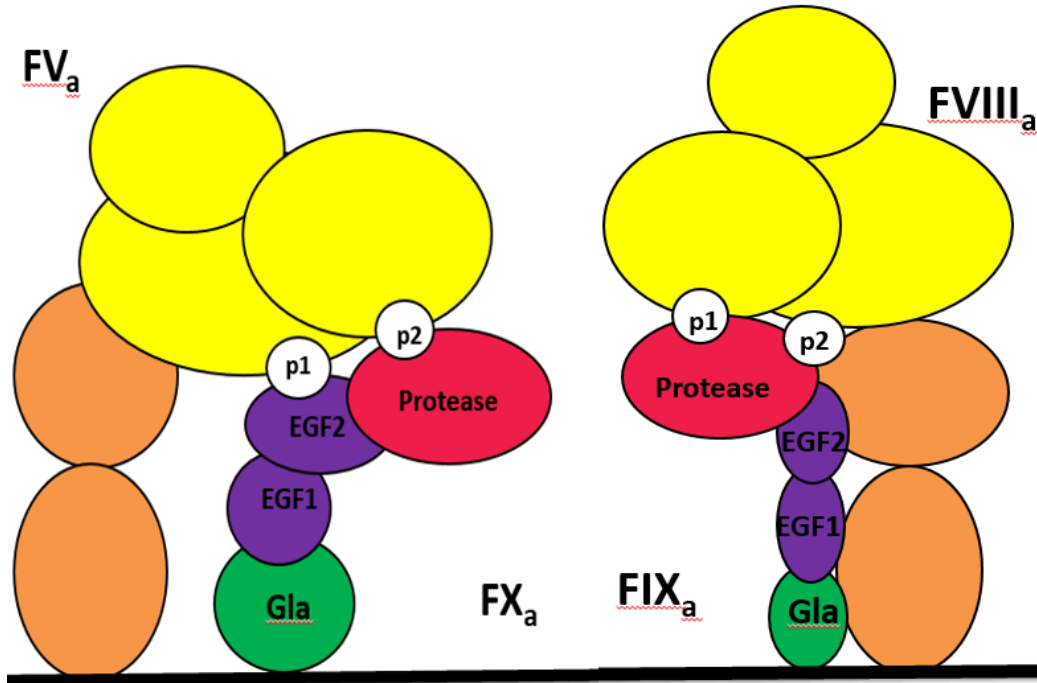
Figure III. Prothrombinase Complex and fVIIIa/fIXa Complex. FV$_a$ .Adapted from (117).

Factor IX_a activates fX partially by forming a complex with its co-factor fVIII then attaching to fX on the surface of platelets and endothelial cells. Its role is critical in encouraging the clotting cascade into the common pathway. In 1952, Rosemary Biggs and her colleagues published an article in the *British Medical Journal* regarding fIX (24). At that time, many of the common characteristics associated with coagulation factors were just being recognized. Biggs *et al* performed several case studies on patients exhibiting the blood disorder, hemophilia, but were unresponsive to traditional hemophilia treatments. These cases were all similar in that their bleeding issues could not be rectified with anti-hemophilic globulin (fVIII), Ca^{2+}, or fVII—known to be critical to coagulation. The ultimate finding was that all cases were deficient in an unknown substance present in normal blood fractions. The unknown substance was identified as Christmas Factor named for case study 1 (24). Now, fIX and Christmas factor are synonymous with one another (20). Further studies by these researchers and others established that hemophilia can manifest in the deficiency of fVIII or fIX. In addition to cleaving fX, fIX_a must diffuse away from cell surfaces before the coagulation process can reach the explosion of thrombin necessary for fibrin deposition. FX_a marks the starting point for the common pathway and plays the largest role in thrombin generation. More detail on fX and its participation in the coagulation cascade are included in <u>The Common Pathway and the Cell-Based Model</u> section.

Fibrinogen, also known as factor I, is a soluble zymogen that is cleaved by fII_a or thrombin. This cleavage catalyzes chemical changes within its composition such that it

becomes fibrin, an insoluble protein that cross-links with platelets to become the final

clot in coagulation (20).

Ca^{2+} is a divalent cation necessary for many biochemical reactions within the

body. It has also been given the title factor IV for its participation in the coagulation

cascade. In particular, Ca^{2+} is a necessary co-factor for most, if not all, coagulation

factors to perform their respective tasks. Table II is a compilation of non-enzyme factors

and their main function.

Coagulation Factors: Cofactors	Function
Factor I Fibrinogen	When cleaved becomes Fibrin and forms hemostatic plug
Factor III Tissue Factor	Receptor and co-factor that complexes with fVII$_a$
Factor IV Ca^{2+}	Divalent cation/Cofactor important in catalysis of proteolytic cleavage
Factor V Pro-accelerin	Cofactor and member of Prothrombinase Complex
Factor VIII Anti-hemophilia A	Cofactor that complexes with fIX$_a$ Deficiency leads to Hemophilia A

Table II. Non-enzymatic Factors and their Functions

Factor Regulation

Normal blood flow is maintained as long as factors in circulation are not exposed

to endothelial components. The system is a tightly regulated culmination of positive and

negative feedback loops. Each factor circulates in zymogen form which keeps them from

activating each other unnecessarily, but there are other constituents in the blood as well

as endothelial cells within vessel walls that regulate certain factors even when vessel

injury has taken place. For instance, thrombomodulin (TMB) is an endothelial surface

receptor and its corresponding ligand is thrombin (fII_a). Binding of thrombin to TMB

triggers the activation of protein C (APC) (20) APC then complexes with Protein S to

remove fV_a and $fVIII_a$ respectively. The removal of each of these protein factors results

in the shutdown of the clotting process by triggering the inactivation of the fV_a and

$fVIII_a$. Other factor regulators include tissue plasminogen activator (tPA) which cleaves

plasminogen. Plasminogen is a zymogen involved in clot breakdown discussed in more

detail in The Blood Coagulation Cascade section.

The Blood Coagulation Cascade

As discussed, the blood coagulation cascade (BCC) is activated by varying stimuli

(2) . An injury to the skin that pierces past the epidermal layer and reaches the

vasculature sets the cascade in motion. Blood coagulation by definition is the cessation

of blood loss from a damaged vessel (2). Blood coagulation is a tightly regulated series

of events that involve the many enzymes and co-factors mentioned previously. These

enzymes work in assembly with their respective cofactors on phospholipid membranes

of platelets and endothelial cells to reach their full pro-coagulant complex potential (22,

25, 26). The early models of the BCC were established within blood vessels as a series of

clotting factor activation events occurring in separate processes known as the extrinsic

and intrinsic pathways. These models were established to explain the mechanisms of

coagulation. Figure IV is a general overview of the coagulation cascade.
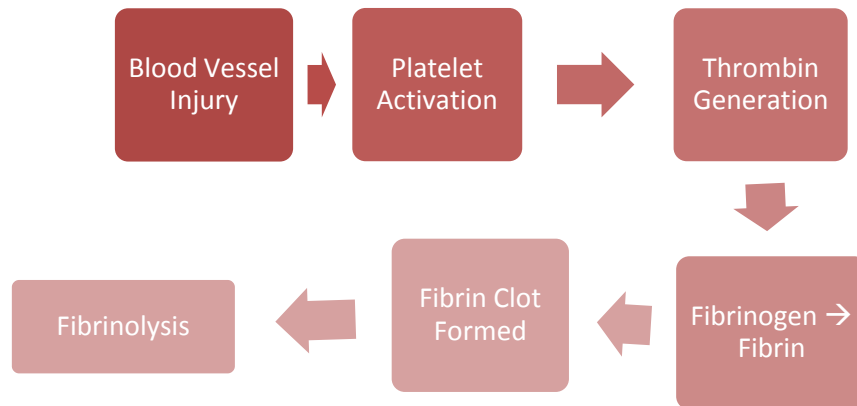
Figure IV. General Overview of the Blood Coagulation Cascade.

Extrinsic Pathway

The extrinsic pathway is ignited when disruption occurs to the endothelium on the outside of the vessel wall. The endothelium contains TF, a glycosylated intrinsic membrane protein. TF, when bound to coagulation factor VII_a, is a powerful activator of the extrinsic pathway. It is found all over most tissues except the lining of the circulatory vessels. It is not released from the endothelium without stimuli from its constituents such as cytokines (27). Exposure to clotting factors in the plasma results only if there is damage to these vessels exposing endothelial components to the blood directly. Because this pathway will only be initiated when the plasma-derived fVII is exposed to the TF which exists outside the blood vessel, this cascade is known as the extrinsic pathway (27, 28).

A classic way to measure the total reaction time for the extrinsic pathway was by using prothrombin time (PT) experiments (20, 28, 29). Quick *et al* developed the prothrombin test in the 1930s as a means of measuring anti-coagulation (29). At that time, the historical 1905 Morawitz *et al* study had based the coagulation process to be centered around 4 plasma proteins (2, 30) . Quick's technique used patient plasma samples to measure the consumption of prothrombin when exposed to thromboplastin which is a lab reagent and protein that converts prothrombin to thrombin. Thromboplastin is a mixture of phospholipids and tissue factor (2). In the early 60s, Glover and Kuzell further developed the technique with the goal of improving anti-coagulant therapies. Experiments for measuring changing levels of prothrombin were done by the Quick one-stage plasma method using whole blood (29). Glover *et al*

employed whole blood from the finger, adjusting measurement temperatures to 37°C

(body temperature), and altering the collection of blood with the administration of

thromboplastin (29).

In addition to TF, the extrinsic pathway involves at least four other factors

including prothrombin, Ca^{2+}, factor X, and thrombin (27, 28). However, this model only

works if the other components located outside the vessel are involved. This is evident in

patients with hemophilia, because their blood could have all four elements present and

still be unable to successfully clot. Although the extrinsic pathway explained a great

deal about the cascade, research has determined that it cannot work to clot the blood

alone. Perhaps the best example of this research is the fact that TF works with $FVII_a$

which circulates in the blood (31). It also does not explain why plasma has the capability

to clot without extrinsic components.
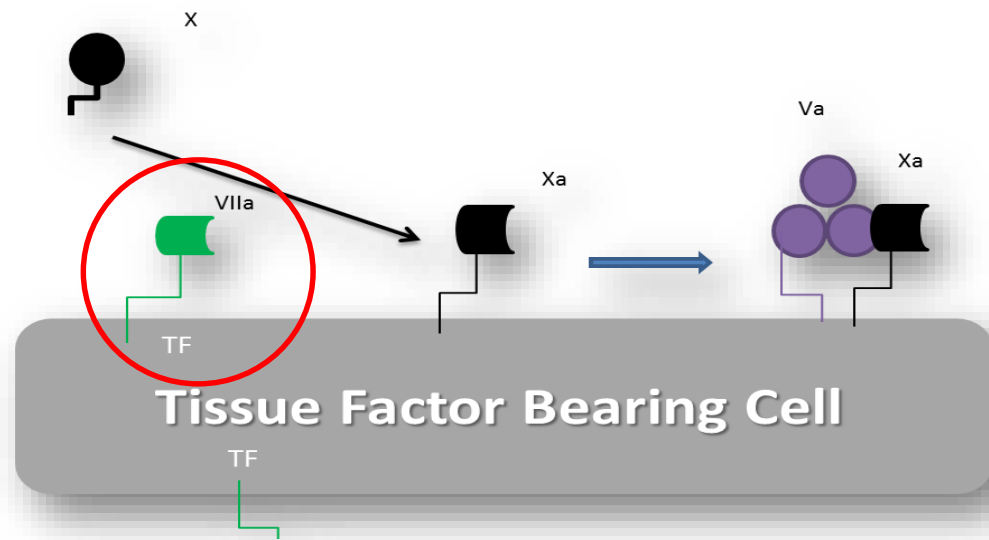
Figure V. TF/fVII$_a$ Complex on membrane surface. Adapted from (20).

Intrinsic Pathway

Classic participants in the intrinsic pathway include the following: fV, fVIII, fIX, Ca^{2+}, and fXI. The pathway begins when thrombin converts fXI to fXI_a. Then, fXI_a cleaves fIX to make it available for attachment of fVIII. While it is now known that neither pathway is sufficient to seal the vessel disruption on its own, the distinction of these pathways remains the widely accepted model in the scientific community. As mentioned, $fVIII_a$, also known as anti-hemophilic factor, serves as the intrinsic co-factor for fIX_a in the activation of fX_a. $FVIII_a$ catalyzes attachment of its associated components to the membrane surface. The phospholipid membrane of TF-bearing cells and platelets acts as the arena for these activities and Ca^{2+} as a reaction catalyzer. This is further depicted in Figure V as the area where platelet activation is controlled. The intrinsic pathway is postulated to maintain its extrinsic counterpart (3, 20). Initial techniques used to study the intrinsic pathway and establish it as a viable part of coagulation is to measure the activated partial thromboplastin time (aPTT). This test was developed in a similar manner to the Quick prothrombin test. Partial thromboplastin is a reagent with similar components to thromboplastin with the exception of TF. This process indicates how fast the intrinsic pathway works with respect to exhausting the factors involved. In other words, aPTT measures the efficiency of the intrinsic pathway. Both the extrinsic and intrinsic pathways occur concurrently and once complete unite into a common pathway.

The Common Pathway & the Cell-Based Model

The common pathway is the process by which factor X catalyzes the activation of pro-thrombin (fII). These events that take place after pro-thrombin becomes thrombin. The common pathway is accepted as a true account of fibrin deposition. However, recent studies in blood coagulation suggest a cell-based model describes the process more accurately (2, 27, 28, 32). The new model focuses greatly on more complicated interactions among vessel endothelium, platelets, and coagulation factors.

Since information regarding coagulation has become more expansive, the cell-based model suggests that coagulation occurs in 3 phases (20, 27, 32). The first phase of the new cell-based model is <u>Initiation</u>. This step begins at the moment TF is exposed to the blood. As the platelets aggregate, TF forms a complex with fVII on endothelial cell surfaces. The TF/fVIIa autocalyzes itself to a fully activated state and then partially activates fIX and fX. As a form of regulation, this complex is diffused away by a special serine protease inhibitor called warfarin discussed in the *Kunitz Inhibitors* section (32). FIX also diffuses away to another anionic phospholipid membrane surface initiating the next phase of the cell-based model, <u>Amplification</u>. Amplification is earmarked by the use of thrombin (fII) as an activator for non-enzymatic factors V and XI. FXI is important for breaking up the complex between $fVIII_a$ and vWF. This step is critical as $fVIII_a$ is a necessary co-factor for fIX. This step is carried out on the surfaces of the platelets that were initially exposed to TF.

The initiation and amplification phases are the priming phases as every factor involved is not fully activated. During the final phase, every factor becomes activated.

The third phase is called <u>Propagation</u>. $FVIII_a/fIX_a$ forms a complex with fX_a on platelet

membranes (called the Tenase Complex) partially activating it and prompting it to then

couple with fV_a (20). The result is a burst of thrombin. Thrombin generation at high

levels cleaves large amounts of fibrinogen into fibrin. Fibrin monomers (or subunits)

clump together with platelets and form a "soft" clot. $FXIII_a$, also known as the enzyme

transglutaminase, seals this soft clot by cross-linking the platelet/fibrin mix. Once this

cross-linking is complete, the hard clot is formed.
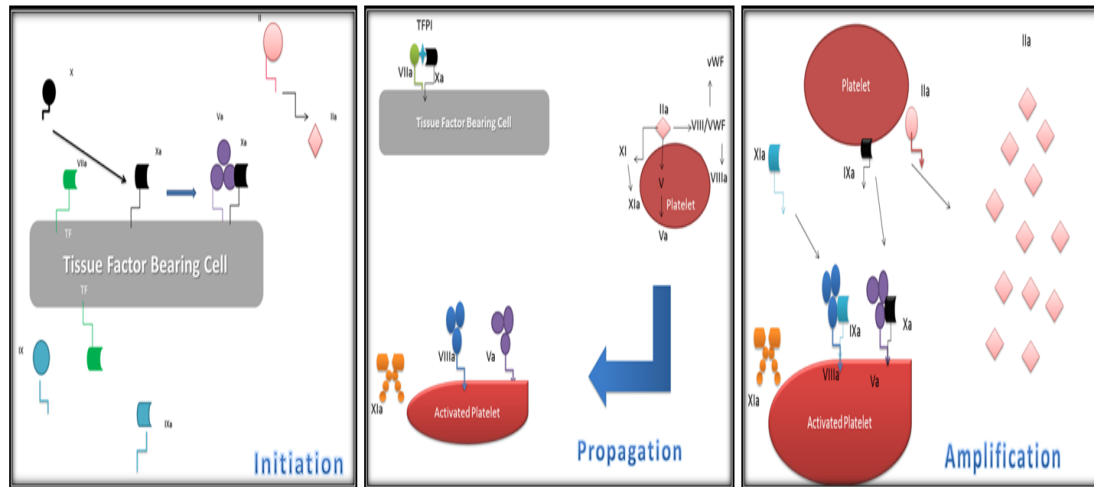
Figure VI. Initiation, Propagation, & Amplification Phases of the Cell-Based Coagulation Model. Adapted from (20).

Breakdown of the clot is an important step in coagulation as well as it marks the stopping point of the clotting cascade. Fibrinolysis occurs when the clot is disintegrated after the vessel disruption is rectified. Plasminogen is a zymogen that is activated by tPA to become plasmin. Tissue plasminogen activator (tPA) and single-chain urokinase plasminogen activator (scuPA) are enzymes necessary to activate plasminogen (20). Plasmin will then cleave fibrin and trigger clot destruction. This step is another form of regulation mechanisms for the maintenance of hemostasis. Protein C and protein S are both participants in the proteolytic deactivation of factors V_a and $VIII_a$. This process will be further explained in the Cascade Regulation section. Plasminogen activator inhibitor-1 (PAI-1) and a_2-antiplasmin are proteins that serve as negative regulators of this process.
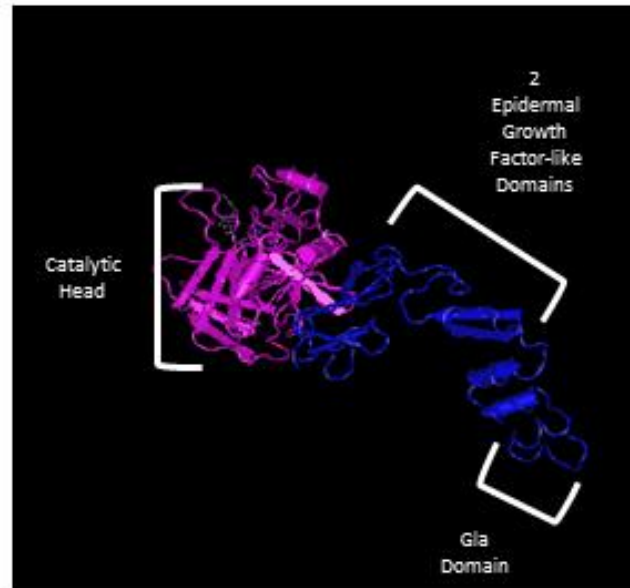
Figure VII. Factor VIIa—catalytic head, 2 EGF Domains, and Gla domain. Adapted from *(116) and PDB ID: 1KLJ.*

Factor Structure

As described, there are over a dozen clotting factors circulating through the blood. These factors each play a significant role in coagulation. Many of them share a structure that contains a catalytic head (similar to trypsin), two epidermal growth factor-like domains, and a Gla domain.

Although they are similar in structure, they are distinct in their degree of specificity for each of their respective co-factors and substrates. In order to understand our purpose in examining fXa, it is important to examine some of the other coagulation factors and their particular role in the BCC cascade. The 1940s marked an era in which many researchers began to focus more closely on the coagulation process (reviewed in (27)). Clotting factors were assigned numbers that correspond with their sequence of discovery rather than their point of interaction within the BCC (23, 33). Factor V_a is activated by proteolytic cleavage. As seen in Figure III, Factor V_a serves as co-factor for fX. It is very homologous with $fVIII_a$, which interacts with the anionic phospholipid surface and requires proteolytic cleavage as well (34). Upon complex formation with fV_a and prothrombin (also known as the prothrombinase complex) on negatively charged phospholipid membranes, fX_a converts prothrombin to thrombin in the final stages of the coagulation cascade (15, 22, 35-38). Although fX is capable of some activity prior to the assembly of the prothrombinase complex, that capability is minimal. This prothrombinase complex enhances the catalytic efficiency of fX greatly because it improves the K_m (Michaelis' constant) and K_{cat} of the substrate activation reaction (38). K_m improvement is credited to the binding of the Gla domain and prothrombin on the

membrane surface. Yang et al conducted a study regarding fV_a and its contribution to

the improvement of K_{cat}. Their findings indicated that among its other tasks fV_a causes

conformational changes of the Na^+ binding loop of fX. Researchers constructed fX

mutants with critical residues Glu-217, Lys-224, and Arg-221$_a$ replaced with an alanine

to study the catalytic function of fX as well as its interactions with the metal ion Na^+.

After replacing these residues, Na^+ interaction was markedly impaired. However, in the

presence of fV_a, the defect in fX catalytic efficiency appeared to be improved. By

binding both fX and prothrombin, fV_a stabilizes the enzyme-substrate complex and

provides ideal docking capabilities for other necessary participants (38). Na^+ plays an

important modulatory role in the structural and chemical functioning of many of the

serine proteases involved in coagulation (39). Figure III is a depiction of how fV_a

complexes with fX to activate it.

Cascade Regulation

Regulation of the BCC is of extreme importance that involves a delicate balance

of all the components described here. This process is that of a negative and positive

feedback loop which works to seal any disruption and maintain blood fluidity. Changes

are seen in blood fluidity as a result of such external or internal stimuli problems as

vessel injury, or increases in blood pressure, or fluctuations in hormones (40, 41). These

increases in blood pressure can occur as a result of cholesterol buildup or chemical

imbalances like sodium or coagulation factors. Table IV is a compiled list of known

cascade regulators.

Coagulation Regulation	Function
Thrombomodulin (TMB)	Receptor, when thrombin is attached coagulation activity is suppressed
Protein C (APC)	Serine protease activated by TMB/thrombin complex ; APC/APS complex dissolves fV_a and $fVIII_a$
Protein S (APS)	Co-factor for Protein C

See above |
Tissue Plasminogen Activator (tPA)	Activates plasminogen for hard clot breakdown
Single-chain Urokinase Plasminogen Activator (scuPA)	
Plasminogen	Cleaves cross-linking for hard clot breakdown

Table III. Cascade Regulation

Protein C and protein S play important roles in regulation when activated as well.

The APC/APS complex is responsible for the dissolution of fV_a and $fVIII_a$ respectively (40).

It is triggered by the attachment of thrombin to one of its receptors called

thrombomodulin (TMB). FV_a and $fVIII_a$ function have been described and are critical to

the continuation of the clotting cascade. Without their presence, blood clotting activity

is suppressed.

Traditional Therapies & Treatments

When the regulation processes of blood described previously are not controlled

properly, the result presents in a number of issues and diseases. In addition to

physiological regulation, diet and genetics both play an integral role in blood regulation.

Although this paper will go into detail on a few of the diseases associated, the focus of

poor regulation of the process is narrowed to genetic de-regulation which can lead to issues including hemophilia or von Willebrand disease.

Hemophilia

Hemophilia is a rare, genetic bleeding disorder in which blood does not clot properly (42). It is an X-linked recessive disorder caused by a functional deficiency in $fVIII_a$ or fIX_a. A deficiency in $fVIII_a$ results in Hemophilia A while deficiency in factor IX leads to Hemophilia B (24, 43, 44) . Each of these factors is a member of the intrinsic pathway necessary for the activation of fX. Deficiencies in either or both result in an abnormal or non-existent clot formation (24, 44) . Patients suffering from this disease usually exhibit excessive bleeding post-injury no matter how minor in many places including the skin, brain, or joints (43, 44). One of the most popular courses of treatment is replacement therapy (42). The clotting factors for which the patient is deficient are replaced in the blood by infusion. Though these factors are harvested in donated blood and have a relatively low ability to incite an immune response in foreign blood, complications can occur including antibody formation against foreign clotting factors. Immune responses can destroy them before they have a chance to work (15, 45) . Clotting factors circulate in the blood and are therefore susceptible to being exposed to other organisms circulating in the blood including viruses and consequently can be affected by them. While these complications are serious, they are rare as the screening process for donated blood is rigorous and thorough (4, 25). Other treatments are available for hemophiliacs including but are not limited to gene therapy as well as anti-fibrinolytic medicines.

Factor V_a Leiden Mutation

The Factor V_a Leiden mutation is another example of a coagulation disorder. Patients inherit the FVL allele which contains a missense mutation. A single G nucleotide is replaced with an A, which alters its functioning (46, 47). The mutation alters the arginine at the 506 position and replaces it with a glutamine. This single residue polymorphism causes fV_a resistance to the degradation that takes place during the APC pathway mediated by thrombomodulin. The Arg-506 is one of the three cleavage sites protein C uses to degrade fV_a during the cessation of the cascade. Ultimately, fV_a becomes resistant to degradation and also gains the ability to perpetuate the cascade. FVL can cause a number of anomalies including venous thromboembolism, deep vein thrombosis, pre-eclampsia, pulmonary embolism, and miscarriage. Van Mens *et al* reported a high prevalence of FVL at 5% in Caucasians (46). The review study these researchers completed was based on understanding the evolution of FVL as well as its advantages and disadvantages. Disadvantages were already mentioned. However, some of the advantages reported by van Mens included decreases in intrapartum blood loss in affected females giving birth, decreased menstrual blood loss, patient survival of severe sepsis, and fecundity (46).

Anti-coagulants

This study is focused on the inhibition of fX_a, which essentially inhibits the coagulation cascade. Therefore, it is important to note how anti-coagulants work. Ticks and leeches naturally produce these anti-coagulation agents, which have powerful

mechanisms to prevent blood from clotting. They do so as a method of feeding on the

blood of its host. Physicians often use anti-coagulants during surgical procedures to

prevent the blood from clotting as a result of an incision to the body or other induced

trauma to the circulatory vessels. Also, they are used as treatments for patients plagued

with thrombotic disorders such as atherosclerosis or prone to strokes (43).

Among some of the most commonly used anti-coagulants are coumarins (48).

These agents work by blocking the activity of vitamin K. Warfarin, a vitamin-K

antagonist, is the most used anti-coagulant in medicine to date (20). Warfarin is a

member of the coumarin family. It is such a great anti-coagulant that it is used as

pesticide, more specifically as a rat poison. As previously stated, serine proteases

including fIX and fX are vitamin-K dependent and susceptible to inhibition by warfarin.

Warfarin decreases γ-carboxylation of Gla residues in fX and other coagulation factors

by blocking the mechanism of the enzyme, vitamin K reductase, needed to replenish

active vitamin K (20). Vitamin K reductase regenerates the vitamin in hepatocytes.

Without a steady stream of active vitamin K, γ-carboxylation is blocked preventing

coagulation factors from docking on membrane surfaces. While it is the most frequently

used clot inhibitor, there are a few issues associated with its effectiveness. For example,

its effects take up to 3 days to act. Additionally, this substance has a lack of specificity in

regard to clotting factors. Its role as a vitamin-K antagonist makes warfarin difficult to

use because of its varying dose requirements and narrow therapeutic range. Its high risk

for serious bleeds especially is what makes proper dosing very difficult. It has been used

to treat deep vein thrombosis and atrial defibrillation (49). For this reason, it is often

distributed alongside a serine protease inhibitor known as heparin. Further discussion

on the significance of heparin can be found in a sub-section below. Though using these

anticoagulants is useful, one drawback is that both of them lack specificity—they

decrease the activity of every component requiring vitamin K.

The Importance of Factor X

Factor X is a very important part of the blood coagulation cascade because its

activation is primarily responsible for thrombin generation. For the purposes of this

study, a particular focus is being placed on fX. As discussed, thrombin is necessary for

the conversion of fibrinogen to fibrin. It is activated by the factor VIII and IX co-factor

complex.

Proteolytic activation within the intrinsic and extrinsic pathways causes

conformational changes in and around the active sites of fX molecules to render the

entire complex as a pro-coagulant. FX cleavage between Arg^{15} and Ile^{16} by the $fVIII_a$/ fIX_a

complex is important in its activation. This site near the N-terminus of the fX substrate

forms a salt bridge at the catalytic head (27, 33, 50). FX_a has both a light chain and a

heavy chain held together by a single disulfide bond. The light chain contains the N-

terminal glutamate domain (called the Gla domain) conserved across many coagulation

factors in addition to two epidermal growth factor-like domains. This Gla domain

contains 11 γ-carboxyglutamic acid residues which mediate electrostatic binding to the

negatively charged phospholipid membrane in the presence of divalent calcium ions.

The heavy chain contains its trypsin-like serine protease domain. At the point of

activation, the BCC has left the extrinsic and intrinsic pathways respectively and entered

the common pathway. The common pathway is where pro-thrombin is converted

through a series of steps to thrombin. Pro-thrombin contains 10 Gla residues. The

prothrombinase complex includes fX_a, fV_a, phospholipids, and Ca^{2+}. Refer to Figure III for

a depiction of the prothrombinase complex.
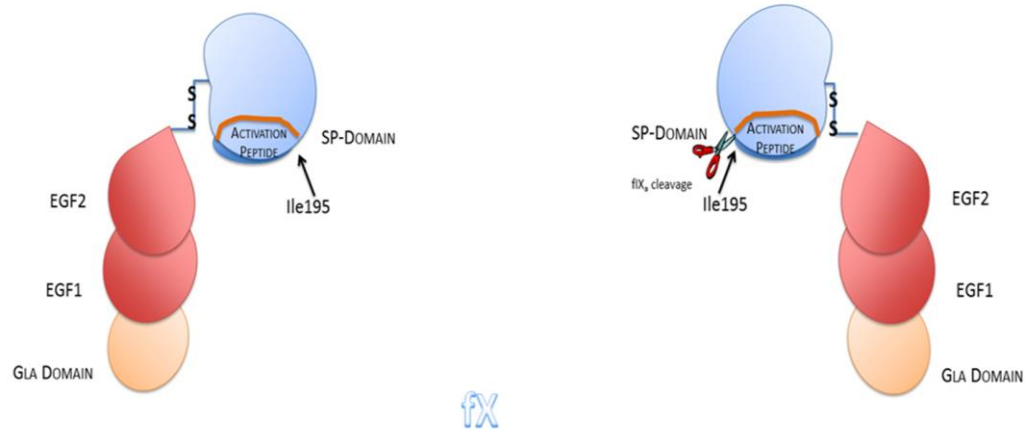
Figure VIII. General overview of fX Activation

fX$_a$ Topography

While the shape and genetic organization of fX$_a$ was mentioned briefly

throughout this report, it is important to go into further detail as it pertains to this

study. Fluorescent Resonance Energy Transfer (FRET) has been used for many years to

study biochemical agents on a molecular level such as fX (51-53) . FRET is defined in

short as a technique used to measure the interaction between two different molecules

(53, 54) . These molecules are labeled with fluorophores which are also known as either

a donor or an acceptor. The donor is said to be the molecule in an excited state and the

acceptor is the molecule nearest to it. It was first discovered by Förster *et al* (55) .

There are different types of FRET including but not limited to emission measurement or

acceptor photo-bleaching. In either case, FRET techniques monitor whether or not the

donor molecule emitted energy and whether the acceptor molecule receives it (53).

Qureshi *et al* measured distances within fX$_a$ using FRET, between donor fluorescein dye

in the active sites of FI-FPR-inhibited fX$_a$ and its N-terminal EGF deletion mutant, fX$_a$-

desEGF1 (56). FI-FPR stands for fluorescein-D-Phe-Pro-Arg-chloromethane. The Qureshi

results indicated that an EGF spacer functions to hold the fX$_a$ active site above

phospholipid membrane surfaces. Not only this, this study found that fV$_a$ alters this

orientation in an unknown but significant conformation (56). This information is integral

to understanding more about fX$_a$ itself as well as its topography.

FX$_a$ inhibition can be measured via enzymatic kinetics as seen in (57-60).

Competitive inhibition is measured using solution-based chromogenic substrate

competition assays. Chromogenic substrates are colorless chemical compounds which

react within enzymatic reactions by generating a colored product (61, 62) . This product

is usually generated because the substrate has been cleaved during the reaction. The

product of the chromogenic substrate is known based on which substrate is used. The

absorbance of the product is noted and is a point of reference during data analysis. This

technique has been used many times to characterize protease functioning (61, 62). By

measuring the rate of substrate hydrolysis (i.e. *consumption*). Additionally, Budzynski *et*

al. explains that chromogenic substrates have also been very useful in determining the

zymogen form of these proteases as well (62). With the appropriate activator,

controlled chromogenic assays can be used to monitor activated enzyme amount to the

inactivated enzymes in the area.

By measuring the rates of chromogenic substrate cleavage, other factors

involved in these mathematical calculations include inhibitor concentration, Michaelis'

constant for substrate usage (K_m), steady-rate velocity without the inhibitor (Ki), steady

rate velocity with the inhibitor, experimental substrate concentration, and the

asymptote or remaining saturating inhibitor present (58). The Michaelis Menten

constant represents the substrate concentration at which 50% of maximal enzymatic

activity in an experiment is measured. The K_i represents the point at which 50% of the

enzymatic activity is inhibited. To calculate this one would measure inhibition of the

desired activity takes place until 100% inhibition is reached. Then, calculate the slope of

each experiment and use that information to determine the K_i. The slopes of these

experiments will be useful to plot a linear graph which can indicate the relationship of

the enzymatic inhibition over time. The enzymatic inhibition time is directly linked in

determining fX topography as it relates to the distance at which fX can be inhibited using this construct and others constructed after it.

Studies report that the enhancement in the catalytic activity of fX_a in the prothrombinase complex appears to arise from two orders of magnitude improvement in the K_m or the concentration of substrate to activate prothrombin. This is because of the Ca^{2+} dependent interactions between the protease/substrate complex via their Gla domains on the same negatively charged membrane surfaces. There are a total of 5 orders of magnitude increases. Thus, the remaining three orders of magnitude improvement in the catalytic efficiency of fX_a is attributed to the co-factor function of fV_a which improves the k_{cat} (or catalytic rate) of the activation reaction by interacting with the protease and/or the substrate on the membrane surface (56).

Figure IX. A ribbon diagram of fX. Adapted from (115) and PDB ID: 2BOK.

FX Regulation

<u>Serpins</u>

While regulation of coagulation factors was discussed briefly already, it is necessary to understand the level of importance in relation to fX specifically. Clotting factors are inhibited in a number of ways. Serpins are a group of serine protease inhibitors, hence the breakdown of the word *ser* for serine, *p* for proteases, and *ins* for inhibitors. They are produced mainly in the liver and comprise almost 10% of plasma proteins. Similarly to the clotting factors, any number of serpins float freely in the blood as needed to control hemostasis. KPIs or Kunitz-type protease inhibitors are classified as serpins. This group includes anti-thrombin III (ATIII) and anti-trypsin as well. ATIII is a protease inhibitor produced by the liver which targets serine proteases including fIX_a, fX_a, and thrombin. ATIII is a small glycoprotein that contains an arginine within its active center that reacts irreversibly with a catalytic-site serine in thrombin; this creates an inactive complex [63] .

<u>Heparin</u>

Heparin, a known anti-coagulant polymer, potentiates ATIII activity [64-69] . A more specific definition of heparin would be that it is a sulfated polysaccharide. Because heparin works well and is very efficient at completing its task, it is often coupled with warfarin in clinical settings. Surgeons often employ it to decrease incidence of clotting during procedures. Enoxaparin is a pentasaccharide form of heparin [70]. It is classified as a low molecular weight heparin used to measure its own potentiation characteristics as compared with heparin. One of the benefits of this particular heparin is it has

specificity for fX. Heparin and its derivatives is a powerful anti-coagulant that works specifically by binding to a basic amino group in the ATIII active site such as arginine. When complexed with ATIII, the inactivation rate of thrombin increases significantly (71). Therefore, thrombin remains deactivated for a longer period of time than under normal circumstances, which leads to a decrease in coagulation. Once thrombin is bound to fibrin it is resistant to the inactivating capabilities of ATIII or the ATIII/heparin complex. Thus, ATIII can directly or indirectly inhibit fX_a. ATIII blocks thrombin via conformational change from forming a clot in the blood. In addition to its interactions with ATIII, heparin serves as a regulation mechanism for fX *vide infra*. Because warfarin can take as long as 72 hours to act, heparin is often used to accompany it as an additional treatment. It is said to have the highest negative charge of any natural polymer in the system and is very closely related to another polymer known as heparan sulfate located on epithelial surfaces of blood vessels because it contains structural similarities including repeating disaccharide units.

As discussed, heparin has many benefits, but it has some negative characteristics as well. Not only does high molecular weight heparin lack specificity, it can also cause clinical issues such as heparin-induced thrombocytopenia (HIT). HIT is characterized by excessive clotting within the patient along with a dramatic decrease in circulating platelets (i.e. thrombocytopenia) associated with the presence of excess heparin (20). Heparin has the ability to bind platelet factor 4 (PF4) which triggers an immune response within the body. This immune response causes the body to classify its own cells as foreign resulting in an attempt to remove them. To reverse these effects,

doctors remove heparin from the blood by filtration or allowing the body to do so via metabolic mechanisms and use another anti-coagulant.

Many studies including Deadmond *et al* found heparin to be a powerful modulator of activity of coagulation factors as well. In an effort to establish the relationship between fIX$_a$ and heparin, the Deadmond study used slow-binding kinetics and serine protease inhibitors including basic pancreatic trypsin inhibitor (BPTI), tissue factor pathway inhibitor, and nexin to determine how heparin is increasing fIX$_a$ reactivity in their presence. Each of these substances are serpins capable of inhibiting a specific portion of the coagulation process. BPTI targets fXa, TFPI targets the extrinsic pathway via tissue factor and factor VIIa. Nexin targets thrombin and has been used in recent studies to be useful with regard to central nervous system circulation (72). Results indicated that heparin modulates a specific loop at active site 99 within fIXa that allows for better inhibition by BPTI (57).

The Role of Kunitz Inhibitors

The initial discovery of Kunitz-type protease inhibitors (KPI) described them as globular soybean proteins, which act to inhibit trypsin much like the ones that regulate fX. Since then, it has been established that not all KPIs are soybean proteins. Some are human proteins; thus, KPIs are conserved across phyla structurally and functionally. KPIs operate to inhibit a number of processes within the body including digestion, blood coagulation, and inflammation. In digestion, they serve to stop or slow trypsin and chymotrypsin (48). Their influence on chymotrypsin is significantly less than its actions on trypsin. In inflammation, they work to decrease the activity of the inflammatory

enzyme, elastase allosterically (73). In blood coagulation, KPIs inhibit various factors such as kallikrein and fX_a (74).

Studies published by M. Kunitz in 1946 established the chemical properties of these inhibitors and were therefore named for him (48, 74). KPIs were characterized as stable globular proteins with estimated molecular weights of up to 24,000 Daltons (Da) and the ability to inhibit the actions of trypsin. It was also reported that the inhibitor is active only in its native or unaltered state (48). The interactions of these inhibitors in combination with their respective substrates take place on the lipid membrane to obtain full enzymatic activity. There have been so many types of KPIs isolated and reported since that time that it is commonly referred to as a superfamily. Some examples of KPIs include basic pancreatic trypsin inhibitor, substilisin inhibitors, and anti-thrombin III.

Inhibitor Structure

The molecular structure of these inhibitors contains varying combinations of ≤ 200 amino acid residues. Many KPIs contain conserved regions of disulfide bonds consisting of four cysteine residues within a single or double polypeptide chain (74) . Two distinct characteristics in the KPI structure are its secondary and tertiary make-up. The secondary KPI structure is described as devoid of an α-helix; rather, it has 12 anti-parallel β strands connected together via long loops instead. Thus, the tertiary structure is that of tri-folds of these β strands. The active site on these inhibitors is usually located near the lysine or arginine residue within the structure.

<u>Basic Pancreatic Trypsin Inhibitor</u>

Basic pancreatic trypsin inhibitor (BPTI) is a kunitz-protein inhibitor which is of particular interest in this study. BPTI is approximately 58 amino acid residues in length. It is a single-chain polypeptide, which has a molecular weight of about 6,512 Da and a size of 30Å (73, 75). It is string-like in structure and folds upon itself twice and is held together by 3 disulfide bonds. It is one of the smallest and simplest of the globular proteins (76).
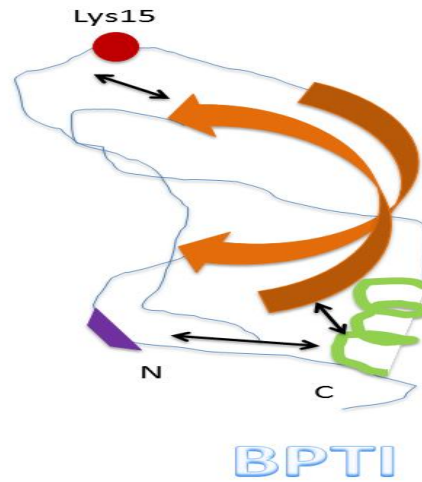
Figure X. BPTI structure including the Lys15 region, N-terminus, C-terminus, and three disulfide bonds denoted by the black double arrows. Adapted from (74) and PDB ID: 9PTI.

It functions to inhibit a broad spectrum of digestive enzymes. It works in particular to suppress the breakdown of proteins into its building blocks (i.e. amino acids) by inhibiting trypsin produced by the bovine pancreas (73) . This serves to carefully control trypsin and assure that important proteins are not degraded arbitrarily. BPTI has been heavily studied which means that its structure and mechanisms are well-characterized. It is stabilized by a small hydrophobic core formed by an internal packing of non-polar amino acid side chains. Its 3 disulfide bonds add further stability to the molecule.

Understanding how BPTI works has been extremely instrumental in understand protein-protein interaction and molecular recognition on a much clearer level. This interaction and the information it has provided prompted this study regarding the actions of fX more closely. The normal inhibition rate for fX_a with non-native BPTI ranges depending on the system and the co-factors involved and works well as a tool for comparative purposes. However, previous inhibition studies have suggested that it takes place in a two-step inhibition reaction involving a reversible binding step with a K_i of about 1.13μm/s as well as an irreversible alkylation step with a rate of k_i of about 0.65μm/s. This information is based upon fX_a interaction with fV_a and BPTI in solution or on membranes (34).

Research Design

Current Information and Fluorescence Studies

Fluorescence studies indicate that *distance of closest approach* to the membrane of the active site of several of various clotting proteases is highly important. In other words, the topology of these pro-coagulant complexes is an important concept to grasp especially because it could provide insight into their specificities (*see Future Aims for more information*). However, an understanding of topology and reactivity of these complexes is limited and, therefore, a critical gap that needs to be filled. In multiple instances, substrate binding has been demonstrated to alter the distance of closest approach. X-ray crystallography shows the distance of closest approach to be similar to the length of these coagulation proteins. Thus, these enzymes (i.e. *clotting factors*) appear to orient themselves in a vertical manner on the membrane surface with little or no bending. This inference has not, however, been confirmed using fluorescence resonance energy transfer-based studies on actual distance for optimal pro-coagulant activity. It does not provide any information on the elasticity (ability to change) of these pro-coagulant complexes or its ability to stretch in the vertical direction. Specifically, we believe that the fX_a pro-coagulant complex may achieve a maximally-effective pro-coagulant capacity when held at a specific orientation above the phospholipid membrane in its cofactor/substrate complex. Ultimately, **this study will examine and compare the range of reactive heights of the fX_a alone and fX_a/fV_a pro-coagulant complex above the membrane surface by using an inhibitor substrate (recombinant BPTI with a linker) that is anchored at specific heights above the phospholipid surface.** The length of the anchor, which gives maximal inhibition will indicate the distance of the maximally reactive catalytic site above the membrane.

Previous Studies and Study Goals

The goal of this study is to gain understanding as to the topography of fX_a in the presence of BPTI. The following are studies done by other laboratories which provide insight regarding the reactivities of the pro-coagulant complex using proven methods planned for use in this project.

<u>*Proof of concept using lipid-tethered inhibitors.*</u>

The viability of anchoring Kunitz-based inhibitors to lipid membranes for analysis was tested using Surface Plasmon Resonance (SPR) (77). The SPR experiments measure the binding of any two molecular partners in real-time without the requirement of labels via changes in the refractive index (57). In particular, researchers generated a phosphatidylcholine (PC) lipid monolayer containing nickel-chelating head groups (used for neutralization purposes) and a 6-histidine tagged BPTI was successfully attached. Soluble proteases were then flowed over the surface, and their capture by the inhibitor was monitored in real time. The results of these studies indicated that BPTI is capable of binding target proteases under these conditions (78). Though lipid-tethered KPIs can potentially retain their function with these proteases, functioning is optimal when the proteases are in a soluble state. In addition to those results, the on-rates of binding were much lower than expected based on solution-phase studies done prior (79). Negative steric effects derived from holding the KPI close to the lipid surface may be the explanation for this phenomenon as well as preference of enzymes for maintaining a vertical orientation on the membrane. Results from previous studies such as this

indicate that slow binding is predicted to increase substantially when elevated from the membrane.

Expression and purification of an active inhibitor

BPTI and other known protease inhibitors were directionally cloned into *p*ET11a DNA system using techniques outlined in Anderer *et al* (80). Recombinant inhibitor DNA was confirmed using sequencing. Next, each was expressed as inclusion bodies in BL21(DE3), an expression strain of *Escherichia coli* (81-83). The methods used in the Deadmond study are outlined here as a template for use in this study. These cells were grown in terrific broth (TB) and 50 µg/ml Carbenicillin at 37°C until log phase was reached. Carbenicillin is a common substitute for ampicillin if unavailable or the need for a more stable antibiotic is necessary (84). Terrific broth is a mixture of peptone (a soluble protein), yeast extract, and glycerol which is rich in nutrients for bacterial growth (85). After log phase was reached, 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was used to induce protein expression over a 4-hour incubation period at 37°C. Using techniques as outlined by Steinle et al, the inclusion bodies were collected and solubilized (86). Next, the solution was clarified using centrifugation. Then, a protein preparation of the inhibitor was exposed to oxidative refolding. Oxidative refolding is a process by which disulfide bonds are forged in the desired protein. Techniques as outlined by Clark *et al* were used. The 6-hour process was allowed to take place with a coordinated stirring step until the protein was properly folded. Exhaustive dialysis of the folded protein into buffer fluid was prepared for ion-

exchange chromatography. Column chromatography was used for protein expression

and purification purposes. Columns designed specifically for BPTI inhibitor affinity were

used to elute the protein. First, a column was designed to contain bound trypsin. It was

used followed by a 0-1M NaCl gradient wash. Then, the inhibitor was eluted by

deactivating bound trypsin with a very low pH elution buffer with Ca^{2+}. Then, the eluate

(expected inhibitor) was neutralized immediately using 2M Tris buffer. SDS-PAGE was

used to determine the protein concentration at 95% purification. Factors involved in

the clotting cascade including fIX_a and fX_a were activated using Russell's Viper Venom

(RVV) (87, 88). RVV activates these factors as they would if exposed to extrinsic

components. Then, standard single-stage clotting assays with a coagulometer were

performed. The reactive sites of recombinant BPTI were then titrated. This method

described the inhibitory activity of the inhibitory protein (79). FIX_a enzymes were

incubated in 150 µM biotin-EGR-ck in tricine, NaCl, $CaCl_2$, and ethylene glycol. The

active sites of these biotin-EGR-fIX_a enzymes were collected as described by Mann *et al*.

The enzyme-linked immunosorbent assay was used to quantify these enzymes with goat

anti-fIX polyclonal capture antibody as well as alkaline phosphatase-conjugated

Streptavidin. Streptavidin is a commercially available protein with a very high affinity for

biotin. Once both the reactive sites of the BPTI inhibitor and the active sites of the fIX_a

were collected, equilibrium enzyme inhibition assays were completed. Specifically,

equilibrium of fIX_a amidolytic activity (cleavage of an amide) was measured using

reversible competitive inhibition. The equation for calculating the equilibrium constant

or K_{ieq} was as follows:

$$v_s = v_O \frac{K_{ieq} \ (1 + S \ /K_m)}{1 + K_{ieq} \ (1 + S/ \ K_m)}$$

V_o describes the steady state rate obtained without inhibitor present. V_s represents the steady state rate at each concentration of the inhibitor. S represents the experimental substrate concentration, and the K_m is Michaelis' constant for substrate hydrolysis. The equilibrium rates were used to determine the activity rates based on the slow-binding enzyme inhibition assays that followed. Kinetic parameters for these assays were obtained from Morrison *et al* and Copeland *et al* (89, 90) . The conditions of these experiments were very similar to that of the equilibrium assays except that the fIX$_a$ enzymes were incubated with or without enoxaparin (low molecular weight heparin mentioned previously) before they were used in the reaction mixture. Varied concentrations of the inhibitor were co-incubated with a pre-determined quantity of trypsin. At equilibrium, a chromogenic substrate was added to measure absorbance at 405 nm for 30 minutes. These measurements indicated substrate hydrolysis over time. The results were tallied from the lowered rates of color and compared to one another to generate a curve used to calculate the amount of viable inhibitor. The lowered rates indicated trypsin inhibition. A computer software called KINEMAX was used to generate data curves for the analysis of slow inhibition. The series of equations used in this study allowed for the calculation of reactivity using variables including the time, initial absorbance, initial rate of substrate hydrolysis, and so on. This information allowed researchers to generate data plots, use the slopes of each k_{obs} calculated, and measure how close k_{obs} is to the equilibrium data.

Construction of a base vector

LAGC Consensus Sequence

An expression plasmid was constructed to include the BPTI inhibitor as well as a bacterial lipophilic tag located after the consensus sequence at the C-terminal called *LAGC*. This is the common sequence for lipid-anchored bacterial proteins named for [LVI][ASTVI][GAS]C amino acid sequences (83). Biological systems including *Staphylococcus aureus* and *E. coli* bacteria have developed this machinery as a means of survival. Nutrient uptake, signal transduction, growth, and development are all processes dependent upon many membrane-bound proteins (83). Additionally, ELISA or enzyme-linked immunosorbent assays and other biosensors use membrane-bound proteins in order to successfully complete tasks such as diagnostics and substance identification. The Kamalakkannan study demonstrated that cloning a target gene sequence behind the LAGC sequence led to lipid-anchored expression of otherwise normally secreted proteins (83). These researchers used two respective strategies to genetically alter a non-lipoprotein into a functional lipoprotein. This motif is recognized by bacterial machinery, more particularly an enzyme called diacylglyceryl transferase. This enzyme modifies the moiety sequence in a two-step reaction leading to the cleavage between the Gly and Cys residues. Then, there is an addition of an N-acyl, s-diacylglyceryl moiety to the N-terminal Cys (91-93) . The moiety is highly hydrophobic and incorporates in the lipid membrane, and its following peptide sequence is expressed in the periplasmic space. Figure XI is a depiction of the chemical structure of this moiety.
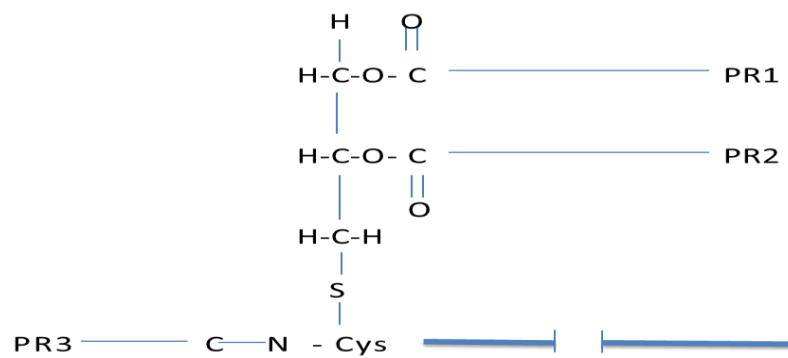
Figure XI. N-acyl, s-diacylglyceryl moiety group at the N-terminal Cys group associated with the LAGC consensus sequence. PR1 and 3 are the palmitoyl residues. PR2 may be a palmitoyl or oleayl residue. Adapted from (83).

Inhibitor Ruler Proof-of-Principle

The Deadmond *et al.* plasmid developed was modeled exactly as the

Kamalakkanan vector with a *p*ET11a backbone modified to contain a ribosome binding

site, a 10 base-pair spacer, the sequence coding for the 23 amino acid leader sequence,

and followed by sequences coding for Leu, Ala, Gly, and Cys. The leader sequence is of

significant importance as it was taken from the apyrase gene (83). *Shigella* apyrase is a

model protein used in the Kamalakkanan study for a number of reasons. It is easy-to-use

and assists in the release of phosphate groups from organic and inorganic

pyrophosphates (93). It guides the protein into the periplasmic space, which supplies

the oxidative environment needed for the inhibitor to spontaneously fold. Finally, the

construct contained a restriction enzyme site along with the sequence for BPTI.

Verification to confirm this construct was built optimally was done via sequencing.

Relipidation of recombinant proteins

Recombinant BPTI with the LAGC-tag was relipidated with 100%

phosphatidylcholine lipid vesicles. The relipidation process is completed by incubating

the recombinant DNA in the presence of phosphatidlycholine as well as detergent

composed of phospholipids and glycopyranoside. It is necessary in order to ensure that

the recombinant proteins will work as expected to that of the body. More specifically, in

experiments performed on prothrombotic surfaces attempting to mimic *in vivo* injuries,

it is necessary that modified factors be relipidated for attachment to surface bound

collagen fibers as seen in (94). After that, the mixture is combined with a solution of

chloroform and methanol and dried onto a thin film in liquid nitrogen. It is then placed

under a vacuum for a minimum of 2 hours to remove any undissolved organic solvents.

Next, the film is dissolved in a mixture of glycopyranoside, Tris-HCl, and apoprotein.

Upon incubation for at least an hour, the detergent is removed by dialysis. The

relipidated protein is treated for up to 2 days with buffer until it is ready for use. Protein

confirmation and concentration was verified by gel filtration chromatography. In this

experiment, a large portion of recombinant BPTI co-eluted with a lipid fraction.

Whereas, native (non-lipid-anchored) BPTI elution profiles eluted after the lipid fraction

using similar conditions.

EA$_3$K Linker Sequence

The EA$_3$K linker sequence is the portion of the recombinant plasmid that serves

as the molecular ruler. Its sequence codes for a string of repeated amino acid residues

that read (using single letter coding) as *E-A-A-A-K*. They are rigid and helical in structure.

The structure is important as they allow for better control regarding the length in which

the inhibitor will be raised off of the membrane surface. Each linker is approximately

10Å in length. The structural height of the linker is about 7.5Å as they attach to one

another in a unique diagonal manner (*refer to* Figure XII). Arai *et al* used the EA$_3$K linker

to enhance protein fusion experiments (95). The linkers were an important tool in

bridging α and β domains between each protein to create a fully functional chimeric

protein. The oligonucleotide sequence designed for this study contains ten of these

linkers. This means that the recombinant BPTI would be raised 75Å from the membrane

surface.

In addition to the E-A-A-A-K repeats, the sequence also contains a G_4S flexible linker. This linker as suggested by its name codes for peptide sequence, *G-G-G-G-S*. One important reason these linkers are expected to work well in the recombinant DNA is because they have been used successfully in the past and do not typically alter protein activity. Su *et al* used this linker to allow for recombinant erythropoietin designed for the study to enable the moiety of the protein the ability to form its native three-dimensional structure properly as well as function in its environment as naturally as possible (96). Its hydrophilic and neutral chemical composition allows these processes to take place. Ultimately, the G_4S linker provides the protein with the ability to float in a flexible manner as it would in the environment and increase its chances of attachment to serine proteases in the area (97).
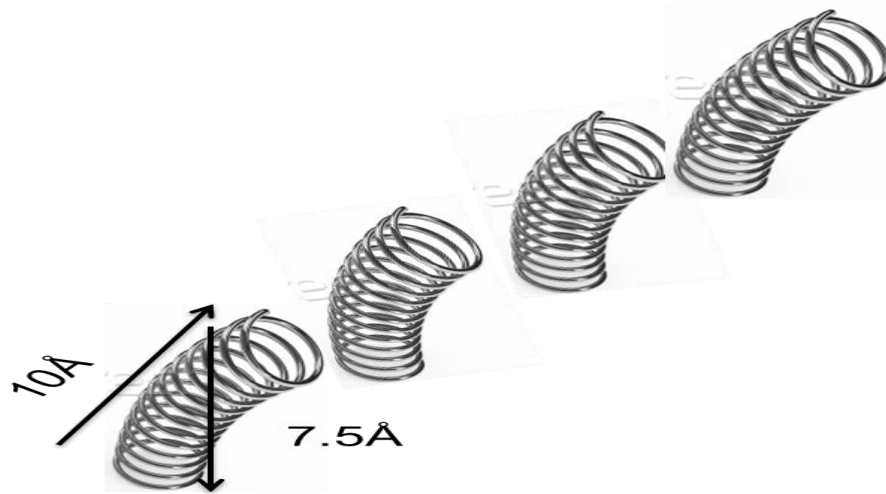
Figure XII. EA$_3$K Linker Structure. This is a theoretical depiction of what the EA$_3$K linker sequence resembles. The length is demarcated here as well.

Overall Study Aim

This study proposes that as the inhibitor is raised from the membrane to an optimal level binding is increased; therefore, its functioning is improved. In an attempt to confirm this hypothesis the following steps were taken.

Specific Aims:

<u>Specific Aim 1: Construct a plasmid using a *p*ET11d backbone containing the LAGC and EA₃K sequences respectively</u>

Preliminary results obtained from Neuenschwander *et al*. demonstrate proof-of-principle for generation of lipid-anchored BPTI (57, 79). The construct will generate sequences encoding a bacterial lipid-anchor consensus site separated by a set number of linker sequences. More specifically, a segment coding for an LAGC leader sequence and EA$_3$K linker sequences will be directionally cloned into *p*ET11d *E. coli* DNA. Below is a simplified image of what the generated construct will look like:
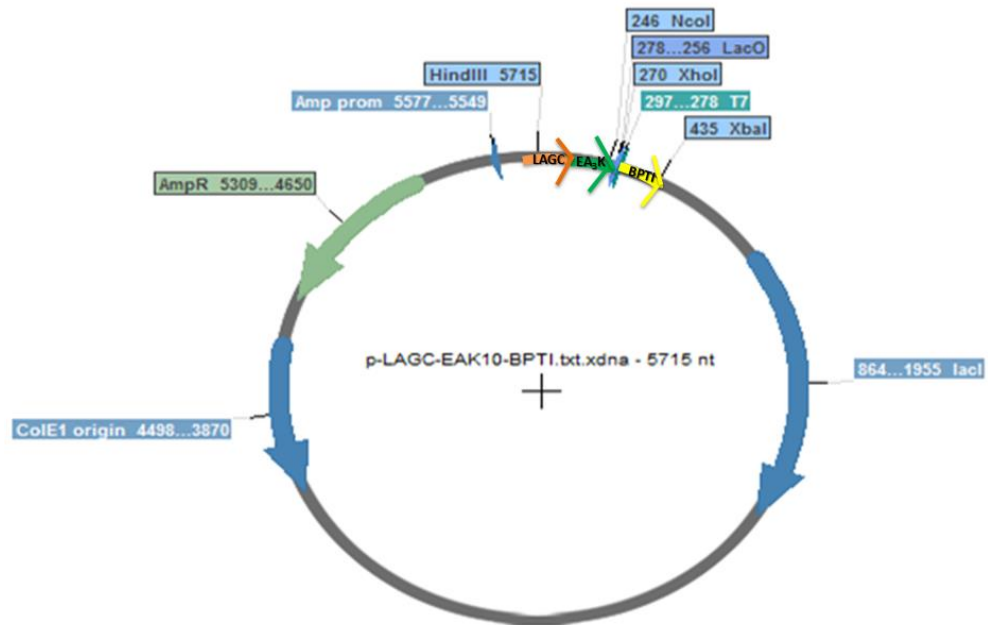
FigureXIII. Projected graphic map of p-LAGC-EAK10-BPTI. LAGC sequence is denoted in the color orange, EA$_3$K in green, and BPTI in yellow. Restriction enzyme sites are noted in the black outlined blue boxes. The ampicillin promoter (Amp prom) and repressor (AmpR) region is noted. The lacI region is a part of the pET11d is a mutated promoter region that controls the lac operon. ColE region of this bacterial plasmid that aids in the survival of the plasmid.

Specific Aim 2: Use the p-LAGC-EAK10 plasmid to incorporate BPTI inhibitor creating the lipid-anchored "inhibitor ruler". Previous research in topography and analysis of serine proteases indicate that their range of attachment lies within this distance from the membrane surface (56, 69, 98, 99). The engineered constructs will be sent for sequencing for verification. Once there is confirmation that this construct has been properly cloned, it will be transfected into bacterial cells for expression. Purification will be performed using affinity chromatography as was done in the Neuenschwander study. Affinity chromatography is a technique in which biochemical agents are separated based on specific interaction between antigen and antibody or enzyme and substrate, for example. Specifically, we will immobilize the enzyme trypsin to the solid support, and use this to separate, purify, and elute our newly generated inhibitor product from the remainder of the mixture. The solution-phase inhibitory activity of each of these fX_a inhibitors will be characterized and compared with the unmodified inhibitor. Once this is complete, it will be used in the next phase of the study.

Materials, Methods, and Results:

Specific Aim 1: Construction of a plasmid using a pET11d bacterial backbone containing the LAGC and EA_3K sequences respectively (p-LAGC-EAK10). To generate a lipid-tethered inhibitor construct as a tool to study distances of fully pro-coagulant complex active sites, standard molecular cloning techniques were used (57, 78, 79, 83, 100). Standard experiments in cloning usually require at least seven steps. These steps are as follows and include but are not limited to: 1) choosing a host organism and cloning vector, 2)

vector DNA purification and preparation, 3) design, preparation, and insertion of the desired DNA to be cloned, 4) confirmation that recombinant DNA has been created, 5) introduction of new DNA into host organism, 6) selection of colonies possibly containing new DNA, and finally, 7) screening of said clones for the appropriate biological properties (57, 78, 79, 83, 100).

Choosing a Host Organism and Cloning Vector

For this study, *Escherichia coli* was used as the host organism. *E. coli* is a bacteria used commonly in molecular cloning because of its versatility, availability, and ability to grow quickly (101) . It is these same reasons that make it a great system to use for these experiments.

Upon choosing this bacterial system, the *p*ET11d vector system was selected as the cloning vector (102). The *p*ET11d system contains an ampicillin-resistance gene that allows selection for transformants. Other studies have used this system successfully not only to create recombinant DNA but for protein expression as well (101). In addition to antibiotic resistance, *p*ET11d also contains unique restriction endonuclease sites, in particular, *Hin*dIII and *Nco*I sites which allows directional cloning of the LAGC and EA$_3$K linkers into the vector system respectively. In Figure XIV below, a depiction of the *p*ET11d vector system has been provided. Note the many cleavage sites available for manipulation particularly those at the *Hin*dIII, *Nco*I, *Bam*HI, and *Xba*I sites. The design of each DNA insert (i.e. LAGC and EA$_3$K) was based on these areas within *p*ET11d (see next page).
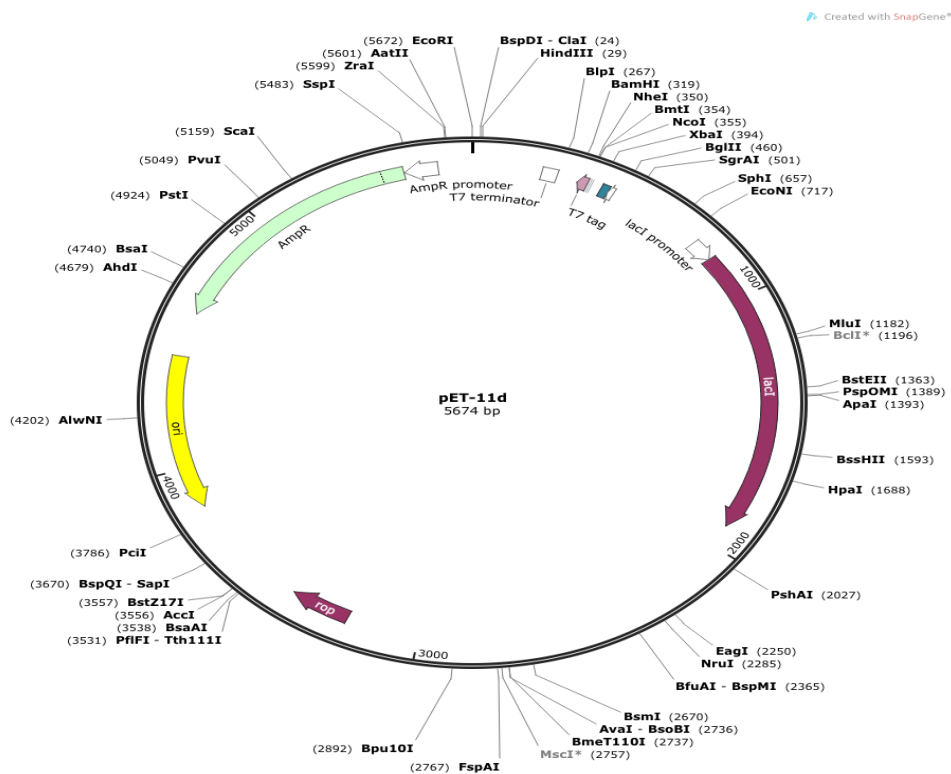
Figure XIV. Graphic DNA map of pET11d vector. Depicted in this map of the 5,674 base pair plasmid are the ampicillin promoter and repressor region, lacI promoter, and termination sites. www.snapgene.com/pet11d

Preparation of Vector DNA

<u>Competent Cell Generation</u>

Competent cells were generated using frozen glycerol stocks of DH5α cells (82). While still frozen, the cells are scraped onto a metal loop using sterile technique. The cells are then streaked onto an LB plate and incubated overnight at 37°C. The next day, a single well-isolated colony was selected and inoculated into 5 ml of LB in a 10-ml Falcon© tube. The inoculated colony was allowed to grow overnight while shaking in a 37°C incubator at 125 rpm. Then, 1 ml of the overnight culture media was inoculated into 100 ml of fresh LB media. This 250-ml Erlenmeyer flask was shaken for 3 hours at 37°C and 125 rpm to ensure the cells reached OD_{600nm} of 0.4. Next, the cells were placed on ice for 10 minutes. The cells were then transferred into pre-chilled 50 ml conical tubes. Cells were centrifuged at 3000 x *g* in a 4° C centrifuge for 10 minutes. The supernatant was removed and the pellet resuspended in 1.6 ml of ice cold 100 mM $CaCl_2$. The cells were incubated on ice for 20 minutes. After the cells are washed twice, 0.5 ml of cold 80% glycerol was added to the suspension. Finally, 500 μl aliquots of cells were prepared in 1-ml cryogenic vials and placed into a -80°C freezer for use.

<u>Plate Preparation</u>

To prepare LB + antibiotic plates, 800 ml of deionized water was placed in a large flask on a stir plate. Then, 25 g of LB mix including 10 g of tryptone, 10 g of NaCl, and 5 g of yeast were weighed and added to the flask (85). Next, 15 mg of agar was added to the flask. All components were stirred until they appeared evenly mixed. Then, about 200 ml of deionized water was added to the mixture and allowed to stir. Once

that is completed, the LB/Agar mixture was placed in an autoclave for one hour for sterilization. After sterilization, the mixture was allowed to cool until 60°C or less is reached. Then, 10 ml of ampicillin (100 mg/ml) was added to the flask. Finally, about 25 ml of LB agar + Ampicillin was poured into each plate. The recipe makes up to 40 plates.

DNA Extraction and Purification

pET11d DNA was obtained from BL21(DE3) expression strain DNA gold stocks in the laboratory provided generously by Dr. Francis Jenney, Philadelphia College of Osteopathic Medicine—Georgia Campus. The strain was inoculated into 5 ml of Luria broth (LB) containing a 1:10 dilution of ampicillin (10 mg/ml) and shaken overnight at 37°C (85). Using the Strataprep™ Plasmid Miniprep kit (Agilent Technologies, Santa Clara, CA), the plasmid DNA was purified using the manufacturer protocol (103).

This purified plasmid DNA was transformed into DH5α growth strain competent cells. First, previously cultured DH5α competent cells were obtained from the -80° C freezer and thawed on ice. While the cells thawed, super optimal broth with catabolise repression (SOC) media was prepared using sterile super optimal broth (SOB), 1M $MgCl_2$, and 40% glucose solution. Media such as this is an optimized version of LB used by researchers attempting to grow and express DNA plasmids in *E. coli* and other bacteria (82). SOB broth containing a mixture of bacto-tryptone, bacto-yeast extract, NaCl, KCl, $MgCl_2$, $MgSO_4$, and deionized water (85). Then, 50 µl of thawed competent cells were pipetted into a 1.5 ml eppendorf tube on ice. Next, 10 ng of pET11d DNA [20 ng/µl] was pipetted into the tube and incubated on ice for 30 minutes. Once this

incubation was complete, the cells were heat shocked at 42°C in a water bath for 30 seconds. Then, the cells were incubated on ice for 2 minutes. Next, 1 ml of the prepared SOC medium was added to the tube. The tube was then placed into a 37°C incubator for 1 hour. After incubation, 100 µl of the cell solution was spread onto an LB + Ampicillin agar plate. The plate was placed into the 37°C incubator to incubate overnight.

The *p*ET11d DNA was extracted from these DH5α colonies in the laboratory using the PureYield™ Plasmid Midiprep System (103, 104). It was found that the DNA recovery yielded using this kit was less than ideal for this study. Thus, the Strataprep EF Plasmid Midiprep Kit (Cat#400721) from Stratagene was used for the remainder of the study (105). The protocol associated with this kit was followed using the steps described here. First, a single colony of DH5α cells was inoculated into 200 ml of LB culture media and vigorously shaking overnight at 37°C. The next day, the bacterial culture was pelleted by centrifugation in 50-ml conical tubes at 4750 x *g* for 1 hour. The clarified supernatant was drained and discarded. Next, the pellets were vortexed for resuspension in the small amount of culture medium remaining in the tubes. After that, the resuspended pellets were combined into one tube. Then, 7 ml of Solution 1 was added to the tube and mixed vigorously with the vortex until the brown mixture turned blue in color. *Solution 1* is a mixture of 50 mM Tris HCl, 10 mM EDTA, 50 ug/ml of RNase A, and 0.5% (w/v) SDS. This solution contains agents which degrade any RNA present in cells. Next, 7 ml of *Solution 2* was added to the tube. Contents were mixed by inverting the tube several times until the contents uniformly turned purple in color. This solution contains 0.2 M NaOH and 1% (w/v) SDS. Solution 2 is responsible for degrading all protein in the

cells. The contents in the solution have been used in other studies to degrade proteins

(106). After this, 2 ml of *Solution 3* was added to the tube. The contents were once

again mixed by inversion until the color changed to blue. Solution 3 is a proprietary

reagent. Therefore, exact components are not available for public information. Once all

3 solutions were added, the tube was centrifuged at 4750 x *g* for 15 minutes. This

centrifugation resulted in a compact, light blue pellet at the bottom of the tube, a thin

blue film on the surface of the supernatant. The supernatant was pipetted into a fresh

50-ml conical tube taking care not to collect any of the pellet of thin film.

Next, 10 ml of *Solution 4* was added to the tubes and the contents mixed by

inversion. Solution 4 is also proprietary, but it is a chaotropic salt which assists in

precipitating all DNA present within the cells. After the contents were mixed properly,

the mixture was added to a midi-spin cup seated in a 50-ml conical tube with the cap

tightened loosely. Sample was then centrifuged at 1500 x *g* for 10 minutes. The midi-

spin cup containing the plasmid DNA was removed from the 50-ml tube and liquid

discarded. The midi-spin cup was then placed back into the 50-ml conical tube. Next, 20

ml of wash buffer was added to the receptacle. The wash buffer contains 10 mM Tris

HCl, 100 mM NaCl, 2.5 mM EDTA, and 10 mM Ethanol. As indicated by its name, it

serves as a way to cleanse the DNA of any impurities that may have been caught in the

fiber matrix of the midi-spin cup. The sample was spun at 1500 x *g* for 3 minutes. The

liquid was discarded in the same manner as mentioned, and the tube was spun once

more to ensure all wash buffer and any impurities it may contain was removed. The

midi-spin cup was then transferred to a fresh 50-ml conical tube. Next, 500 ml of elution

buffer (i.e. nuclease free water) was added directly onto the fiber matrix at the bottom of the midi-spin cup. This was incubated at room temperature for 5 minutes. After the time elapsed, the tube was centrifuged at 1500 x g for 3 minutes. The purified plasmid DNA eluted into the receptacle was transferred to a 1.5 ml eppendorf tube.

   To determine the concentration of this DNA, gel electrophoresis was used. A 0.8% agarose gel was used to run the negatively charged DNA toward the positive electrode on the electrophoresis apparatus. Agarose gels are commonly used as they are composed mainly of agarose, water, and 1M Tris and EDTA buffer. Agarose is a polysaccharide found in algae. This composition makes it easy for DNA to travel through the hole-filled gel. The percentage of this gel was used as it was recommended as the ideal percentage size of the DNA being separated (roughly 5000-6000 base pairs in size). About 10 μl of DNA and bromophenol blue stain (0.0025 mg/ml) were loaded onto the gel and run at 100V (constant voltage?) for 30 minutes. The bromophenol blue stain solution is composed of bromophenol sodium salt and deionized water. New England Biolabs Quick-load 1kb ladder used to determine the DNA size and concentration. Figure XV below is the DNA extracted using the aforementioned Stratagene kit.
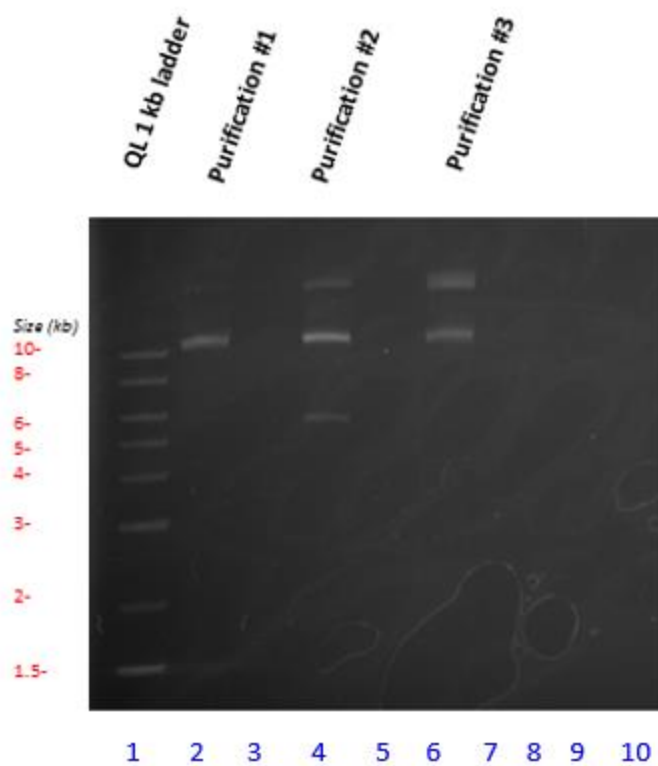
Figure XV. 0.8% Agarose Gel used to determine the concentration of pET11d DNA extracted from Stratagene DNA Purification Kit. Lane 1 contains 10 ul of New England Biolabs Quick-load 1kb DNA Ladder. Lanes 2, 4, and 6 contain DNA collected from 3 separate purification experiments. It was determined that the DNA in lanes 2 and 6 are *p*ET11d.

<u>Restriction Digests</u>

Restriction endonucleases were used to prepare the purified vector DNA for insertion of the custom LAGC and EA$_3$K sequences ordered from Integrated DNA Technologies. These sequences were introduced at the same time and were designed to insert into the vector DNA in a certain order. This is known as directional cloning. Therefore, two sites were selected to accommodate for the sticky ends that correlate with these recognition sequences. For directional cloning, *Nco*I and *Hin*dIII sites were selected as the areas to be digested for insertion. Cloning in this manner has been completed successfully by a number of studies over the last few decades (107).

Restriction enzymes generate sticky ends within a vector which allow for the insertion of foreign DNA. These sites were selected because they are unique within *p*ET11d and, therefore, should cut at its only respective cleavage recognition site. The recognition site for *Nco*I from 5' to 3' is *C/CATGG* (107). For this reason, the EA$_3$K sequence contains a sticky ends coding for the complementary site to *Nco*I. The LAGC sequence was designed with sticky ends that correlate with the *Hin*dIII recognition site *A/AGCTT*. To ensure that both sequences attached well with one another sticky ends for *Bam*HI were included in each. The *Bam*HI recognition site is *G/GATCC* (108). This site is important not only to incorporate these 2 sequences, but also because this *Bam*HI site was already within the *p*ET11d vector. Cleaving the DNA with both *Nco*I and *Hin*dIII should have removed that site. Replacing this region allowed for screening of the final product to ensure that the foreign DNA cloned in properly. Restriction digests involve the following components mixed together in one tube: DNA, 10X NEB buffer, bovine

serum albumin (BSA), ≥ 10 units of desired restriction enzyme with attention to star

activity, and deionized water. Bovine serum albumin is used as a reagent used to

stabilize enzymes in restriction digests. A restriction digest using purified *p*ET11d DNA

was prepared using the reaction conditions listed below in Table I. These components

were combined at the volumes listed below into a 1.5 ml eppendorf tube. The

restriction enzyme was added last. The tube was then incubated for 1 hour at 37°C.

After incubation, 5 µl of 1M Tris/0.1M EDTA (i.e. *stop solution*) was added to cease the

reaction.

Reaction Components	Restriction Digest with *Nco*I (µl)	Restriction Digest with *Nco*I (µl)	Restriction Digest with *Nco*I (-) Control (µl)
BSA	0.5 100 µg/ml	0.5 100 µg/ml	0.5 100 µg/ml
NEBuffer 3 *[I] = 10X*	5 1X	5 1X	5 1X
ddiH2O	32	32	43.5
DNA *[Final] = 60ng/ul*	10 600 ng	10 600 ng	1 60 ng
Restriction Enzyme **10 units/µl**	2.5 25 u	2.5 25 u	2.5 25 u
Total	50	50	50

Table IV. Restriction Digestion with *Nco*I. This table represents the reaction conditions
used to prepare pET11d for DNA insertion.

After the digestion was complete, the digested DNA was loaded onto a 0.8%

agarose gel and run on a gel electrophoresis apparatus at 100V for 45 minutes. Upon

viewing the gel using the [Alpha Imager], the desired band was excised from the gel

using a razor and placed into a 15 ml conical tube. After excision, the Strataprep DNA

Gel Extraction Kit (Cat #400766) was used to extract the DNA from the agarose gel.

Specifically, 6.36 ml of extraction buffer was added to the 15-ml conical tube. Then, the

tube was incubated in a 50°C water bath for 10 minutes (mixing occasionally). After the gel was completely dissolved, the mixture was transferred to a 2-ml receptacle with a microspin cup seated in it. Due to volume, 825 µl of the mixture was transferred at a time. After the mixture was transferred, the tube is centrifuged in a microcentrifuge at 14,000 x g for 30 seconds. The DNA is retained in the fiber matrix of the microspin cup. Thus, after centrifugation, the liquid was decanted. This process was repeated until all the liquid had been centrifuged and decanted. Then, 750 µl of wash buffer (with 100% ethanol added) was pipetted into the microspin cup. The tube was centrifuged at 14,000 x g for 30 seconds. The wash buffer was decanted, and the tube was centrifuged at 14,000 x g for 30 seconds ensure that all wash buffer was removed. The micro-spin cup was then transferred to a fresh 2-ml receptacle tube. Then, 50 µl of elution buffer was added directly on top of the fiber matrix of the micro-spin cup and incubated for 5 minutes. Lastly, the tube is centrifuged at 14,000 x g for 30 seconds. The extracted pET11d + NcoI DNA was seated in the 2-ml receptacle tube. Figure XVI below is a depiction of the NcoI digest that was gel extracted.
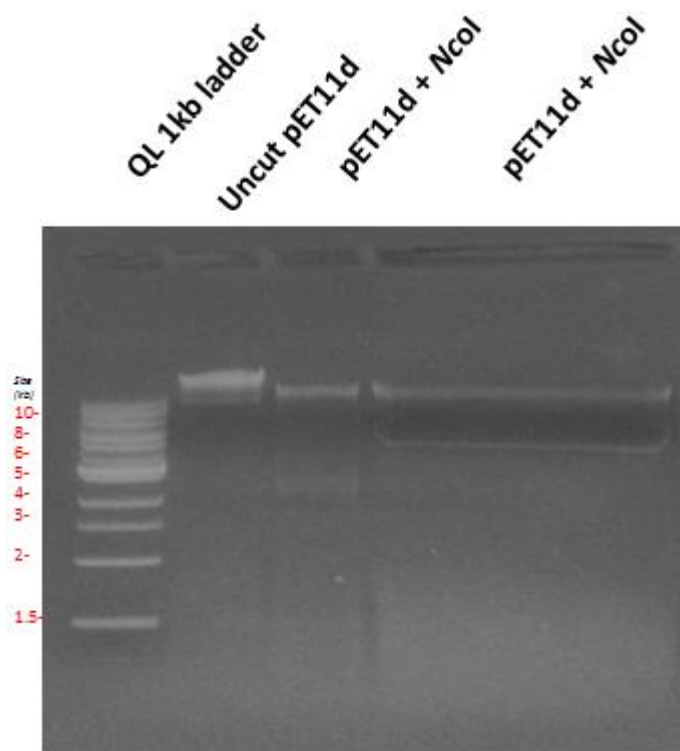
Figure XVI. Restriction Digest with *Nc*oI. 0.8% agarose gel of *p*ET11d digested with *Nc*oI and gel extracted using Strataprep DNA Gel Extraction kit.

Next, the gel extracted DNA was digested with *Hin*dIII. This experiment was conducted in the exact manner as the *Nco*I digest. However, a gel electrophoresis experiment and extraction were not necessary as this second digestion would be indistinguishable from the first digestion.

<u>Dephosphorylation</u>

Next, the *p*ET11d + *Nco*I + *Hin*dIII was treated using New England Biolabs Calf Intestinal Alkaline Phosphatase (AP-CIP). This was done as a precautionary measure to ensure that the sticky ends generated by the restriction enzyme digests could not close before any foreign sequences could be introduced. Alkaline phosphatase works by removing the phosphate group (PO_4^{-3}) from the 5' end of DNA strands (109). DNA treatment with AP-CIP is carried out in a similar manner to a restriction enzyme digestion. Therefore, Table V below is a representation of the reaction conditions the DNA was exposed to for dephosphorylation.

Reaction Components	Amount used (ul)
10X NEB Buffer 3	11
	1X
Calf Intestinal Alkaline Phosphatase	1
(10 units/µl)	10 u
DNA	96
5.5 ng/µl	528 ng
dd$_i$H$_2$O	2
Total	110

Table V. Dephosphorylation of pET11d + *Nco*I + *Hin*dIII

Water, NEB buffer 3, AP-CIP, and DNA were combined in 1.5 ml eppendorf tube and

incubated for 1 hour in a 37°C incubator. Figure XVII A/B is an agarose gel depicting

double digested *p*ET11d after treatment with AP-CIP and gel extracted for use.
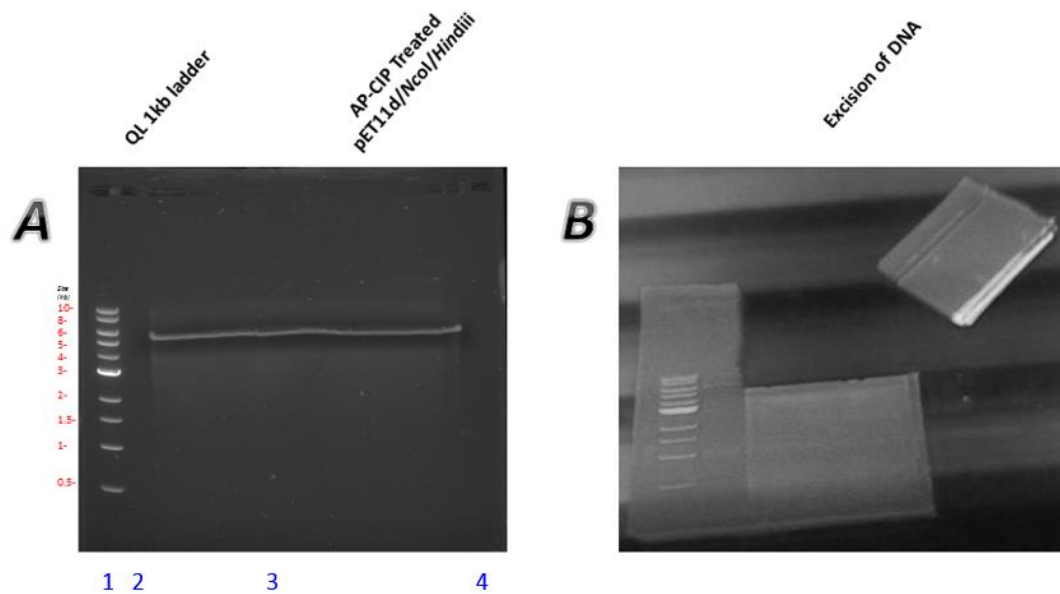
Figure XVII A and B. 0.8% agarose gel with alkaline phosphatase treated DNA. Lane 1 is QL 1kb ladder and Lane 3 is dephosphorylated *p*ET11d backbone DNA.

Preparation of Insert DNA

As mentioned previously, the LAGC and EA$_3$K linker oligonucleotide sequences were designed and purchased through Integrated DNA Technologies. Among its services, this company provides custom oligonucleotide synthesis using artificial gene synthesis. Gene synthesis techniques have been used since the 1970s to generate usable, customizable DNA constructs. Nucleoside phosphoramidites are synthetic building blocks used to construct oligonucleotides added one at a time to create a chain. Currently, oligonucleotide sequences can be ordered at a length of up to 200 base pairs each. Goeddel *et al* generated synthetic insulin using synthetic techniques including PCR and radioimmunoassay (110). These synthetic DNA sequences are generated in forward and reverse sequences which are shipped in lyophilized conditions at a 20 nmole concentration. Thus, it was necessary to re-suspend each one in the appropriate buffer. Figure XVIII is a depiction of oligonucleotide synthesis and how oligonucleotides with sticky ends anneal to each other.
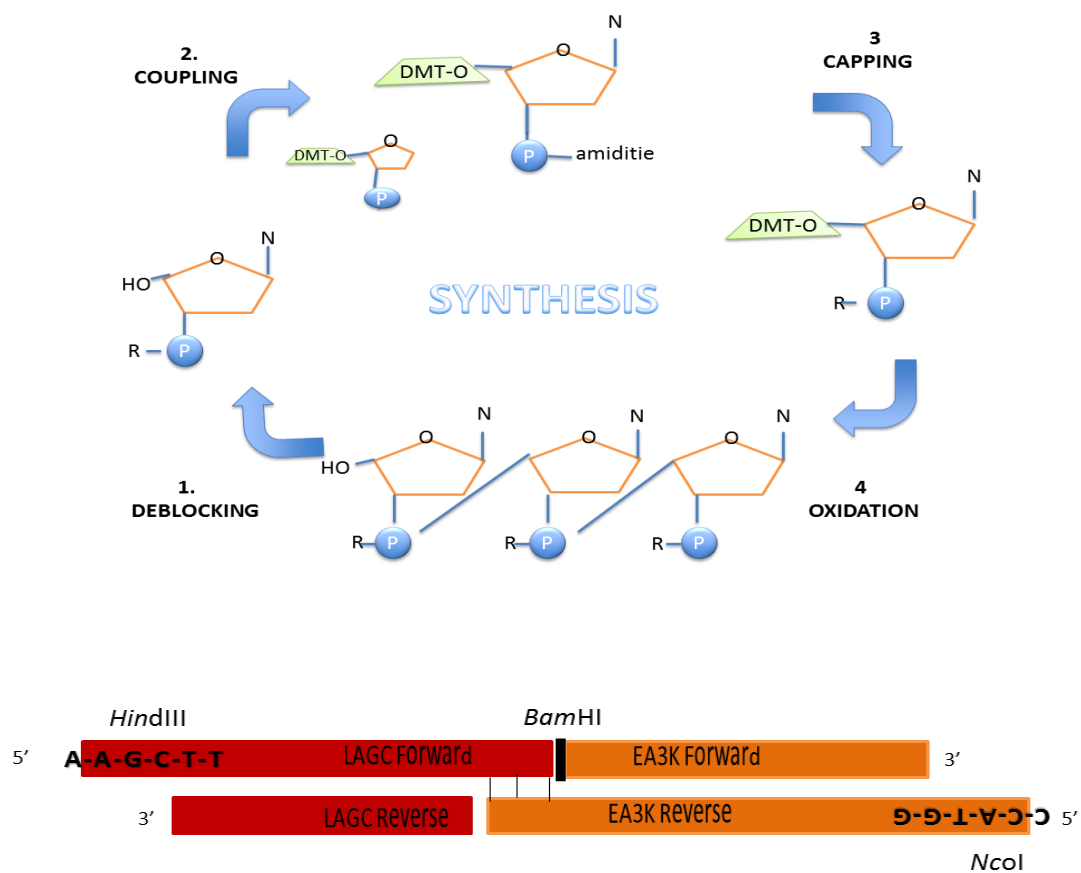
Figure XVIII A and B. A—Oligonucleotide Synthesis. B--General Annealing of Oligonucleotides with Sticky Ends.

Fresh, sterilized 10 mM Tris/ 1mM EDTA buffer was selected as the re-suspension buffer. This media was recommended by the manufacturers because its composition provides a constant pH environment for the synthetic DNA. First, each oligo tube was centrifuged at 14,000 x *g* for 30 seconds to ensure none of the lyophilized product is lost when the tube is opened. Then, approximately 200 µl of this buffer was added to each lyophilized oligo pellet for a final concentration of 100 uM respectively. This amount was added based on IDT recommendations to suspend as follows:

`# nmole x 10 µl/nmole = x µl TE buffer to be added for [100 uM]`

After re-suspension, the oligonucleotides were placed in the -20°C freezer. For use, each oligo forward and reverse sequence was annealed at a 1:1 molar ratio. This is completed as indicated by Table III below. First, 500 ml of water was heated in a beaker until it reached 95°C. Then, 10 µl of nuclease free water was added to an eppendorf tube. Next, 5 µl of DNA per oligo in µl was placed into the tube. Once these components are combined, the tube containing the combined oligonucleotide sequences was placed into the water beaker which was heated until the temperature of the water was 95°C. Annealing began once that tube was placed into the beaker and the water temperature was allowed to decline until a temperature of 30°C was reached. Confirmation that annealing occurred was verified using gel electrophoresis as depicted below in Figure XIX. A successfully annealed oligonucleotide will display a band at the expected size on the agarose gel. In this case, a band would be visible near the 100 bp rung for the LAGC oligonucleotide and near the 200 bp rung for the EA$_3$K linker. If annealing did not take

place, it is possible that a band may not be visible on the gel at all. Furthermore, an

incomplete or incorrect annealing may be denoted by a smear on the gel.

LAGC	EA₃K Linker
100 µM LAGC-Fwd 5 µl	100 µM Linker-Fwd 5 µl
100 µM LAGC-Rev 5 µl	100 µM Linker-Rev 5 µl
10 uL ddiH2O	10 ul ddiH2O
[Final] = 50 µM 20 µl	[Final] = 50 µM 20 µl

Table VI. This table contains the components used
to anneal the oligonucleotides. Components were
combined and heated in a 95°C water beaker until
the water reached 30°C.
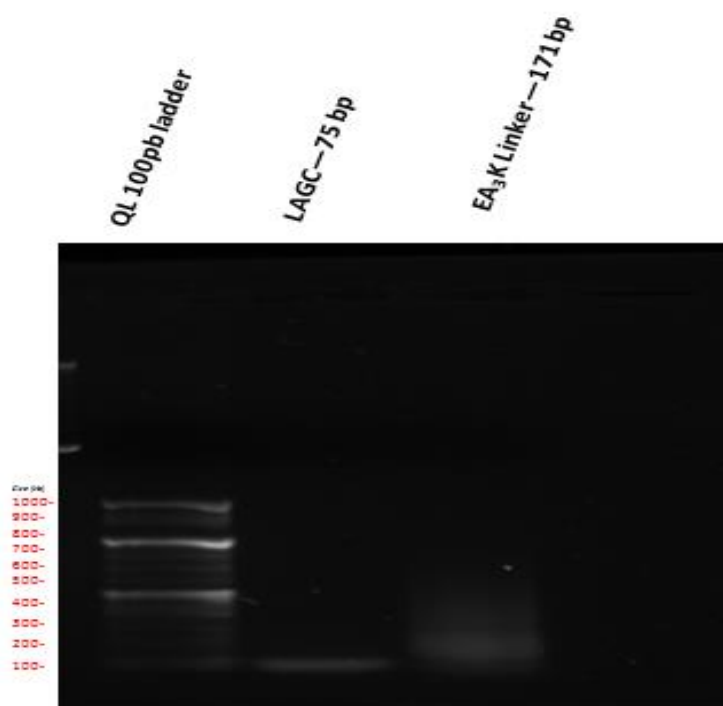
Figure XIX. 0.8% Agarose Gel with Annealed LAGC and EA$_3$K Linker Oligonucleotides.

Insertion of LAGC and EA₃K Linker DNA into pET11d

<u>Ligation</u>

The dephosphorylated *p*ET11d + *Nco*I + *Hin*dIII, LAGC, and EA₃K Linker DNA were combined in the attempt to create recombinant DNA using T4DNA ligase (New England Biolabs, Ipswich, MA). This reaction was set up such that a 3:1 insert-vector ratio was used. The exact reaction components and volumes are listed below in Table IV. Once all calculations were complete to ensure the insert-vector ratio was as expected, the components were mixed together making sure to pipet each gently into a 0.5 ml strip eppendorf tube. The tubes were then placed into the Thermocycler© PCR machine to be incubated overnight at 16°C.

REACTION COMPONENTS	LIGATION #1	LIGATION #2 (CONTROL)
10X T4 DNA LIGASE BUFFER* ~NEB#3	3 μl [Final] = 1X	3μl [Final] = 1X
VECTOR DNA (5.674 KB)	20 μl 100 ng	20μl 100 ng
LAGC INSERT DNA (75 BP)	0.34 μl 300 ng	0.34 μl 300 ng
EA₃K LINKER INSERT DNA (171 BP)	0.8 μl 300 ng	0.8 μl 300 ng
DDᵢH₂O	4.86 μl	5.86 μl
T4 DNA LIGASE 10 UNITS/μL	1 μl 10 u/ μl	0 μl 0 u/ μl
TOTAL	30 μl	30 μl

Table VII. Ligation of *p*ET11d backbone DNA with LAGC and EA₃K Linker

Introduction of Recombinant DNA into Host Organism

The tubes were retrieved from the thermocycler and chilled on ice. DH5α competent cells retrieved from the -80°C freezer were thawed on ice for 10 minutes. An aliquot of 50 µl of these thawed cells were placed into 14 ml tubes for ligation 1, ligation 2 (- control), and pUC18 DNA (+ control/transformation efficiency). This is transformation process is very closely related to the one done for obtaining *p*ET11d DNA as mentioned previously. About 3 µl of DNA (or 10 ng) from ligation 1, ligation 2, and pUC18 were added to its respective tube carefully. The tubes were then incubated on ice for 30 minutes. Then, the cells were heat shocked at 42°C for exactly 30 seconds. Next, tubes were placed on ice for an additional 2 minutes. Then, 250 µl of pre-warmed, pre-prepared SOC without antibiotics was added to each tube. Next, the tubes were placed in a 37°C incubator to be shaken for 1 hour at 225 rotations per minute (RPM). While this shaking was taking place, six LB + Ampicillin plates were labeled and prepared for plating of the cells. When the one hour incubation was complete, a volume of cells were spread on each plate in the as described in Table III below. Once cells were spread on each plate, they were allowed to dry and placed into the 37°C incubator overnight.

LB + Ampicillin Plate	Ligation #1	Ligation #2 (- control)	*p*UC18 (+ control)
1	100 µl cells only	100 µl cells only	100 µl cells only
2	100 µl LB + 10 µl cells	100 µl LB + 10 µl cells	100 µl LB + 10 µl cells

Table VIII. This table correlates to the six plates total from this transformation experiment.

Selection of Colonies Possibly Containing Recombinant DNA

The next day, the six plates were collected and observed for growth of colonies. A

number of colonies was observed on four of the six plates total. These four plates

included the set from ligation 1 as well as the pUC18 plate. Figure XX below is one

representative plate from each set of plates.
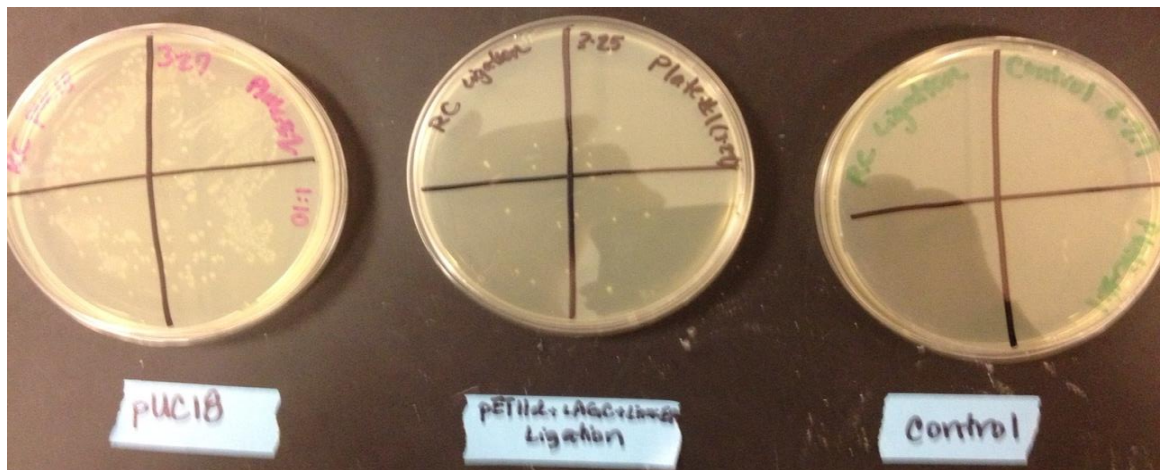
Figure XX. Observation of LB/AMP plates containing colonies with possible recombinant *p*ET11d DNA.

For organizational purposes, ligation plate 1 containing possible recombinant *p*ET11d

DNA was divided into quadrants A, B, C, and D. After selecting quadrants, each well-

isolated colony (WICs) in each quadrant was counted and given a number. The number

of WICs per quadrant is listed as follows: A-6, B-5, C-6, and D-4. Table VI below indicates

the total number of colonies counted on each plate regardless of whether or not they

were well-isolated or not.

Ligation Plates	Plate #1	Plate #2
*p*ET11d + LAGC + Linker	31	0
control	0	0
*p*UC18	TMC	TMC

Table IX. Ligation Colony Count. Plates with colonies too numerous to count are listed

Each of these WICs was streaked on LB + Ampicillin agar plate and grown overnight at

37°C. Then, the cells were collected by sterile technique and inoculated into 5 ml of

sterilized LB broth with ampicillin (50 ug/ml). These inoculated colonies were allowed to

grow overnight in a 37°C shaking incubator. The next day, DNA purification using the

Strataprep© EF Plasmid Miniprep kit was performed on each colony. The protocol for

this kit was followed as listed in a previous section. Figure XXI has been provided to

accurately depict each quadrant on the LB + Ampicillin plate in this experiment.
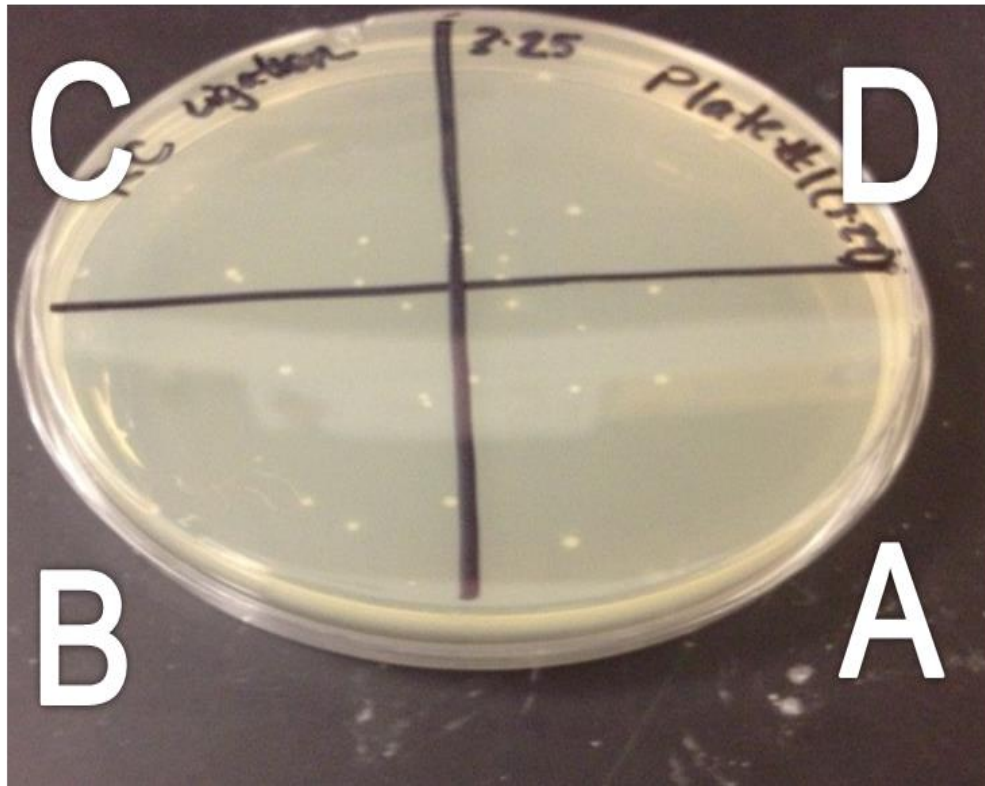
Figure XXI. Ligation 1 plate 1 quadrants A-D and their associated colony growth.

Screening for Clones with Appropriate Biological Properties

As mentioned previously, the design of this recombinant DNA included a number of unique restriction sites within the plasmid that would allow the identification of plasmids containing the correct sequence. The *Bam*HI site within recombinant *p*ET11d indicates that ligation and transformation efforts were successful.
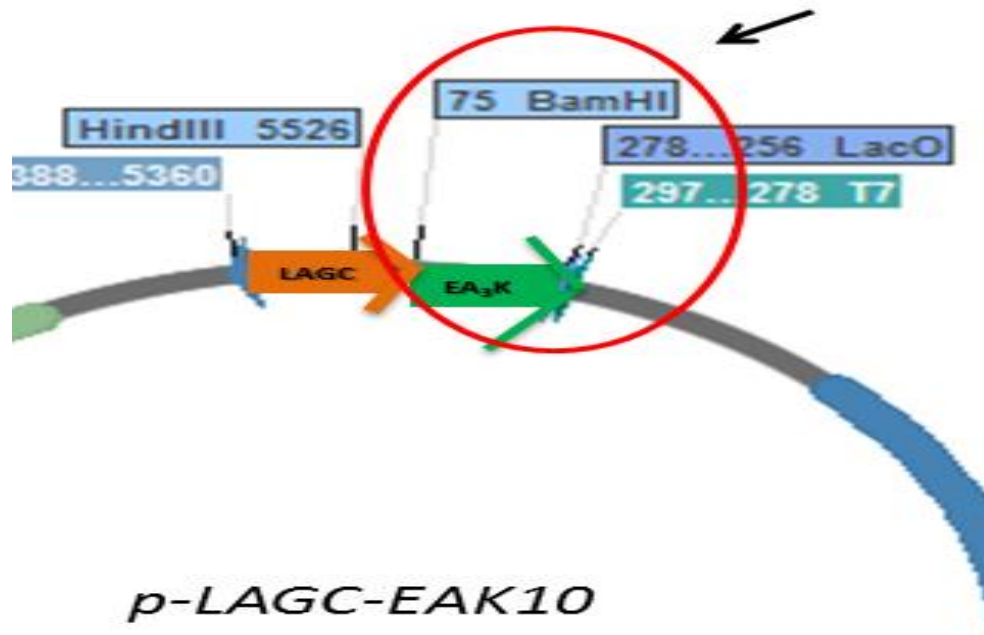
Figure XXII. Vector Map of Predicted Recombinant DNA including *Bam*HI site.

Thus, upon DNA purification, each colony was digested with *Bam*HI. The protocol for

this restriction digest is nearly exact to that mentioned in the restriction digest section

above. Table V below is a depiction of the conditions of which the purified recombinant

DNA was treated.

Components	A-D purified colonies (ul)
BSA	0.5
NEB Buffer 3	5
DNA [ ] = 40 ng/ul	10
BamHI	2
ddiH2O	25
Total	30

Table X. Restriction Digest with *Bam*HI

Pre-Screening of Recombinant Colonies

   Each colony was successfully digested with *Bam*HI. Of the 21 colonies collected and

purified, a total of 4 appeared to linearize the DNA at the expected size for the

recombinant *p*ET11d DNA. These colonies containing possible recombinant DNA will be

referred to as *p*-LAGC-LINKER for the remainder of the publication. Figure XV A-D

represents the restriction digests correlating to quadrants A, B, C, and D (see Figure XIII).
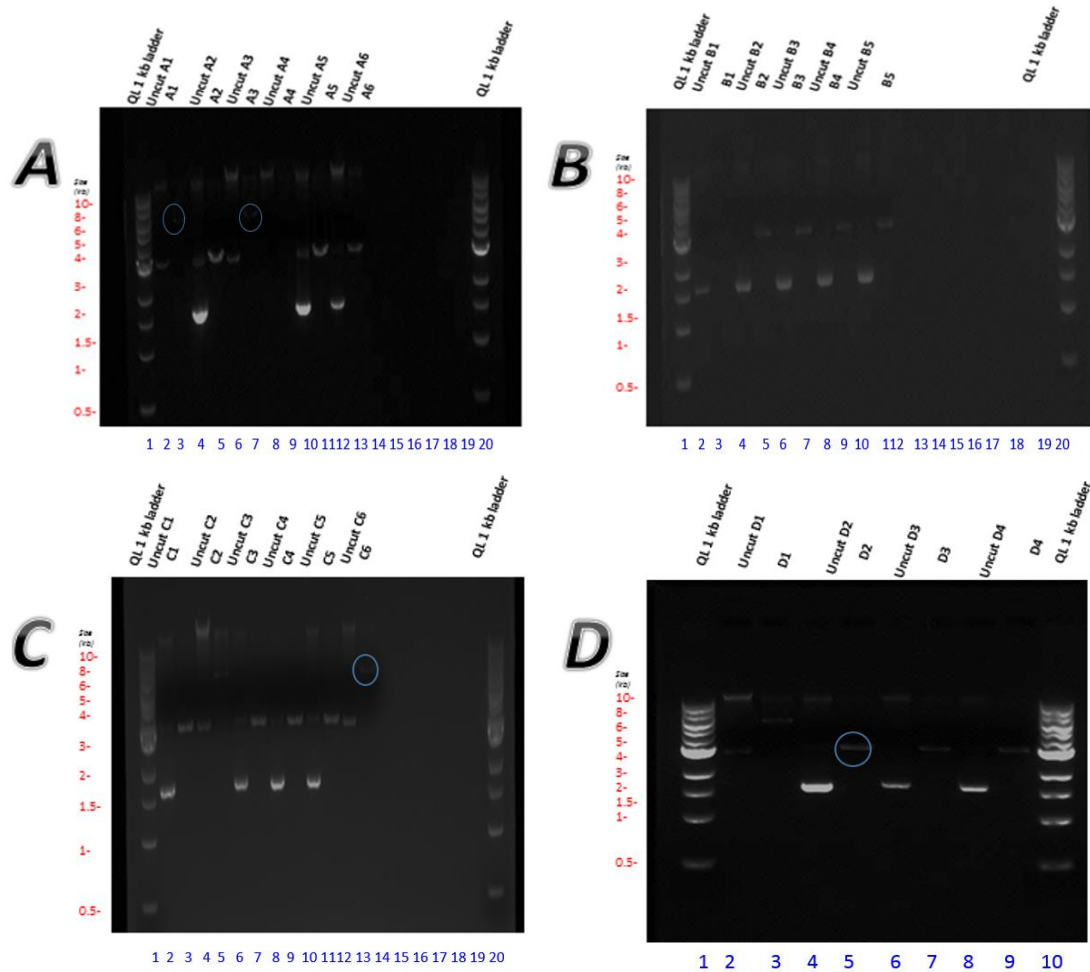
Figure XV A, B, C, and D. Each of these 0.8% agarose gels correlate with their corresponding letters. A restriction digest with *Bam*HI was performed on each colony. Undigested colonies are listed as *uncut*. The lanes of each gel are denoted in blue below. The colonies expected to contain the appropriate sequence are circled.

Table XI depicts which colonies were expected to contain *p*-LAGC-LINKER DNA.

They are circled and indicated in the table below. It appeared that quadrant A contained

at least two colonies that could possibly be the correct sequence. Quadrant B did not

appear to have a single colony digest the DNA at the appropriate size. Both quadrants C

and D contained one colony that demonstrated a linearized band at the correct size.

Restriction Digest with *Bam*HI						
Quadrant	Colony 1	Colony 2	Colony 3	Colony 4	Colony 5	Colony 6
A	+	-	+	-	-	-
B	-	-	-	-	-	None
C	-	-	-	-	-	+
D	-	+	-	-	None	None

Table XI. Pre-Screening Colonies with BamHI. + indicates linearized DNA band at
expected size. – denotes that DNA did not linearize at the expected size.

After the pre-screening tests were completed, the purified DNA from all of these

colonies was sent for DNA sequencing at Retrogen Technologies. Because the *p*ET11d

vector contains a T7 promoter primer (5' TAA TAC GAC TCA CTA TAG GG 3') as well as a

T7 terminator sequence primer (5' GCT AGT TAT TGC TCA GCG G 3') on either side of the

targeted insert site, The data from Retrogen can be found in the *Appendix*: *Sequencing

Data* section of this publication. The data indicate that colony A1 contained the

sequence correlating with what was expected should the LAGC and EA$_3$K Linker

sequences have incorporated properly. Figure XXIV below is a depiction of this data.
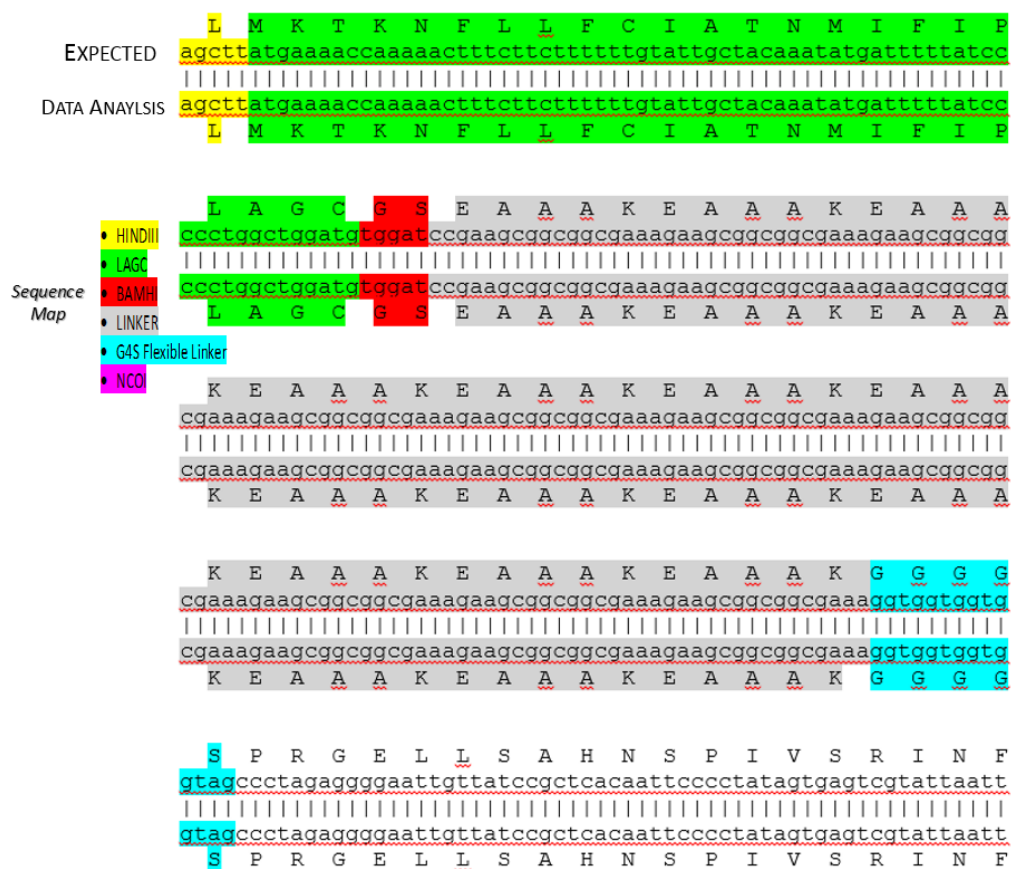
Figure XXIV. Data Analysis of recombinant DNA received from Retrogen Technologies. A color coded sequence map is included.

Upon confirmation that the colony A1 contained p-LAGC-LINKER DNA, the plasmid was given the name *p-LAGC-EAK10*.

Specific Aim 2: Use the p-LAGC-EAK10 plasmid to incorporate BPTI inhibitor creating the lipid-anchored "inhibitor ruler".   Once it was confirmed that the LAGC and EA$_3$K sequences were properly introduced into pET11d generating a new plasmid named *p*-LAGC-EAK10, an attempt to introduce the BPTI sequence into the recombinant DNA downstream from the EA$_3$K linker region was done. This process is identical to that of techniques performed in ***Specific Aim #1***. A graphic map of the current plasmid can be found in Figure XXV below depicting the unique restriction sites within the vector.
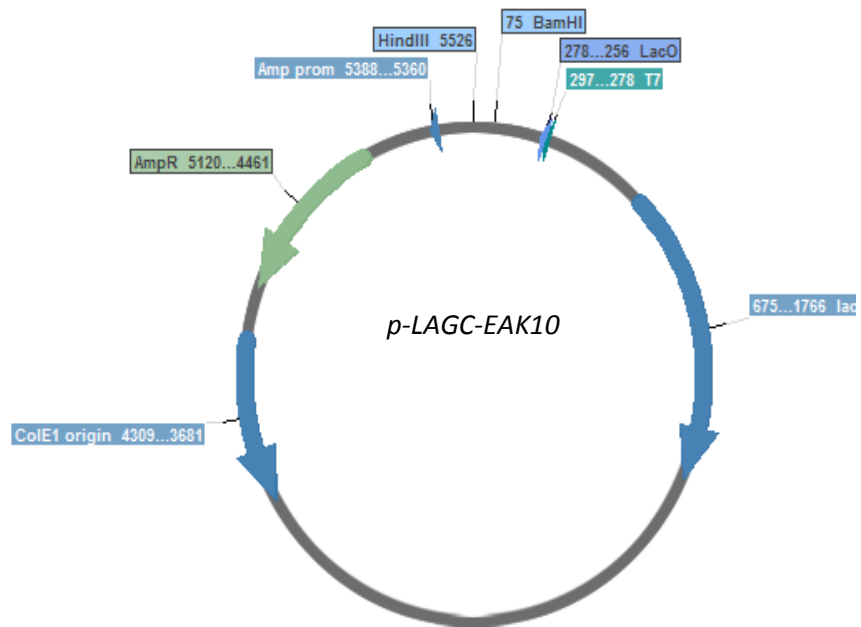
Figure XXV. Graphic map of *p*-LAGC-EAK10
Serial Cloner 2.6

p-LAGC-EAK10 DNA Purification

Using techniques as outlined in Specific Aim #1 in the DNA purification section,

DH5α cells containing *p*-LAGC-EAK10 DNA was grown in 500 ml of LB broth with

ampicillin, and DNA harvested as described in *Materials and Methods* section. To

confirm this DNA had been properly purified, it was separated on an agarose gel at 100V

for 45 minutes. The plasmid DNA was observed on the gel exhibiting two crisp bands

below the 8,000 bp rung, which indicated that the DNA was successfully purified.

Multiple bands are common with circularized DNA as it is often supercoiled. One band

ran very high, while the other was observed much lower on the gel suggesting nicked

DNA.

*Sequential Restriction Digests using Nco*I *and Xb*aI

BPTI inhibitor DNA insert oligonucleotides were designed to contain sticky ends

correlating to unique cutting sites of *Nco*I and *Xb*aI within recombinant p-LAGC-EAK10

DNA. Therefore, recombinant p-LAGC-EAK10 was cleaved open beginning with the

restriction endonuclease *Xb*aI.

Reaction Components	Restriction Digest with *Xb*aI (µl)	Restriction Digest with *Xb*aI (-) Control (µl)
BSA	0.5 100 µg/ml	0.5 100 µg/ml
NEBuffer 3 [I] = 10X	5 1X	5 1X
ddiH2O	22	43.5
DNA [Final] = 50ng/ul	20 1000 ng	1 50 ng
Restriction Enzyme **10 units/µl**	2.5 25 u	0 0 u
Total	50	50

Table XII. *p*-LAGC-EAK10 Restriction Digest with *Xb*aI

The DNA and all necessary components as listed above were combined in a 1.5 ml eppendorf tube and incubated at 37°C for 1 hour. Upon digestion, stop solution (containing EDTA) was added to cease enzymatic activity. Then, DNA was loaded onto a 0.8% agarose gel and run at 100V for 45 minutes. After gel separation, the band corresponding to the size of linearized DNA (≈ 5700 bp) was excised and gel extraction was performed.

Reaction Components	Restriction Digest with *Nco*I (µl)	Restriction Digest with *Nco*I (-) Control (µl)
BSA	0.5 100 µg/ml	0.5 100 µg/ml
NEBuffer 3 [I] = 10X	5 1X	5 1X
ddiH2O	18	43.5
DNA [Final] = 21ng/ul	24 500 ng	1 21 ng
Restriction Enzyme **10 units/µl**	2.5 25 u	0 0 u
Total	50	50

Table XIII. *p*-LAGC-EAK10/*Xba*I Restriction Digest with *Nco*I

Once extracted, the DNA was combined with all necessary components as listed above and digested with *Nco*I. Once this process was completed, the *p*-LAGC-EAK10 DNA was dephosphorylated in a 37°C incubator using AP-CIP as described below.

Reaction Components	Amount used (ul)
10X NEB Buffer 3	11 1X
Calf Intestinal Alkaline Phosphatase **(10 units/µl)**	1 10 u
DNA **5 ng/µl**	96 480 ng
ddiH2O	2
Total	110

Table XIV. *p*-LAGC-EAK10/*Xba*I/*Nco*I Restriction Digest with AP-CIP

BPTI insertion into p-LAGC-EAK10 recombinant DNA

As mentioned, the BPTI forward and reverse oligonucleotides were designed to contain sticky ends correlating to the recognition sites for *Nco*I and *Xba*I. The recognition site for *Xba*I is *T/CTAGA*. Because this sequence was purchased as forward and reverse sequences as the other oligonucleotides were, it was necessary to anneal it using the same technique as listed in Specific Aim #1. The size of the annealed product should be 189 base pairs. Figure XXVI is a 0.8% gel depicting the annealed DNA that correlates to the size of the designed BPTI insert. Smearing around the predominant band can be explained by the overloading of the DNA, misalignment of the annealed product, or potentially degraded DNA.
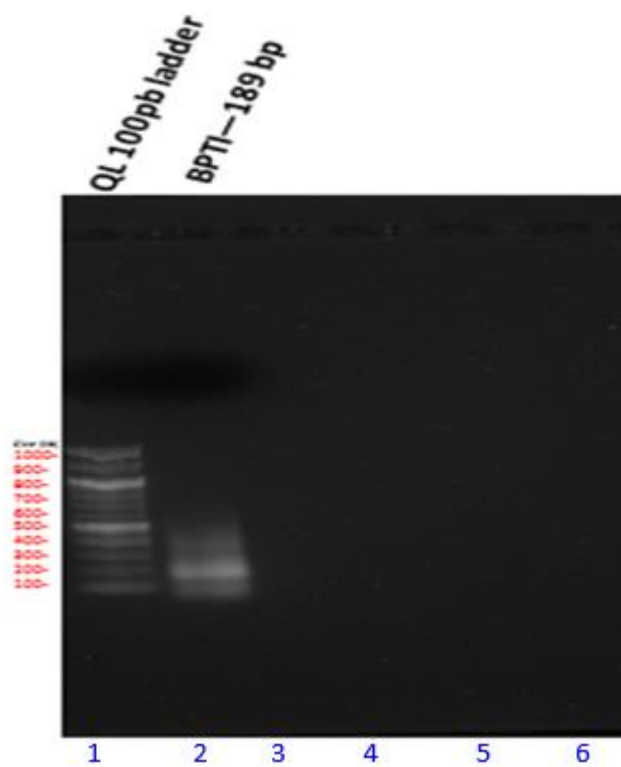
Figure XXVI. Annealed BPTI product on a 0.8% agarose gel. Lane 1 is QL 1kb ladder. Lane 2 is 100 bp ladder. Lane 3 is annealed BPTI product about 189 bp in size.

The reaction components of this ligation are listed below. This ligation was conducted

using the protocol as outlined in the previous sections.

REACTION COMPONENTS	3:1 LIGATION	P-LAGC-EAK10/XBAI/NCOI
10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3µl [Final] = 1X
VECTOR DNA (5.674 KB) (5 ng/µl)	20 µl 100 ng	20µl 100 ng
BPTI INSERT DNA (189 BP) (177 ng/µl)	1.7 µl 300 ng	1.7 300 ng
DD₁H₂O	4.3 µl	4.3 µl
T4 DNA LIGASE 10 UNITS/µL	1 µl 10 u/ µl	0 µl 0 u/ µl
TOTAL	30 µl	30 µl

Table XV. Ligation of *p*-LAGC-EAK10 and BPTI

Introduction of recombinant p-LAGC-EAK10 DNA into Host Organism

After the ligation was complete, a transformation was performed using the

techniques outlined in a previous section. DH5α competent cells were used in this

experiment as in the transformation prior to this. Once the transformation was finished,

five LB + Ampicillin plates were used to spread the ligation and control cells for

overnight incubation in a 37°C incubator.

Selection and Screening of recombinant p-LAGC-EAK10 Colonies

The next day, the LB + Ampicillin plates were collected and observed for growth of

any colonies. Of all the plates, none of them contained colonies for screening. Ligation

experiments were repeated as described as well as with the addition of troubleshooting steps. Multiple troubleshooting attempts were made to remedy any possible reasons for unsuccessful colony growth.

Troubleshooting

Because the plates from the initial ligation of p-LAGC-EAK10 and BPTI were void of colony growth, it was necessary to evaluate for possible issues that would lead the experiment to be unsuccessful.

<u>*Attempt 1*</u>

In an effort to increase the chances of insertion, a second ligation experiment using insert-vector ratios of 3:1. 6:1, and 9:1 were completed. All other conditions and components remained the same. The experiment was carried out as described in the *Materials and Methods* section. To ensure that the issue was not the DH5α competent cells pUC18 DNA was transformed into the cells. In addition to the pUC18 DNA, a ligation using no insert was used as a control. No colonies were observed on those plates. While no colonies were observed on any of the ligation plates including the ligation without insert, no growth was observed on the *p*UC18 plate either. Photos of this attempt were not recorded.

Reaction Components	3:1 Ligation	6:1 Ligation	9:1 Ligation	p-LAGC-EAK10/XbaI/NcoI
10X T4 DNA Ligase Buffer*	3 µl [Final] = 1X	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X
Vector DNA (5.674 kb) (5 ng/µl)	20 µl 100 ng	20 µl 100 ng	20 µl 100 ng	20µl 100 ng
BPTI Insert DNA (189 bp) (177 ng/µl)	1.7 µl 300 ng	3.4 µl 600 ng	5.1 µl 900 ng	1.7 300 ng
DD₁H₂O	4.3 µl	2.6 µl	0.9 µl	4.3 µl
T4 DNA Ligase 10 units/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 10 u/ µl	0 µl 0 u/ µl
Total	30 µl	30 µl	30 µl	30 µl

Table XVI. Ligation Attempt 1

Attempt 2

Because no growth was observed on any of the plates, the viability of the competent cells was in question. A set of competent cells were prepared using the techniques described in the *Materials and Methods* section. After the cells were prepared, the viability of the competent cells was confirmed by transformation of *p*UC18 DNA as described previously. Another ligation attempt was made by protocol using the conditions in Attempt 1 previously. Though the *p*UC18 positive control plate set contained colony growth, ligation colonies were not observed.

REACTION COMPONENTS	3:1 LIGATION	6:1 LIGATION	9:1 LIGATION	P-LAGC-EAK10/XBAI/NCOI
10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X
VECTOR DNA (5.674 KB) (5 ng/µl)	20 µl 100 ng	20 µl 100 ng	20 µl 100 ng	20µl 100 ng
BPTI INSERT DNA (189 BP) (177 ng/µl)	1.7 µl 300 ng	3.4 µl 600 ng	5.1 µl 900 ng	0 0 ng
DDIH$_2$O	4.3 µl	2.6 µl	0.9 µl	5.0 µl
T4 DNA LIGASE 10 UNITS/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 0 u/ µl
TOTAL	30 µl	30 µl	30 µl	30 µl

Table XVII. Ligation Attempt 2

Attempt 3

Once the viability of the competent cells was re-established but not colonies growing on the ligation plates, it was suspected that there may be an issue with the p-LAGC-EAK10 plasmid DNA itself. So, an experiment was performed using the same components as above with *p*UC18 DNA as a positive control along with uncut *p*-LAGC-EAK10 plasmid DNA and p-LAGC-EAK10/*Xb*aI/*Nc*oI with all other components except oligonucleotide inserts.

REACTION COMPONENTS	3:1 LIGATION	6:1 LIGATION	9:1 LIGATION	P-LAGC-EAK10/XBAI/NCOI	PUC18	P-LAGC-EAK10
10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X	N/A	N/A

VECTOR DNA (5.674 KB) (5 ng/ µl)	20 µl 100 ng	20 µl 100 ng	20 µl 100 ng	20µl 100 ng	N/A	N/A
BPTI INSERT DNA (189 BP) (177 ng/µl)	1.7 µl 300 ng	3.4 µl 600 ng	5.1 µl 900 ng	0 0 ng	N/A	N/A
DD₁H₂O	4.3 µl	2.6 µl	0.9 µl	5.0 µl	N/A	N/A
T4 DNA LIGASE 10 UNITS/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 0 u/ µl	N/A	N/A
TOTAL	30 µl	30 µl	30 µl	30 µl	N/A	N/A

Table XVIII. Ligation Attempt 3

This ligation attempt was made by protocol using the conditions (1 hour, 37°C) previously. Though the *p*UC18 and uncut p-LAGC-EAK10 positive control plate sets contained colony growth, ligation colonies were not observed on any other plate.

Attempt 4

No colonies were observed on the ligation plates including the control plate without insert. This plasmid demonstrated viability in past experiment, but it was possible that there was an issue with any one of the ligation components. The protocol associated with the T4 DNA ligase enzyme states that it contains ATP (adenosine triphosphate) which catalyzes the ligation of the DNA strands. It also states that multiple freezing and thawing of this enzyme is not recommended as ATP becomes deactivated after multiple thaws. For this reason, a set of ligations at 3:1, 6:1, 9:1, and 12:1 insert-

vector ratios was set up using a fresh vial of T4 DNA Ligase and using LB broth alongside

a set cultured in SOC. All other components remained the same.

REACTION COMPONENTS	3:1 LIGATION	6:1 LIGATION	9:1 LIGATION	12:1 LIGATION	P-LAGC-EAK10/XbAI/NcoI	pUC18
NEW 10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3 µl [Final] = 1X	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X	N/A
VECTOR DNA (5.674 KB) (5 ng/µl)	20 µl 100 ng	20 µl 100 ng	20 µl 100 ng	20 µl 100 ng	20µl 100 ng	N/A
BPTI INSERT DNA (189 BP) (177 ng/ul)	1.7 µl 300 ng	3.4 µl 600 ng	5.1 µl 900 ng	6.8 µl 900 ng	0 0 ng	N/A
DD₁H₂O	4.3 µl	2.6 µl	0.9 µl	0 µl	5.0 µl	N/A
T4 DNA LIGASE 10 UNITS/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 0 u/ µl	N/A
TOTAL	30 µl	30 µl	30 µl	30.8 µl	30 µl	N/A

Table XIX. Ligation Attempt 4

After the overnight incubation at 37°C, colonies cultured in SOC were observed

on the 9:1 plates. A lawn of colonies was recorded on these plates. Figure XXVII below is

the image of this plate.
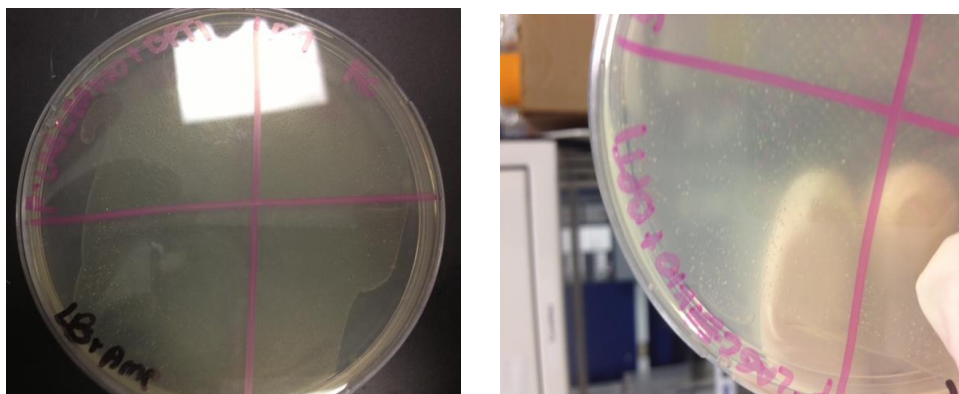
Figure XXVII. 9:1 Insert-Vector Ratio Ligation Plate

It was necessary to re-plate these cells at a 1:100 dilution for more well-isolated colonies. The next day, the plate was obtained and observed for colonies. No colonies were present. It can be surmised that the transformed DNA was plated at a concentration that was too high which allowed for a lawn to grow successfully on the plate. It is possible antibiotic concentration played a role as well. Therefore, when plated at a lower concentration no cell growth occurred. Another possibility there may have been confusion between the positive control and ligation sample by technician error. Finally, there is a possibility that a lawn grew because the ampicillin concentration was too high or too low to allow colony growth at the appropriate rate.

Attempt 5

Although all these adjustments to the ligation experiment had not induced much successful colony growth, it was determined over the last few experiments that 6:1 and 9:1 ratios were the most effective in colony growth. Also, the fresh T4 DNA ligase appeared to have a small role in the growth of colonies seen in Attempt 4. So, a set of ligations using 6:1 and 9:1 insert-vector ratios was set up using T4 DNA ligase and additional ATP from a stock solution concentrated at 10 mg/ml. All other components and conditions remained the same.

REACTION COMPONENTS	6:1 LIGATION	9:1 LIGATION	P-LAGC-EAK10/XBAI/NCOI	PUC18
10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X	N/A
ATP 10 MG/ML	1 µl 0.1 mg/ml	1 µl 0.1 mg/ml	1 µl 0.1 mg/ml	N/A

Vector DNA (5.674 kb) (5 ng/µl)	20 µl 100 ng	20 µl 100 ng	20µl 100 ng	N/A
BPTI Insert DNA (189 bp) (177 ng/µl)	3.4 µl 600 ng	5.1 µl 900 ng	0 0 ng	N/A
DDₗH₂O	1.6 µl	0.9 µl	4.0 µl	N/A
T4 DNA Ligase 10 units/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 0 u/ µl	N/A
Total	30 µl	31 µl	30 µl	N/A

Table XX. Ligation Attempt 5

The following day colonies were observed on both 6:1 and 9:1 plates. There was a total of 4 colonies are organized in plates E and F. Plate E contained 3 well-isolated colonies while plate 4 contained a single well-isolated colony.

Pre-screening and Data Analysis for Possible Recombinant DNA

All well-isolated colonies were cultured and collected for DNA purification using the Strataprep™ kit (see protocol in *DNA Purification* section). Once the DNA from these colonies was properly extracted, pre-screening the DNA with *Xho*I was necessary. As indicated in Figure XXVIII, BPTI contains a unique *Xho*I site within the sequence of the expected vector. The recognition site for *Xho*I *is C/TCGAG*. Any colony DNA exhibiting a linearized band between 5 and 6kb could potentially contain the desired recombinant DNA. The results from these experiments are listed below.
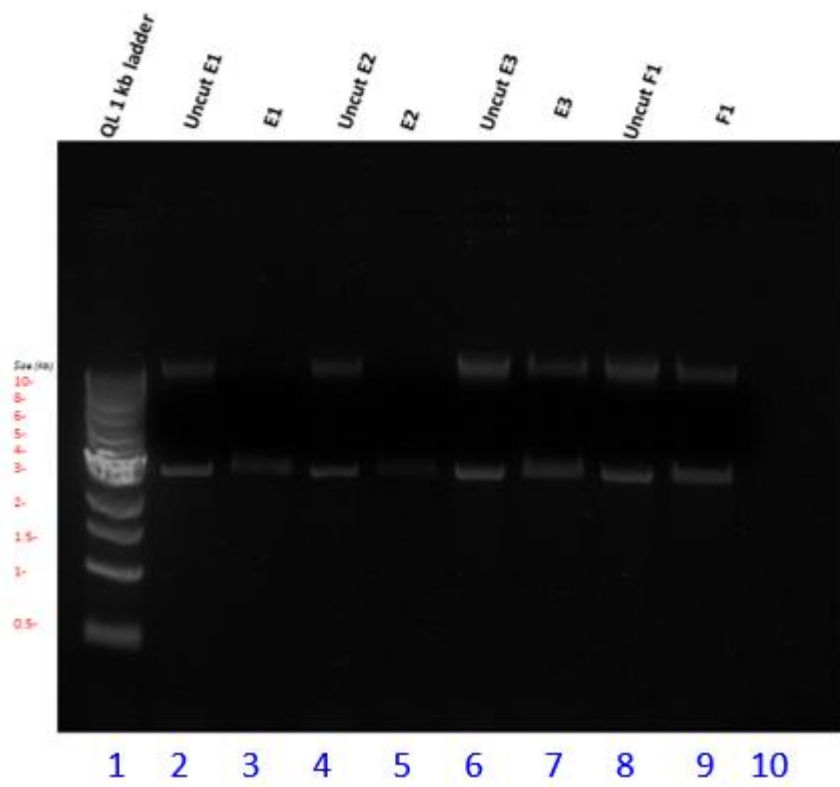
Figure XXVIII. Ligation colonies digested with *Xho*I.

Screening results suggested that though DNA was successfully ligated, none of the

colonies incorporated the BPTI DNA into the vector. Though the results suggested the

ligation was unsuccessful, the purified DNA colonies were sent to Retrogen for

sequencing. The data collected at Retrogen yielded the same conclusion.

Attempt 6

The addition of ATP to this ligation appears to have assisted the reaction

successfully. Thus, a final ligation attempt was made using the exact conditions as

Attempt 5 in anticipation that the results would be more successful.

REACTION COMPONENTS	6:1 LIGATION	9:1 LIGATION	P-LAGC-EAK10/XBAI/NcoI	PUC18
10X T4 DNA LIGASE BUFFER*	3 µl [Final] = 1X	3 µl [Final] = 1X	3µl [Final] = 1X	N/A
ATP 10 MG/ML	1 µl 0.1 mg/ml	1 µl 0.1 mg/ml	1 µl 0.1 mg/ml	N/A
VECTOR DNA (5.674 KB) (5 ng/µl)	20 µl 100 ng	20 µl 100 ng	20µl 100 ng	N/A
BPTI INSERT DNA (189 BP) (177 ng/µl)	3.4 µl 600 ng	5.1 µl 900 ng	0 0 ng	N/A
DD₁H₂O	1.6 µl	0.9 µl	4.0 µl	N/A
T4 DNA LIGASE 10 UNITS/µL	1 µl 10 u/ µl	1 µl 10 u/ µl	1 µl 0 u/ µl	N/A
TOTAL	30 µl	31 µl	30 µl	N/A

Table XXI. Ligation Attempt 6

After transformation and overnight growth, the six plates were retrieved and observed for colony growth. Of the six plates, four plates including one of the 6:1 and one of the 9:1 contained colonies. The other two plates were the controls pUC18 and *p*-LAGC-EAK10/*Xb*aI/*Nc*oI without insert. The plates were divided into plates G and H. Plate G represented the 6:1 ratio colonies. Plate H was the 9:1 ratio plates. Figure XXIX is a depiction of these plates.
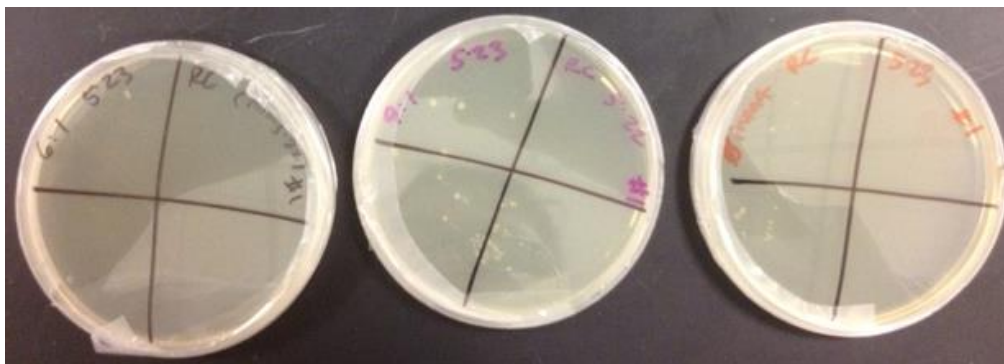
Figure XXIX. Ligation Plates G, H, and *p*-LAGC-EAK10 without insert.

These colonies were cultured and DNA purified as described previously. Screening of

these colonies by restriction digest with *Xh*oI was performed. Figure XXX A and B is a

depiction of the results of this digest.
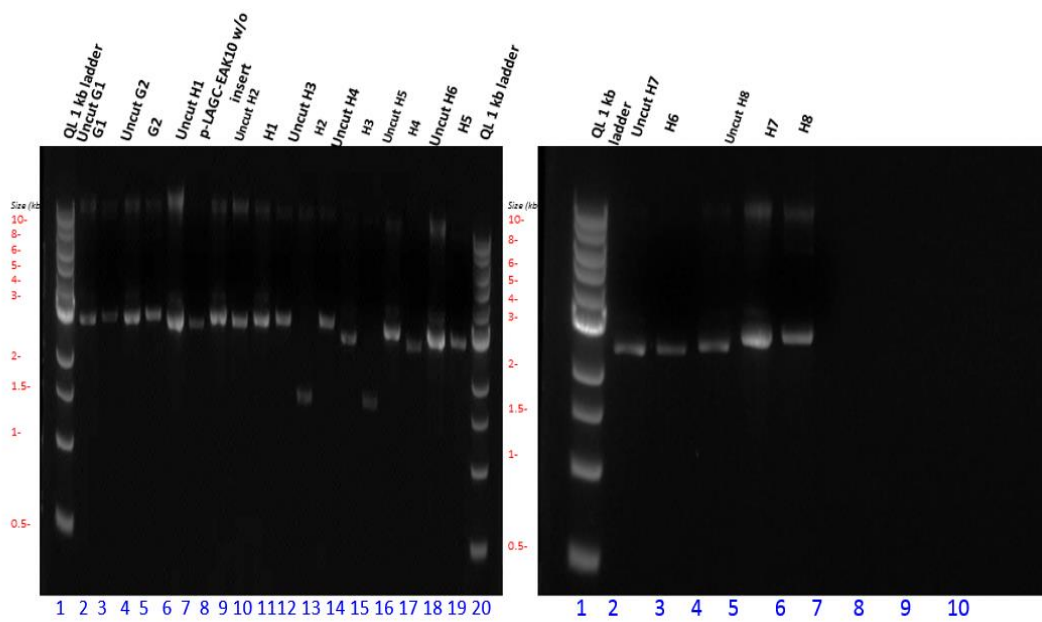
Figure XXX. Restriction Digest with *Xh*oI. Lanes are denoted in blue.

According to the screening results, H6 appeared to be the only colony that could possibly contain the recombinant DNA as the plasmid compared to the undigested plasmid generated a crisp band at the expected digestion site. All the colonies were sent for sequencing at Retrogen. The data analysis indicates that no recombinant DNA was obtained. One possible reason for this is that the annealed BPTI product contained smears when observed on a 0.8% agarose gel. This suggests that the DNA misaligned and therefore would not allow the sticky ends to associate properly with the linearized *p*-LAGC-EAK10 vector.

Discussion:

Molecular cloning is a multi-step process involving several that are time consuming and unpredictable. As mentioned previously, generating recombinant DNA involves choosing a host organism and orchestrating conditions in which to linearize the vector, insert a foreign insert, and circularize the DNA. Upon circularization of recombinant DNA, sequencing is necessary to confirm the incorporation of foreign DNA insertions. This project was limited in the time frame allowed to complete experiments. While *p*-LAGC-EAK10 was generated successfully during the course of this project, several unsuccessful attempts to incorporate BPTI into the p-LAGC-EAK10 vector were made.

Possible issues for this have been listed previously including non-viable competent cells, deactivated ligation enzyme, and a low concentration of ATP necessary for ligation. Other possible issues to address could be the potentially misaligned

oligonucleotide annealed product. Perhaps, adjusting the annealing protocol by temperature or time frame would allow for a more successful annealing of the forward and reverse sequences.

Future Aims:

BPTI Incorporation into p-LAGC-EAK10

As this study ended with unsuccessful attempts to incorporate BPTI into the p-LAGC-EAK10 plasmid, the following are suggestions regarding the continuation of this research. First, increasing the insert to vector ratio would allow the annealed oligonucleotide more opportunities to incorporate properly into the plasmid.

Expression & Purification of Inhibitor Ruler

Next, expression of the BPTI-lipid anchored "inhibitor ruler" in the BL21(DE3) expression strain followed by detection of the protein using Western Blotting-SDS-PAGE with a goat polyclonal antibody that would recognize BPTI.

Deadmond *et al* (57) optimized the procedure for the induction of protein expression in BL21(DE3) cells. Fresh, sterilized TB broth with carbencillin (50 µl/ml) and glycerol would be used to culture the *p*-LAGC-EAK10-BPTI DNA shaking at 300 rpm in a 37°C incubator until the A_{600} (absorbance) reaches 1.5-2.0. Next, the temperature is decreased to 25°C and the culture is allowed to continue incubating overnight. The next day, the cultures would be transferred into 1-L bottles and centrifuged at 3000 *x g* for 25 minutes at 4°C. The supernatant would be decanted off and the cells washed with 250 ml of ice cold TBS/Azide solution. This solution is a Tris saline based azide often used as

blocking buffer in western blotting assays. It is used here to remove any impurities that may be present. It is composed of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.02% NaN$_3$. The cells are centrifuged again for 25 minutes at 3000 x g at 4°C. Next, after the supernantant is decanted, the cell pellets should be weighed. They are then frozen at -80 C for an hour or more. Next the pellets should be thawed at 37°C. Then, 50 ml of lysis buffer per gram of cell pellet should be added. The lysis buffer is a mixture of 20 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100, 0.02% NaN3, and 1 mg/ml of lysozyme. The re-suspended cell pellets should be mixed well then incubated on a rocker at 4°C for one hour. Next, poly-ethylamine solution should be added at a final concentration of 0.1% and mixed well. This is allowed to stand at 4°C for 10 minutes. The poly-ethylamine solution is composed of 5% (w/v) poly-ethylenimine in water and NaN$_3$ concentrated at 0.02%. The cells are centrifuged at 3000 x g for 25 minutes at 4°C. If clarity is not achieved, it is suggested that the cells be treated with 5 µg/ml DNAseI for at least 20 minutes and centrifuged at 14,000 x g for 25 minutes at 4°C. The crude cell lysate yielded from this centrifugation should contain the BPTI protein.

After detection by SDS-PAGE (sodium dodecyl polyacrylamide gel electrophoresis), the next step would be purification of the p-LAGC-EAK10-BPTI inhibitor ruler using affinity chromatography with trypsinized columns. SDS-PAGE works by separating proteins based on molecular weights using gel electrophoresis. Because proteins with differing molecular weights usually have differing charges as well, separating them on an electrical field requires that the charges all be negative such that they migrate at the same time on the gel (111). The SDS detergent is used to do this.

Neuenschwander *et al* also provided proof-of-principle methods for expression and

purification of the membrane-tethered BPTI constructs. Expression and purification can

be done by the methods these researchers used in a bacterial system to engineer a

construct containing a lipid-tethered inhibitors extending 75Å above the membrane

surface (57). Trypsin is used because as mentioned previously, serine proteases are very

similar to trypsin. BPTI and trypsin have a very high affinity for one another. This means

that any non-specific proteins in the cell lysate will elute out leaving only BPTI. Trypsin

must be covalently bound to the column using techniques adapted from the *BioRad™*

Activated Immunoaffinity Chromatography kit. Then, the 10-ml column is equilibrated

with trypsin. A minimum of 5 column volumes of equilibration buffer (20 mM Tris-HCl,

100 mM NaCl, and 0.1% Triton-X100) is flowed over the column at a flow rate of 1 ml/40

seconds. Then, the tubing containing the crude cell lysate should be loaded and flowed

over the column at a rate of 1 ml/ 2.5 minutes. The flow-through should be saved

because it should contain the desired product. About 50 ml of wash buffer (1 mM Tris-

HCl, 100 mM NaCl) should be loaded into the tubing and run over the column at 1

ml/min. A high-salt wash removes any non-specific proteins that may bind to the

column leaving only BPTI theoretically. Next, a fraction collector with 40 tubes should be

set up. Each tube should contain 20 μl of 1M Tris-HCl to neutralize eluate upon

collection. A fraction collector for 1 ml fractions should also be set up. Next, the tubing

should be filled with elution buffer containing 10 mM HCl and 500 mM KCl. This elution

buffer should be flowed over the column at a flow rate of 1 ml/min. Eluate should be

collected in the fraction tubes that were set up. These fractions can be read at A_{280} in a quartz cuvette. The relevant fractions should be kept and the irrelevant ones discarded.

Once these steps have been completed, the p-LAGC-EAK10-BPTI inhibitor ruler will be relipidated and verified by gel filtration chromatography as described previously. Then, it should be ready for use to assist in the determination of the optimal reactive height for FX_a alone as well as the prothrombinase complex (including fX_a, fV_a, and Ca^{2+}). Using an equilibrium-based chromogenic enzyme assay, researchers could measure the rates and extent of inhibition for both FX_a and the FX_a/pro-coagulant complex. These experiments can be used to determine the reactivity of the complex to each modified inhibitor. As described previously, experiments such as this have been carried out successfully by a number of researchers including Deadmond *et al (57)*.

Comparing the reactivity for this inhibitor at a height of 70Å off the membrane surface will provide insight into the elasticity (ability to reach the cell surface) of these complexes, as well as suggest potential optimal reactive conformation of the fX_a complex. Reactivity at 70Å will indicate the level at which fX_a is inhibited from the membrane surface because this is the height previously predicted to be the location of the active site (57). As the height of this inhibitor ruler is adjusted, the height at which the inhibitor is the most reactive can be determined. Also, the height that is too short to cause inhibition can be determined. This information is indicative of how close to the simulated vessel surface fX can reach. This information will give confirmation as to the conclusions about fX_a topography and exactly how it carries out its role in fibrin clot deposition.

Enzyme Reactivity Experiments

Rates of inhibition will be measured using solution-phase and surface-based equilibrium inhibition. Solution-phase enzyme inhibition assay and surface-based enzyme inhibition can be used to characterize inhibitor activity (i.e. its K_i and inhibitory potential) as compared its native state. Using both of these experiments will indicate fX_a enzymatic activity as it floats in solution as well as its activity when BPTI is attached to the membrane surface. As the study continues, site-directed mutagenesis can be used to generate *p*-LAGC-EAK-BPTI constructs with one fewer linker at a time to determine the range of optimal reactivities of FX_a.

Solution-phase equilibrium inhibition

Purified recombinant BPTI should be incubated with trypsin for 30 minutes or more until equilibrium is reached. Then, a chromogenic substrate can be added to measure enzymatic activity. A similar experiment using membrane-bound BPTI/fX_a and soluble (free-floating) BPTI/fX_a will need to be performed as well. The K_i of BPTI/trypsin, anchored BPTI/fX_a, and soluble BPTI/fX_a will be compared to one another. These comparisons will indicate whether or not the predicted reactivity height was accurate.

Rates can be recorded and plotted in comparison to the inhibitor concentration to calculate the K_i. Data from the inhibition plots/graphs derived from a series of slope calculations can be used to examine fX_a and prothrombinase complex reactivity. A comparison of the modified and unmodified inhibitor could provide some information on inhibitory activity and the effect of the linker region within the engineered construct.

Solution-phase equilibrium as described above can be used to measure fX_a reactivity in the presence of *un-anchored* BPTI. Using anchored BPTI is a plausible experiment too, as it can be done using only the fX_a catalytic head and measuring activity of BPTI attached to a neutral phospholipid surface to ensure the process is not altered by a negative membrane surface. K_i values obtained from each set of inhibitor rulers can be compared. It is important to ensure that modifications added to BPTI did not interfere or alter factor activity and also to correct any differences recorded between the modified and unmodified versions of the inhibitor.

Surface-based equilibrium inhibition

Each isolate of the inhibitor complexes can be incorporated separately into liposomes for the purposes of comparing the inhibition at variable-lengths. These liposomes will contain percentages of phosphatidyl choline (PC) (roughly 90%) and phosphatidylserine (PS) (roughly 10%) necessary for optimal binding of fX_a. Smith & Morrissey developed the protocol we will use to construct the liposomes by way of detergent removal from lipid-protein mixture using adsorbent beads (112). The fX_a, lipid vesicles containing recombinant BPTI, and pro-thrombinase complex members fV_a and Ca^{2+} can be added and incubated with the membrane. Once this forms the pro-coagulant complex and reaches equilibrium, a chromogenic substrate will be added and absorbance measured at 405 nm. This experiment takes place over a monitored time frame to establish the initial rate of substrate hydrolysis and indicate the remaining enzyme activity when equilibrium is reached. Rates recorded during this experiment can be plotted as described previously. Analysis regarding the ratio of anchored BPTI/fX_a and

soluble BPTI/fX$_a$ K$_i$ rates will assist in making conclusions about the optimal height of active sites above the membrane. For instance, a K$_i$ ratio of greater than 1 suggests a non-optimal distance separating BPTI and the fX$_a$ active site. Conversely, a K$_i$ ratio of lesser than or equal to 1 indicates an optimal distance is reached. Any changes noted between these two data sets are also helpful with further data analysis on the proximity of BPTI and the FX$_a$ active site.

Clinical Significance

Venous thromboembolism, deep vein thrombosis, and other thrombotic disorders is the cause of over 100,000 deaths each year (113). Patients with these conditions and prone to atrial fibrillation are at the highest risk of death as a result of the anticoagulant treatment they need to maintain hemostasis. As mentioned, current treatments for thrombotic disorders are limited due to the specificity of drug targets and effective medical techniques. Clinicians are currently prescribing thrombotic patients warfarin, low molecular weight heparin, protease inhibitors, and other vitamin-K inhibitors. However, many of these treatments lack specificity in that each of them target more than one portion of the process. Whereas research indicates direct fX$_a$ or thrombin anticoagulants are much more effective at inhibiting harmful clotting. These two components are the largest proponents of coagulation. Therefore, specifically targeting one or the other has been shown to greatly reduce clotting without disturbing other biochemical processes. A review published by S. Aditya in *the Journal of Post-graduate Medicine* reports newer anticoagulant treatments are effective at targeting specific parts of the coagulation process but put the patient at risk of excessive

coagulation as well. Though these treatments are an improvement, they are still not as ideal as targeting fX_a (114).

Information characterizing fX_a topography more clearly would greatly improve researchers' ability to generate drugs which can be used to target the two most committed steps to coagulation, activation of fX_a and thrombin bursts. Data obtained from these experiments will be invaluable in gaining knowledge about the rational design of effective membrane-based inhibitors. In addition, findings could be very useful in clinical medicine as well in that these membrane-based inhibitors could be used for the treatment of thrombosis and other thrombotic disorders. The information will provide some guidance on specificity in regard to effective distances of inhibition on the membrane surface which could lead to a number of clinical applications. Among these applications are drugs designed to work more accurately to prevent adverse responses to thrombosis symptoms.

Acknowledgements

I would like to acknowledge a number of individuals and entities without which this project would have been impossible. I would like to thank CCDA and the Philadelphia College of Osteopathic Medicine-Georgia Campus for resources to carry out this study. I would also like to thank my mentor, Dr. Kimberly J. Baker, for her patience and guidance. I would like to thank both Dr. Francis E. Jenney and Dr. Harold Komiskey who served not only on the review committee for this project but also as great advisors. Each of them have provided guidance and assistance that was above and beyond what they were called to do.

Next, I'd like to acknowledge my sweet friend, Siri L. Chirumamilla. She has been invaluable to this project. She is an angel. She dedicated a great deal of her time to seeing this project through. I am certain that I would not have succeeded without her kind words, time, and experience. I would also like to mention Ms. Trina Bursey who has listened to me and offered me sanctuary every time I needed it. I would be remiss if I didn't acknowledge Betty Ma for everything she has done toward the completion of this project. Also, I'd like to thank my family, friends, and supporters including but not limited to Jennifer Conley, Amelie Bottex, Stacy D. Arrington, LaChanda D. Wright, Shalisa Sanders, Gerald Holifield, Terra Jones-Steele and a host of others. Let me also extend my gratitude to my GA-PCOM family Mary Keith, Christal Santos, Cynthia Brooke Woods, Kristen Britnni Robertson, Linda Williams, Zicole Browne, Atijah Collins, Anthony Cooper, Austin Russell, Indi Shelton, Simone Pitts, Kimberly Lucier, and Robert Bonds. I want to recognize my family: the Jacksons, Blanks, and Sanders families. They have

provided spiritual, physical, and financial support for all of my endeavors. Their love and

support cannot be replaced. Lastly, I would like to acknowledge the GA-PCOM

Biomedical Sciences program. This is including and especially Dr. Brian Matayoshi and

Linda G. Williams for giving me the opportunity to learn and develop my skills as a

scientist, lifelong learner, and researcher. I am one step closer to achieving my dreams

and it is because of this program.

Appendix I: Truncated pET11d Vector DNA and Protein Sequence

HINDIII NCOI BAMHI XbaI

Serial Cloner 2.6: Alignment pET11d Sequence

Similarity: 5674/5674 (100.00 %)

```
        F  S  C  L  T  A  Y  H  R  *  A  L  M  R  *  F  I  T  V  K
pET11d  ttctcatgtttgacagcttatcatcgataagctttaatgcggtagtttatcacagttaaa  60
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  ttctcatgtttgacagcttatcatcgataagctttaatgcggtagtttatcacagttaaa  60


        L  L  T  Q  S  G  T  V  Y  E  I  *  Q  C  A  H  R  H  P  R
pET11d  ttgctaacgcagtcaggcaccgtgtatgaaatctaacaatgcgctcatcgtcatcctcgg  120
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  ttgctaacgcagtcaggcaccgtgtatgaaatctaacaatgcgctcatcgtcatcctcgg  120


        H  R  H  P  G  C  C  R  H  R  L  G  Y  A  G  T  A  G  P  L
pET11d  caccgtcaccctggatgctgtaggcataggcttggttatgccggtactgccgggcctctt  180
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  caccgtcaccctggatgctgtaggcataggcttggttatgccggtactgccgggcctctt  180


        A  G  Y  P  D  I  V  P  P  F  S  K  K  P  L  K  T  R  L  E
pET11d  gcgggatatccggatatagttcctcctttcagcaaaaaacccctcaagacccgtttagag  240
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  gcgggatatccggatatagttcctcctttcagcaaaaaacccctcaagacccgtttagag  240


        A  P  R  G  Y  A  S  Y  C  S  A  V  A  A  A  N  S  A  S  F
pET11d  gccccaagggggttatgctagttattgctcagcggtggcagcagccaactcagcttccttt  300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  gccccaagggggttatgctagttattgctcagcggtggcagcagccaactcagcttccttt  300


        R  A  L  L  A  A  G  S  D  P  F  A  V  H  Q  S  C  *  P  W
pET11d  cgggctttgttagcagccggatccgacccatttgctgtccaccagtcatgctagccatgg  360
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  cgggctttgttagcagccggatccgacccatttgctgtccaccagtcatgctagccatgg  360


        Y  I  S  F  L  K  L  N  K  I  I  S  R  G  E  L  L  S  A  H
pET11d  tatatctccttcttaaagttaaacaaaattatttctagaggggaattgttatccgctcac  420
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pET11d  tatatctccttcttaaagttaaacaaaattatttctagaggggaattgttatccgctcac  420
```

Appendix II: BPTI DNA & Protein Sequence

```
Serial Cloner 2.6: Alignment BPTI Sequence


          W  M  R  P  D  F  C  L  E  P  P  Y  T  G  P  C  K  A  R  I
BPTI      CATGGATGCGTCCGGACTTCTGCCTCGAGCCGCCGTACACGGGTCCCTGCAAAGCGCGCA  60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTI      CATGGATGCGTCCGGACTTCTGCCTCGAGCCGCCGTACACGGGTCCCTGCAAAGCGCGCA  60


          I  R  Y  F  Y  N  A  K  A  G  L  C  Q  T  F  V  Y  G  G  C
BPTI      TCATCCGTTACTTCTACAACGCTAAAGCAGGCCTGTGCCAGACCTTCGTTTACGGTGGCT  120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTI      TCATCCGTTACTTCTACAACGCTAAAGCAGGCCTGTGCCAGACCTTCGTTTACGGTGGCT  120


          R  A  K  R  N  N  F  K  S  A  E  D  C  M  R  T  C  G  G  A
BPTI      GCAGAGCTAAGCGTAACAACTTCAAATCCGCTGAAGACTGCATGCGTACCTGCGGTGGTG  180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
BPTI      GCAGAGCTAAGCGTAACAACTTCAAATCCGCTGAAGACTGCATGCGTACCTGCGGTGGTG  180


          *  *  X
BPTI      CTTAATAAT  189
          |||||||||
BPTI      CTTAATAAT  189
```

Appendix III: Oligonucleotides

LAGC—75 base pairs
Molecular Weight—46,366 g/mol

Forward:

5'/5Phos- AGC TTA TGA AAA CCA AAA ACT TTC TTC TTT TTT GTA TTG CTA CAA ATA TGA TTT TTA TCC CCC TGG CTG GAT GTG-3'

Reverse:

5'/5Phos- GAT CCA CAT CCA GCC AGG GGG ATA AAA ATC ATA TTT GTA GCA ATA CAA AAA AGA AGA AAG TTT TTG GTT TTC ATA-3'

Protein Sequence:

LMKTKNFLLFCIATNMIFIPLAGCX

LINKER—171 base pairs
MolecularWeight—105,725 g/mol

Forward:
5'/5Phos-
GATCCGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGAAGCGGCGGCGA
AAGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAGAAGCGGCGGCGAAAG
AAGCGGCGGCGAAAGAAGCGGCGGCGAAAGGTGGTGGTGGTAGCC-3'

Reverse:
5'/5Phos-
CATGGGCTACCACCACCACCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCG
CCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTC
TTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCG–'3

ProteinSequence:
SEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKEAAAKGGGGSX

BPTI—189 base pairs
MolecularWeight—116,819 g/mol

Forward:

5'/5Phos-
CATGGATGCGTCCGGACTTCTGCCTCGAGCCGCCGTACACGGGTCCCTGCAAAGCGCGCATCATCCG
TTACTTCTACAACGCTAAAGCAGGCCTGTGCCAGACCTTCGTTTACGGTGGCTGCAGAGCTAAGCGT
AACAACTTCAAATCCGCTGAAGACTGCATGCGTACCTGCGGTGGTGCTTAATAAT-3'

Reverse:

5'/5Phos-
CTAGATTATTAAGCACCACCGCAGGTACGCATGCAGTCTTCAGCGGATTTGAAGTTGTTACGCTTAG
CTCTGCAGCCACCGTAAACGAAGGTGTGGCACAGGCCTGCTTTAGCGTTGTAGAAGTAACGGATGA
TGCGCGCTTTGCTGGGACCCGTGTACGGCGGCTCGAGGCAGAAGTCCGGACGCATC-3'

ProteinSequence:
WMRPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCG
GAX**

Appendix IV: p-LAGC-EAK10 Plasmid DNA

```
Serial Cloner 2.6: Alignment p-LAGC-EAK10 Sequence

Similarity: 5526/5526 (100.00 %)


             L  M  K  T  K  N  F  L  L  F  C  I  A  T  N  M  I  F  I  P
p-LAGC-EAK10 agcttatgaaaaccaaaaactttcttctttttttgtattgctacaaatatgattttttatcc   60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 agcttatgaaaaccaaaaactttcttctttttttgtattgctacaaatatgattttttatcc   60


             L  A  G  C  G  S  E  A  A  A  K  E  A  A  A  K  E  A  A  A
p-LAGC-EAK10 ccctggctggatgtggatccgaagcggcggcgaaagaagcggcggcgaaagaagcggcgg  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 ccctggctggatgtggatccgaagcggcggcgaaagaagcggcggcgaaagaagcggcgg  120


             K  E  A  A  A  K  E  A  A  A  K  E  A  A  A  K  E  A  A  A
p-LAGC-EAK10 cgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcgg  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 cgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcgg  180


             K  E  A  A  A  K  E  A  A  A  K  E  A  A  A  K  G  G  G  G
p-LAGC-EAK10 cgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcggcgaaaggtggtggtg  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 cgaaagaagcggcggcgaaagaagcggcggcgaaagaagcggcggcgaaaggtggtggtg  240


             S  P  R  G  E  L  L  S  A  H  N  S  P  I  V  S  R  I  N  F
p-LAGC-EAK10 gtagccctagagggggaattgttatccgctcacaattcccctatagtgagtcgtattaatt  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 gtagccctagagggggaattgttatccgctcacaattcccctatagtgagtcgtattaatt  300


             A  G  S  R  S  R  S  S  T  P  D  A  S  W  P  A  S  P  A  P
p-LAGC-EAK10 tcgcgggatcgagatctcgatcctctacgccggacgcatcgtggccggcatcaccggcgc  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 tcgcgggatcgagatctcgatcctctacgccggacgcatcgtggccggcatcaccggcgc  360


             Q  V  R  L  L  A  P  I  S  P  T  S  P  M  G  K  I  G  L  A
p-LAGC-EAK10 cacaggtgcggttgctggcgcctatatcgccgacatcaccgatggggaagatcgggctcg  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 cacaggtgcggttgctggcgcctatatcgccgacatcaccgatggggaagatcgggctcg  420


             T  S  G  S  *  A  L  V  S  A  W  V  W  W  Q  A  P  W  P  G
p-LAGC-EAK10 ccacttcgggctcatgagcgcttgtttcggcgtgggtatggtggcaggccccgtggccgg  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
p-LAGC-EAK10 ccacttcgggctcatgagcgcttgtttcggcgtgggtatggtggcaggccccgtggccgg  480
```

Appendix V: Sequencing Data

Display Results for 26796-27 A1-T7

3730 Chromatogram

Use the text box to Copy contents to the clipboard.

```
NNNNNNNNNNNNNNNNNNCNCTAGANTAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCTACCACCACCACCTTTCG
CCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGC
TTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCTTTCGCCGCCGCTTCGGAT
CCACATCCAGCCAGGGGGATAAAAATCATATTTGTAGCAATACAAAAAAGAAGAAAGTTTTTGGTTTTCATAAGCTTATCGA
TGATAAGCTGTCAAACATGAGAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAA
TAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTC
AAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACAT
TTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAG
ATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCC
CGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAG
CAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCA
TGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGG
ACCGAAGGAGCTAACCGCTTTTTTNNACANATGGGGGATCATGNAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAN
NATACCAAACGANNNNNNNNGNNNNCNCGATGCCTGNAGCAANGGCAACAACNNN
```

References

1. Jackson CM, Nemerson Y. Blood Coagulation. Annu Rev Biochem. 1980;49:765-811.
2. Riddel JP,Jr, Aouizerat BE, Miaskowski C, Lillicrap DP. Theories Of Blood Coagulation. J Pediatr Oncol Nurs. 2007 May-Jun;24(3):123-31.
3. Versteeg HH, Heemskerk JWM, Levi M, Reitsma PH. New Fundamentals In Hemostasis. Physiol Rev. 2013 01;93(1):327-58.
4. Verhamme P, Hoylaerts MF. Hemostasis And Inflammation: Two Of A Kind? Thrombosis Journal. 2009 / 11 / 18 /;7.
5. Holmsen H. Platelet Function – Assessment, Diagnosis And Treatment. Eur J Haematol. 2006 04;76(4):360-2.
6. Shattil SJ, Bennett JS. Platelets And Their Membranes In Hemostasis: Physiology And Pathophysiology. Ann Intern Med. 1981 01;94(1):108-18.
7. MILBURN JA, FORD I, CASSAR K, FLUCK N, BRITTENDEN J. Platelet Activation, Coagulation Activation And C-Reactive Protein In Simultaneous Samples From The Vascular Access And Peripheral Veins Of Haemodialysis Patients. International Journal Of Laboratory Hematology. 2012 02;34(1):52-8.
8. Nachman RL, Jaffe EA, Weksler BB. Immunoinhibition Of Ristocetin-Induced Platelet Aggregation. J Clin Invest. 1977 Jan;59(1):143-8.
9. Chopek MW, Girma JP, Fujikawa K, Davie EW, Titani K. Human Von Willebrand Factor: A Multivalent Protein Composed Of Identical Subunits. Biochemistry. 1986 Jun 3;25(11):3146-55.
10. Santoro SA, Cunningham LW. Fibronectin And The Multiple Interaction Model For Platelet-Collagen Adhesion. Proc Natl Acad Sci U S A. 1979 Jun;76(6):2644-8.
11. Dean WL. Structure, Function And Subcellular Localization Of A Human Platelet Ca2+-Atpase. Cell Calcium. 1989 Jul;10(5):289-97.
12. Kornfeld S, Mellman I. The Biogenesis Of Lysosomes. Annu Rev Cell Biol. 1989;5:483-525.
13. Clemetson KJ. Mini Review: Platelets And Primary Haemostasis. Thromb Res. 2012;129(18-23):220-4.
14. Kinlough-Rathbone R, Packham M, Reimers H, Cazenave J, Mustard J. Mechanisms Of Platelet Shape Change, Aggregation, And Release-Induced By Collagen, Thrombin, Or A23, 187. J Lab Clin Med. 1977;90(4):707-19.
15. Esmon CT. Cell Mediated Events That Control Blood Coagulation And Vascular Injury. Annu Rev Cell Biol. 1993;9:1-26.
16. Jari Petäjä. Inflammation And Coagulation. An Overview. Thromb Res;127(-):S34-7.
17. Weiss HJ. Platelet Physiology And Abnormalities Of Platelet Function. N Engl J Med. 1975 09/11;293(11):531.
18. Tortora GJ, Derrickson B. Principles Of Anatomy And Physiology / Gerald J. Tortora, Bryan Derrickson. Hoboken, N.J. : John Wiley & Sons, C2012; 13th Ed; 2012.
19. Ashford TP, Frieiman DG. The Role Of The Endothelium In The Initial Phases Of Thrombosis. An Electron Microscopic Study. Am J Pathol. 1967 Feb;50(2):257-73.
20. Lieberman M, Allan D. Marks, Marks DB, Colleen Smith. Marks' Basic Medical Biochemistry: A Clinical Approach. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2009.
21. Campbell JE, Brummel-Ziedins K, Butenas S, Mann KG. Cellular Regulation Of Blood Coagulation: A Model For Venous Stasis. Blood. 2010 / 12 / 23 /;116(26):6082-91.
22. Davie EW, Fujikawa K, Kisiel W. The Coagulation Cascade: Initiation, Maintenance, And Regulation. Biochemistry. 1991 Oct 29;30(43):10363-70.

23. Nomenclature Of Blood Clotting Factors. British Medical Journal. 1959;1(5132):1292-1294.

24. BIGGS R, DOUGLAS AS, MACFARLANE RG, DACIE JV, PITNEY WR, MERSKEY. Christmas Disease: A Condition Previously Mistaken For Haemophilia. Br Med J. 1952 12/27;2(4799):1378-82.

25. Hemostasis And Thrombosis: Basic Principles And Clinical Practice. Annals Of Internal Medicine. 1988 March 1;108(3):504-.

26. Dahlbäck B. Blood Coagulation. The Lancet. 2000 5/6; 355(9215):1627-32.

27. Dorothy M. Adcock M. The Revised Model Of Blood Coagulation. Clinical Hemostasis Review. 2002; 16(6):1.

28. Jerry B. Lefkowitz M. Coagulation Pathway and Physiology. Hemostasis Physiology. 2008; Unknown(Unknown):1.

29. GLOVER RP, KUZELL WC. Prothrombin Time. Determination by A Whole Blood Micro-Method For Control Of Anticoagulant Therapy. Calif Med. 1961 Jul;95:24-9.

30. Morawitz P. Die Chemie Der Blutgerinnung. Ergebnisse Der Physiologie. 1905;4(1):307 <Last_Page> 422.

31. Mccallum CD, Hapak RC, Neuenschwander PF, Morrissey JH, Johnson AE. The Location of the Active Site of Blood Coagulation Factor Viia above The Membrane Surface And Its Reorientation Upon Association With Tissue Factor. A Fluorescence Energy Transfer Study. J Biol Chem. 1996 Nov 8; 271(45):28168-75.

32. Broze GJ,Jr. Tissue Factor Pathway Inhibitor And The Revised Theory Of Coagulation. Annu Rev Med. 1995; 46:103-12.

33. Mann Fd. Blood Clotting. Annu Rev Physiol. 1957; 19:205-30.

34. Walker RK, Krishnaswamy S. The Influence Of Factor Va On The Active Site Of Factor Xa. J Biol Chem. 1993 / 01 / 01 /; 268(19):13920-9.

35. Langner M, Kubica K. The Electrostatics of Lipid Surfaces. Chem Phys Lipids; 101:3-35.

36. Mann KG, Jenny RJ, Krishnaswamy S. Cofactor Proteins In The Assembly And Expression Of Blood Clotting Enzyme Complexes. Annu Rev Biochem. 1988; 57:915-56.

37. Rezaie AR. Identification Of Basic Residues In The Heparin-Binding Exosite Of Factor Xa Critical For Heparin And Factor Va Binding. J Biol Chem. 2000 Feb 4; 275(5):3320-7.

38. Yang L, Manithody C, Qureshi SH, Rezaie AR. Factor Va Alters The Conformation Of The Na+-Binding Loop Of Factor Xa In The Prothrombinase Complex†. Biochemistry. 2008;47(22):5976 <Last_Page> 5985.

39. Dang QD, Di Cera E. Residue 225 Determines The Na(+)-Induced Allosteric Regulation Of Catalytic Activity In Serine Proteases. Proc Natl Acad Sci U S A. 1996 Oct 1;93(20):10653-6.

40. Orfeo T, Gissel M, Butenas S, Undas A, Brummel-Ziedins K, Mann KG. Anticoagulants And The Propagation Phase Of Thrombin Generation. Plos ONE. 2011 11;6(11):1-13.

41. Yang L, Manithody C, Qureshi SH, Rezaie AR. Contribution of Exosite Occupancy By Heparin To The Regulationof Coagulation Proteases By Antithrombin. Thromb Haemost. 2009 Dec 18.

42. Liras A, Segovia C, Gabán AS. Advanced Therapies For The Treatment Of Hemophilia: Future Perspectives. Orphanet Journal of Rare Diseases. 2012; 7(1):97.

43. How Is Hemophilia Treated? - NHLBI, NIH [Internet].; Cited 2/9/2013]. Available From:http://www.nhlbi.nih.gov/health/health-topics/topics/hemophilia/treatment.html.

44. Van Dijk K, Van Der Bom JG, Lenting PJ, De Groot PG, Mauser-Bunschoten EP, Roosendaal G, Et Al. Factor VIII Half-Life And Clinical Phenotype Of Severe Hemophilia A. Haematologica. 2005 Apr; 90(4):494-8.

45. Esmon CT, Xu J, Lupu F. Innate Immunity and Coagulation. Journal of Thrombosis & Haemostasis. 2011 07/02;9(7):182-8.

46. Van Mens TE, Levi M, Middeldorp S. Evolution Of Factor V Leiden. Thromb Haemost. 2013 Apr 25; 110(1).

47. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, De Ronde H, Et Al. Mutation In Blood Coagulation Factor V Associated With Resistance To Activated Protein C. Nature. 1994 May 5;369(6475):64-7.

48. Kunitz M. Crystalline Soybean Trypsin Inhibitor: Ii. General Properties. J Gen Physiol. 1947 03/20;30(4):291-310.

49. Wadelius M(1), Chen LY(2), Bumpstead S(2), Ghori J(2), Bentley D(2), Mcginnis R(2), Et Al. Association Of Warfarin Dose With Genes Involved In Its Action And Metabolism. Hum Genet. 2007 / 03 / 01 /; 121(1):23-34.

50. Borensztajn K, Peppelenbosch MP, Arnold Spek C. Review: Factor Xa: At The Crossroads Between Coagulation And Signaling In Physiology And Disease. Trends Mol Med; 14:429-40.

51. Yang L, Qureshi SH, Yegneswaran S, Rezaie AR. FRET Studies With Factor X Mutants Provide Insight Into The Topography Of The Membrane-Bound Factor X/Xa. Biochem J. 2007; 407(3):427.

52. Arai R, Wriggers W, Nishikawa Y, Nagamune T, Fujisawa T. Conformations of Variably Linked Chimeric Proteins Evaluated By Synchrotron X-Ray Small-Angle Scattering. Proteins. 2004 Dec 1; 57(4):829-38.

53. Berney C, Danuser G. FRET or No FRET: A Quantitative Comparison. Biophys J.; 84:3992-4010.

54. Förster T. Intermolecular Energy Migration and Fluorescence. Ann Phys [FRET]. 1948; 437(1-2):55.

55. Sun Y, Rombola C, Jyothikumar V, Periasamy A. Forster Resonance Energy Transfer Microscopy And Spectroscopy For Localizing Protein-Protein Interactions In Living Cells. Cytometry A. 2013 Sep; 83(9):780-93.

56. Qureshi SH, Yang L, Yegneswaran S, Rezaie AR. FRET Studies With Factor X Mutants Provide Insight Into The Topography Of The Membrane-Bound Factor X/Xa. Biochem J. 2007 Nov 1; 407(3):427-33.

57. Neuenschwander, P.F. (1,3), Williamson SR(1), Baker-Deadmond K, Nalian A(2). Heparin Modulates The 99-Loop Of Factor Ixa: Effects On Reactivity With Isolated Kunitz-Type Inhibitor Domains. J Biol Chem. 2006 / 08 / 11 /;281(32):23066-74.

58. Khanin MA, Semenov VV. A Mathematical Model of The Kinetics Of Blood Coagulation. J Theor Biol. 1989 1/23; 136(2):127-34.

59. Huang Z-, Wun T-, Broze Jr. GJ. Kinetics Of Factor Xa Inhibition By Tissue Factor Pathway Inhibitor. J Biol Chem. 1993 / 01 / 01 /; 268(36):26950-5.

60. Rosing J, Tans G, Govers-Riemslag JW, Zwaal RF, Hemker HC. The Role of Phospholipids And Factor Va In The Prothrombinase Complex. J Biol Chem. 1980 Jan 10; 255(1):274-83.

61. Hasan AA, Chang WS, Budzynski AZ. Binding Of Fibrin Fragments To One-Chain And Two-Chain Tissue-Type Plasminogen Activator. Blood. 1992 05/01; 79(9):2313-21.

62. Budzynski A. Chromogenic Substrates In Coagulation And Fibrinolytic Assays. Laboratory Medicine. July 2001; 32(7):365.

63. Burchenal JEB(1), Marks DS(2), Mann JT(2), Schweiger MJ(2), Rothman MT(2), Ganz P(2), Et Al. Effect Of Direct Thrombin Inhibition With Bivalirudin (Hirulog) On Restenosis After Coronary Angioplasty. Am J Cardiol. 1998 / 01 / 01 /;82(4):511-5.

64. Hogg PJ, Jackson CM, Labanowski JK, Bock PE. Binding Of Fibrin Monomer And Heparin To Thrombin In A Ternary Complex Alters The Environment Of The Thrombin Catalytic Site, Reduces Affinity For Hirudin, And Inhibits Cleavage Of Fibrinogen. J Biol Chem. 1996 Oct 18;271(42):26088-95.

65. Becattini C, Agnelli G, Emmerich J, Bura A, Weitz JI. Initial Treatment Of Venous Thromboembolism. Thromb Haemost. 2006 Sep;96(3):242-50.

66. Damus P, Rosenberg R. Anticoagulant Action Of Heparin. Nature. 1973 Dec 7; 246(5432):355-7.

67. Gettins PGW. Serpin Structure, Mechanism, and Function. Chem Rev. 2002; 102(12):4751 <Last_Page> 4804.

68. Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The Anticoagulant Activation Of Antithrombin By Heparin. Proc Natl Acad Sci U S A. 1997 Dec 23; 94(26):14683-8.

69. Johnson DJ, Li W, Adams TE, Huntington JA. Antithrombin-S195A Factor Xa-Heparin Structure Reveals The Allosteric Mechanism Of Antithrombin Activation. EMBO J. 2006 May 3; 25(9):2029-37.

70. Neuenschwander PF. Exosite Occupation By Heparin Enhances The Reactivity Of Blood Coagulation Factor Ixa. Biochemistry. 2004 Mar 16; 43(10):2978-86.

71. WEITZ JI. Factor Xa Or Thrombin: Is Thrombin A Better Target? Journal Of Thrombosis And Haemostasis. 2007; 5:65-7.

72. Vaughan PJ, Su J, Cotman CW, Cunningham DD. Protease Nexin-1, A Potent Thrombin Inhibitor, Is Reduced Around Cerebral Blood Vessels In Alzheimer's Disease. Brain Res. 1994 Dec 30;668(1-2):160-70.

73. Ascenzi P, Bocedi A, Bolognesi M, Spallarossa A, Coletta M, De Cristofaro R, Et Al. The Bovine Basic Pancreatic Trypsin Inhibitor (Kunitz Inhibitor): A Milestone Protein. Curr Protein Pept Sci. 2003 Jun;4(3):231-51.

74. Oliva ML, Sampaio UM. Bauhinia Kunitz-Type Proteinase Inhibitors: Structural Characteristics and Biological Properties. Biol Chem. 2008 Aug; 389(8):1007-13.

75. Anderson S, Kingston IB. Isolation Of A Genomic Clone For Bovine Pancreatic Trypsin Inhibitor By Using A Unique-Sequence Synthetic DNA Probe. Proc Natl Acad Sci U S A. 1983 / 01 / 01 /; 80(22):6838-42.

76. Ning W, Wang Y, Zhang F, Wang H, Wang F, Wang X, Et Al. Beneficial Effects Of Trypsin Inhibitors Derived From A Spider Venom Peptide In L-Arginine-Induced Severe Acute Pancreatitis In Mice. Plos One. 2013 Apr 15;8(4):E61049.

77. Mutucumarana VP, Duffy EJ, Lollar P, Johnson AE. The Active Site Of Factor Ixa Is Located Far Above The Membrane Surface And Its Conformation Is Altered Upon Association With Factor Viiia. A Fluorescence Study. J Biol Chem. 1992 Aug 25; 267(24):17012-21.

78. Neuenschwander PF, Bianco-Fisher E, Rezaie AR, Morrissey JH. Phosphatidylethanolamine Augments Factor Viia-Tissue Factor Activity: Enhancement of Sensitivity To Phosphatidylserine. Biochemistry. 1995 Oct 31; 34(43):13988-93.

79. Neuenschwander PF, Baker-Deadmond KJ, Jones AD. Low Molecular Weight Heparin Modulates The Binding And Reactivity Of Factor Xa Towards Basic Pancreatic Trypsin Inhibitor: A Surface Plasmon Resonance And Kinetics Study. ASH Annual Meeting Abstracts. 2006 November 16;108(11):1623.

80. Anderer FA, Hornle S. CHEMICAL STUDIES ON KALLIKREIN INACTIVATOR FROM BOVINE LUNG AND PAROTID GLAND. Ann N Y Acad Sci. 1968; 146(2 Chemistry, Ph):381 <Last_Page> 385.

81. Chand HS, Schmidt AE, Bajaj SP, Kisiel W. Structure-Function Analysis Of The Reactive Site In The First Kunitz-Type Domain Of Human Tissue Factor Pathway Inhibitor-2. J Biol Chem. 2004 Apr 23; 279(17):17500-7.

82. Hanahan D. Studies On Transformation Of Escherichia Coli With Plasmids. J Mol Biol. 1983 Jun 5;166(4):557-80.

83. Kamalakkannan S, Murugan V, Jagannadham MV, Nagaraj R, Sankaran K. Bacterial Lipid Modification Of Proteins For Novel Protein Engineering Applications. Protein Eng Des Sel. 2004 Oct;17(10):721-9.

84. Standiford HC, Jordan MC, Kirby WM. Clinical Pharmacology Of Carbenicillin Compared With Other Penicillins. J Infect Dis. 1970 Sep; 122:Suppl:S9-13.

85. Growth Media For E. Coli. [Methods Enzymol. 2013] - Pubmed - NCBI [Internet].; Cited 7/2/2014]. Available From: http://www.ncbi.nlm.nih.gov/pubmed/24182923.

86. Steinle A, Li P, Morris DL, Groh V, Lanier LL, Strong RK, Et Al. Interactions Of Human NKG2D With Its Ligands MICA, MICB, And Homologs Of The Mouse RAE-1 Protein Family. Immunogenetics. 2001 May-Jun;53(4):279-87.

87. Mellanby J. The Coagulation of Blood: Part II. The Actions of Snake Venoms, Peptone And Leech Extract. J Physiol. 1909 Jul 2; 38(6):441-503.

88. Selistre-De-Araujo HS, De Souza EL, Beltramini LM, Ownby CL, Souza DH. Expression, Refolding, and Activity Of A Recombinant Nonhemorrhagic Snake Venom Metalloprotease. Protein Expr Purif. 2000 Jun; 19(1):41-7.

89. Copeland RA. Enzymes. . 2000.

90. Morrison JF, Walsh CT. The Behavior and Significance Of Slow-Binding Enzyme Inhibitors. Adv Enzymol Relat Areas Mol Biol. 1988; 61:201-301.

91. Sankaran K, Wu HC. Lipid Modification Of Bacterial Prolipoprotein. Transfer Of Diacylglyceryl Moiety From Phosphatidylglycerol. J Biol Chem. 1994 Aug 5;269(31):19701-6.

92. Gennity J, Goldstein J, Inouye M. Signal Peptide Mutants Of Escherichia Coli. J Bioenerg Biomembr. 1990 06; 22(3):233-69.

93. Babu MM, Kamalakkannan S, Subrahmanyam YVBK, Sankaran K. Shigella Apyrase – A Novel Variant Of Bacterial Acid Phosphatases? FEBS Lett. 2002 2/13; 512(1–3):8-12.

94. Colace TV, Jobson J, Diamond SL. Relipidated Tissue Factor Linked To Collagen Surfaces Potentiates Platelet Adhesion And Fibrin Formation In A Microfluidic Model Of Vessel Injury. Bioconjug Chem. 2011 Oct 19; 22(10):2104-9.

95. Arai R, Ueda H, Kitayama A, Kamiya N, Nagamune T. Design Of The Linkers Which Effectively Separate Domains Of A Bifunctional Fusion Protein. Protein Eng. 2001 Aug; 14(8):529-32.

96. Su L, Chen SS, Yang KG, Liu CZ, Zhang YL, Liang ZQ. High-Level Expression Of Human Stem Cell Factor Fused With Erythropoietin Mimetic Peptide In Escherichia Coli. Protein Expr Purif. 2006 Jun;47(2):477-82.

97. Wen D, Foley SF, Hronowski XL, Gu S, Meier W. Discovery And Investigation Ofo-Xylosylation In Engineered Proteins Containing A (GGGGS)Nlinker. Anal Chem. 2013;85(9):4805 <Last_Page> 4812.

98. Zhong-Fu H, Wun T, Brose G. Kinectics Of Fxa Inhibition By Tissue Factor Pathway Inhibitor. Biochemistry. 1993;286(36).

99. Brandstetter H, Kuhne A, Bode W, Huber R, Von Der Saal W, Wirthensohn K, Et Al. X-Ray Structure Of Active Site-Inhibited Clotting Factor Xa. Implications for Drug Design and Substrate Recognition. J Biol Chem. 1996 Nov 22; 271(47):29988-92.

100. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory; 2001.

101. Sato H, Akama K, Kojima S, Miura K, Sekine A, Nakano M. Expression Of A Zinc-Binding Domain Of Boar Spermatidal Transition Protein 2 In Escherichia Coli. Protein Expr Purif. 1999 8; 16(3):454-62.

102. Pan SH, Malcolm BA. Reduced Background Expression and Improved Plasmid Stability with Pet Vectors In BL21 (DE3). Biotechniques. 2000 Dec;29(6):1234-8.

103. Leite DC, Balieiro FC, Pires CA, Madari BE, Rosado AS, Coutinho HL, Et Al. Comparison Of DNA Extraction Protocols For Microbial Communities From Soil Treated With Biochar. Braz J Microbiol. 2014 May 19;45(1):175-83.

104. Pureyield™ Plasmid Midiprep System Protocol [Internet].; Cited 6/27/2013]. Available From: http://www.promega.com/resources/protocols/technical-manuals/0/pureyield-plasmid-midiprep-system-protocol/?__utma=1.328599151.1372372193.1372372193.1372372193.1&__utmb=1.1.10.1372372193&__utmc=1&__utmx=-&__utmz=1.1372372193.1.1.utmcsr=(direct)|utmccn=(direct)|utmcmd=(none)&__utmv=-&__utmk=157702586.

105. Agilent Technologies. Strataprep EF Plasmid Midiprep Kit. Catalog #400721 And #400722; Revision A.01:1.

106. Klingemann H-, Egbring R, Holst F, Gramse M, Havemann K. Degradation Of Human Plasma Fibrin Stabilizing Factor XIII Subunits By Human Granulocytic Proteinases. Thromb Res. 1982 12/15;28(6):793-801.

107. Bi Y, Qiao X, Hua Z, Zhang L, Liu X, Li L, Et Al. An Asymmetric PCR-Based, Reliable and Rapid Single-Tube Native DNA Engineering Strategy. BMC Biotechnol. 2012 Jul 6; 12(1):39.

108. Jo C, Jo SA. A Simple Method To Construct T-Vectors Using Xcmi Cassettes Amplified By Nonspecific PCR. Plasmid. 2001 1;45(1):37-40.

109. Coleman JE. Structure and Mechanism Of Alkaline Phosphatase. Annu Rev Biophys Biomol Struct. 1992; 21:441-83.

110. Goeddel DV, Kleid DG, Bolivar F, Heyneker HL, Yansura DG, Crea R, Et Al. Expression In Escherichia Coli Of Chemically Synthesized Genes For Human Insulin. Proc Natl Acad Sci U S A. 1979 Jan;76(1):106-10.

111. DNA Electrophoresis : Methods And Protocols - NLM Catalog - NCBI [Internet].; Cited 7/2/2014]. Available From: http://www.ncbi.nlm.nih.gov/nlmcatalog/101620321.

112. Smith SA, Morrissey JH. Rapid And Efficient Incorporation Of Tissue Factor Into Liposomes. J Thromb Haemost. 2004 Jul;2(7):1155-62.

113. Lankeit M, Konstantinides S. Mortality Risk Assessment and The Role Of Thrombolysis In Pulmonary Embolism. Crit Care Clin. 2011 Oct;27(4):953,67, Vii-Viii.

114. Aditya S. Oral and Parenteral Anticoagulants: New Kids On The Block. J Postgrad Med. 2012;58(4):275.

115. Morgenthaler M, Schaerer K, Paulini R, Sander UO, Banner DW, Schlatter D, Et Al. Factor Xa - Cation. . 2005.

116. Madej T, Addess KJ, Fong JH, Geer LY, Geer RC, Lanczycki CJ, Et Al. MMDB: 3D Structures And Macromolecular Interactions. Nucleic Acids Res. 2012 Jan; 40(Database Issue):D461-4.

117. Stoilova-Mcphie S. 3-Dimensional Structure of Membrane-Bound Coagulation Factor VIII: Modeling Of The Factor VIII Heterodimer Within A 3-Dimensional Density Map Derived By Electron Crystallography. Blood. 2002; 99(4):1215 <Last_Page> 1223.