Cloning, Characterization, and Analysis of the Complete

Bovine Factor XIIa inhibitor cDNA.

by

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ABSTRACT

The bovine homologue of the human C1 esterase inhibitor is called the bovine

factor XIIa inhibitor. Like the human C1 esterase inhibitor, bovine factor XIIa inhibitor is

a serine protease inhibitor with a reported molecular weight of between $\approx$72,000 and

92,000 Da. To determine the primary structure of bovine factor XIIa inhibitor, the cDNA

was cloned and sequenced. Bovine total liver RNA was reverse transcribed using an

$_{oligo}$dT$_{17}$ primer to produce cDNA. This single stranded cDNA was then used as a

template for polymerase chain reaction amplification experiments. The primers were

designed using amino acid sequence data from tryptic fragments of purified plasma-

derived bovine factor XIIa inhibitor and human C1 esterase inhibitor. The bovine factor

XIIa inhibitor cDNA was amplified in two overlapping pieces and each piece was cloned

separately. The cDNA sequence is 1608 bp long. The identity of the bovine factor XIIa

inhibitor cDNA was established by comparison of the cDNA sequence with sequences

derived from the bovine and human protein and the human cDNA. The 5-prime end of the

cDNA starts at the initiation codon, ATG. The 3-prime end extends 179 base pairs past

the stop codon, TAG, to the polyadenylation recognition signal AATAAA, followed 14 bp

later by the poly-A tail. An open reading frame of 1404 bp encodes the bovine factor XIIa

inhibitor polypeptide. Translation of the bovine factor XIIa inhibitor cDNA nucleotide

sequence into amino acid sequence results in a 468 amino acid polypeptide that includes a

23 amino acid leader peptide. The theoretical molecular weight of the 445 amino acid

non-glycosylated protein, is 49,217 Da. Comparison of the predicted amino acid

sequences of the bovine factor XIIa inhibitor and human C1 esterase inhibitor

polypeptides shows an overall sequence identity of 60%. Both the human and bovine

proteins have leader peptides, 22 amino acids and 23 amino acids respectively, which are

90% identical. The N-terminal region of the plasma bovine factor XIIa inhibitor, comprising the next 99 amino acids, shows only 17% identity with the same region of the human C1 esterase inhibitor. The remainder of the proteins encode the serpin regions and share 68% identity. The mature human protein is 33 amino acids longer than the bovine protein; these extra residues occur as two insertions or amplifications with respect to the bovine protein. The first lies between residues 95 and 96 of the bovine protein and is 27 amino acids long; the second lies between residues 385 and 386 of the bovine protein and is 6 amino acids long. The 27 amino acid insertion comprises the bulk of a region in the human protein consisting of nine tetra-peptide repeats, seven of which conform to the consensus Glx-Pro-Thr-Thr. This region, in the human C1INH protein is associated with O-linked glycosylation. The six amino acid insertion lies at the very 3-prime end of exon 7 of the human gene. There is not enough data at this time to interpret the significance of the six amino acid insertion.

It was unclear from the cDNA sequence if the 99 amino acid N-terminal region of the bovine factor XIIa inhibitor protein was merely a region of low identity or a novel exon that had been acquired by the bovine protein or deleted from the human protein. Experiments were performed to elucidate the genomic organization of the 5' region of the bovine factor XIIa inhibitor gene. The results show that while the N-terminal sequences of the human and bovine proteins are non-identical, the genomic organization in the 5-prime region of both genes is the same. Thus, the 27 amino acid insertion is the result of an amplification or deletion of an existing sequence rather than the acquisition or loss of an (novel) exon.

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LIST OF ABBREVIATIONS

A	absorbance
aa(s)	amino acid(s)
ADP	adenosine diphosphate
Amp	ampicillin
AT-III	antithrombin III
BFXIIaINH	bovine factor XIIa inhibitor
bis	N,N'-methylenebisacrylamide
C1INH	Human C1-esterase inhibitor
bp(s)	basepair(s)
Ca^{++}	calcium ions
cDNA	complementary deoxyribonucleic acid
cm	centimeters
DIC	disseminated intravascular coagulation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EtBr	ethidium bromide
FII(a)	factor II/prothrombin(activated factor II/thrombin)

FV(a)	factor V(activated)
FVII(a)	factor VII(activated)
FVIII(a)	factor VIII(activated)
FIX(a)	factor IX(activated)
FX(a)	factor X(activated)
FXI(a)	factor XI(activated)
FXII(a)	factor XII(activated)
g	force of gravity
Gla	γ-carboxyglutamic acid
h	hour(s)
HAE	hereditary angioedema
HMWK	high molecular weight kininogen
IPTG	isopropylthiogalactoside
kDa	kilodaltons
kbp(s)	kilo base pair(s)
Klenow fragment	E. coli DNA polymerase (large fragment)
LB	Luria-Bertoni broth
min	minute(s)
Mr	molecular weight
mRNA	messenger ribonucleic acid
NaOAc	Sodium acetate
OAc	acetate
OD	optical density
orf	open reading frame

PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RGD-X	Arg-Gly-Asp-X where X may vary but is usually Ser or Val
RNA	ribonucleic acid
RNase A	ribonuclease A
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
sec(s)	second(s)
sscDNA	single-stranded complementary deoxyribonucleic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane base
UTR	untranslated region
UV	ultra-violet light
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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I. INTRODUCTION

A. WHY A COW?

The choice of the cow as an animal model for studying the human process of blood clotting is based on the following criteria: evolutionary proximity, access to tissues for experimental purposes, and experimental design considerations. *Evolutionary Proximity.* Humans and cattle are closely related evolutionarily. Because of this, the bovine clotting systems and our own are very similar in organization and function. Therefore, if properly designed, experiments on the clotting system of cows provide results that can legitimately be applied to the human system. Furthermore, comparison of the molecular and functional characteristics of the two systems can be exploited to learn about the structure function relationships in the molecules being studied. *Access to Material.* Because cows are husbanded for the purpose of eating (among other things), we have a ready supply of large quantities of all types of tissue including blood. *Experimental Design.* The ethical constraints that we work under, with regards to human subjects, do not all apply to the use of cows as experimental subjects; it is therefore possible to design experiments using cattle that we could not do using humans.

B. OVERVIEW OF HAEMOSTASIS AND THROMBOSIS

Vertebrates have evolved a closed, pressurized circulatory system that is required for a number of essential physiological processes including transport of oxygen and nutrients, removal of metabolic waste, and the functioning of the immune system. Haemostasis (literally the stoppage of blood flow) is the process of maintaining blood flow and volume by arresting blood loss at sites of vascular damage. Haemostasis involves the interaction of four different processes - the vascular endothelium, vasoconstriction, platelet activation and plug formation, blood coagulation and fibrinolysis (Thompson, &

Harker, 1983). The first three of the above mentioned processes result in the formation of a blood clot, whereas the last is involved in the removal of the clot during the subsequent phases of tissue repair. These reactions occur in two phases (Davie, *et al.*, 1991). The primary phase comprises the first two processes while the secondary phase consists of the latter two processes. In the smallest blood vessels primary haemostatic reactions are enough to control bleeding (Bloom, *et al.*, 1990). Damage of larger vessels requires activation of the secondary phase; this involves activation of the coagulation sequence, the generation of thrombin and the conversion of fibrinogen to fibrin, followed by fibrinolysis. In the primary phase, platelets adhere to damaged endothelium and/or components of the subendothelial matrix exposed by the damage to the endothelium. Platelets adhere to the endothelial tissue through interaction with adhesive proteins, e.g. von Willebrand factor (vWF), via vWF receptors on the subendothelium and on activated platelets (Girma, *et al.*, 1987; Ruggeri, and Zimmerman, 1987; Lopez, *et al.*, 1988; and Hickey, et al., 1989). Activated platelets are bound to each other by fibrinogen via fibrinogen receptors, thereby stabilizing the platelet plug (Bennet, et al., 1982; Savage, & Ruggeri, 1991). Aggregated, activated platelets then act to potentiate and concentrate coagulation reactions at the site of damage by exposing binding sites for coagulation factors (Bloom, *et al.*, 1990) (see figure. 1A). The secondary phase of haemostasis involves the activation of the clotting cascade. The basic model proposed independently by Davie and Ratnoff, and MacFarlane, in 1964 is that blood coagulation results from an ordered interaction of serine protease enzymes. These enzymes circulate as zymogen precursors which are activated by limited proteolysis (Davie, & Ratnoff, 1964; and MacFarlane, 1964). Activation of these coagulation factors, in the presence of co-factors, results in the sequential amplification of the triggering stimulus, the generation of thrombin, the conversion of fibrinogen to fibrin, and the formation of a platelet-fibrin plug (see figure 2). The fibrinolytic system regulates deposition of fibrin and its removal during healing (Majerus, 1987). Thrombin plays a

central role in these processes. The activities of these serine proteases are regulated by serine protease inhibitors, (SERPINS), including anti-thrombin III, heparin co-factor II, and C1-esterase inhibitor (the bovine homologue of which is factor XIIa inhibitor (Thornton & Kirby, 1987; Muldbjerg, *et al.*, 1994; Edmeston, *et al.*, *unpublished*)). Vertebrates require a closed circulatory system for a number of essential physiological processes, this in turn requires haemostasis. Haemostasis is the process of maintaining the functional integrity of the blood circulatory system and it takes place on the inner surface of the vasculature. The process can be divided into four steps that occur in two phases, central to which is the coagulation cascade. Blood coagulation is a complex regulatory process that involves the cooperative interactions of many factors and cofactors. After outlining the role of the vascular endothelium, I shall briefly describe each of these steps in turn.

1. The Vascular Endothelium.

The vascular endothelium (see figure 1), originally thought to play a passive role in the process of haemostasis, is now known to play both active and passive roles in controlling and regulating the process (Thompson, & Harker, 1983). The vessel wall forms the barrier between the circulating blood and the underlying tissues. The intact vessel wall provides diverse anticoagulant mechanisms. The cells which line the luminal surface of the blood vessels are involved with the regulation of vascular permeability, maintaining smooth blood flow (Guyton, 1976) and preventing abnormal clot formation. The vascular endothelium is able to synthesize, secrete, and bind different coagulation and fibrinolytic components, lipid compounds, and glycosaminoglycans, each of which plays a role in the regulation of the processes of haemostasis (Jaffe, *et al.*, 1984). These result in the endothelial cells presenting a highly thromboresistant surface under normal physiological conditions (see figure 1B), and a highly reactive surface for the induction of

the haemostatic response if the cell surface is damaged or severed. This results in the minimization of inappropriate thrombosis, and the localization and concentration of the haemostatic reactions at the areas of damage and blood loss (Preissner, 1990). Platelets do not normally adhere to intact vascular endothelial cells (Wight, 1980), and the non-turbulent blood flow helps prevent platelet activation by mechanical means such as shearing (Guyton, 1976). In addition to the mechanical anti-coagulant properties of the endothelial tissue, there are hormonal and enzymatic factors synthesized and secreted that inhibit coagulation. These include prostaglandin I_2, (Bloom, 1990), nitric oxide (Palmer, *et al.*, 1987, 1988) thrombomodulin, heparin and plasminogen activators (Colman, *et al.*, 1987). The anti-coagulant activities of intact vascular endothelial cells is briefly described below (see figure 1).

Endothelial cells synthesize prostaglandin I_2 which is a powerful inhibitor of platelet aggregation (Weksler, *et al.*, 1977) and a potent vasodilator. In addition, endothelial cells express thrombomodulin and synthesize heparin sulfate (Esmon, & Owen, 1981). When thrombin binds to thrombomodulin it becomes an anti-coagulant and changes its substrate specificity from fibrinogen to protein C. Protein C is activated by the thrombin/thrombomodulin complex by limited proteolysis. Activated protein C proteolytically inactivates the pro-coagulation co-factors Va and VIIIa(Esmon, & Owen, 1981). The activity of anti-thrombin III is greatly enhanced by the presence of heparin sulfate, expressed on the cell surface of intact vascular endothelium. Thrombin also induces endothelial cells to release prostaglandin I_2. When the vessel surface is damaged, either mechanically by crushing, abrasion or severance, or enzymatically by bacterial endotoxins, the surface becomes a substrate for coagulation. When the luminal lining of the blood vessels is damaged or disrupted, the subendothelial connective tissue matrix is exposed, including banded fibrils of collagen to which platelets strongly adhere. The

Figure 1. Roles played by the vascular endothelium in controlling haemostasis. A) Damaged endothelium provides a substrate for platelet adhesion and activation, while providing a surface where coagulation can initiate. B) Intact endothelial tissue inhibits platelet activation, aggregation, and coagulation.

Outside blood vessel. | Luminal (inside) of blood vessel.

A) Damaged vascular endothelium activates platelets and provides a surface where coagulation can initiate.

Activated platelets

Prothrombin

Activated factors V and X

Fibrinogen

Thrombin

Fibrin

B) Intact vascular endothelium inhibits platelet activation and blood coagulation.

PGI$_2$, inhibits platelet activation.

Plasminogen

Protein C

Plasminogen Activator

Plasmin

Lysis of Fibrin

Thrombomodulin. Thrombin.

Activated protein C

Inactivated co-factors V and VIII

Thrombin

Heparin Sulfate Anti-thrombin III

Inactivated Thrombin

binding of the platelets to the subendothelial extracellular matrix results in their activation. Activated platelets interact with each other and with other platelets to form a platelet plug. In addition damaged cells secrete tissue factor, a lipoprotein which in cooperation with factor VII (FVII) promotes coagulation through the activation of factor X (FX), on a phospholipid surface, in the presence of calcium (Walsh, 1987; Bloom, 1990).

2. Vasoconstriction at the Site of the Wound.

In response to injury there is an immediate, intense, and transient, constriction of blood vessels. Vasoconstriction is not required for haemostasis but is critical in preventing excess blood loss immediately after severance of vessels (Guyton, 1976). Control of the reflex is mediated locally, can be observed in denervated vessels, and is probably dependent upon stretch receptors in smooth muscle fibres (Davies, & McNicol, 1984). The duration of these vasoconstriction effects is increased by factors such as serotonin, ADP, and platelet derived thromboxane A_2 (Mustard, *et al.*, 1987) that are secreted at the site of injury by activated platelets.

3. Platelet Activation and Aggregation.

The main function of platelets is to maintain haemostasis. To perform this function, platelets undergo activation, adherence, and aggregation reactions and interact with the clotting cascade. These steps are presented separately but occur in vivo as a cooperative series of overlapping interactions.

a. Activation. The exact mechanism of platelet activation in vivo is not known, but probably includes exposure to any and all of the following factors: adhesive molecules present in the subendothelial matrix, e.g. vWF, fibrinogen, fibronectin, collagen, laminin, and vitronectin (Bloom, 1990) and or exposure to ADP, thrombin, or thromboxane A_2 (Colman, *et al.*, 1987). Platelet activation consists of three main things: adherence to de-

endothelialized sites in the circulation to cover the de-nuded areas, and maintain the integrity of the circulatory system. Exposure or expression of "latent" vWF, and fibrinogen receptors on the platelet allow the platelets to bind to the damaged areas and to each other respectively. Secretion of active agents recruits other platelets and release of contents of intracellular granules that contain, ADP, fibrinogen, vWF, thromboxane A_2, and FV (Holmsen, 1987; Weksler, 1987; and Colman, *et al.*, 1987). Changes in lipid structure of the platelet membrane allow interaction with clotting factors and provide a vital catalytic element in fibrin production. (Bloom, 1990).

b. Adhesion. Platelets do not normally adhere to undamaged endothelium but in haemostatic reactions, platelets adhere to exposed subendothelium. Recent evidence (Ginsberg, *et al.*, 1988; and Hawiger, 1987) indicates that the interactions between fibrinogen, fibronectin and vWF are mediated by RGD-X (Arg-Gly-Asp-X, where X may vary but is usually Ser or Val) receptors. Upon activation, platelets change shape from lenticular disks to pseudopod studded spheres followed eventually by spreading so that the activated adherent platelets form a mono-layer over the de-nuded area.

c. Aggregation. Once a layer of platelets has adhered to the subendothelium the platelets aggregate. Aggregation consists of Ca^{++}-and fibrinogen-dependent platelet-to-platelet adherence (Davies, & McNicol, 1984). Aggregation can be induced in vitro by exposing platelets to ADP. This results in the exposure of fibrinogen binding sites (Colman, & Walsh, 1987). In vivo there are several Ca^{++} dependent pathways including an ADP mediated one (Bloom, 1990). Platelets contain α-granules (containing PDGF, fibrinogen, fibronectin, FV, high molecular weight kininogen, and vWF) and dense bodies (that contain ADP, serotonin, and Ca^{++}); aggregation of the platelets stimulates the release of the contents of the granules. Adherent platelets become activated by contact with collagen and the action of thrombin as well as by several extracellular agonists. These result in shape change, secretion of various platelet constituents, exposure of platelet

protein receptors, and the flip-flop mechanism for bringing phospholipid to the external surface for participation in coagulation. Under physiological conditions the formation of an aggregated plug takes place simultaneously with activation of the coagulation system, so that the processes interact in a co-ordinated fashion to establish normal haemostasis. The effect of these changes is to localize the platelet aggregation, activate other platelets in the immediate vicinity (causing them to aggregate at the wound site) and to provide a surface that allows the formation of the prothrombinase complex, thus leading to the acceleration of blood coagulation. Platelets interact with and contribute to the coagulation cascade in at least two different ways. The most important is the catalytic function which they provide in the generation of thrombin by the activated FX (FXa) complex and in the generation of FXa by the intrinsic pathway (see figure 2). The presence of phospholipids from the platelet membrane accelerates both reactions many thousand fold, probably by concentrating the reactants into optimal orientations (Davies, & McNicol, 1984). In addition, platelets interact with the coagulation mechanism through an inherent capability to bind and activate various clotting factors. Factor XII (FXII), factor XI (FXI), and FX are activated by washed suspensions of platelets (Walsh, 1972; 1987), though it is unclear if the FXIa promoting activity is completely distinct from the intrinsic activation system. Platelets have been shown to bind specifically to activated factor Va (FVa) which in turn acts as a high affinity binding site for FXa (Kane, 1980; Tracy, *et al.*, 1981; Bloom, 1990). Platelets also act as a shield for the early stages of thrombin generation by protecting the early clotting factors from inactivation by plasma inhibitors e.g. the release of platelet factor four which neutralizes the effects of heparin.

4. The Clotting Cascade.

The clotting cascade involves the enzymatic amplification of the initial triggering signal; the amplification consists of the stepwise activation, by limited proteolysis, of a

series of circulating serine protease zymogens to serine proteases. Because each activated participant in the cascade is an enzyme whose substrate is the next zymogen in the cascade, the signal strength is amplified at each step. The clotting cascade is the term used to describe the enzymatic amplification pathway that starts in response to vascular injury and results in the formation of a stable fibrin clot (see figure 2). The cascade is comprised of two separate pathways: the intrinsic pathway, so named because all of the required components are intrinsic to blood, and the extrinsic pathway, named because some of the initiating factors are released by damaged endothelium and are thus extrinsic to blood (MacFarlane, 1964; and Davie, & Ratnoff, 1964). The relative physiological significance of the two pathways is not fully understood. The two pathways converge at the activation of FX. FXa then activates prothrombin (factor II) to thrombin (factor IIa). Thrombin cleaves fibrinogen to fibrin which forms the basis of the stable clot. In the following sections, the steps of the two pathways will be discussed separately, though, *in vivo*, they overlap and act together to affect the stoppage of blood loss.

a. The Intrinsic Pathway. The intrinsic pathway, also known as the contact activation pathway, has been principally investigated *in vitro* . The contact activation pathway is so called because it was initially observed, *in vitro*, to occur upon blood *contact* with a negatively charged surface, e.g. glass, or kaolin sulfate (Colman, *et al.*, 1987). *In vivo*, the negatively charged surface may be supplied by the exposed subendothelial collagen at the site of vascular damage (Colman, *et al.*, 1987). There are four proteins involved in the initiation of the contact activation system (see figure 2): FXII, prekallikrein, high molecular weight kininogen, and FXI (Davies, & McNicol, 1984). The contact pathway, *in vitro*, is initiated by FXII. FXII is also known as Hageman factor, after John Hageman for whom the first FXII deficiency was described
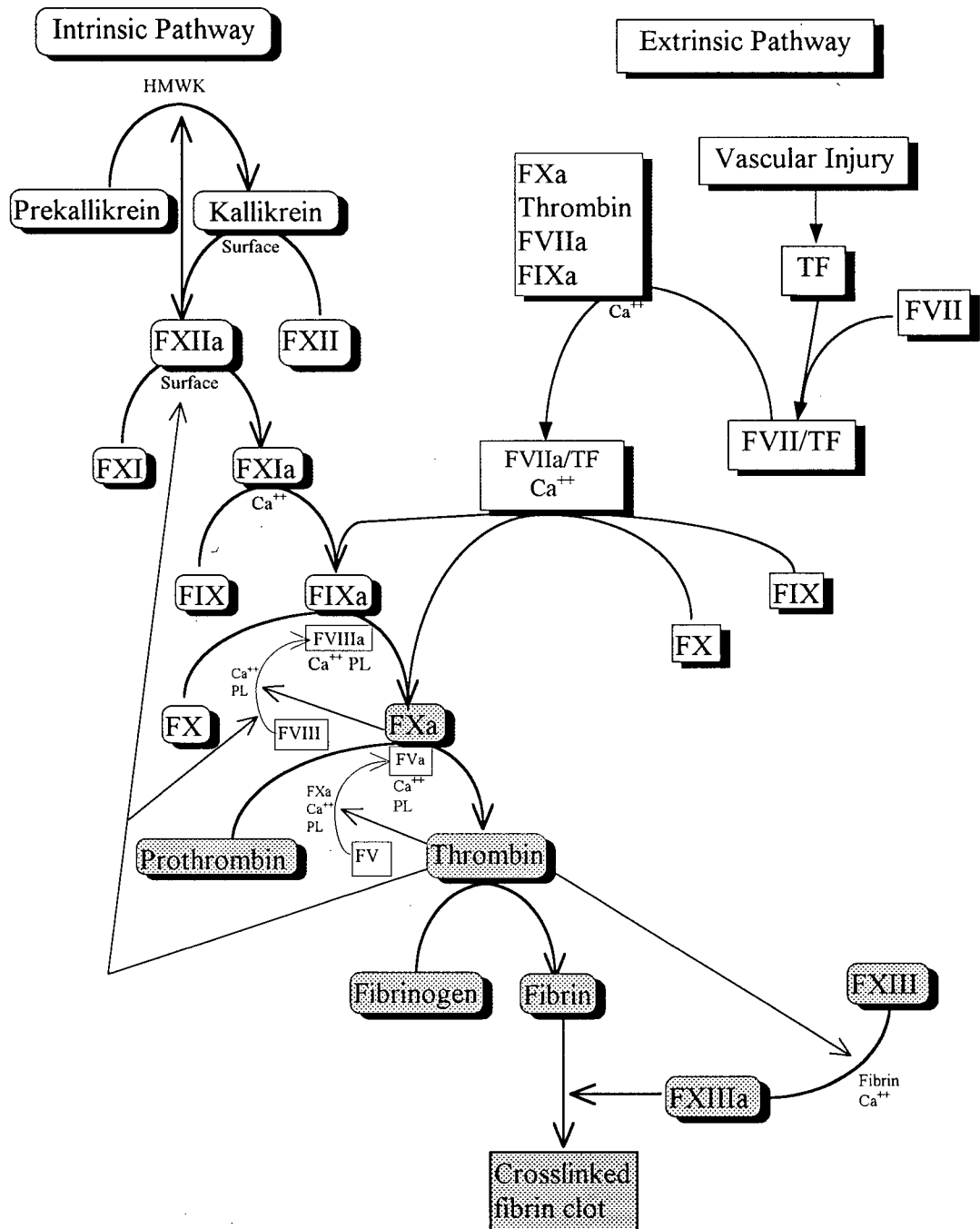
Figure 2. Schematic overview of the clotting cascade. Fibrin clot can be generated by either the intrinsic or extrisnic pathway. Initiation of the intrinsic pathway (⬤) occurs *via* activated FXII. Activation of the extrinsic pathway (⬤) follows vascular injury and the exposure of tissue factor to the blood. The two pathways converge into a common pathway (⬤) at the generation of activated FX. The possible new role for thrombin in the activation of the intrinsic pathway, *via* FXI, is shown. Clotting factors are designated "F" followed by their number, in roman numerals, and activated factors are designated by a lowercase a. PL is phospholipid; Ca^{++} is calcium 2+; and TF is tissue factor.

(Ratnoff, & Colopy, 1955). John Hageman did not suffer any bleeding disorders; in addition, chickens and whales are apparently naturally devoid of FXII activity, but, do not suffer bleeding disorders either (Ratnoff, & Rosenblum, 1958; Robinson, et al., 1969). Therefore the exact physiological importance of the initiating steps of the intrinsic pathway with regards to blood clotting is not fully understood; however, the severe bleeding disorders that result from deficiencies in the latter steps involving factors IX, X, and VII, clearly reveal their importance.. The exact mechanism and order of steps involved in the initiation of the intrinsic pathway are unclear (Jackson, & Nemerson, 1980), but include the following steps (see figure 2): the activation of FXII, probably reciprocal activation of FXII and prekallikrein (Rosenberg, 1987), in the presence of cleaved high molecular weight kininogen, and/or by autoactivation once it is bound to a negatively charged surface, and/or by kallikrein. Activated FXII (FXIIa) a serine protease, then goes on to activate prekallikrein (to kallikrein), FXI (to FXa) and to cleave high molecular weight kininogen releasing bradykinin, FXII can also activate itself to FXIIa. In addition, FXIIa is known to interact with other pathways (see figure 5). FXIIa can activate FVII (see figure 2), thus activating the extrinsic pathway. Cleavage of high molecular weight kininogen by FXIIa results in the release of bradykinin which is a potent vasodilator (Kluft, et al., 1987). This results in the reduction of blood pressure which stimulates the release of tissue-type plasminogen activator by endothelial cells (Hekman, 1987). Tissue-type plasminogen activator, FXIIa, and kallikrein activate plasminogen to plasmin, which proteolytically lyses the fibrin clot (Colman, et al., 1987). In the presence of Ca^{++}, FXIIa activates factor IX (FIX) to FIXa by limited proteolysis. In a complex with Ca^{++}, phospholipid, and factor VIIIa (FVIIIa), FIXa then activates FX to FXa. This is where the intrinsic and the extrinsic pathways converge and all subsequent steps are common to both pathways. Present evidence suggests that the intrinsic pathway plays an important role in the growth and maintenance of fibrin formation in the coagulation

cascade, while the extrinsic pathway is critical in the rapid initiation of fibrinogen formation (Davie, *et al.*, 1991).

b. *The Extrinsic Pathway.* Initiation of the extrinsic pathway requires tissue factor (TF) (see figure 2), which is located in the tissue adventitia and comes in contact with the blood only after vascular injury (Maynard, *et al.*, 1975, 1977; Weiss, *et al.*, 1989; Wilcox, *et al.*, 1989). TF is an integral membrane protein (Bach, *et al.*, 1981; Broze, *et al.*, 1985; Guha, *et al.*, 1986) that is tightly associated with phospholipid (Pitlick, & Nemerson, 1970). TF also has a high affinity for plasma FVII (Broze, 1982; Bach, *et al.*, 1986; Fair, & McDonald, 1987; Sakai, *et al.*, 1989). When vascular injury occurs, the two proteins form a one-to-one complex. This results in the conversion, by limited proteolysis (Nemerson, & Repke, 1985; Rao, & Rapaport, 1988; Sakai, *et al.*, 1989), of FVII to the serine protease, activated FVIIa. This reaction is Ca^{++} dependent and is catalyzed by the presence of trace amounts of proteases circulating in the blood, e.g. FXa, thrombin, FVIIa, FIXa, or an unidentified plasma or cellular enzyme (Radcliffe, & Nemerson, 1976; Kisiel, *et al.*, 1977b; Masya, *et al.*, 1982; Wildgoose, & Kisiel, 1989; Pedersen, *et al.*, 1989). In the presence of Ca^{++} and phospholipid the FVIIa-tissue factor complex, then converts FX to activated FX, FXa. This is accomplished by limited proteolysis of a single Arg_{52}-Ile peptide bond in the amino-terminal end of the heavy chain (DiScipio, *et al.*, 1977). Tissue factor functions as a cofactor in the activation of factors VII and FX, and greatly accelerates these reactions, apparently by causing a conformational change in FVII to FVIIa (Nemerson, & Gentry, 1986). FXa is a serine protease that is part of the prothrombinase complex, consisting of FVa, prothrombin, Ca^{++}, and phospholipid (Tracy, *et al.*, 1981). In the prothrombinase complex, FXa activates prothrombin (FII) to thrombin (FIIa). FVa participates in this reaction as a cofactor that accelerates the V_{max} of the reaction about 1000-fold (Davie, *et al.*, 1991). Activation of FV is essential for its normal participation in the generation of thrombin, and probably occurs initially *via* FXa,

in the presence of Ca^{++}, and phospholipid (Monkovic, & Tracy, 1990), and finally *via* the activity of thrombin, once it has been generated (Nesheim, & Mann, 1979; Esmon, 1979; Susuki, *et al.*, 1982).

5. The Common Pathway: Fibrin Production.

When thrombin is generated (see figure 2), it converts fibrinogen to fibrin (see figure 5) by limited proteolysis. Fibrinogen is a glycoprotein that exists as a dimer of two identical halves each comprised of three polypeptide chains, Aα, Bβ and γ, connected by disulphide bridges. The two halves are connected by disulphide bridges at their N-terminal ends, forming a linear molecule with globular domains at either end (the C-termini) and in the middle (the N-termini). The formation of fibrin is due to the cleavage of a peptide bond in each of the two α chains and β chains (Blomback, & Blomback, 1972). This cleavage releases four fibrinopeptides (two A and two B) and gives rise to fibrin monomers that have novel amino-terminal sequences in both the α and β chains (Davie, *et al.*, 1991). The new N-terminal sequences can interact with C-terminal domains of other fibrin molecules, resulting first in the formation of a dimer of fibrin molecules arranged in a half-overlap and side-to-side manner (see figure 5), and later as more molecules of fibrin are added in the formation of polymers of varying length and thickness (Laudano, & Doolittle, 1980, 1984, & 1987). The polymerization of fibrin results in the proper alignment of γ chains, in the terminal domains, for cross-linking by factor XIIIa (FXIIIa). In addition to forming fibrin, thrombin also catalyses the conversion of FXIII to activated FXIII (FXIIIa) in the presence of Ca^{++} (Lorand, & Konishi, 1964; Naski, *et al.*, 1991). Plasma FXIII circulates as a precursor comprising four polypeptide chains. FXIIIa is a transglutaminase and crosslinks fibrinogen, firstly *via* their γ chains then by their α chains (Folk, & Finlayson, 1977). These covalent cross links lead to the formation of a very

strong fibrin clot. FXIIIa will also cross-link other plasma proteins such as fibronectin and may be responsible for anchoring the clot to the extracellular matrix (Davie, *et al.*, 1991).

6. Fibrinolysis.

The fibrinolytic system is the primary pathway for fibrin clot removal by enzymatic degradation of the fibrin polymers (Colman, *et al.*, 1987). Degradation of fibrin can be affected by plasmin and some leukocyte derived proteases (Francis, & Marder, 1987). Plasmin is a two chain proteolytic enzyme that is derived from the single chain precursor called plasminogen. Plasminogen has an N-terminal glutamic acid residue and is called Glu plasminogen. Once generated, plasmin cleaves the Glu plasminogen exposing a Lys at the N-terminal; the resulting Lys plasminogen has a higher affinity for fibrin than Glu plasminogen. Five kringles at the N-terminal of plasmin mediate the binding to fibrin (Bloom, 1990).

There are two main types of plasminogen activators: 1) urokinase, and 2) tissue plasminogen activator (tPA). Urokinase is produced by the endothelium and in the kidneys as a single chain precursor. tPA is produced by the endothelium and is the main activator of plasminogen (Hekman, 1987). tPA is secreted as a single chain which is cleaved by plasmin to a two-chain molecule. In the presence of fibrin, tPA-catalyzed activation of plasminogen is markedly increased. The luminal deposition of fibrin thus seems not only to stimulate tPA release, but promotes the formation of a mutually interactive ternary complex with plasminogen. Release of tPA may be triggered by physical exercise, venous occlusion, or infusion of adrenaline and vasopressin or its analogues. Patients with thromboembolic disease may have an impaired tPA response to these stimuli. tPA can also be activated through interaction with the contact phase of blood coagulation, by a mechanism called the FXII dependent fibrinolytic pathway

(Colman, *et al.*, 1987). The FXII dependent fibrinolytic pathway involves the interactions of FXII, prekallikrein, high molecular weight kininogen, and FXI.

The activity of plasmin is regulated in two ways: (1) by regulating the activation of plasminogen to plasmin, and (2) by regulating the activity of plasmin directly. Activation of plasminogen to plasmin is affected primarily by plasminogen activator inhibitor type 1. Plasminogen activator inhibitor type 1 is produced by endothelial cells as well as other cells (Sprengers, *et al.*, 1987), and acts with tPA in regulating fibrinolysis, and in the inflammatory deposition of fibrin. Other plasminogen activator inhibitors exist in placenta and urine (Bloom, 1990). The main inhibitor of plasmin is α_2-antiplasmin. Plasmin bound to fibrin is protected form inactivation, but α_2-antiplasmin rapidly inactivates free plasmin.

C. OVERVIEW OF THE COMPLEMENT CASCADE, CLASSICAL PATHWAY

The complement system is one of the secondary defense systems that the body uses to protect itself, primarily against bacterial pathogens. The primary defense systems comprise the mechanical barrier presented by the skin (epithelial or endothelial cells) and/or surface secretions, such as lysozyme (Greenwood, 1984). Complement comprises a set of proteins that work to eliminate microorganisms and other antigens from tissues and the blood. This task is achieved either by complement components alone or by complement components together with antibodies and/or cells that express complement receptors, (Ross, & Medof, 1985; Kinoshita, 1991). In the complement system, the activating components circulate as inactive zymogens that are activated, by limited proteolysis, by the enzyme "upstream" from them in the sequence. Once activated, the enzymes, most of which are serine proteases, then activate the next zymogen in the pathway in the same manner. This results in an enzymatic amplification of the signal from the triggering stimulus to the end point of the terminal pathway, the assembly of the membrane attack complex, MAC. The MAC is composed of the five terminal

complement proteins C5b, C6, C7, C8 and C9 in the formation C5b-8,9$_n$, where $0 \leq n \leq 4$, (Müller-Eberhard, 1986; Esser, 1990; & 1991) or $0 \leq n \leq 18$ (Fearon, & Collins, 1983). The assembled C5b-C8 molecules have cytolytic properties that are greatly enhanced by the addition of one or more C9 molecules (Kinoshita, 1991). The number of C9 molecules, for each type of target, are necessary and/or sufficient is not clear. The cytolytic properties of the MAC are probably derived either from the induction of a "leaky patch" in the target membrane in the vicinity of the MAC insertion (Esser, 1991) or by the formation of a pore in the target membrane by the insertion of the assembled MAC (Bhakdi, & Tranum-Jensen, 1991). The complement cascade performs three major functions, targeting microorganisms and other antigens to complement-receptor bearing cells, recruiting phagocytic cells to the area where complement activation is occurring, and destroying target membranes, by the MAC (Müller-Eberhard, 1986). Similarly to the clotting cascade, there are two activation pathways for complement that converge on a common pathway (called the terminal pathway) which leads to the formation of the MAC. In complement the two activation pathways are called the classical and alternative pathways. In this thesis, I will only describe the classical activation pathway.

1. The Classical Pathway.

Antigen-antibody complexes initiate the classical pathway of the complement system (see figure 3) by generating a C3 convertase from the early acting complement components. The C1 component of complement is a complex of six of each of three sub-components, C1$_q$, C1$_r$, and C1$_s$, held together by Ca^{++} ions. The C1$_q$ subunit is the one that interacts with the antibody while the C1$_r$ and C1$_s$ subunits are the ones that exhibit the serine protease activity. C1 can be activated by a single IgM molecule but requires at least two molecules of IgG, and often aggregates of IgG are required. The main regulating component of C1 is the SERPIN, human C1-esterase inhibitor. Human C1-esterase

inhibitor also inhibits FXIIa and kallikrein. I shall discuss human C1-esterase inhibitor in more detail in sections E & G, below. When C1 is bound to IgG or IgM antibody complexed with antigen on a membrane surface, it is activated to a serine protease, C1 which catalyzes activation of C4 and C2. Together these form C42, a C3 convertase. C42, as well as cleaving C3 into its two active fragments, C3a, and C3b, combines with C3b to form C423, another protease, which can, by limited proteolysis, activate C5. Activation of C5 is the last enzymatic step in the complement cascade, and is the first step in the terminal pathway, as discussed earlier.

2. Bradykinin Production.

As stated above, the three main functions of the complement system are marking of foreign antigens for clearance by immune cells with complement receptors, recruiting immune cells to the area of complement activation, and formation of the MAC. A further effect of activation of the classical pathway of complement is the cleavage of C2 into two fragments, C2a, and C2b. C2a is the fragment that combines with the C4 component to form the C3 convertase C42. The other fragment released is C2b; C2b is an extremely potent vasodilator known as C2-bradykinin. It is the role of the human C1-esterase inhibitor in regulating the inflammatory effects, including the production of C2-bradykinin, *via* the regulation of the activation of the classical complement pathway that is of interest for the purposes of this thesis. The important aspects of this, as well as the interactions with the intrinsic pathway of blood clotting will be explored in section G.

Classical Complement Pathway
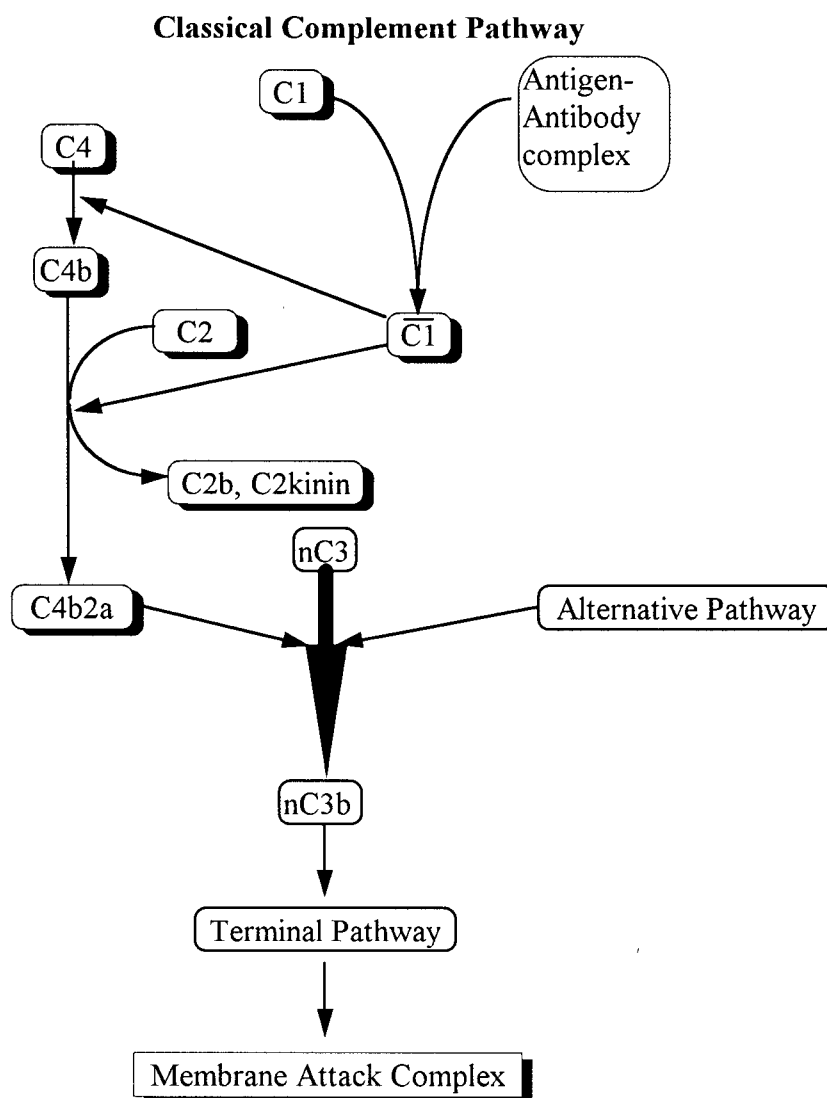
Figure 3. Classical pathway of complement cascade. The first complement component C1 is activated upon binding with the Fc portions of either IgM (one molecule is sufficient) or IgG (up to five molecules are required) antibodies that are bound to antigens. Activated C1, designated $\overline{C1}$, is a serine esterase, that cleaves the next complement components C4 and C2. Cleavage of C2 releases C2kinin, a potent vasodilator. The classical and alternative pathways converge at the cleavage of complement component C3 in the terminal pathway that ultimately leads to the formation of the membrane attack complex, MAC.

D. THE FAMILY OF SERINE PROTEASES

1. Archetypes: Trypsin & Subtilisin.

The serine proteases are a class of proteolytic enzymes characterized by the presence of a uniquely reactive serine side chain as the catalytic residue in the active site. Serine proteases are of extremely widespread occurrence and diverse function. The trypsin family and the subtilisin family have been identified based on their similar mechanisms of action(Kraut, 1977; Neurath, 1984). Members of both the trypsin & subtilisins families have been identified in organisms from prokaryotes to vertebrates. The trypsin-like serine proteases are more widely distributed in the animal kingdom than the subtilisin like proteases (Brenner, 1988; Mizuno, *et al.*, 1988; Fuller, *et al.*, 1989a/b; Bresnahan, *et al.*, 1990; van de Ven., *et al.*, 1990; van den Ouweland, *et al.*, 1990; Smeekens, & Steiner, 1990).

2. General Features.

The two families of enzymes share the same mechanism of catalysis, but not sequence homologies or three dimensional structures. The original work by Blow and co-workers (Matthews, *et al.*, 1967; Blow, *et al.*, 1969; Henderson, *et al.*, 1971) who elucidated the structure of this catalytic system, suggests that the invariant residues, Asp_c, His_c, Ser_c (where c denotes a catalytic residue), form a catalytic triad, that is involved in a charge relay system of the form, $Asp_c^- \text{-} His_c \text{-} Ser_c \rightarrow Asp_c \text{-} His_c \text{-} Ser_c^-$ as a factor that increases the nucleophilicity of Ser_c.

Prothrombin
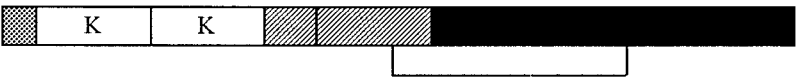
Factor IX

Factor VII

Factor X

Factor XII
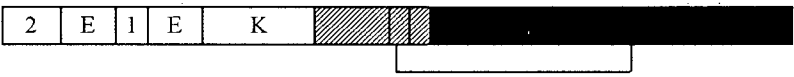
Factor XI
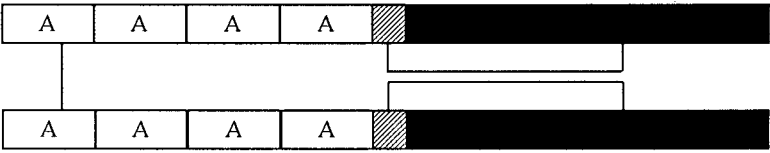
Trypsin

Figure 4. Schematic comparison of the amino acid sequence homologies of some serine protease clotting factors to Trypsin. The solid black bar (■) represtents the protease domain, the hatched region (▨)represents the activation peptide, and the shaded region (▨)represents the Gla domain. The letters A, E and K and the numbers 1 and 2 denote the apple domains, the epidermal growth factor like regions, the kringle domains, and the fibronectin type I and II like regions respectively. The solid lines underneath the proteins indicate the presence of disulphide bridges, but do not show their locations. The lengths of the bars are approximately proportional to the lengths of the polypeptide chains of the molecules (adapted from D.K. Banfield, 1991)

3. Clotting Factors.

In higher vertebrates serine proteases play roles in a diverse number of physiological processes including blood clotting, fibrinolysis, complement activation, digestion, and neuropeptide and post-translational processing (Brenner, 1988; Mizuno, *et al.*, 1988; Fuller, *et al.*, 1989a/b; Bresnahan, *et al.*, 1990; van de Ven., *et al.*, 1990; van den Ouweland, *et al.*, 1990; Smeekens, & Steiner, D.F., 1990). All of the trypsin-like serine proteases share sequence identity. The coagulation factor serine proteases differ from trypsin in that they possess long amino terminal non-catalytic sequences (see figure 4). These non-catalytic sequences are thought to be involved in binding the proteases or their zymogens to their substrates or other macromolecules and by these means regulate the coagulation, and fibrinolysis cascades (Patthy, 1985).

4. Evolution of the Serine Proteases.

It is reasonable to assume that the two families of serine proteases evolved from different ancestors because their three dimensional structures are totally dissimilar and the structural features thought to function in catalysis are contributed by amino acid residues with different orders of occurrence in the sequence. However, the structural features participating in catalysis are common to both the trypsin and subtilisin like proteases (Wright, *et al.*, 1969; Robertus, *et al.*, 1972). This evolutionary convergence provides powerful evidence that each of the features in question must play an important role in the catalytic function of the enzymes (Kraut, 1977).

E. HUMAN FACTOR XII(a).

1. Introduction.

Human FXII has been more extensively studied than FXII from other organisms and therefore I shall describe it first and discuss bovine FXII after in light of what is known of the human protein. FXII also known as Hageman factor, after John Hageman in whom the first FXII deficiency was described (Ratnoff, & Colopy, 1955). FXII is a single chain glycoprotein of β-globulin mobility with a Mr 80,000 (Dunn, *et al.*, 1982) that is synthesized in the liver and is present in normal plasma at a concentration of approximately 30 μg/mL (Revak, *et al.*, 1974). FXII is secreted and as such has the expected signal peptide required for its transport across the rough endoplasmic reticulumn membrane (Blobel, *et al.*, 1979). Post translational modifications include glycosylation (several N-linked, and at least one O-linked (Harris, *et al.*, 1992)) and removal of the signal peptide to give rise to plasma FXII. The FXII protein has been highly purified from human plasma (Revak, *et al.*, 1974; Griffin, & Cochrane, 1976; Fujikawa, & Davie, 1981; Fujikawa, & McMullen, 1983; McMullen, & Fujikawa, 1985), and the FXII cDNA and gene have been isolated (Cool, *et al.*, 1985; Cool, & MacGillivray, 1987). All three forms of FXII have been sequenced (Fujikawa, & McMullen, 1983; McMullen, & Fujikawa, 1985; Cool, *et al.*, 1985; Tripodi, *et al.*, 1986; Que, & Davie, 1986; Cool, & MacGillivray, 1987) and all the sequences agree with one another.

2. Structural Homologies with other Proteins.

Comparison of residues 1-276 of FXII with other known protein sequences reveals extensive sequence identity with regions of both fibronectin and tPA (Cool, *et al.*, 1985). The amino terminal region of FXII shares sequence homology with the type II homology regions of fibronectin (Cool, *et al.*, 1985). The type II homologies probably comprise the

collagen binding site in fibronectin and by analogy the amino terminal region of FXII, may be involved in the collagen binding properties of FXII (Yamada, 1983; Cool, *et al.*, 1985). Two regions of FXII are homologous to an epidermal growth factor (EGF) like sequence that has been found in many proteins including tPA, and several blood clotting factors; however the function of the EGF-like homology is unknown (Blomquist, *et al.*, 1984; Doolittle, *et al.*, 1984). Between the two EGF-like regions is a region that shares limited sequence homology with the type I regions of fibronectin; the function of the type I "finger" domain is unclear but in fibronectin may be involved in fibrin-binding (Petersen, *et al.*, 1983; Yamada, 1983; Cool, *et al.*, 1985). A kringle domain is also found in FXII; kringles have been found in tPA (Pennica, *et al.*, 1983), plasminogen (Sottrup-Jensen, *et al.*, 1978), and prothrombin (Magnusson, *et al.*, 1975). Kringles are thought to be involved, *in vivo*, in protein-protein interactions (Esmon, & Jackson, 1974; van Zonneveld, *et al.*, 1986; Verheyen, *et al.*, 1986), and the presence in FXII of both a type I, finger domain, and a kringle domain suggests that the fibrin binding capacity of FXII may be important *in vivo*. Between the kringle region and the catalytic region of FXII there is a proline rich region that bears some homology with calf thymus high-mobility group protein 17 (Walker, *et al.*, 1977); the significance of this region and the observed homology are unknown. The carboxy-terminus of FXII contains the catalytic region that shares homology with other trypsin like serine proteases (Cool, *et al.*, 1985). Like all other known serine proteases, FXIIa employs the charge relay system of Asp-His-Ser, to catalyze the hydrolysis of peptide bonds. Like all trypsin-like proteases, FXIIa cleaves specific residues, usually following a positively charged residue such as Arg or Lys.

3. Physiological Relevance.

a. Clotting.

i. Activation. *In vivo*, the exact mechanism(s) of activation of FXII and the intrinsic (contact) coagulation pathway are unclear. *In vitro*, FXII may be activated by a number of substances which present a negatively charged surface to FXII for binding; these include glass, kaolin, celite, dextran sulfate, and ellagic acid (Colman, *et al.*, 1987). Physiological activators may include collagen, and vascular subendothelial basement membrane (Colman, *et al.*, 1987). *In vivo* it is possible that trace amounts of FXIIa circulate continuously, and that under appropriate conditions, this triggers the contact activation pathway; it is also postulated that in the presence of high molecular weight kininogen and a negatively charged surface, FXII may autoactivate as a result of a conformational change, induced by binding to the surface (Griffin, 1978; Silverberg, *et al.*, 1980; Dunn, *et al.*, 1982; Colman, 1984). Surface bound FXII can also activate prekallikrein; the resulting kallikrein cleaves FXII to FXIIa; FXIIa and kallikrein participate in a reciprocal activation feedback amplification loop (Wiggins, *et al.*, 1977; Thompson, *et al.*, 1977, 1979; Keribirou, & Griffin, 1979).

ii. Substrates. FXII circulates as an inactive zymogen and is activated by limited proteolysis to FXIIa, a serine protease of identical molecular weight as the zymogen, and consisting of two chains held together by a disulphide bond (Revak, *et al.*, 1974). As substrates FXIIa recognizes itself (Griffin, 1978; Dunn, *et al.*, 1982), prekallikrein (Mandle, & Kaplan, 1977), high molecular weight kininogen (Wiggins, 1983), FXI (Kurachi, & Davie, 1977), plasminogen (Colman, 1969), FVII (Radcliffe, *et al.*, 1977), and can activate the classical complement pathway by activating C1 (Donaldson, 1968; Ghebrehiwet, *et al.*, 1983). Prekallikrein and FXI circulate in plasma as inactive zymogens complexed with high molecular weight kininogen (Mandle, *et al.*, 1976; Thompson, *et al.*, 1977, 1979; van der Graaf, *et al.*, 1982). Upon exposure to a

thrombogenic surface *in vivo* the high molecular weight kininogen-prekallikrein complex, the high molecular weight kininogen-FXI complex and FXII bind to the exposed subendothelium at the site of injury. Surface bound FXII activates prekallikrein and FXI (that are complexed with high molecular weight kininogen) (Wiggins, *et al.*, 1977; Thompson, *et al.*, 1977, 1979; Keribirou, & Griffin, 1979).

    iii. FXIIa independent activation of factor XI. Recent *in vitro* evidence shows that thrombin can, in the presence of a negatively charged surface, activate FXI directly, in the absence of FXIIa (Naito, & Fujikawa, 1991); further work *in plasmo* disputes these findings and suggests that under physiological conditions, thrombin activation of FXI in an FXIIa independent manner does not occur (Brunnée, *et al.*, 1993). This does not however rule out another still unidentified FXI activator or pathway and there is some evidence to indicate that platelets may play a role by providing a platelet FXI (Walsh, 1987; Kitchens, 1991). FXII can also activate FVII leading to activation of the extrinsic pathway (Rapaport, *et al.* 1955; Radcliffe, *et al.*, 1977). Contact activation leads to the activation of the fibrinolytic pathway via the activity of FXIIa on plasminogen to form plasmin, although the contribution of FXIIa is < 15% of the total plasminogen activator activity in human plasma (Kluft, *et al.*, 1987), and kallikrein and FXIa are also involved.

    b. Interactions of factor XII with other pathways (see figure 5).

    i. Inflammation. FXIIa and kallikrein may also be involved in the stimulation of neutrophil aggregation and degranulation (Wachtfogel, *et al.*, 1983; Wachtfogel, *et al.*, 1986). The proteolytic cleavage of prekallikrein and FXI requires the presence of high molecular weight kininogen and occurs *in vivo* while bound to a membrane. This results in their activation to kallikrein and FXIa, both of which are serine proteases. Activation of FXI results in the initiation of intrinsic coagulation. As mentioned in the previous section there is some evidence if FXII independent activation of FXI by thrombin (Naito, & Fujikawa, 1991; Gailani, & Broze, 1991).

ii. Extrinsic pathway. FXIIa interacts with the extrinsic coagulation pathway through its activation of FVII. The exact physiological role of FXII remains unclear, as FXII-deficient individuals do not have bleeding problems and are generally asymptomatic (Ratnoff, & Colopy, 1955). There are also examples of non-human vertebrate and mammalian species (whales and birds) that appear to be completely and/or functionally devoid of factor XII (Ratnoff, & Rosenblum, 1958; Vroman, 1958; Lewis, *et al.*, 1969; Robinson, *et al.*, 1969; Ratnoff, *et al.*, 1976). It is clear however that factor XII does have a number of roles to play, but, their overall and relative importances are unclear. Factor XII was first discovered, *in vitro* by its ability to activate factor XI and promote blood coagulation via the intrinsic pathway. As mentioned above, factor XII deficient individuals are generally asymptomatic but factor XI deficient individuals suffers from bleeding disorders (Colman, *et al.*, 1987); this suggests that factor XI is essential to proper physiological blood clotting and suggests that there may be another physiological activator or activators of factor XI, besides factor XIIa.

ii. Bradykinin Production. Cleavage of high molecular weight kininogen by factor XIIa and kallikrein releases bradykinin which stimulates the release of tPA from endothelial cells (Kluft, *et al.*, 1987). Factor XII and the contact activation system also play roles in the processes that lead to and mediate septic shock. Patients with septicemia often develop disseminated intravascular coagulation (DIC), but die from circulatory collapse as a result of hypovolemia, rather than from the direct effects of the infection. The causes of this are thought to be over stimulation by the infecting organisms, of the intrinsic clotting and classical complement pathways. This results in over activation of factor XII, prekallikrein, and human C1-esterase, which in turn results in the overproduction of bradykinins that result in disseminated vasodilation, hypovolemia, circulatory collapse and death. In this case it is thought that human C1-esterase inhibitor (the regulatory inhibitor of factor XIIa, kallikrein, and human C1-esterase activity) is

swamped by the over stimulation of the two pathways; in addition, some bacteria produce proteases that specifically cleave the human C1-esterase inhibitor rendering it inactive (Pixley, *et al.*, 1993). In some animal models, bacterial proteases can activate factor XII as well as human C1-esterase (Matsumoto, *et al.*, 1984; Kamata, *et al.*, 1985; Molla, *et al.*, 1989; Semba, *et al.*, 1992). Pixley, *et al.*, 1993, showed that in a baboon animal model, pretreatment of the animal with an anti-factor XII antibody that blocks activation of factor XII before the induction of hypotensive bacteremia with *E. coli*, resulted in improved survival from reduction of the hypotensive effects, but did not reduce the extent of DIC. It has also been shown in the guinea-pig septic shock model that depletion of the components of the factor XII/kallikrein-kinin systems prevents shock (Khan, *et al.*, 1991). Factor XIIf, a proteolytic fragment of factor XII that is missing almost the entire heavy chain including the surface binding site but includes the serine protease domain and is still active (Kaplan, & Austen, 1970; Dunn, & Kaplan, 1982; Fujikawa, & McMullen, 1983) is known to activate the first component of the classical pathway of complement, human C1-esterase (Ghebrehiwet. *et al.*, 1981). The activity of factor XIIa is regulated *in vivo* primarily by human C1-esterase inhibitor which accounts for approximately 90% of factor XIIa inactivation in plasma. The rest of the inhibition in plasma is accounted for by the activities of antithrombin III, α_2-antiplasmin, and α_2-macroglobulin.
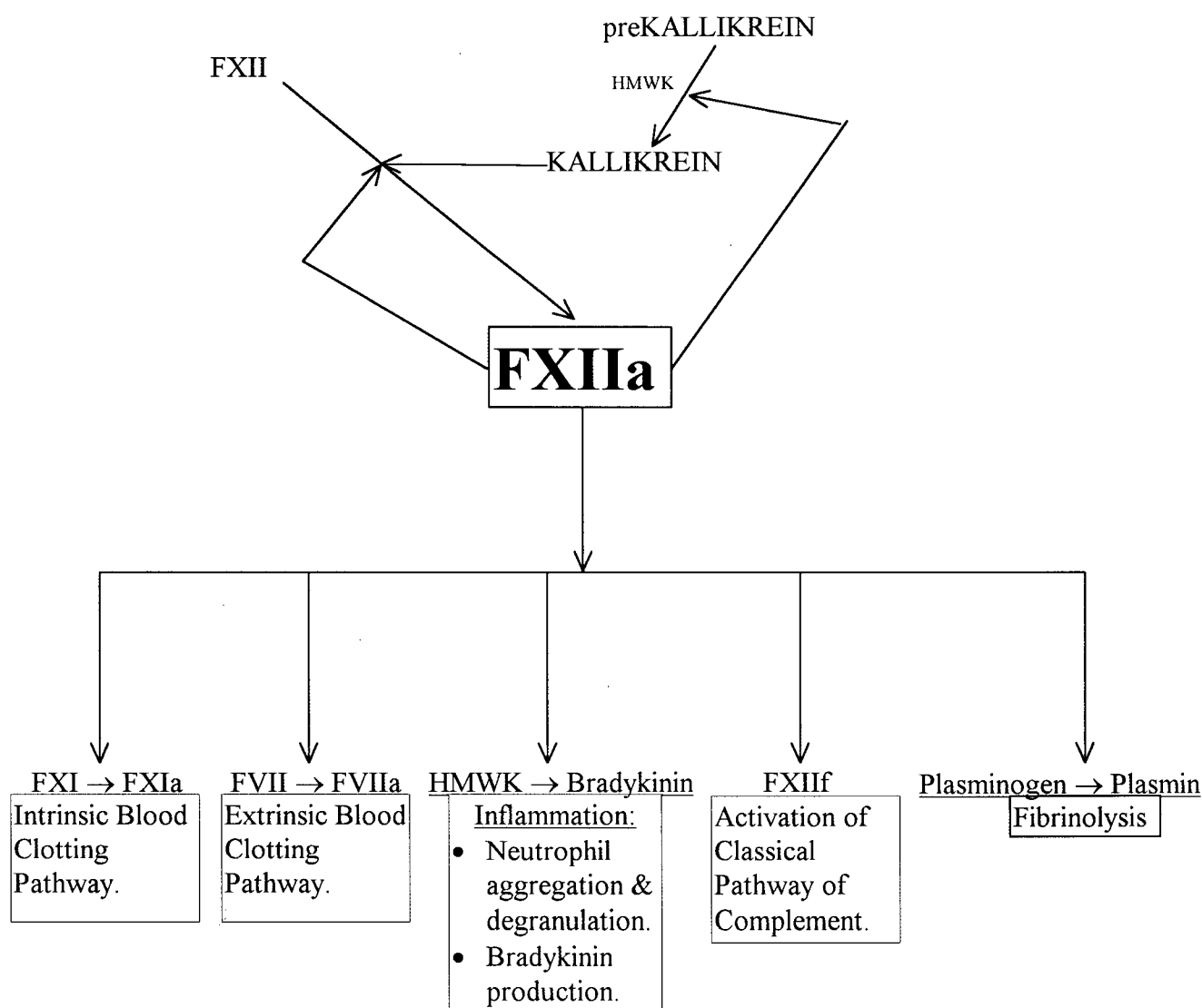
Figure 5. Interactions between factor FXII and other biochemical pathways. The bulk of its effects are through FXIIa and activation of the kallikrein/kinin pathway but another fragment of FXII, FXIIf, acts to activate the classical pathway of complement. FXIIa stimulates fibrinolysis directly by activation of plasminogen to plasmin or indirectly by cleaving HMWK to release bradykinin which stimulates the release of tPA from endothelial cells.

4. Bovine Factor XII.

The bovine and human factor XII proteins are very similar in size, and range of physiological activities. Bovine factor XII has a molecular weight of 78,000 (Fujikawa, 1988) and circulates in a single chain glycosylated form as an inactive zymogen of a trypsin like serine protease that participates in the intrinsic or contact phase of blood coagulation (Davie, *et al.*, 1979; Griffin, & Cochrane, 1979; Ratnoff, & Saito, 1979; Colman, 1984). Under reducing conditions, the active form shows two polypeptide bands SDS-PAGE, a heavy and a light chain of 46,000 and 28,000 Mr respectively (Fujikawa, 1988). Bovine factor XII does not autoactivate (Sugo, *et al.*, 1982), but, in normal bovine plasma and in the presence of an anionic surface such as kaolin (Kirby, & Mcdevitt, 1983), dextran sulfate (Fujikawa, *et al.*, 1980), or ellagic acid (Bock, *et al.*, 1981), the contact phase of clotting is activated. In the presence of high molecular weight kininogen and when bound to a negatively charged surface, there is a reciprocal activation feedback amplification loop between factor XII/factor XIIa prekallikrein/kallikrein as in the human system and this leads to the activation of factor XI and ultimately to the generation of fibrin. As in the human system both factor XIIa and kallikrein can cleave high molecular weight kininogen, causing the release of a vasoactive peptide bradykinin (Kato, *et al.*, 1988) also as in the human case, the identity of the physiological negatively charged surface is thought to be activated platelets and or exposed subendothelial matrix of the vascular endothelium (Walsh, & Griffin, 1981).

F. THE FAMILY OF SERINE PROTEASE INHIBITORS, SERPINS.

1. Overview.

The serpins (*ser*ine *p*rotease *in*hibitors, term coined by Carrell & Travis in 1985) and related proteins constitute one of the earliest described gene super families,

recognized by Hunt and Dayhoff in 1980, and the most recently defined of a limited number of proteinase inhibitor super families (Carrell, & Travis, 1985; Davis, 1988; Huber, & Carrell, 1989; Travis, *et al.*, 1990; Perkins *et al.*, 1992; Remold-O'Donnel 1993). Members of the serpin family are widespread in higher organisms and have also been described in plants and viruses, such that more than 40 proteins have been designated as serpins (Huber, & Carrell, 1989). Not all serpins have inhibitory activity e.g. ovalbumin, angiotensinogen, and corticosteroid (Stein, *et al.*, 1989; Pemberton, *et al.*, 1988). Serpins are a majority constituent of the blood proteins comprising over 10% of the total protein in human plasma (Travis, *et al.*, 1990). With the exception of α_2-macroglobulin, and inter-α-trypsin inhibitor, all of the major plasma protease inhibitors, e.g. α_1-antitrypsin, α_1-antichymotrypsin, human C1-esterase inhibitor, antithrombin III, α_2-antiplasmin, heparin cofactor 2, and activated protein C inhibitor, are serpins (Perkins, *et al.*, 1992). These plasma serpins each play a vital and often interdependent role in the regulation of serine protease activity in a number of distinct physiological roles (see table IV), e.g. coagulation, complement activation, fibrinolysis, kinin release and phagocytosis (Travis, *et al.*, 1990; Perkins, *et al.*, 1992). Characteristically, the protease inhibitors of the serpin family present a reactive site region as a proteinase accessible loop, thus mimicking an ideal substrate for the target proteinase (Carrell, & Travis, 1985).

2. General Features.

    a. Structure. All native SERPINs are single chain molecules. While many possess unique amino terminal or carboxy terminal extensions, all contain a conserved domain of about 390 residues (Huber, & Carrell, 1989). The reactive site region lies about 40 residues from the carboxy terminal end of this domain. The serpins share the same overall tertiary structure; over 80% of the amino acid residues are contained in secondary elements consisting of three β-sheets surrounded by eight or nine α-helices (Stein, *et al.*,

1990; Wright, *et al.*, 1990; Loebermann, *et al.*, 1984; Mottonen, *et al.*, 1992; Baumann, *et al.*, 1991). This structure is also found in the non-inhibitory serpins like ovalbumin (Hopkins, *et al.*, 1993). As with other inhibitors, the residue that binds at the primary specificity pocket of the target proteinase is denoted the P_1 residue (Schechter, & Berger, 1967). The reactive-site loop of serpins extends from P_{15} to P_5', and the amino acid sequences show low identity except for the region P_9-P_{15}, named the hinge region. The P_9-P_{12} residues are usually short chain amino acids, predominantly alanines; P_{13} is glutamic acid; P_{14} is often a serine or threonine; and P_{15} is almost always glycine (Hopkins, *et al.*, 1993).

b. Mechanism. All native serpins are single chain molecules. While many have unique amino or carboxy-terminal extensions, all contain a conserved domain of about 390 residues with the reactive site loop lying approximately 40 residues upstream from the carboxy terminal end of the conserved region (Huber, & Carrell, 1989). The reactive centre loop is formed by the 19 amino acid residue segment from P_{15}-P_5', and takes the forms of a three turn α-helix which protrudes from the main body of the protein on two peptide stacks each of about four residues (Stein, *et al.*, 1990). The external position of the loop is required for function, so that it can interact with the target protease, but also makes it vulnerable to proteolytic cleavage by non-target proteases. In this way serpins are readily inactivated by a variety of bacterial, snake venom, and other proteases (Kress, & Catanese, 1981). Upon cleavage between the P_1 and P_1' residues in the reactive centre loop, the new amino terminal end changes its secondary structure from an α-helix to a β-sheet and folds over and inserts into the A β-sheet of the body of the inhibitor molecule forming one of its central strands. This change as the basis of the mechanism was first proposed by Lobermann, *et al.*, in 1984, on the basis of the structure of cleaved α_1-antitrypsin. This change results in the reorientation of the P_1 and P_1' residues to opposite poles of the molecule and separated by about 70 Å, as a result of the cleavage of this bond

the molecule realizes significant stabilization from many small improvements in the geometry of the hydrogen bonds that constitutes the secondary structure of the serpin fold (Perkins, *et al.*, 1992).

3. Mechanism of Inhibition, the Mouse trap.

Serpins are suicide substrates of their target proteases (Patson, *et al.*, 1991). They are also known as mechanism-based, Trojan horse inhibitors, or enzyme activated irreversible inhibitors (Walsh, 1984; Fersht, 1985; Knight, 1986). The reaction pathway for suicide inhibitors is branched after the formation of the reversible Michaelis complex between the target enzyme and the inhibitor, after which an intermediate complex is formed which is probably tetrahedral (Matheson, *et al.*, 1991). From this point there are two possible outcomes: either the inhibitor merely acts as a substrate and is cleaved and released as an inactivated inhibitor, or the inhibitor becomes covalently linked, probably via an ester bond between the new C-terminal residue, P_1 and the active site serine of the inhibitor (Longas, & Finlay, 1980). When serpins act as substrates for proteinases, limited proteolysis of the reactive site region occurs, with the primary sites of cleavage being found within a domain of approximately 11 residues, from P_{10} to P_1'. As a result of this cleavage, serpins that possess inhibitory potential e.g. antithrombin III, α_1-antitrypsin, and human C1-esterase inhibitor, undergo a major conformational change (Bruch, *et al.*, 1988; Bock, *et al.*, 1987; Stein, *et al.*, 1989), which results in the newly formed C-terminus moving approximately 7 nm to the opposite pole of the molecule. This results in significant stabilization of the cleaved compared to the intact molecule. In contrast, the noninhibitory serpins ovalbumin, and angiotensinogen do not undergo the conformational change or stabilization as a result of reactive site cleavage (Bruch, *et al.*, 1988; Gettins, 1989; Stein, *et al.*, 1989). This large rearrangement has no effect on the overall molecular structure. At the lowest structural resolution, the overall structure of the serpin fold is

SERPIN	C1-esterase	FXIIa	Kallikrein	Trypsin	Thrombin	Plasmin
C1INH	++^{b}, ++^{c}	+^{b}, +^{c}	+^{b}, -^{c}	-	-	±
BFXIIaINH	++^{b}, ++^{c}	++^{b}, ++^{c}	++^{b}, -^{c}	+^{c}	±^{b}	±^{b}
ATIII	-	+^{b}, ND^c	+^{b}, ND^c	+	++	+
α1PI	-	±^{b}, ND^c	+^{b}, ND^c	+	+	+
α2AP	-	±^{b}, ND^c	+^{b}, +^{c}	+	+	++

Table I[a]. Major SERPINS and their targets. [a], Adapted from Thornton, & Kirby, 1987. [b], tested with human protease. [c], tested with bovine protease. ND, not determined. -, does not inhibit; ±, weakly inhibits; +, inhibits; ++, strongly inhibits.

unaffected by the cleavage of the scissile bond (P_1-P_1') at the reactive site. Evidence for this was obtained using synchrotron X-ray scattering, which showed that under physiological conditions, the gross folded structures of the native and cleaved α_1-antithrombin and human C1-esterase inhibitor were closely similar (Smith, *et al.*, 1990; and Perkins, *et al.*, 1990).

4. Mutations Affecting Function.

    a. Hinge Region. There is good evidence that the failure of the nonihibitory serpins to undergo the conformational change upon cleavage in the reactive site loop is due to substitutions in a conserved row of alanines and small bulk residues that form the base of the reactive centre loop (Wright, *et al.*, 1990). This is consistent with the findings in a series of antithrombin III mutants that are rendered nonfunctional as an inhibitor by changes in this conserved area (Perry, *et al.*, 1989; Mohlo-Sabatier, *et al.*, 1989; Caso, *et al.*, 1991; Skiver, *et al.*, 1991). The conservation of the hinge-region amino acid residues between different members of the serpin family also indicates that they play the same important role in all members, and are critical in the functioning of the serpins as inhibitors.

    b. Reactive-Site Loop. The region of a serpin that binds to the active site of its target proteinase is an exposed loop that has been called the reactive-site loop. The reactive site loops of small proteinase inhibitors, such as those of the Kunitz family, are held in a tightly constrained conformation by interactions with the body of the inhibitor. The tertiary structure of this loop is conserved between inhibitors of different families, and this structure has been called the canonical form. The conformation of the reactive site loop of small inhibitors is essentially the same in the free and bound inhibitor, and it is thought that the stabilization of its conformation by the interactions with the body of the inhibitor is important for the formation of the tight complex with the cognate proteinase

(Bode, & Huber, 1991). In contrast to the tightly constrained loop of the small inhibitors, the serpins have a "mobile" reactive centre loop which is able to move in and out of the A β-sheet (Bode, & Huber, 1992; Carrell, & Evans, 1992). It has been proposed that this mobility is required for serpin function in that it allows the partial insertion of the loop into the A β sheet, which results in a smaller, more tightly constrained loop that could adopt the canonical form similar to that of the reactive site loop of the Kunitz inhibitors (Bock, 1990; Bode, & Huber, 1992; Carrell, *et al.*, 1991).

i. P_1-P_1'. Serpins are globular proteins with exposed surface loops that contain idealized proteinase substrate sequences, the specificities of which are primarily determined by the residues at the P_1 and P_1' positions. Mutations at the P_1 position often result in retention of inhibitory ability, but result in a change in specificity. Examples of naturally occurring P_1 and P_1' mutations are (1) α_1-antitrypsin Pittsburgh (Met$\rightarrow$Arg at P_1, makes the P_1, P_1' residues the same as in antithrombin III) where the specificity of the inhibitor is changed from trypsin to thrombin (Owen, *et al.*, 1983); and (2) when the P_1 Leu of α1-antichymotrypsin is changed to an Arg the result is again an altered specificity from chymotrypsin to thrombin (Rubin, *et al.*, 1990). A recombinant α_1-antitrypsin where both the P_1 and P_2 residues are changed from Met to Arg and Pro to Ala respectively, (these are the same residues as are found in the P_1 and P_2 positions of human C1-esterase inhibitor) exhibits a change in specificity from trypsin to kallikrein and FXIIa. This double mutant of α_1-antitrypsin is a more effective inhibitor than the native human C1-esterase inhibitor (Shapira, *et al.*, 1987). Secondary structure changes stabilize the reactive-centre cleaved form of serpins (Perkins, *et al.*, 1992).

G. HUMAN C1-ESTERASE INHIBITOR/BOVINE FACTOR XIIa INHIBITOR.

1. Introduction.

The bovine homologue of human C1-esterase inhibitor is the bovine factor XIIa inhibitor (Thornton, & Kirby, 1987; Muldbjerg, *et al.*, 1993). Human C1-esterase inhibitor belongs to the *serpin* superfamily of proteins (see above). The human C1-esterase inhibitor is the more fully studied of the two molecules and is the basis of comparison for analysis of the bovine factor XIIa inhibitor; therefore, I shall describe the human C1-esterase inhibitor first then compare the bovine factor XIIa inhibitor to it.

2. Human C1-esterase inhibitor.

a. Protein: Structure & Function. The human C1-esterase inhibitor protein (C1INH) is a single chain, 478 residue, heavily glycosylated ($\approx$35% carbohydrate) α_2-globulin with an apparent molecular weight, by SDS-PAGE, of 112 Kda and 105 Kda under non-reducing and reducing conditions respectively. The apparent difference in molecular weight between reduced and non-reduced forms of the protein is thought to be from differences in tertiary structure conferred by the presence or absence of the two disulphide bonds. The secretion of C1INH is mediated by a 22 amino acid signal peptide, the cleavage of which constitutes the only post translational proteolytic processing that occurs (Pensky, *et al.*, 1961; Haupt, *et al.*, 1970; Harpel, & Cooper, 1975; Reboul, *et al.*, 1977; Harrison, 1983; Bock, *et al.*, 1986). C1INH has two disulphide bonds, and an estimated 17 carbohydrate prosthetic groups (both glucosamine and galactosamine based) most of which ($\approx$10) occur in the first 120 amino acids of the protein. The glycosylation consists of both the N and O linked types. C1INH is synthesized mainly in the liver, and has inhibitory activity in four systems: complement, intrinsic (contact) pathway of coagulation, fibrinolysis, and kinin-generating (Cullmann, *et al.*, 1986). C1INH is the

major homeostatic component of the classical pathway of complement activation, inhibiting the proteolytic activity of activated C1 and thus the activation of C2 and C4 and therefore the production of C2 bradykinin. Human C1-esterase inhibitor is also the main physiological inhibitor of factor XIIa activity (Schreiber, *et al.*, 1973; de Agostini, *et al.*, 1984; Pixley, *et al.*, 1985). C1INH is a serpin that interacts with its target enzymes in a stoichiometric fashion forming covalent, denaturation resistant, inactive 1:1 molar complexes that involve the active site serine of the target enzymes and Arg and Thr residues in the P_1 and P_1' positions of the C1INH (Harpel, & Cooper, 1975; Sim, *et al.*, 1979b, 1980; Arlaud, *et al.*, 1979; Salvesen, *et al.*, 1985). While C1INH was first described as an inhibitor of activated C1, it has long been known to be active against other serine proteases such as clotting factors XIIa and XIa, (Forbes, *et al.*, 1970) and components of the fibrinolytic and kinin systems (Ratnoff, *et al.*, 1969; Gigli, *et al.*, 1970). Kallikrein is inhibited also by α_2-macroglobulin. It is likely that the most important physiological role of C1-esterase inhibitor is as a regulator of the inflammatory response through its inhibition of kinin production by the classical complement, and kallikrein/kinin pathways *via* C1 and FXIIa respectively.

 b. Structure of the gene and cDNA. The gene, cDNA, and primary structures for C1-esterase inhibitor have been determined (Bock, *et al.*, 1986; Tosi, *et al.*, 1986; Carter, *et al.*, 1988 & 1991). The cDNA encodes an open reading frame of 1500 bp; this translates into 500 amino acids, and includes the 22 amino acid signal peptide. The single stop codon, TGA, is followed 246 bp later by the polyadenylation signal AATAAA which is followed 15 bp later by the poly-A tail (Proudfoot, & Brownlee, 1976; Bock, *et al.*, 1986). The gene is located on chromosome 11, p11.2-q13, is 17,159 bp long and is split by seven introns with junctions of phases zero and one. The 5' untranslated region of the gene does not have an obvious promoter site of the TATA box type, but does contain regions of structure similar to that found in the *c-myc* gene. As identified by an open

reading frame, the 5′ untranslated region also contains an untranslated exon. Comparison of the cDNA and primary amino acid structures indicates that C1INH, like other serpins is the product of divergent evolution from a common ancestral gene. C1INH has also been invaded by successive waves of *Alu* repeats, the sites of which are the insertion and deletion points in several known lesions in the C1INH gene (Bock, *et al.*, 1986; Tosi, *et al.*, 1986; Carter, *et al.*, 1988, & 1991).

3. Human C1-esterase inhibitor deficiency.

Angioedema is associated with low levels of C1INH protein or activity (Donaldson, & Evans, 1963; Rosen, *et al.*, 1965, 1971). There are two types of lowered functional activity of C1INH, inherited and acquired. The inherited form comes in two forms, type I, and type II, where type I is characterized by insufficient production of C1INH, and type II is a result of functionally defective protein (Rosen, *et al.*, 1965). The etiology of acquired C1INH deficiency is not fully understood, but has been associated with lymphoproliferative disorders (Caldwell, *et al.*, 1972) or the presence of anti- C1INH autoantibodies (Jackson, *et al.*, 1986; Alsenz, *et al.*, 1987; Malbran, *et al.* 1988). The mechanism that leads to the angioedema in either the inherited or acquired form of C1-INH deficiencies is still a debated issue; however in either of the cases a low level of functional C1-INH may lead to unopposed activation of C1 of the classical complement pathway, which then results in the release of C2kinin (Melamed, *et al.*, 1986) or activation of the intrinsic clotting pathway which in turn activates the kallikrein/kinin pathway with the concomitant production of bradykinin (Strange, *et al.*, 1988). Either the C2kinin, the bradykinin or both are assumed to be the cause of the increased vascular permeability that leads to the angioedema (Proud, & Kaplan, 1988).

4. Bovine FXIIa Inhibitor.

Bovine factor XIIa inhibitor (BFXIIaINH) was first described in 1987 by Thornton & Kirby and appears to be similar to a bovine proteinase inhibitor with C1 inhibitor activity described by Van Nostrand & Cunningham also in 1987. The N-terminal sequences of the BFXIIaINH prepared according to Thornton & Kirby and by Muldbjerg *et al.*, are identical and are the same as the published 10 N-terminal amino acid residues of the C1-inhibitor purified from bovine plasma by Van Nostrand & Cunningham. This indicates that these inhibitors may be identical. By SDS-PAGE, BFXIIaINH has an apparent M_r of 85 Kda and 92 Kda, reduced and non-reduced, respectively and contains between 17 % and 42 % carbohydrate (see discussion). The differences in apparent molecular weight are probably from different tertiary conformations attributable to the presence or absence of a disulphide bond (see above). BFXIIaINH inhibits bovine and human FXIIa, C1-esterase, and human kallikrein, but does not inhibit bovine kallikrein or trypsin or human plasmin or thrombin. This activity is most similar to human C1-esterase inhibitor and was identified as the bovine homologue of that protein. The amino acid composition of the two molecules is similar but can not be directly compared because of the differences in molecular weight. Rabbit antisera raised against either the human or bovine protein did not cross react with the molecule from the other species. This could be due to the fact that the two molecules do not share any epitopes (at least not the ones tested) or that the molecules were so similar to the molecule in the animal producing the antibodies that the antibodies were raised to small differences in the protein (Thornton, & Kirby, 1987). The bovine inhibitor of FXIIa interacts with FXIIa to form a very stable complex with 1:1 stoichiometry. The active site of FXIIa, located on the light chain, is directly involved in the interaction and complex formation between BFXIIaINH and FXIIa and can be blocked by diisopropylfluorophosphate, corn trypsin inhibitor or the chromogenic substrate S2302. The complex does not spontaneously dissociate and is

stable in thiocyanate, acid, hydroxylamine, and boiling SDS. Additionally a cleaved form of the inhibitor is observed. These data indicate that like other serpin type inhibitors BFXIIaINH operates as a suicide type or mechanism based inhibitor (see part E above) (Thornton, & Kirby, 1988). In 1992, Muldbjerg, et al. did further studies of BFXIIaINH. N-terminal sequence analysis revealed a unique sequence without homology with any other known proteins. The C-terminal region showed homology with human C1-esterase inhibitor with a part showing 70% identical residues. The presence of N- and O-linked glycosylation was confirmed. The reactive site comprises an Arg-Asn bond in the P_1-P_1' position, and this is the first example of an Arg in the P_1 position of a serpin with well documented inhibitory activity.

5. Objectives of this study.

As pointed out by Thornton and Kirby in their first paper on BFXIIaINH in 1987, BFXIIaINH and C1-esterase inhibitor are similar, "but there are differences between the two proteins". The complete primary sequence of BFXIIaINH was not known and therefore full comparisons of the structures and features of the BFXIIaINH and C1-esterase inhibitor were not possible. It is the objective of this study to elucidate what these differences are and to understand how the respective molecules function in their respective roles. To this end the complete cDNA for BFXIIaINH was cloned and sequenced, the primary sequence of the protein was predicted and then compared with the primary sequence of the human C1-esterase inhibitor. To determine the possible evolutionary mechanism(s) that led to the observed differences between the human and bovine proteins, the genomic organization of the 5' end of the bovine gene for the FXIIaINH protein was determined for comparison with the intron/exon organization of the human C1-esterase inhibitor gene.

Residue	Mol %		
	Bovine factor XIIa inhibitor[a]	Bovine factor XIIa inhibitor[a,b]	Human C1-inhibitor[a,c]
Asx	9.5	9.3	9.4
Glx	9.8	11.3	10.3
Ser	9.0	7.2	10.0
Gly	5.8	6.2	2.7
His	2.6	3.1	2.1
Arg	5.1	5.1	2.5
Thr	7.3	5.6	11.1
Ala	7.1	7.7	6.3
Pro	5.9	5.1	6.7
Tyr	1.5	1.6	1.5
Val	6.7	5.6	6.9
Met	1.5	1.6	2.7
Cys[a,d]	0.5	2.1	0.8
Ile	4.3	4.1	3.8
Leu	11.7	11.3	11.5
Phe	5.8	6.7	5.0
Lys	5.1	5.6	6.1
Trp[a,e]	0.9	1.0	0.6

Table II. Comparison of amino acid compositions of bovine factor XIIa inhibitor and human C1-inhibitor. [a], Taken from Muldbjerg, et al., 1994. [b], Taken from Thornton & Kirby, 1987. [c], Taken from Bock, et al., 1986. [d], Determined as S-carboxymethycysteine. [e], Determined spectrophometrically using the method of Edelhoch.

II. MATERIALS AND METHODS

A. MATERIALS

1. Reagents. Pure, sterilized Ampicillin was purchased from Ayerst. Tris base, and SDS, Ultrapure, and special grades respectively were purchased from Boehringer Mannheim. Acetic acid (glacial), ammonium acetate, ammonium sulfate, calcium chloride, EDTA) glucose, potassium acetate, potassium chloride, sodium acetate, and sodium hydroxide, all analytical grade, were purchased from BDH. NaI, and glass milk for isolation of DNA from agarose gels were purchased from Bio 101. Acrylamide, N,N'-methylenebisacrylamide (bis), urea, APS, and TEMED, all electrophoresis grade were purchased from Bio-Rad Laboratories. Boric acid and magnesium sulfate, certified grade were from Fisher Scientific. X-Gal, was prepared as a stock solution in 100% dimethylformamide at a concentration of 20 mg/mL. IPTG was prepared as an aqueous stock solution, (20% w/v). X-Gal and IPTG were purchased from $5' \rightarrow 3'$ Inc. Agarose (ultraPURE, electrophoresis grade), and phenol were from Gibco BRL. Phenol was equilibrated with TRIS buffered solution at the appropriate pH or water as required. α-[thio-^{35}S]-dATP was purchased from New England Nuclear (Dupont), at a starting specific activity of 43.3 TB/mMol. Deoxy-,and dideoxyribonucleotides were purchased from Pharmacia, and re-suspended in water at the appropriate concentrations. EtBr, DMSO, DTT, and Ribonuclease A, were purchased from Sigma Chemicals. EtBr was prepared as an aqueous stock solution at a concentration of 10 mg/mL, 1 μL/100 mL gel solution was used. All other reagents were reagent grade or better and were purchased from BDH, Fisher Scientific, or Sigma Chemicals. Kodak X-Omat and Kodak XAR film was used for autoradiography.

2. Primers. Oligodeoxyribonucleotide and oligodeoxythymidine-XbaI-SacI-PstI (T17$_{XSP}$) primers, for polymerase chain reaction (PCR) amplification and sequencing, were

synthesized on an Applied Biosystems 391 PCR Mate DNA synthesizer, using standard ABI reagents. The primers were cleaved, deprotected, and dried according to the ABI protocol. The primers were re-suspended in 200 µL dH$_2$0, the A$_{260}$ of a 1:200 dilution (dH$_2$0) was measured; the total yield of the synthesis was calculated using 1 unit A$_{260}$ = 33 µg/mL and the volume was adjusted to achieve a concentration of one µg/µL. This was stored at -20°C. A 1:10 dilution of the stock solution was made to 100 ng/µL as a working solution and also stored at -20°C.

3. Enzymes. Taq polymerase was purchased from Perkin-Elmer Cetus/ABI. Restriction enzymes and the *E.coli* DNA polymerase large fragment (Klenow) were purchased from either Pharmacia, New England Biolabs (NEB) or Gibco BRL. T4 ligase and T7 polymerase were purchased from Gibco BRL. Sequenase was purchased from USB. RNase A was purchased from Pharmacia and prepared according to manufacturers instructions.

4. Buffers. The restriction enzyme, ligation, and reverse transcription buffers supplied by the manufacturer were used when available. The PCR buffer consisted of: 67 mM Tris, pH 8.8, 16.6 mM (NH$_4$)$_2$SO$_4$, 10 mM β-mercaptoethanol, 1.0 mM MgSO$_4$ (D.K. Banfield, 1991). Klenow buffer was made according to Maniatis et al. 5X sequencing buffer was made as follows: 200 mM Tris, pH 7.5, 100 mM MgCl$_2$, 250 mM NaCl. 1 X TAE buffer was used for agarose gel electrophoresis, and 1 X TBE buffer was used for DNA sequencing (polyacrylamide) gel electrophoresis. Glucose buffer for the rapid plasma isolation protocol was 50 mM glucose, 25 mM Tris•HCl, pH 8.0 at 4°C, 10 mM EDTA.

5. Tissues. Bovine liver was harvested freshly and used in the preparation of genomic DNA. Human blood was collected, freshly, from a thumb prick, and used in the preparation of control genomic DNA (Lewis & Stewart-Haynes, 1992).

6. DNA/RNA. Bovine total RNA and single stranded cDNA (sscDNA), used as a template for PCR amplification of the BFXIIaINH cDNA, were gifts from Dr. D. K. Banfield. Bovine genomic DNA, used as template for the genomic organization experiments, was a gift from Jeff Hewitt. Human genomic DNA was prepared as described by Lewis and Stewart-Haynes, (1992).

B. Bacterial Strains, Media and Vectors.

1. Bacterial strains. The *E.coli* strain DH5αF' (Hanahan, 1983), from Stratagene, was used for the production of competent cells, transformation, and propagation of the pBluescript plasmid. The DH5αF' cells were made competent, by the calcium chloride method, transformed, and recombinants selected according to the protocols laid out in Maniatis *et al* (1982).

2. Media. Nutrient broth mixes, agar, yeast extract, casamino acids, bacto-tryptone, and bacto-agar were purchased from Difco Laboratories. The liquid medium for growth and selection of plasmid containing bacteria was LB broth supplemented with 100 μg/mL AMP (Maniatis *et al.*, 1982). For the selection of plasmid containing bacteria, clones were plated on LB-agar (1.5% w/v) plates supplemented with 100 μg/mL AMP, 25 μg/mL IPTG, and 50 μg/mL X-GAL.

3. Vectors. Both Cloning and sequencing were done in the pBluescript (KS+) plasmid vector, from Stratagene.

C. Equipment and Software

1. Equipment. Polymerase chain reaction experiments were carried out in a Perkin Elmer Cetus Corporation DNA Thermal Cycler 480, using Taq polymerase. Bio-Rad horizontal submarine slab gel apparatuses and power supplies were used for non-

denaturing agarose gels. Denaturing polyacylamide sequencing gels were run on apparatuses from Stratagene, with power supplies from Bio-Rad.

    2. Software. Sequence alignments, comparisons and analysis as well as all primer design and analysis were done using PCgene, release 6.8, from IntelliGenetics and/or Oligo version 4.0, from Primer Analysis Software. All analyses were performed on an IBM compatible personal computer.

D. Methods

1. Polymerase Chain Reaction.

    a. Primers. PCR amplification of the 3′ and 5′ overlapping clones of the BFXIIaINH cDNA was done using the primer sets PCR1/T17XSP and PCR2/PCR3 respectively (see Table II and figure 7). The genomic organization PCR experiments were done with nested primer sets PCR4/PCR5/PCR6 (detection and sequencing of the exon/intron junctions between exon 2 and exon 3), and PCR7/PCR8 (assay for novel intron within exon 3 and restriction mapping of exon 3.) (see Table II and figures 9 and 13). Identity of the DNA fragments was established by sequencing the ends of cloned PCR products using the plasmid primers M13, forward & reverse, and T7 or T3. Primers required to complete sequencing of the cDNA were synthesized as needed based on the sequence data from the previously sequenced regions of the cDNA. Nucleic acid sequences derived from amino acid data are uncertain because there can be more than one codon for a given amino acid (except for Met and Trp) and most of the codon variability is manifest in the third position. Primers for polymerase chain reaction amplification experiments were designed based on regions of amino acid sequence identity between the human and bovine proteins (See Table I. and Figure 6). In the event of

```
1 N P Ṇ A T S S S S Q D P E S L Q D R G E G K V A T 25
                                              S   T V S L T
26 T V I S K M L F V E P I L E V S S L P T   T Ṇ S T T  50
     X M V T S F Y P                 T Q P A X E - T L Q
51 N S A T K I T A Ṇ T T D E P T   T Q P T T E P T T Q  75
       I T I Q P   T A E P F C L A P V
76 P   T I Q P   T Q P T T Q L P T D S P T Q P T T G S F 100
                                A E A V L G E A L T
101 C P G P V T L C S D L E S H S T E A V L G D A L V 125
      D F S L R L Y Q D F S V V K
126 D F S L K L Y H A F S A M K K V E T N M A F S P F 150
                                              L V
151 S I A S L L T Q V L L G A G Q N T K T N L E S I L 175
      S Y P Q Ṅ F S C V H H A L R A F M S E G F T S F S
176 S Y P K D F T C V H Q A L K G F T T K G V T S V S 200
      Q I F H S S D L T I R           L Y G S S
201 Q I F H S P D L A I R D T F Ṇ A S R T L Y S S S 225
      P R P L G Ṅ D S T A S L E L I N D W V A K
226 P R V L S Ṇ N S D A N L E L I N T W V A K N T N N 250
251 K I S R L L D S L P S D T R L V L L N A I Y L S A 275
276 K W K T T F D P K K T R M E P F H F K N S V I K V 300
            K Y P V A S F T D R                 L
301 P M M N S K K Y P V A H F I D Q T L K A K V G Q L L 325
      Q L S H Ṅ L S F V I N V P G P V K
326 Q L S H N L S L V I L V P Q N L K H R L E D M E Q 350
                                F Ṅ P T Ṅ L T
351 A L S P S V F K A I M E K L E M S K F Q P T L L T 375
      M P R     V Q S S Q D M L - - - - - - D Y F D F I
376 L P R I K V T T S Q D M L S I M E K L E F F D F S 400
      Y D V N L C G L T E D P D V Q V S G K
401 Y D L N L C G L T E D P D L Q V S A M Q H Q T V L 425
                                      N L L L F E
426 E L T E T G V E A A A A S A I S V A R T L L V F E 450
      V Q Q P F L F L L W D Q Q H K F P V F M G P V Y D
451 V Q Q P F L F V L W D Q Q H K F P V F M G R V Y D 475
      P
476 P R A 478
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Figure 6. Alignment of the peptide sequences obtained from BFXIIaINH with the amino acid sequence of C1INH. Identical residues are boxed. ↑ between Arg444 and Thr445 indicates the reactive site of bovine C1-inhibitor. • shows the positions of N-linked carbohydrate in human C1-inhibitor and ○ the potential N-linked glycosylation sites in bovine factor FXIIa inhibitor. Reproduced from Muldbjerg, *et al.*, 1994.

Table III. Amino acid sequence of tryptic fragments of BFXIIaINH polypeptide. X designates unidentified residues and (N) indicates potentially glycosylated Asn residues. Repetitive yields varied between 90% & 95%. Reproduced from Muldbjer, *et al*, 1994.

Peptide	Amino acid sequence	Homologous positions in human C1-inhibitor	Degree of identity (%)
I	STVSLTXMVTSFYP	Thr45-Ala58	28
II	RQPA(N)ETLOITIOPPAEPXFLAPV	Thr66-Asp90	41
III	SAEAVLGEALTDFSLR	Ser115-Lys130	69
IV	LYQDFSVVK	Leu131-Lys140	56
V	LVSYPO(N)FSCVHHALR	Ile174-Lys189	56
VI	AFMSEGFTSFSQIFHSSDLTIR	Gly190-Arg211	64
VII	LYGSSPRPLG(N)DSTASLEINDWVAK	Leu221-Lys246	73
VIII	KYPVASFTDR	Lys307-Gln316	70
IX	LQLSH(N)LSFVINVPQPVK	Leu325-Lys342	78
X	F(N)PT(N)LTMPR	Phe369-Arg378	70
XI	VQSSQDMLDYFDFIYDVNLCGLTEDPDVQVSGK	Val381-Met419	71
XII	NLLLFEVQQPFLFLLXDQQHKFPVFMGPVYDP	Thr445-Val473	48
XIII	DQVQVDGIR	No homology	
XIV	NSMQEGN(N)YTXVVMXEALXN(N)YTQR	No homology	
N-term	DMIVGPGNLQEGESEGDSQKGGILDGESIQGNED	No homology	

mismatches between the human and bovine sequences at the nucleic acid level the bovine codon was used (NAR codon bias table Spring, 1992). To improve annealing properties of the primers the 3′ base of the last codon in the primer was deleted in 5′ primers while in 3′ primers the 5′ base of the first codon was deleted. This was done to avoid mismatches that might affect annealing of the primer and or binding of the polymerase to the primer template complex, See Table II for a list of all the primers used in this study; for details of uses of specific primers please consult the Results section.

b. Reaction conditions. In a total reaction volume of 50 μL, 3μL sscDNA template (containing 5-10 ng of sscDNA), 1 μL dNTP's final concentration of 200 μM (5 mM each dNTP, 20 mM total, 20 mM MgCl$_2$), 1 μL at 100 ng/μL of each primer (equivalent of 20 pmoles of each primer), 5 μL 10X PCR buffer (see Materials: Buffers), 0.2 μL Taq polymerase (1 unit), 5 μL DMSO (final concentration = 10%), and 33.8 μL dH$_2$0 were added to a standard 650 μL Eppendorf tube. All the reactants except the Taq polymerase were added together on ice, mineral oil was overlaid on the reaction mix, and the tubes were placed in the Perkin Elmer Cetus Corporation DNA Thermal Cycler model 480 with the heater block pre-heated to 94°C. The tubes were incubated at 94°C for 4 minutes, then the tubes were opened, the Taq polymerase was added and the cycling was started.

c. The thermal profile. The thermal profile used was: a melting temperature of 94 °C for 15 seconds, an annealing temperature of 50 °C or 52 °C for 20 seconds, and an extension temperature of 72 °C for 90 sec. Typically, 25-30 cycles were performed. In addition to the conventional thermocycler profiles, a thermocycler profile called a "Touch Down" profile was also used. Touch Down PCR (Don, *et al.*, 1991) thermocycling consists of starting the annealing temperature at approximately 10 °C above the calculated theoretical annealing temperature. The annealing temperature is then incrementally reduced by 2 °C every second cycle down to the theoretical annealing temperature. The touchdown profile finishes with 10 cycles at the theoretical annealing temperature.

Table IV. Oligodeoxynucleotide primers used in this study for polymerase chain reaction amplification and DNA sequencing experiments. Primer T_{17xsp} and all primers starting with "PCR" were used for polymerase chain reaction experiments. The T3, T7, M_{13}-20, M_{13} reverse primers and all primers starting with "SEQ" were used for sequencing. The number in brackets following the primer sequence indicate the location, in base pairs, of the primer in the bovine cDNA, while the arrow indicates the priming direction of the primer.

Primer name	Sequence (position and direction):
T_{17XSP}	5′-ACACTGCAGGAGCTCTCTAGATTTTTTTTTTTTTTTT-3′ (1609←1625)
PCR1	5′-ATCAACGACTGGGTGGCCAA-3′ (706→725)
PCR2	5′-GCCGA(C/T)GTGGCCGCCCA(G/A)ATG-3′ (-18→3)
PCR3	5′-TTCATCATGGGCACTTTGAT-3′ (880←899)
PCR4	5′-ACCCTCCTGCTGCTGCTGCT-3′ (24→41)
PCR5	5′-GATCTCCGAGTCAGAGCAG-3′ (309←327)
PCR6	5′-CCCTTCTTGTAAGTTCCCTGG-3′ (85←105)
PCR7	5′-CTCAGATATGATCGTCGGCCCA-3′ (66→87)
PCR8	5′-AAGCAGGATTTGGGTGAGGA-3′ (452←471)
M_{13}-20	5′-GTAAAACGACGGCCAGT-3′
M_{13} reverse	5′-AACAGCTATGACCATG-3′
T3	5′-ATTAACCCTCACTAAAG-3′
T7	5′-AATACGACTCACTATAG-3′

Table II, continued:

Primer name:	Sequence (position and direction):
SEQ1	**5′-CTCCTGCTGCTGCTGCT-3′** (28→44)
SEQ2	**5′-TCCATTCAAGGCAACGAGGAC-3′** (151→171)
SEQ3	**5′-GCCGATAACTAACCTGA-3′** (183→194)
SEQ4	**5′-AAGAGGGAGACCAACTTC-3′** (406→423)
SEQ5	**5′-CAGATCTTCCACAGCTCA-3′** (589→606)
SEQ6	**5′-TTCACCTCAAATCCTCTG-3′** (859→877)
SEQ7	**5′-CAGCTTTGTGATCCTGGT-3′** (982→999)
SEQ8	**5′-CCAAGTTCCATCCCACT-3′** (1089→1105)
SEQ9	**5′-CTGGAGCTGACGGAGAGCGG-3′** (1244→1263)
SEQ10	**5′-GCTCTGCACTTGCCCCTCCC-3′** (1444→1463)
SEQ11	**5′-CACGGTAAGGGAGGAGGGAA-3′** (1512←1531)
SEQ12	**5′-CGCAGTCGCATCCACCC-3′** (1263←1280)
SEQ13	**5′-AGTGGGATGGAACTTGG-3′** (1089←1105)
SEQ14	**5′-TGGTGTTTCACGGTCTGGGG-3′** (1001←1020)
SEQ15	**5′- CAGAGGATTTGAGGTGAA-3′** (859←877)
SEQ16	**5′-TTGGCCACCCAGTCGTTGAT-3′** (706←725)
SEQ17	**5′-ATGCTGAAGGGGGAAAAG-3′** (426←443)
SEQ18	**5′-TGGTAGAGCTTCAGGGAGAA-3′** (357←376)
SEQ19	**5′-GACTCTCCAAGTTGAGAGCAG-3′** (309←327)
SEQ20	**5′-CTTGAATGGACTCACCATCG-3′** (141←160)

Starting annealing temperatures for the oligodeoxyribonucleotide primers used in PCR were estimated using the following equation, modified from Maniatis, *et al.*, 1982,:

$$T_A = \{4 \text{ °C(GC)} + 2 \text{ °C(ATI)}\} - 10 \text{ °C}$$

Where T_A is the estimated annealing temperature, A, C, G, T, and I are the numbers of adenine, cytosine, guanine, thymine and inosine bases, respectively, occurring in the primer, and all temperatures are in degrees Celsius. The temperatures derived using this formula were found to be a good starting point for primers up to 30 nucleotides in length. The optimum annealing temperatures would ultimately be determined empirically.

2. Isolation and Purification of DNA

   a. Plasmid isolation. plasmids were isolated using a modified alkaline lysis, ethanol precipitation method (Nucleic Acids Research, 7 (1979): 1513). In a sterile, 15 mL snap top culture tube, 5 mL of LB broth (100 µg/mL AMP) was inoculated with a single colony of *E.coli* containing the plasmid of interest. The culture was incubated in a shaking air bath at 37 °C until an OD_{600} of 0.5-0.6 or overnight. The cells were collected by centrifugation at 5000 xg for 10 min at 4 °C. The nutrient broth was decanted, and the tubes inverted onto paper towel and let air dry on bench at RT for 10 min Any excess culture broth was carefully wiped off and the pellet was resuspended in 200 µL Glucose buffer. To each tube 400 µL freshly prepared Alkaline-SDS solution was added and mixed by inversion. Next, 300 µL 3 M NaOAc, pH 4.8 was added, the tube contents were mixed by inversion. If a clot formed, the entire mix was transferred to a 1.7 mL Eppendorf tube and microfuged at 17,000 xg for 5 min. If no clot formed, 100 µL of phenol (dH$_2$0 saturated) was added, then the tube was vortexed and microfuged as above. To a clean Eppendorf tube 750 µL of the supernatant were transferred and 450 µL

isopropanol were added, the mixture was vortexed. The tubes were microfuged at 17,000 xg for 10-20 min, supernatant decanted and remaining liquid removed with a "drawn out" Pasteur pipette. The pellet was resuspended in 200 µL dH$_2$0. After incubating at 68 °C for 5 min the tubes were microfuged at 17,000 xg for five min at RT, and the supernatant was transferred to a clean Eppendorf tube. Final precipitation was performed by the addition of 100 µL of 7.5 M NH$_4$OAc, pH 7.5, and 1 mL ice cold EtOH, vortexing and microfuging at 17,000 xg for 10-20 min. The liquid was decanted, and the tubes were inverted and let air dry on bench for 15 min, remaining liquid was removed with a "drawn out" Pasteur pipette, and the pellet was resuspended in 100 µL dH$_2$0. If the plasmid was to be used for sequencing it was further purified by extracting once with phenol and twice with phenol/chloroform (1:1).

b. Purification of the PCR products. The total volume of the PCR reaction mix was removed from under the mineral oil vapour barrier, and the volume of the reaction mixture was adjusted to 50 µL. The PCR product was extracted once with an equal volume of phenol/chloroform mixture (1:1).

c. Precipitation of DNA. The aqueous (top) layer was removed to a clean Eppendorf tube, 1/2 a volume of 7.5 M NH$_4$AOc (pH 7.5), and 2 volumes of 95% EtOH were added, and the DNA precipitate was collected by centrifugation at 17,000 xg for 20 minutes. The 95% EtOH was decanted, the pellet was washed once with 70% EtOH, air dried for 15-20 minutes, and resuspended in 20 µL dH$_2$0. 20 µL dH$_2$0 was added to the tube, and incubated at 37 °C for 10 min. vortexing twice to resuspend the PCR product.

d. Gel purification. DNA , PCR products, or restriction enzyme fragments, were purified by gel electrophoresis according to the Bio 101 gene clean kit protocol, with the following modification: the NEW (NaCl, ethanol, water) wash was made by mixing 2 X TAE and 95% ethanol 1:1. When purifying the blunt end repaired PCR products, the entire reaction mixture was loaded onto the gel. The purified, blunt ended DNA was

precipitated with ethanol as above and resuspended in 20 μL dH2O, 10 μL of this preparation was used per ligation reaction.

3. Cloning

a. Blunting the PCR product. None of the primers used in the PCR experiments had restriction sites added to facilitate cloning; therefore, they all had to be blunt-ended before they could be cloned. The gel purified PCR products were made blunt-ended as follows: 2.0 μL 10X Klenow buffer and 1.0 μL Klenow (large fragment, 6 units/μL) were added to the resuspended PCR product, this mixture was incubated at 37 °C for 2 minutes, after which time 1 μL dNTPs (10 mM, aqueous) was added and the mixture was incubated at 37 °C for an additional 5 minutes.

b. Ligation of PCR products into pBluescript (KS+). For DNA sequence determination and restriction endonuclease mapping, PCR products were ligated into the pBluescript (KS+) vector. The ligations were carried out with 10-20 ng of restriction endonuclease digested vector DNA and 25-50 ng of insert DNA (in a 3:1 molar ratio of ends, insert:vector) in a reaction volume of 15 μL. The gel purified, blunt ended PCR product (10 μL) was added to 1 μL of gel purified, Hinc II cut, pBluescript vector, in the presence of 1 μL T4 ligase (1 unit), and 3 μL of 5X ligase buffer (supplied by the manufacturer). The reaction mixture was incubated for 4 hours at room temperature or overnight at 15 °C. If not used immediately, the ligations were stored at -20 °C.

c. Transformation of DNA into Competent Bacteria. Competent DH5αF' cells were prepared using the calcium chloride method described by Maniatis et al., 1982, and stored in 15 % glycerol at -70°C. Half of the ligation reaction (see above) (7.5 μL), was added to 40 μL of the competent cells and incubated on ice for 30 minutes. Sterile LB broth (400 μL) was added to each transformation reaction, the transformed cells were transferred to a shaking air bath at 37 °C and incubated for 30 minutes. The cells were

centrifuged at 16,000 xg for 10 seconds, the LB decanted, and the cell pellet was resuspended in the remaining LB (100-200 μL). Half of the cells were each plated onto an LB agar plate containing ampicillin (100 mg/mL), X-Gal (50 μg/mL), and IPTG (25 μg/mL). The plates were left on the bench, covered, right side up for 15 minutes, then inverted and placed, overnight (12-18 hours), in a 37 °C incubator.

d. Selection of recombinants. Recombinants were identified on the basis of α-complementation (blue/white colour selection). Recombinants were grown overnight in 5 mL cultures of LB broth that contained ampicillin at 100 mg/mL, and harvested at an optical density of 0.6-1.0 units/mL. Plasmid DNA from selected recombinants was prepared as described above.

4. GEL ELECTROPHORESIS

a. Non-Denaturing agarose gels. DNA fragments were separated according to size by electrophoresis in agarose. The buffer for native agarose gel electrophoresis was 1X TAE, 0.02 μg/mL EtBr (Maniatis, *et al.*, 1982). DNA samples were mixed with 1/10th volume 10X loading dye (3 % ficoll, 0.02 % xylene cyanol, and 0.02 % bromophenol blue) to a final concentration of 1X, and separated by electrophoresis at 5-10 volts/cm. DNA fragments were visualized by EtBr fluorescence under UV light.

b. Denaturing polyacrylamide gels. The DNA products of the sequencing reactions were separated on 0.3 mm thick denaturing polyacrylamide gels. The gels were prepared in 1X TBE, 8.3 M urea with the appropriate concentration of acrylamide, usually either six or eight percent, and varied by adjusting the amount of acrylamide/bis stock solution (acrylamide:bis 38:2) and dH$_2$O added. Polymerization was initiated by the addition of APS (10 % w/v stock solution made fresh for each gel(s)) and TEMED to final concentrations of 0.066 % and 0.024 % (w/v) respectively. For sequencing, DNA fragments were denatured in the total reaction mix (see below) by boiling for 2 min., snap

cooled on ice for 2 min., then loaded onto the sequencing gel. The DNA fragments were separated by electrophoresis in 1X TBE running buffer at 55 watts constant power for 2-3 hours. The gels were dried at 80 °C under vacuum and exposed at RT on either Kodak X-Omat or XAR films.

5. DNA Sequence Analysis

a. DNA Sequence Determination of cloned, double stranded plasmid DNA.

DNA sequences of double stranded PCR products cloned into pBluescript (KS+) were determined using the enzymatic chain termination method (Sanger, *et al.*, 1977). Sequencing reactions were carried out using the ratio of chain terminating dideoxy- and deoxyribonucleotides recommended by the manufacturer. Template DNA (3-5 µg) was denatured in 200 mM NaOH, 2 mM EDTA in the presence of 2 pmoles sequencing primer (see Table II) by boiling in dH_2O for 2 min. The denatured plasmid DNA was then precipitated by the addition of $1/10^{th}$ volumes of 3M NaOAc, pH 5.2, and 2 volumes cold 95 % EtOH (−20 °C) mixed by gentle vortexing. The DNA precipitate was collected at 17,000 xg for 20 min at RT. The DNA pellet was washed once with −20 °C 75 % EtOH and all remaining EtOH was removed with a "drawn out" Pasteur pipette. The DNA pellets were resuspended in 2 µL of 5X reaction buffer (see above) and 8 µL dH_2O. The samples were vortexed and incubated at 37 °C for 10 min to allow annealing of the primers to the template. The DNA samples were removed from the hot dH_2O bath and 1 µL of 100 mM DTT, 2 µL of a $1/8^{th}$ dilution of *Sequenase*, 2 µL of labeling mix (9 µM dATP, 9 µL dCTP, 9 µL dGTP, and 9 µL dTTP) and 0.5 µL of α-[thio-^{35}S]-dATP (3000 Ci/mMol) was added to each sample. Sample contents were mixed and incubated at RT for 2-5 min. Aliquots of 3.5 µL of each sample were mixed with 2.5 µL of the termination mixes (80 µM dATP, 80 µM dCTP, 80 µM dGTP, 80 µM dTTP, 8 µM ddNTP) and incubated on a block heater at 37 °C for 5-10 min. Reactions were stopped by the

addition of 4 μL 98 % formamide, 10 mM EDTA, 0.02 % xylene cyanol, and 0.02 % bromophenol blue. Sequencing reactions were loaded right away (see above) or stored at −20 °C for up to one week.

  b. DNA Sequence Data Analysis. DNA sequence data were collected from the autoradiograms using a sonic digitizer connected to an IBM compatible PC, with PCGENE software. DNA sequence data was aligned and analyzed using the PCGENE suite of programs version 6.8, from IntelliGenetics.

III Results:

A. *Isolation of the Bovine cDNA for Factor XIIa inhibitor in two overlapping fragments.*

1. 3′ half of the BFXIIaINH cDNA.

As discussed earlier, the human homologue of Bovine factor XIIa inhibitor (BFXIIaINH) is C1-inhibitor (C1INH). The C1INH protein and cDNA have been previously isolated and their sequences determined (Bock, *et al.*, 1986). BFXIIaINH protein had been previously isolated, purified and partially sequenced (Muldbjerg, Markussen, Magnusson, & Halkier, 1993). These data were used to design PCR primers (see fig 7 for PCR strategy) that were used to amplify the BFXIIaINH cDNA. The first PCR amplification experiments were done with each of three different PCR primers in the 5′ position and the T_{17XSP} primer in the 3′ position. The pair that worked, PCR1/T_{17XSP}, is shown in table II and a picture of the agarose gel of the product is shown in fig. 8. The template for the reaction was first strand cDNA reverse transcribed from total bovine liver RNA using the T_{17XSP} primer. This starting material was supplied by Dave Banfield. The product of the PCR amplification experiment was a 940 bp fragment (including primers and restriction endonuclease site on the T_{17XSP} primer). The fragment was made blunt ended and cloned into the Hinc II site of the Bluescript KS+ polycloning site. The complete nucleotide sequence of the fragment was determined. There is a single stop codon at bp 1407 followed 178 bp later by a poly adenylation signal. The polyadenylation signal is followed 14 bp later by the poly-A tail. The fragment contained an ORF 699 bp long that translated to a predicted 233 amino acids. The nucleotide and amino acid sequences of the bovine fragment were compared with the sequences from the C1INH, and confirmed that a BFXIIaINH cDNA had been isolated (see later section).
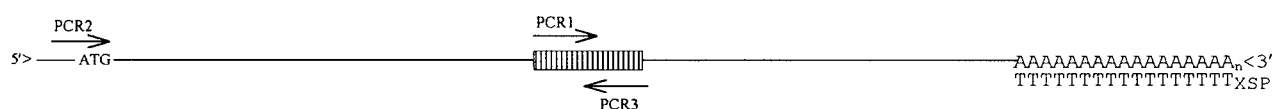
Figure 7.   PCR amplification strategy for the BFXIIaINH cDNA molecule. The complete bovine factor XIIa inhibitor cDNA molecule was amplified in two overlapping pieces from bovine sscDNA. Primers, except T_{17XSP}, are shown as horizontal arrows $\rightarrow$ or $\leftarrow$, to indicate the priming direction. The relative primer locations are shown, not to scale. Primers PCR1 and T_{17XSP} were used together as were PCR2 and PCR3. The horizontal line represents the bovine sscDNA. The 5′ ATG is shown and marks the 3′ three bases of primer PCR2. The 3′ As represent the poly A tail of the first round product of PCR amplification of the cDNA. The T_{17XSP} primer is shown binding to the beginning of the poly A stretch as indicated by sequencing data. The shaded box represents the area of overlap but is not to scale. The T_{17} in the T_{17XSP} primer represents the number of Ts present and the XSP stands for the restriction endonuclease sites Xba I, Sac I, and Pst 1. For primer design details please see the Materials and Methods section.
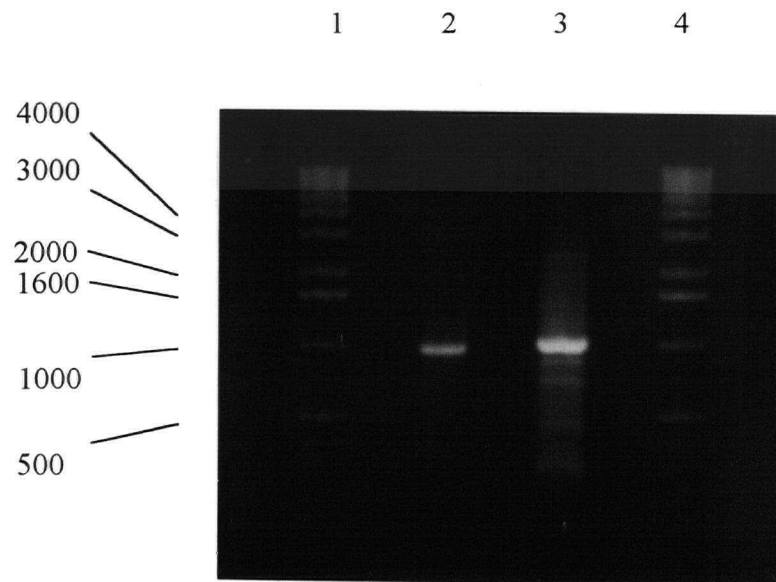
Figure 8. Agarose gel showing the product of the PCR experiment to amplify the 3′ half of the BFXIIaINH cDNA. Lanes 1 and 4 show molecular weight markers, lane 2 shows the product of the PCR amplification experiment using bovine total sscDNA as the template, lane 3 shows the same primer pair used on human total RNA as the template. In both experiments the primer pair PCR1/T$_{17XSP}$ was used and the PCR reaction was completed in 10% DMSO.

2. 5′ half of the Bovine Factor XIIa inhibitor cDNA.

From the sequence data derived from the 3′ fragment PCR primers were designed from the 5′ end of the fragment that would prime in the 5′ direction of the cDNA (see table II and fig. 7). Sequence data from the 5′ end of the human cDNA was used to design a primer for the bovine cDNA that primed in the 3′ direction (see fig. 9). Figure 10 shows the results of these experiments. There are three different sized products shown. These correspond to the three different 3′ primers used. The intermediate sized fragment was chosen to give a good overlap with the 3′ fragment while minimizing the amount of sequencing required. The fragment was made blunt ended and cloned into the Hinc II site of the Bluescript KS+ cloning vector.

3. Sequencing the cDNA fragments.

The fragment was sequenced. The fragment was 917 bp long (including primers) and encodes an orf of 305 amino acids. The 5′ fragment has a 192 bp overlap (64 amino acids) with the 3′ fragment. Figure 11 shows the sequencing strategy and primer location used to sequence the complete cDNA. The complete cDNA sequence with predicted amino acid sequence is shown in figure 12. The complete cDNA sequence is 1608 bp long (excluding primers and poly-A tail) and encodes a 1404 bp orf. Translation of the orf into its predicted amino acid sequence results in a polypeptide of 468 amino acids.

B. Determination of the genomic organization of the 5′ region of the gene for BFXIIaINH.

1. PCR amplification of the region spanning the first two exons of BFXIIaINH.

The N-terminal region of the BFXIIaINH cDNA and protein (Muldbjerg, *et*

PCR primer PCR2 includes the ATG start codon. Used to amplify the 5' fragment of the BFXII cDNA.

Start codon

5' UTR

1>GGGCAGAAGTTTGGAGTCCGCTGACGTCGCCGCCCAGATGGCCTCCAGGC< 50

1>CCCGTCTTCAAACCTCAGGCGACTGCAGCGGCGGGTCTACCGGTGGTCCG< 50

Exon 2   Exon 3

51>TGACCCTGCTGACCCTCCTGCTGCTGCTGCTGGCTGGGGATAGAGCCTCC<100

51>ACTGGGACGACTGGGAGGACGACGACGACGACCGACCCCTATCTCGGAGG<100

End of leader sequence.

101>TCAAATCCAAATGCTACCAGCTCCAGCTCCCAGGATCCAGAGAGTTTGCA<150

101>AGTTTAGGTTTACGATGGTCGAGGTCGAGGGTCCTAGGTCTCTCAAACGT<150

Cleavage site of leader peptide, beginning of mature plasma protein.

Figure 9. Nucleotide sequence of the 5' end of the human C1INH cDNA. The 5' 150 bps of the human C1-inhibitor cDNA are shown. Design of the PCR2 primer used to amplify the 5' half of the bovine factor XII cDNA molecule was based on the the human cDNA sequence. The shaded box shows the PCR primer location. UTR stands for untranslated region. The demarcation between exons two and three (human notation) is indicated as is the start point of the mature circulating protein.
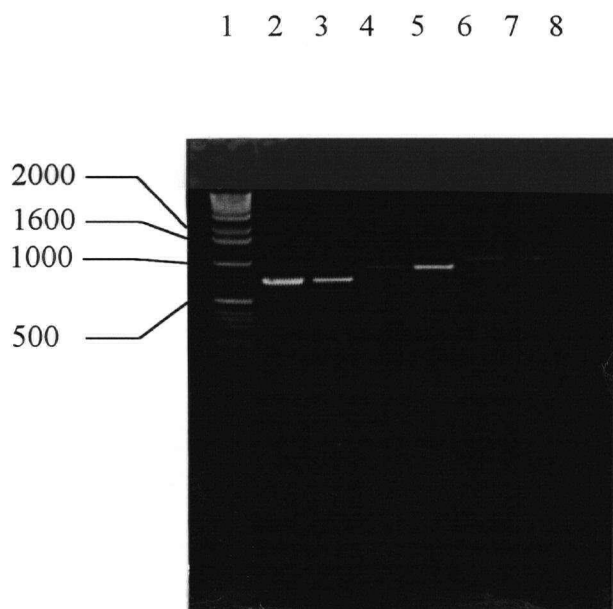
Figure 10. Agarose gel showing the products of the PCR experiment to amplify the 5′ half of the BFXIIaINH cDNA. First strand cDNA of total liver RNA was made uisng RT and T_{17XSP} primer. This was used as the template for PCR with the primer pairs PCR2/5P1, PCR2/5P2, or PCR2/5P3. Lane 1 contains the molecular weight standard, lanes 2 and 3 show PCR products with primer pair PCR2/5P1, lanes 4 and 5 show PCR products with primer pair PCR2/5P2 and lanes 6 and 7 show PCR products with primer pair PCR2/5P3. All - and + DMSO respectively. Lane 8 contains a negative control (dH2O was added as the template). The sizes of the fragments,estimated from the human cDNA for the respective primer combinations, were 819 bps, 993 bps, and 1113 bps. The product of the primer pair PCR2/5P2 was chosen for further experiments to achieve a reasonable overlap with the 3′ half of the bovine cDNA while minimizing the amount of sequencing required.
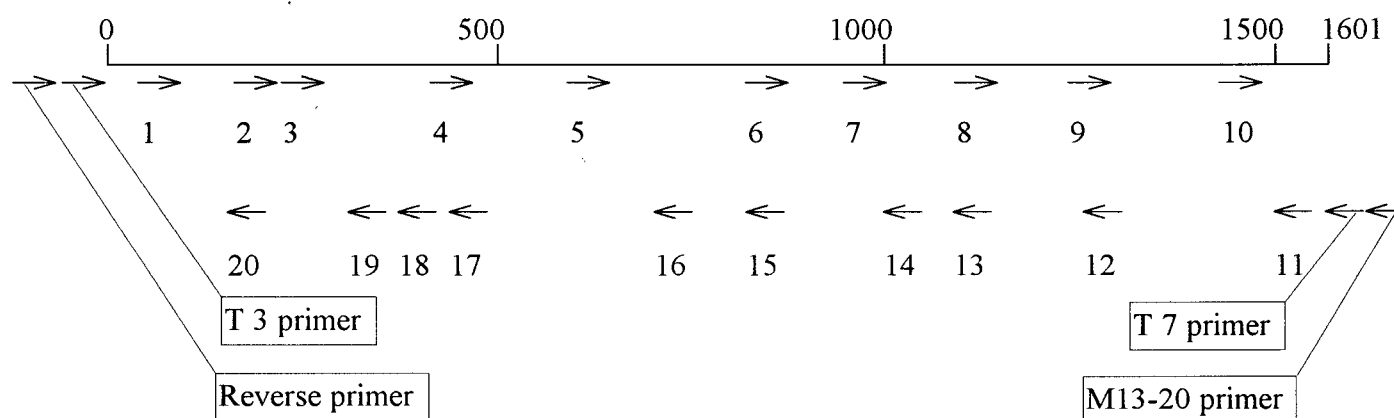
Figure11. Sequencing strategy and location of primers used to sequence the BFXIIaINH cDNA. Horizontal line represents the cDNA, numbers above the line show the length in base pairs. Horizontal arrows show location and direction of primers. Numbers below each primer correspond to the primer number in table II. For details of sequencing and primer use please see Materials and Methods. Distances, primer sizes and locations are not exactly to scale.

```
  1>ATGGCCTCCAGGCTGACCCCGCTGACCCTCCTGCTGCTGCTGCTGCTGGCTGGGGACAGA<  60
     M  A  S  R  L  T  P  L  T  L  L  L  L  L  L  L  A  G  D  R

 61>GTCACCTCAGATATGATCGTCGGCCCAGGGAACTTACAAGAAGGGGAAAGTGAAGGAGAC< 120
     V  T  S  D  M  I  V  G  P  G  N  L  Q  E  G  E  S  E  G  D

121>AGCCAGAAGGAGGTATTCTCGATGGTGAGTCCATTCAAGGCAACGAGGACTCTCCCACC< 180
     S  Q  K  G  G  I  L  D  G  E  S  I  Q  G  N  E  D  S  P  T

181>TTGCCGATAACTAACCTGACCGTCGTGCCAGCCACTGTCACCAAGCCCTTCAGCCAGCCT< 240
     L  P  I  T  N  L  T  V  V  P  A  T  V  T  K  P  F  S  Q  P

241>GCCACTGAACCGGTCCAATCAACTATCCAGCCCACTGCTGAGCCCTTCTGCCTGGCTCCC< 300
     A  T  E  P  V  Q  S  T  I  Q  P  T  A  E  P  F  C  L  A  P

301>GTCACCTCCTGCTCTGACTCGGAGATCCGCTCAGCAGAGGCGGTGCTGGGGGAGGCTTTG< 360
     V  T  S  C  S  D  S  E  I  R  S  A  E  A  V  L  G  E  A  L

361>ACAGACTTCTCCCTGAGGCTCTACCAGGACTTCTCAGTGTTGAAGAAGAGGGAGACCAAC< 420
     T  D  F  S  L  R  L  Y  Q  D  F  S  V  L  K  K  R  E  T  N

421>TTCATCTTTTCCCCCTTCAGCATTGCCAGCCTCCTCACCCAAATCCTGCTTGGGGCTGGA< 480
     F  I  F  S  P  F  S  I  A  S  L  L  T  Q  I  L  L  G  A  G

481>GGAGAAACCAGGGTCAGCCTGGAGCACCTCCTCTCTTACCCCCAGAACTTCAGCTGTGTC< 540
     G  E  T  R  V  S  L  E  H  L  L  S  Y  P  Q  N  F  S  C  V

541>CACCACGCCCTGAGGGCCTTCATGTCCGAAGGTTTCACCTCGTTCTCTCAGATCTTCCAC< 600
     H  H  A  L  R  A  F  M  S  E  G  F  T  S  F  S  Q  I  F  H

601>AGCTCAGACCTGACCATCAAGGACACCTTCGCCGAGGCCTCTCAGAGGCTCTATGGCAGC< 660
     S  S  D  L  T  I  K  D  T  F  A  E  A  S  Q  R  L  Y  G  S

661>AGCCCGAGACCCCTGGGAAATGACAGCACAGCCAGCTTGGAGCTTATCAACGACTGGGTG< 720
     S  P  R  P  L  G  N  D  S  T  A  S  L  E  L  I  N  D  W  V

721>GCCAAGAAGACCAACCTCAGGATCAGGCGGCTGCTGGACAGCCTGCCTGAGGACACCCGC< 780
     A  K  K  T  N  L  R  I  R  R  L  L  D  S  L  P  E  D  T  R

781>CTCATCCTCCTCAACGCTGTCGCCCTGAGCGCCAAGTGGAAGATAGCCTTTGATAAAGGC< 840
     L  I  L  L  N  A  V  A  L  S  A  K  W  K  I  A  F  D  K  G

841>AGAACAAGCACAAAGCCCTTTCACCTCAAATCCTCTGCCATCAAAGTGCCCATGATGAAC< 900
     R  T  S  T  K  P  F  H  L  K  S  S  A  I  K  V  P  M  M  N

901>AGCAAGAAGTACCCTGTGGCCTCTTTCACAGACCGGACTCTGAACCGCCCGGGTGGGCGC< 960
     S  K  K  Y  P  V  A  S  F  T  D  R  T  L  N  R  P  G  G  R

961>CTGCAGCTGTCTCACAACCTCAGCTTTGTGATCCTGGTGCCCCAGACCGTGAAACACCAT<1020
     L  Q  L  S  H  N  L  S  F  V  I  L  V  P  Q  T  V  K  H  H

1021>CTTCAAGACCTGGAGCAGGCTCTCAGCACCGCCGTCTTCAAAGCTGTCATAAAGAAGCTG<1080
      L  Q  D  L  E  Q  A  L  S  T  A  V  F  K  A  V  I  K  K  L

1081>GAGATGACCAAGTTCCATCCCACTCACCTGACGATGCCTCGCATCAAAGTGCAGAGCAGT<1140
      E  M  T  K  F  H  P  T  H  L  T  M  P  R  I  K  V  Q  S  S
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1141>CAGGACATGCTGGATTACTTTGACTTCATCTACGACGTCAACCTGTGCGGGCTGACGGAG<1200
      Q  D  M  L  D  Y  F  D  F  I  Y  D  V  N  L  C  G  L  T  E

1201>GACCCAGACGTGCAGGTGTCTGGGATCCGGCACCAGGCCACCCTGGAGCTGACGGAGAGC<1260
      D  P  D  V  Q  V  S  G  I  R  H  Q  A  T  L  E  L  T  E  S

1261>GGGGTGGATGCGACTGCGGCCTCGGTCGTCTCCGTGGCCCGCAACTTGCTGCTCTTCGAA<1320
      G  V  D  A  T  A  A  S  V  V  S  V  A  R  N  L  L  L  F  E

1321>GTGCAGCAGCCCTTCCTCTTCCTGCTCTGGGACCAGCAGCACAAGTTCCCGGTCTTCATG<1380
      V  Q  Q  P  F  L  F  L  L  W  D  Q  Q  H  K  F  P  V  F  M

1381>GGTCGGGTGTACGACCCCAAGGGCTGAGCCCCCCTGGGGCCTCAGCTCACCAGCTGCA<1440
      G  R  V  Y  D  P  K  G

1441>GCCGCTCTGCACTTGCCCCTCCCCACCTCCCACCTCAGGCCTGCCTCCACCGAAGGGCTC<1500

1501>CCCTGGGGTCCTTCCCTCCTCCCTTACCGTGACCCCTCCAAAGCACTTTTTGCCACCTTC<1560

1561>TCTGGTACAGATACAGCGGACTTCACAAATAAAACCTGGCAAACCGTG<1608
```

Figure 12. Complete nucleotide sequence and predicted amino acid sequence of the BFXIIaINH cDNA. The predicted amino acid sequence is shown, in single letter code, below the nucleic acid sequence. Start (ATG) and stop (TGA) codons and the polyadenylation recognition sequence (AAATAAAA) are boxed. The open arrow indicates the probable propeptidase cleavage site, as the mature circulating peptide sequence begins with the following D residue. The shaded box indicates the position of the SERPIN signature sequence. The first two residues, R & N, of the SERPIN signature sequence are the P1 and P1' residues of the putative reactive site and the closed arrow shows the location of the bond cleaved during complex formation with target proteases. This is the only known example of a SERPIN with well documented inhibitor activity that has an asparagine residue in the P1' position.

al., 1993) show a very low degree of identity with human C1INH protein and cDNA. It was possible that the different sequence was contained in a novel exon that had been acquired by the bovine gene, or been lost from the human gene. To see if the sequence differences were reflected in the genomic organization a series of PCR experiments were carried out (see figure 14). PCR primers were designed by comparing the BFXIIaINH cDNA sequence data and with the sequence and organization known for the human gene (Carter, *et al.*, 1988; Carter, *et al.*, 1991). Two sets of primers were designed, one to amplify the intervening intron between the known exons and the other to amplify within the exon number three (human numbering) (see figure 13). If the different sequence was due to a new intron or exon (see bovine case II figure 13) then we the PCR products amplified from the bovine genomic DNA would be different in sized from those predicted from the human gene sequence. If the BFXIIaINH gene organization was similar to the human gene we would expect similarly sized PCR products but their restriction patterns would be different.

1. PCR amplification across the intron/exon junctions using bovine genomic DNA as template.

PCR primers PCR4/PCR6 (of the nested set PCR4/PCR5/PCR6) produced a smear with three distinct bands showing in the first round PCR (see figure 14 lane 5). In the second round PCR primers PCR4/PCR6 produced the same pattern of three bands as seen (see figure 14 lane 2) in round 1; however the molecular weights were lower by the amount predicted by the difference in 3' primer location, and there was some smearing below the lowest band. If the bovine and human genomic organizations were the same the PCR product was expected to be at least 1.6 kb, therefore the upper band (estimated to be around 2.1 kb by comparison to the molecular weight standard figure 14, lane 1) was cloned into the cloning vector pBluescript KS+ and the ends were sequenced.
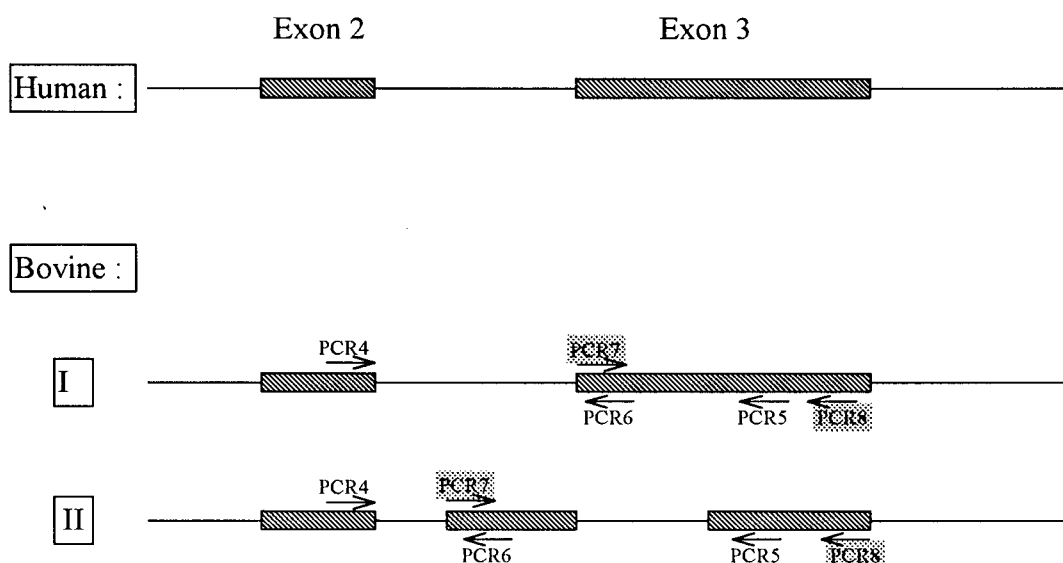
Figure 13. PCR amplification strategy for determining the genomic organization of the 5′ end of the BFXIIaINH gene. The horizontal lines represent intronic sequences. The shaded boxes represent exons. The exon numbering is shown above and is taken from the human gene. The horizontal arrows represent the respective PCR primers and show relative location and direction. The primer numbers correspond to the primers in table II. The shaded primers were used as a set as were the unshaded primers. The drawing in the Human case represents the known organization of the human gene. The two drawings for the bovine case represent the two possible organizations that are being discriminated. A nested PCR amplification strategy, unshaded primers used in the order numbered, was used to generate the intron containing fragment in bovine case I. The first PCR amplification experiment was done using primer combination PCR4/PCR5 on bovine genomic DNA, the product from this experiment was used as the template in the follow up PCR amplification with primer pair PCR4/PCR6. See Results for a discussion of the details of the experiments.
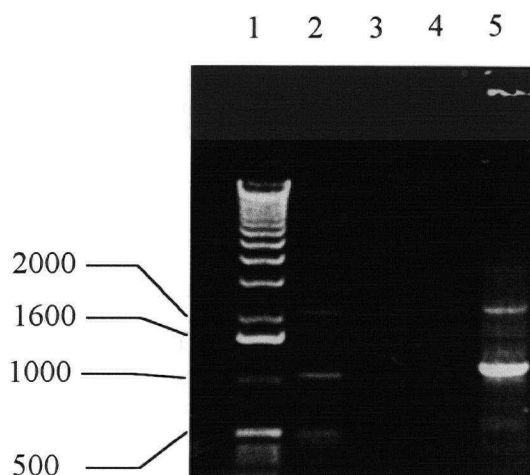
Figure 14. Agarose gel showing results of the nested PCR amplification experiment between exons 2 and 3 using bovine genomic DNA as template. Lane 1 molecular weight standard, lane 2 PCR product using primer pair 4 and 6 on the product shown in lane 5 (see table IV), lanes 3 and 4 are blank, lane 5 shows the product of primers PCR4/PCR5 on bovine genomic DNA. 2μL out of 20μL loaded. Touch down PCR was used to generate these fragments. First PCR reaction was with PCR4/PCR5 on bovine genomic DNA. The product of this reaction was used as a template for a second PCR reaction using the PCR4/PCR6 primer combination (see Fig. 13). PCR5 lies 224 bps 3′ of PCR6. The PCR product of the PCR4/PCR6 amplification experiment was cloned and the ends were sequenced.

2. Sequence of the intron/exon junctions in the 5′ region of the BFXIIaINH gene.

The results summary of sequencing the cloned PCR product are shown in figure 15. The exonic sequences matched the bovine cDNA sequences perfectly, up to the exon/intron junction. The conserved GT/AG sequence was observed at the beginning and end of the intronic sequence. Only the first 220 bp and the last 123 bp of the intron were sequenced. The intron sequence was aligned with the human intron sequence but no significant identity was seen. This was as expected as there is no selection pressure to maintain a given sequence in the intron.

3. PCR assay for the presence of a novel intron within exon 3 of the BFXIIaINH gene.

The second primer pair (see fig. 13) was predicted to generate a PCR product of 405 bp using bovine genomic DNA if it did not contain an extra intron. Figure 16 shows the results of the experiment; a single band of the expected size was produced. This band was harvested and purified for subsequent restriction mapping.

4. Restriction endonuclease mapping of the BFXIIaINH exon 3.

To rule out the possibility of contamination with human material restriction enzymes that gave differential patterns were chosen. The human and bovine cDNA sequences were restriction mapped using the PCgene program and appropriate enzymes chosen based on the results of that analysis. Figure 17 shows the results of these experiments. Hpa II and Mbo II were chosen because they do not cut the human sequence in this area. Lane three of fig. 17 shows the uncut size of the fragment; it is at the 405 bp calculated size. Lanes four and five show the restriction patterns from cutting with Hpa II and Mbo II respectively. The Hpa II digest was calculated to give two bands of sizes 185 bp and 220 bp respectively, which can be seen clearly. The Mbo II digest was

expected to produce restriction fragments 348 bp and 58 bp respectively. It was not expected that the 58 bp band would be resolved under these conditions. There is a faint band above the 348 bp band in lane five by comparison with the molecular weight marker and the uncut band in lane three which is thought to be undigested original fragment. The results of these experiments support the hypothesis that the genomic organization of the bovine gene in the 5' region investigated is the same as that of the human C1-inhibitor gene. Thus the difference in N-terminal sequences between the bovine and human genes is due to an amplification or deletion of existing exonic sequence rather than the acquisition of a novel exon by the human gene or the loss of a pre-existing exon by the bovine gene.
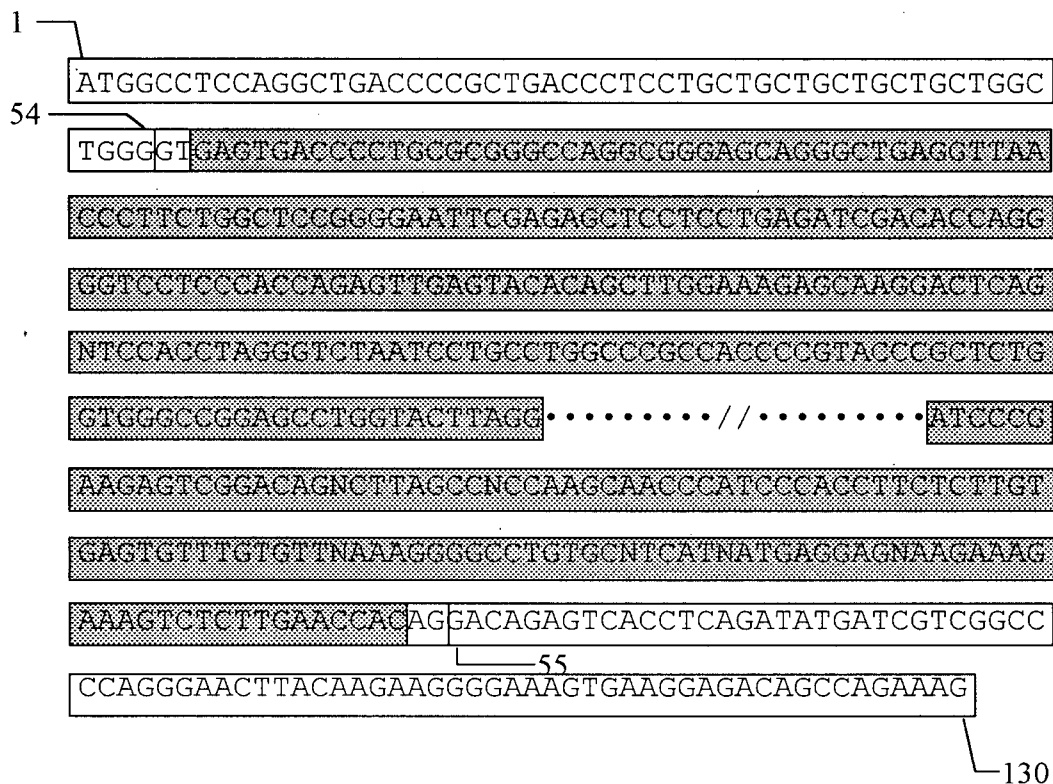
Figure 15. Sequence of intron/exon junctions between exon 2 and 3 of the BFXIIaINH gene. The unshaded boxes contain exon sequence. The shaded boxes contain the available intron sequence. The consensus GT & AG beginning and ending intron sequences are show in the clear boxes. The numbers are taken from the bovine factor XIIa inhibitor cDNA sequence. The •••//••• symbol indicates an indeterminate amount of intervening intron sequence.
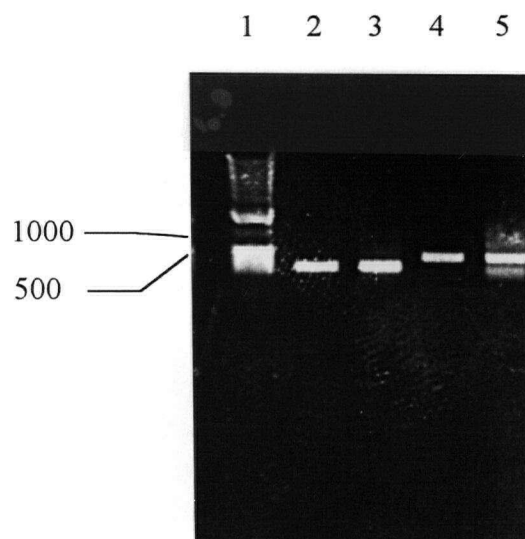
Figure 16. Agarose gel showing the products of the PCR amplification experiment on bovine genomic DNA using primer pair PCR7/PCR8. The PCR primer pair 7/8 was designed to amplify the region between the beginning and the end of the bovine equivalent of exon 3 in the human C1-inhibitor gene. Lane 1 contains the molecular weight standard, lanes 2 & 3 contain the positive controls (primers that amplified a portion of exon 14 of human blood clotting factor IX were used on a template of human genomic DNA), lanes 4 & 5 contain the PCR amplification products of primer pair PCR7/PCR8 with bovine genomic DNA as the template. The experiments were carried out in duplicate with two different PCR buffers (see materials and methods).
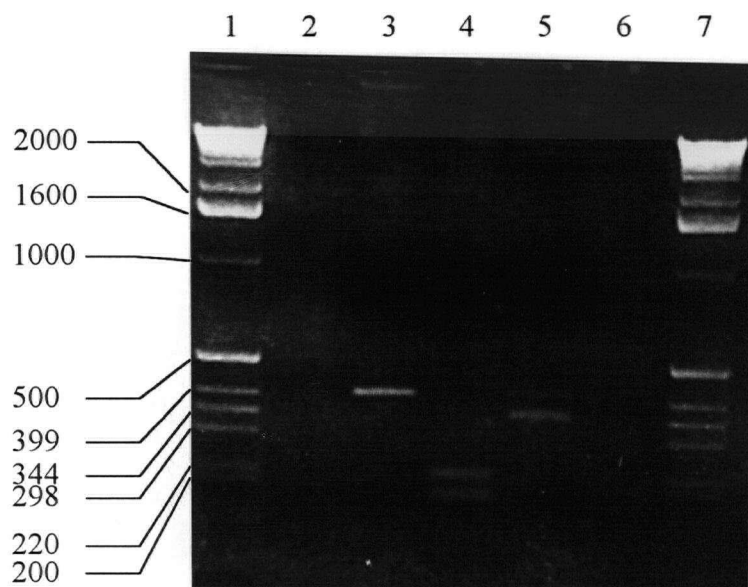
Figure 17. Restriction endonuclease digests of the PCR product from the 5′ genomic organization experiment using PCR primer pair PCR6/PCR7. Lanes 1 & 7 contain the molecular weight standard (sizes shown on the right in base pairs), lanes 2 and 6 are blank, lane 3 contains the uncut PCR product, lane 4 contains Hpa II cut PCR product, and lane 5 contains Mbo II cut PCR product. The top band in lane 5 is uncut PCR product. The expected sizes of the fragments are Hpa II cut → 220 bps & 185 bps, Mbo II cut → 348 bps & 58. The 58 bp band is not visible.

```
            -20        -10         1          10         20        30
        ------  --------  -----      _    -   -- -         _           _
HSC1INHA: MASRLTLLTLLLLL-LAGDRASSNPNATSSSSQDPESLQDRGEGKVATTVISKMLFVEPI
BFXIIAIN: MASRLTPLTLLLLLLLLAGDRVTSDMIVGPGNLQEGESEGDSQKGGILDGESIQGNEDSPT
HS_ATIII:
HS_α1-AT:
Chk_Oval:
                            ↑

            40      _   50         60         _  ---  --  70
            --  -                   -  ---  --
HSC1INHA: LEVSSLPTTNSTTNSATKITANTTDEPTTQPTTEPTTQPTIQPTQPTTQLPTDSPTQPTT
BFXIIAIN: LPITNLTVVPATVTKPFSQPATEPVQSTIQPTAEP------------------------
HS_ATIII:                              HGSPVDICTAKPRDIPMNPMCIYRSPEK
HS_α1-AT:              ,                                        EDP
Chk_Oval:

              80         90        100        110        120
            --   ---  ---  -  ------+-  ++--*--  +*-  ---   +-  -+*++++**-**+++
HSC1INHA: GSFⓄPGPVTLⓄSDLESHSTEAVLGDALVDFSLKLYHAF-SAMKKVETNMAFSPFSIASLL
BFXIIAIN: --FⓄLAPVTSⓄSDSEIRSAEAVLGEALTDFSLRLYQDF-SVLKKRETNFIFSPFSIASLL
HS_ATIII: KATEDEGSEQKIPEATNRRVWELSKANSRFATTFYQHLAD-SKNDNDNIFLSPLSISTAF
HS_α1-AT: QGDAAQKTDTSHHDQDHPTFNKITPNLAEFAFSLYRQLA--HQSNSTNIFFSPVSIATAF
Chk_Oval:                         GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSAL

          130     140        150             160        170
          ++-+**-  -*---+---+-+--  -  ---      +--  -*-----++  ---    - -
HSC1INHA: TQVLLGAGENTKTNLESILSYPKDFTⓄVH----QALKGFTTKGVTSVSQ-----------
BFXIIAIN: TQILLGAGGETRVSLEHLLSYPQNFSⓄVH----HALRAFMSEGETSFSQ-----------
HS_ATIII: AMTKLGACNDTLQQLMEVFKFDTISEKTSD---QIHFFFAKLNC-RLYRKANKSSKLVSA
HS_α1-AT: AMLSLGTKADTHDEILEGLNFNLTEIPEA---QIHEGFQELLRTLNQPDSQLQLTTDGG
Chk_Oval: AMVYLGAKDSTRTQINKVVRFDKLPGFGDSIE---AQCGTSVNVHSSLRDILNQITKPND

                180        190        200        210        220
          +*-+--*-+ +++---+- **-+---   -+-----+---+**-**-+-
HSC1INHA: ----------IFHSPDLAIRDTFVNASRTLYSSSPR----VLSNNSDANLELINTWVAKN
BFXIIAIN: ----------IFHSSDLTIKDTFAEASQRLYGSSPR----PLGNDSTASLELINDWVAKK
HS_ATIII: NR--------LFGDKSLTFNETYQDISELVYGAKLQPL--DFKENAEQSRAAINKWVSNK
HS_α1-AT: ----------LFLSEGLKLVDKFLEDVKKLYHSEAFTV--NFGDTEEAKKQ-INDYVEKG
Chk_Oval: VYSFSLASR-LYAEERYPILPEYLQCVKELYRGGLEPI--NFQTAADQARELINSWVESQ

              230        240        250        260        270
          *++-*--++  -++--+*-++*+*+-++++*+  -*----*-  +-*+--  ----+*+**-
HSC1INHA: TNNKISRLL--DSLPSDTRLVLLNAIYLSAKWKTTFDPKKTRMEPFHFKNS-VIKVPMMN
BFXIIAIN: TNLRIRRLL--DSLPEDTRLILLNAVALSAKWKIAFDKGRTSTKRFHLKSS-AIKVPMMN
HS_ATIII: TEGRITDVIPSEAINELTVLVLVNTIYFKGLWKSKFSPENTRKELFYKADGESCSASMMY
HS_α1-AT: TQGKIVDLV--KELDRDTVFALVNYIFFKGKWERPFEVKDTEEEDFHVDQVTTVKVPMMK
Chk_Oval: TNGIIRNVLQPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMY

          280        290        300             310        320        330
          --+--+-  -  -+-  -++-*----+-          +-+-++*---*-  *--+*--*--++-
HSC1INHA: SKKYPVAHFIDQTLKAKVGQLQLSHNL-----SLVILVPQNLKHRLEDMEQALSPSVFK-
BFXIIAIN: SKKYPVASFTDRTLNRPGGRLQLSHNL-----SFVILVPQTVKHHLQDLEQALSTAVFK-
HS_ATIII: QEG-KFR-YRRVAEGTQVLELPFKGDDI----TMVLILPKPEKS-LAKVEKELTPEVLQ-
HS_α1-AT: RLGM-FNIQHCKKLSSWVLLMKYLGNA-----TAIFFLPDEGK--LQHLENELTHDIIT-
Chk_Oval: QIGL-FRVASMASEKMKILELPFASGTM----SMLVLLPDEVSG-LEQLESIINFEKLT-

            340      - - --+*+--+*----++----+- -+-----*---+ *-*-++---
          -----++-
HSC1INHA: -AIMEKLEMSK-FQPTLLTLPRIKVTTSQDMLSIMEKLEFFDFSYDLN-IⓄGLTEDPD--
BFXIIAIN: -AVIKKLEMTK-FHPTHLTMPRIKVQSSQDML------DYFDFIYDVN-IⓄGLTEDPD--
HS_ATIII: -EWLDELEEMM--LVVHMPRFRIEDGFSLKE-QLQDMGLVDLFSPEKSKLPGIVAEGRDD
HS_α1-AT: -KFLENEDRRS--ASLHLPKLSITGTYDLKS-VLGQLGITKVFSNGAD-LSGVTEEAP--
Chk_Oval: -EWTSSNVMEERKIKVYLPRMKMEEKYNLTS-VLMAMGITDVFSSSAN-LSGISSAES--

              390        400        410         +++-   -*----****----+--+
          *-+* +-*+*-*-*  *-**+++++----
HSC1INHA: LQVSAMQHQTVLELTETGVEAAAASAISVA--RTLL------VFEVQQPFLFVLWDQQHK
BFXIIAIN: VQVSGIRHQATLELTESGVDATAASVVSVA--RNLL------LFEVQQPFLFLLWDQQHK
HS_ATIII: LYVSDAFHKAFLEVNEEGSEAAASTAVV-IAGRSL--NPNRVTFKANRPFLVFIREVPLN
HS_α1-AT: LKLSKAVHKAVLTIDEKGTEAAGAMFLEAIP-MSIP-----PEVKFNKPFVFLMIEQNTK
Chk_Oval: LKISQAVHAAHAEINEAGREVV-GSAEAGVDAASV-----SEEFRADHPFLFCIKHIATN
                                            ↑

            440   445
          -+-*****--*  -
HSC1INHA: FPVFMGRVYDPRA
BFXIIAIN: FPVFMGRVYDPKG
HS_ATIII: TIIFMGRVANPCVK
HS_α1-AT: SPLFMGKVVNPTQK
Chk_Oval: AVLFFGRCVSP
```

Figure 18. Amino acid Alignment of BFXIIaINH with C1INH and three other SERPINs. Human antithrombin III[a] (HS_ATIII), α1-antitrypsin[a] (HS_α1-AT), and Chicken ovalbumin[a] (Chk_Oval). Leader sequences for HSC1INHA and BFXIIaINH are shown in bold. The sequences are numbered according to the bovine sequence. Cysteines known to be involved in secondary structure in C1INHA and their proposed homologous residues in BFXIIaINH are circled. The SERPIN signature sequence is boxed. The open arrow indicates the putative cleavage site of the signal peptidase. The closed arrow indicates the bond between residues P1 and P1′ of the proposed reactive site that is cleaved during complex formation with the target proteases. The horizontal dashes within the sequences are gaps that have been placed in the sequences to maximize the sequence identity, and in some of the sequences common gaps were inserted to maintain sequence numbering. Two large insertions are shown in the BFXIIaINH sequence, 27 amino acids between residues 72 & 73 and 6 amino acids between reidues 361 & 362. These gaps were placed here to best align the bovine sequence with the human. The symbols above the sequences represent the following degrees of homology: -, identity in two proteins, +, identity in three proteins, and *, identity in ≥ four proteins. [a], sequences and alignments taken from Bock, S.C., *et al.*, 1986.

IV. DISCUSSION

A. Analysis of the BFXIIaINH cDNA.

1. Comparison of BFXIIaINH with C1INH and other serpins.

a. Molecular weight. The observed M_r of human C1INH, by SDS-PAGE, is between 105-116 kDa (Harrison, 1983; Bock, *et al.*, 1986) and the carbohydrate content is estimated at 34% (Haupt, *et al.*, 1970). There is an apparent change in M_r under reduced and non reduced conditions (Odermatt, *et al.*; 1981; Schmaier, *et al.*, 1985) thought to be due to the highly asymmetrical shape of the molecule conferred by its internal disulphide bonds. Deglycosylation of the protein yields a M_r of between 68,250 and 78,000. The human circulating protein is 478 amino acids long with a calculated molecular weight from the cDNA of 52,869 Da. This equals between 73% and 77% of the observed deglycosylated M_r. However the difference between the observed and calculated M_rs suggests that glycosylation accounts for $\approx$49% of the observed M_r. This difference may be accounted for by incomplete deglycosylation reactions.

As estimated from SDS-PAGE, the observed M_r of the BFXIIaINH is between 75 kDa (Muldbjerg, *et al.*, 1993) and 85 kDa to 92 kDa, reduced and non-reduced, respectively (Thornton, & Kirby, 1987,88). The apparent M_r of the molecule is observed to change under reduced or non reduced conditions by Thornton & Kirby but not by Muldbjerg, *et al.* The reason for these discrepancies is not clear. The variation observed by Thornton and Kirby may be because the native molecule is constrained under nonreducing conditions into a highly asymmetric shape by its disulphide bonds as in the case of the C1INH molecule (Thornton, & Kirby, 1987; Odermatt, *et al.*, 1981; Schmaier, *et al.*, 1985). The variation between the M_rs observed by Thornton & Kirby and Muldbjerg *et al.* may be due to slight variations in isolation conditions that result in differently shaped molecules or different running conditions when doing the gel

electrophoresis (Thornton, & Kirby, 1987, report an observed M_r of 150,000 Da for BFXIIaINH under some running conditions of their SDS-PAGE experiments). Carbohydrate content, estimated from deglycosylation studies, is between 18% and 20% (Thornton, & Kirby, 1987,88; Muldbjerg, *et al.*, 1993).

The calculated molecular weight of the 445 amino acid sequence predicted from the cDNA sequence is 49,217. This is only 82 % of the estimated deglycosylated molecular weight of the protein observed by Muldbjerg *et al.* As in the case of C1INH the difference between observed and calculated M_r suggests a much higher carbohydrate content in this case between 34% and 42% for Thornton & Kirby's or Muldbjerg *et al*'s observed M_rs respectively. This apparent low molecular weight as estimated from the amino acid sequence translated from the cDNA may be due to incomplete deglycosylation of the protein isolated by Thornton & Kirby and Muldbjerg *et al.* There may also be other modifications to the protein *in vivo* that add to its apparent molecular weight as estimated by gel electrophoresis. The difference in calculated M_r and observed deglycosylated M_r are comparable with that observed for the human C1INH molecule (Odermatt, *et al.*, 1981; Schmaier, *et al.*, 1985).

b. Sequence identity with C1INH and other serpins. Bovine factor XIIa inhibitor has been previously assigned to the serpin gene family on the basis of its mechanistic properties and its sequence homology with other family members (Thornton, & Kirby, 1987, 1988; Muldbjerg, *et al.*, 1993). This was previously based on functional assays and only partial amino acid sequence data. The complete sequence of the cDNA finally allows the unambiguous assignment of BFXIIaINH to the serpin gene family. Figure 18 shows the identity of BFXIIaINH with other serpins is not limited to the human C1 inhibitor. The serpins shown in figure 18 exhibit identity that extends past the region that interacts directly with the target protease during complex formation and covers over 80% of the polypeptide chain between ≈ residue 120 and the C-terminal. This extensive homology

shows that these genes are the product of divergent evolution from an ancestral gene. Extensive amino acid sequence identity within a protein family often indicates an equal or greater tertiary structural identity (Birktoft, & Blow, 1972; Sawyer, *et al.*, 1978; James, *et al.*, 1978; Marquart, *et al.*, 1983). At present, the only serpin tertiary structure that has been solved is that of α1-antitrypsin (Loebermann, *et al.*, 1984). Examination of the tertiary structure and the amino acid sequence of α1-antitrypsin allows the assignment of discrete stretches of amino acid sequence to certain structural motifs in the protein. This analysis shows that the non-conserved sequences between serpins correspond almost entirely to surface areas of the molecule (Bock, *et al.*, 1986). Because of the high degree of identity between BFXIIaINH and C1INH and the rest of the serpins (see figure 18) for which there is sequence data this same pattern can be expected to exist in BFXIIaINH as well. The reactive site residues are also exposed on the surface of the molecule and can be expected then, along with other surface residues to contribute to the target specificity of the inhibitor. BFXIIaINH is the homologous protein to C1INH, therefore the things that apply to C1INH with regard to homology to serpins apply also to BFXIIaINH.

c. cDNA length and N-terminal differences between BFXIIaINH and C1INH.

The human cDNA for the C1INH protein is 1801 bp long and contains a 1500 bp ORF. This translates into a 500 amino acid polypeptide. There is a 22 amino acid leader sequence that when subtracted from the total polypeptide length yields a circulating polypeptide of 478 amino acids. The cDNA of the homologous bovine protein BFXIIaINH is 1740 bp long with an ORF of 1404 bp. This translates into a polypeptide 468 amino acids long including a leader sequence of 23 amino acids. Removal of the leader sequence leaves a 445 amino acid polypeptide. There is a difference of 33 amino acids between the two polypeptides. These extra residues occur as two insertions or amplifications with regard to the bovine protein. The first lies between residues 72 and 73 of the bovine polypeptide and is 27 amino acids long; the second lies between residues 361

and 362 and is six amino acids long. The 27 amino acid insertion comprises the bulk of a region in the human protein that is associated with O-linked glycosylation (Bock, *et al.*, 1986). In the human protein this region consists of nine tetra-peptide repeats, seven of which conform to the consensus Glx-Pro-Thr-Thr. These repeats are thought to be sites for O-linked glycosylation (Bock, *et al.*, 1986). The six amino acid insertion occurs at the very 3′ end of exon seven of the human gene. There are not enough data at this time to interpret the significance of the six amino acid insertion. Carbohydrate plays no role in the inhibitor activity of the C1INH (Reboul, *et al.*, 1987) which is confined to the C-terminal "serpin" region. The majority of the glycosylation sites occur in the N-terminal regions of the proteins. The region of least identity among the serpins is the N-terminal 120 amino acids (Bock, *et al.*, 1986). The N-terminal region when mapped to the tertiary structure of α1-antitrypsin occupies an area on the surface of the molecule. The difference in cDNA lengths is mostly accounted for by the difference in lengths of the N-terminal regions. Therefore it is reasonable to speculate that the difference in lengths results in different patterns and amounts of glycosylation in the two polypeptides. Carbohydrate is thought to be involved in mediating protein-protein interactions. Therefore the difference in N-terminal lengths may influence the target specificity of the inhibitors *via* the influence of the carbohydrate content.

It has been previously reported that the N-terminal of BFXIIaINH shows no identity with the human C1 inhibitor (Muldbjerg, *et al.*, 1993). That finding has been confirmed by the cDNA sequence of BFXIIaINH. The N-terminal region of the BFXIIaINH molecule, the first 99 amino acid residues, shows only 17% identity with the human C1-inhibitor. The bovine protein N-terminal amino acid sequence showed no homology with any other protein sequences in the databases. Muldbjerg *et al*, purified BFXIIaINH using Thornton & Kirby's method and using a modified version of Thornton & Kirby's method that they developed. The N-terminal amino acid sequences of the

proteins prepared using these two methods were identical (Muldbjerg, *et al*,, 1993). In addition these N-terminal sequences were identical to the published ten N-terminal amino acid residues of human C1-inhibitor purified from bovine plasma (Van Nostrand, & Cunningham, 1987). All of these sequences were identical with the amino acid sequence derived from the BFXIIaINH cDNA. This indicates that these inhibitors may be identical. The fact that there is no identity between the human C1-inhibitor and the BFXIIaINH is not unexpected as there appears to be little or no identity between any of the serpin in this region. Comparison of serpin amino acid sequences reveals that the N-terminal ends of serpins are dissimilar in both length and sequence and thus may represent separate domains evolved from different protein families (Bock, *et al.*, 1986). The differences between the different serpin proteins is not unexpected given their different target specificities.

BFXIIaINH and C1-inhibitor are the homologous proteins from their respective species from this point of view the differences between them are greater than expected. There are two differences between the human and bovine proteins in the N-terminal region. First the sequences are non-identical, second the lengths are significantly different: there is an insertion in the human sequence with respect to the bovine protein, or a deletion with respect to the human protein. One of three possibilities is that the human N-terminal extension has no counterpart in the bovine molecule. This difference may reflect different protein functions in their respective species for the two molecules.

The second explanation is that the N-terminal regions of the two molecules are unrelated. As suggested by Bock *et al*, the N-terminal regions may represent separate domains evolved from different protein families. As such this would mean that the alignment of the two (or more) N-terminal regions does not reflect true relatedness. Therefore the homology between the human and bovine (and possible all serpins) molecules, at the level of amino acid sequence, may start at the point where all members

of the serpin family become homologous.

The third explanation is that the extra 27 amino acid residues found in the human inhibitor with respect to the bovine inhibitor are the product of a deletion event in the bovine species or an amplification event in the human species. Different structures may have different functions associated with them and these structures may occur in discrete domains and be encoded in discrete regions of DNA, perhaps even on separate exons. These functional motifs may be shuffled or exchanged to give rise to new proteins with altered function from the original molecule. This might be the case in the difference between the human and bovine proteins. The PCR amplification experiments were conducted to test this possibility. The results show that this is not the case as the entire N-terminal region is coded for by a single exon. The nucleotide and amino acid sequences of the bovine exon show sufficient identity at the 3′ and 5′ ends with the human exon to rule out the possibility of the extra sequence present in the human protein being encoded on an extra exon. The bulk of the extra region in the human protein is comprised of a series of tetra-peptide repeats. Deletions and insertions can result from unequal crossing over during homologous recombination between repeat elements oriented in the same direction (Lehrman, *et al.*, 1986). The human C1 inhibitor at the DNA level in the N-terminal region has a series of 12 bp repeats. The nucleotide sequence of the gene for human C1 inhibitor has revealed the presence of an unusually high number of *Alu* repeats (Carter, *et al.*, 1991). These repeats are oriented in both directions and are thought to be the basis of some of the observed genetic lesions that give rise to clinical complications resulting from abnormally low levels of C1 inhibitor. The molecular defects are thought to be based on the above mentioned mechanism of unequal crossing over during homologous recombination based in these regions of high density of *Alu* repeats. This however could not be a factor in the difference between the human and bovine molecules because the *Alu* repeats are present in the introns and the resulting rearrangements involve the deletion or

insertion of entire exons, not the middle section of a single exon. Over all I think the evidence favours the first possibility. Even if the two N-terminal regions were the same length they still show little or no identity. While it may be likely that different molecules acquired separate N-terminals as discrete domains that evolved from different protein families, it is less likely that this occurred in homologous proteins of different species. Furthermore none of the serpins show any identity with each other in the N-terminal region.

   d. Comparison of the intron/exon junctions of the 5' region of the BFXIIaINH gene and the human C1INH gene. There is no identity between the serpins so far examined in the level of genomic organization. The genomic organization of the human C1 inhibitor gene has been elucidated (Carter, *et al.,* 1988, 1991). This was used as the basis of comparison for the BFXIIaINH cDNA. The reason for this examination is the difference observed between the human and bovine proteins in the N-terminal region. The intron exon junctions in the N-terminal region of the bovine polypeptide were mapped. PCR amplifications were done on bovine genomic DNA within the exon that contains the difference and between the exons that would flank the difference. The PCR products from these experiments were of the predicted sizes based on the human genomic organization. These PCR products were cloned, sequenced, and mapped with restriction endonucleases. The intra exon amplification product was mapped with restriction endonucleases that would cut the known bovine sequence (from the cDNA sequence) but not cut human sequences and shown to be contiguous and not contain any new introns. The inter exon amplification product had the ends sequenced and were shown to contain ≈40 bp of the expected exon sequences and ≈200 bp of the intron sequences. The consensus GT/AG intron start and end sequences are present and the intron sequence did not show any sequence identity with either the human intron sequence or any other sequence in the databases. Comparison of the bovine genomic sequence data with the human genomic

data show that the location of the junctions was the same as in the human protein. This is further evidence that the N-terminal region differences are a result of divergent evolution.

The origin of the difference (did human gene gain a piece or the bovine gene lose a piece) is open to speculation. It is known that repeats of sequence homology can be the basis for either insertions or deletions of homologous sequences at that point *via* unequal crossing over during recombination (Lehrman, *et al.*, 1986). In this region of the bovine cDNA sequence there are no repeats to build on. The human sequence exhibits limited repeating sequences of this sort in the region of the tetra-peptide repeats Glx-Pro-Thr-Thr. It is impossible to tell if these 9 repeat sequences grew by some mechanism of duplication out of an ancestral originator gene. The entire 81 bp region may have been deleted, based on the sequence homology of these repeats, or added to give rise to the bovine gene or human gene respectively. It is known that the entire region containing the region of difference between the two genes is encoded by a single exon so the mechanism involved is not one of simple exon shuffling. Bovine and human factor XIIa have different characteristics *in vivo* and they also have different primary structures. The inhibitors of bovine and human factor XIIa proteins act upon these respective proteins and in addition have a limited range of other substrates. The range of these substrates is not the same for each inhibitor. The differences in the proteases and their respective inhibitors may be used to assign certain functions or activities to discrete structural motifs or domains.

Both human and bovine inhibitors inhibit human kallikrein, neither inhibits bovine kallikrein, therefore a look at the human and bovine kallekreins may give more information as to the structure/function relationships in these molecules.

B. Clinical implications of the BFXIIaINH/C1INH inhibitors.

Gram-negative bacteremia causes disseminated intravascular coagulation (DIC) and hypovolemia resulting in septic shock and sometimes death from circulatory collapse

in humans and in a lethal baboon model. Pixley, R., *et al*, have shown the contact activation system *via* the activity of factor XIIa contributes to the hypotensive effects but not the DIC. They have shown that, in the baboon model, using an antifactor XII monoclonal antibody (mAb) to block the activation of factor XII they can stop activation of the contact system and prevent the hypovolemia and circulatory collapse due to vasodilation. The blockage of factor XII activation results in the blockage of activation by factor XIIa of factor XI and kallikrein. However the contact system is still activated as evidenced by the intravascular coagulation. This indicates that there may be other activators of the coagulation system and or the contact system *in vivo* in baboons. This may be by the direct activation of factor XI by thrombin or some other unidentified FXI activator. The evidence for this *in vivo* is still unclear (Walsh, 1987; Kitchens, 1991; Gailani, & Broze, 1991; Naito, & Fujikawa, 1991; Brunnée, *et al.*, 1993).

Pixley, *et al.*, have shown that administration of antifactor XII mAb can prevent death from circulatory collapse in the baboon model. There may be similar applications for the administration of FXIIaINH/C1INH. Exogenous supplies of the inhibitor might prevent depletion of the endogenous stores. It might be possible to administer it in combination with the antifactor XII mAb.

While the picture of the explicit roles of these inhibitors and their target proteases is still unclear, the approaches used in this study can be used to learn more about their respective functions. By studying homologous molecules from different species we are in effect observing the results of an experiment in structure function analysis that has been carried out for us.

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