THE ROLE OF HISTIDINE-RICH GLYCOPROTEIN IN COAGULATION & FIBRINOLYSIS

THE ROLE OF HISTIDINE-RICH GLYCOPROTEIN IN COAGULATION & FIBRINOLYSIS

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Masters of Science

McMaster University

MASTERS OF SCIENCE (2007)
(Biochemistry and Biomedical Sciences)

McMaster University
Hamilton, ON

TITLE: The Role of Histidine-rich Glycoprotein in Coagulation & Fibrinolysis

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NO. OF PAGES: xiii, 116

ABSTRACT

The fibrinolytic system has an important role in maintaining vascular patency by restricting fibrin clot formation to prevent occlusion of the blood vessel. Plasminogen activation is the central event in fibrinolysis and is tightly regulated by activators and inhibitors. Histidine-rich glycoprotein (HRG) is an abundant plasma protein that has been proposed to have a regulatory role in many biological processes, including fibrinolysis. Approximately 50% of plasminogen in the blood circulates in complex with HRG. Conflicting reports dispute the role of HRG in fibrinolysis, specifically whether it promotes or inhibits plasminogen activation. To elucidate the role of HRG in fibrinolysis, we isolated HRG from human plasma and analyzed its effect on plasminogen activation by tissue-type plasminogen activator in a kinetic assay. HRG had no significant effect on plasminogen activation by tissue-type plasminogen activator once contaminating plasminogen was eliminated from our HRG preparations. Based on these results, the focus of our research was redirected to analyzing the effect of HRG on additional plasminogen activators, namely urinary-type plasminogen activator and factor (F) XIIa. HRG inhibited plasminogen activation by both activators. HRG had the greatest inhibitory effect on FXIIa activity. This novel finding led us to explore the relationship between HRG and FXIIa by measuring the affinity of HRG for FXIIa by surface plasmon resonance, and by analyzing the effect of HRG on FXIIa activity in various contact pathway reactions. $ZnCl_2$ was also included in these reactions because it plays an important role in enhancing both HRG- and FXII-mediated interactions and is released by activated platelets. In the presence of 12.5 μM $ZnCl_2$, FXIIa bound to the histidine-rich region of HRG with very high affinity (K_d = 56 ± 8.9 pM). Interestingly,

HRG does not bind to FXII. Functional analysis of HRG revealed that it significantly inhibits a number of contact pathway reactions, including FXII autoactivation, kallikrein-mediated FXII activation, and FXIIa-mediated FXI activation. Conversely, HRG enhanced FXIIa-mediated prekallikrein activation. Based on these findings, we hypothesize that HRG binds to an exosite on FXIIa, which is not expressed by the zymogen FXII, and alters FXIIa activity. The mechanism of HRG-mediated FXIIa inhibition is not fully understood and needs to be further analyzed by both binding and functional assays. These observations raise the possibility that the main function of HRG is to modulate FXIIa activity, rather than plasminogen activation. Because of its abundance, HRG may function as a modulator of haemostasis through its effect on coagulation and fibrinolysis.

ACKNOWLEDGEMENTS

I would like to thank the people who were committed to helping me succeed in completing my thesis. These people largely contributed to my success as a graduate student.

To Dr. Jeffrey Weitz, my supervisor, for providing me with this opportunity that has allowed me to grow as a student and develop confidence in my work and myself. You have also provided me much support and guidance in all aspects of my graduate work, and for that I am greatly thankful.

To the members of my supervisor committee, Dr. Patricia Liaw and Dr. Geoff Werstuck, for their academic guidance.

To the entire Weitz laboratory for their ongoing support. To Alan Stafford, Beverly Leslie and Colin Kretz for their valuable assistance and advice in the laboratory. To Dr. Jim Fredenburgh for his insightful knowledge, helpful discussions, and who has provided many hours of editing written work and offering insightful comments. I am greatly thankful.

To my parents for their consistent encouragement, love and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	Absorbance
BK	Bradykinin
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAPS	3-cyclohexylamino-1-propane-sulfonic acid
COOH	Carboxy terminus
CTI	Corn trypsin inhibitor
Da	Daltons
Є	Extinction coefficient
EACA	ε-amino-n-caproic acid
EC_{50}	Half maximal effective concentration
EDTA	Ethylene diaminetetra acetic acid
Fg	Fibrinogen
FI	Fluorescence Intensity
F	Factor
a	Activated factor
g	Gravitational force
Glu-Pg	NH_2-terminal glutamic acid plasminogen
HBS-Tw20	10 mM Hepes-OH, 150 mM $NaCl_2$ pH 7,4, containing 0.005% Tween20
HK	High molecular weight kininogen
HRG	Histidine-rich glycoprotein
HRR	Histidine-rich region

IC_{50}	Half maximal inhibition concentration
IOD	Integrated optical density
K_d	Dissociation constant
Lys-Pg	NH_2-terminal lysine plasminogen
min	Minutes
MW	Molecular weight
NH_2	Amino terminus
Ni	Nickel
NO	Nitric oxide
OD	Optical density
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PFR-AMC	Pro-Phe-Arg-4-methylcoumrin
PGI_2	Prostacyclin
Pg	Plasminogen
PK	Prekallikrein
PKa	Kallikrein
Pm	Plasmin
PRR	Proline-rich region
PVDF	Polyvinylidene difluoride
RU	Response units
sc	Single-chain

SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SPR	Surface plasmon resonance
TAFI	Thrombin activatable fibrinolysis inhibitor
TBS	20 mM Tris-HCl, 150 mM NaCl pH 7.4
TFPI	Tissue factor pathway inhibitor
tc	Two-chain
tPA	Tissue-type plasminogen activator
U	Units
uPA	urinary-type plasminogen activator
VFKck	D-Val-Phe-Lys chloromethyl ketone
Xase	Tenase complex
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength

1 INTRODUCTION

1.1 Haemostasis

The haemostatic system is an essential physiological process that is exquisitely regulated to maintain blood fluidity and vascular integrity. Haemostasis reflects a continuum of interrelated responses of the vasculature, circulating platelets, coagulation proteins and the fibrinolytic systems. Under normal conditions, blood coagulation proteins and cells involved in hemostasis circulate in the blood in inactive forms. When damage occurs at the endothelial layer, there is exposure of the subendothelial matrix. Disruption of the endothelium exposes circulating platelets to collagen and plasma factor VII to extravascular tissue factor. Tissue factor can also be expressed upon activation of the endothelium by chemicals, cytokines or inflammatory processes (Weiss *et al.*, 1989). Once tissue factor is exposed to the blood, zymogens of the coagulation pathway are converted sequentially to enzymes and thrombin is rapidly formed, initiating fibrin formation. These events are accompanied by the exposure of phosphotidylserine on the membrane surface of activated endothelium, which provides a surface for coagulation protein assembly and activation. Activated platelets also play an important role in the amplification of the coagulation cascade by providing a thrombogenic surface. The burst in thrombin generation leads to the rapid conversion of fibrinogen (Fg) to fibrin and its polymerization into a fibrin clot. Various coagulation factors as well as other components involved in wound healing initiate the conversion of plasminogen (Pg) to plasmin, which then proceeds to degrade the fibrin clot in a timely manner. This system is precisely regulated, such that the fibrinolytic system is closely linked to coagulation,

which ensures that the processes of clot formation and dissolution are balanced to maintain a normal haemostatic response (Mosnier and Bouma, 2006, Schmaier, 1998).

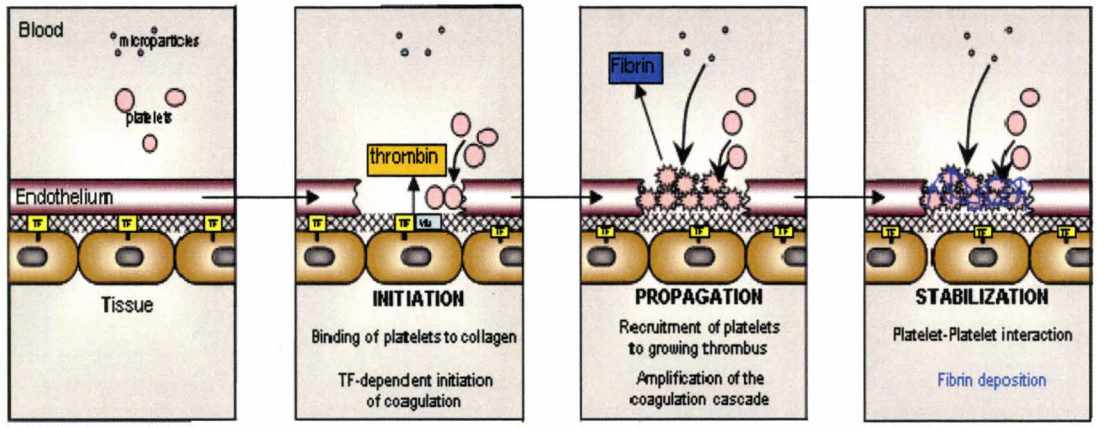
1.2 Coagulation

Coagulation is an important part of haemostasis and has been traditionally divided into intrinsic and extrinsic pathways, which were initially believed to represent unique physiological mechanisms for the initiation of normal coagulation. Each pathway involves the sequential activation of clotting proteins and converge at a "final common pathway" with the activation of Factor X (FX) (Colman *et al.*, 2001). Over the years, it became evident that such a division does not occur *in vivo* and this has prompted a revision of the coagulation model that depicts clotting as a multi-phase event, involving initiation, amplification and propagation of fibrin clot formation.

1.2.1 *Revised Coagulation Model*

The classical extrinsic pathway is believed to be the principal initiating mechanism of coagulation *in vivo*. This pathway involves components that are found in blood and in the extravascular space. The principal initiating factor of this pathway is tissue factor (TF), which is an integral membrane glycoprotein expressed by specific cells found in the subendothelium that become exposed to the blood following vascular injury (Morrissey *et al.*, 1987, Weiss *et al.*, 1989). TF functions as a cofactor by forming a stoichiometric complex with FVII on a phospholipid surface in the presence of calcium. TF facilitates the autolytic conversion of FVII to FVIIa. This requires a small amount of

pre-existing factor VIIa (Davie *et al.*, 1991, Fair and MacDonald, 1987), which comprises less than 1% of total FVII in plasma (Karalapillai and Popham, 2007). The newly formed FVIIa/TF complex (extrinsic factor Xase) activates the zymogens, factor IX and factor X (Komiyama *et al.*, 1990, Silverberg *et al.*, 1977). FVIIa/TF converts FX to FXa by the cleavage of a single peptide bond in the heavy chain of FX (Di Scipio *et al.*, 1977). Inhibition of this pathway by tissue factor pathway inhibitor (TFPI), an inhibitor of the FVIIa/TF complex, results in extremely low levels of FXa generation during the initiation phase of coagulation (Baugh *et al.*, 1998, Rapaport, 1991). TFPI can also inhibit the FVIIa/TF complex in a FXa-dependent process. TFPI binds and inhibits FXa and subsequently binds to the FVIIa/TF complex to prevent further FX activation (Broze, Jr. *et al.*, 1988). Low levels of FXa and the absence of its cofactor FVa prevent measurable fibrin clot formation. The initiation phase of coagulation is defined by the generation of small amounts of thrombin from its precursor prothrombin (Mann *et al.*, 2003) (Figure 1). In this phase, only trace amounts of thrombin are generated by FXa. However, there is sufficient thrombin to activate platelets and the cofactors FV and FVIII (Butenas *et al.*, 1997, Lawson *et al.*, 1994). Activated FIX, generated by the FVIIa/TF complex, and its cofactor FVIIIa form the intrinsic tenase complex on a phospholipid surface provided by platelets, microparticles, or endothelial cells (Rosing *et al.*, 1985, Stern *et al.*, 1985, Tans *et al.*, 1991). This complex activates FX at a rate approximately 50-100-fold higher than TF-FVIIa (Ahmad *et al.*, 1992, Mann *et al.*, 1992).



Modified from: Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007; 27: 1687.

Figure 1. Formation of a fibrin clot at the site of vessel injury. When the endothelial layer is intact, tissue factor (TF) expressed by smooth muscle cells and fibroblasts in the extravascular space is not exposed to blood. When damage occurs to the endothelium, collagen in the vessel wall and tissue factor become exposed to circulating vascular components. During the initiation phase of coagulation, platelets bind to collagen and VIIa/TF complexes are formed, which initiates the coagulation cascade and the generation of thrombin. During the propagation phase, there is the recruitment of additional platelets to the site of injury and amplification of thrombin generation. Thrombin converts Fg to fibrin, which becomes incorporated into the growing platelet plug to form a stable thrombus.

The VIIa/TF complex also activates small amounts of FIX and FXI (Butenas *et al.*, 1997, Lawson *et al.*, 1994). During the early stages of coagulation, FIXa and FXIa generated by FVIIa/TF are insufficient to sustain coagulation (Asakai *et al.*, 1991, Gailani and Broze, 2001). Feedback activation of FXI by thrombin provides sufficient FXIa to drive FIX activation by FXIa (Oliver *et al.*, 1999). Thrombin also activates the FIXa cofactor, FVIIIa, that together with FIXa forms the intrinsic Xase complex (Butenas *et al.*, 1997, Lawson *et al.*, 1994). This complex amplifies FXa generation, which generates sufficient FXa to drive substantial thrombin formation and maintain clot formation during the propagation phase of coagulation (Ofosu *et al.*, 1996).

FXa generation by the extrinsic or intrinsic Xase complex represents the classic common pathway, which forms the "prothrombinase complex" on the membrane surface together with its cofactor FVa. This complex is responsible for converting prothrombin to thrombin (Nesheim *et al.*, 1979). Thrombin further amplifies its own generation by activating FXI (Oliver *et al.*, 1999) and with the additional activation of platelets, FV and FVIII (Butenas *et al.*, 1997, Pieters *et al.*, 1989). During the final stages of blood coagulation, thrombin cleaves Fg to produce fibrin monomers that spontaneously polymerize to form a insoluble fibrin network (Bailey *et al.*, 1951, Mosesson, 1992). Thrombin also converts FXIII to FXIIIa, which introduces covalent cross-links between the fibrin monomers to further stabilize the clot (Muszbek *et al.*, 1996).

1.3 The Contact System and Intrinsic Coagulation

The plasma contact system mediates the activation of the classic intrinsic coagulation pathway through activation of the plasma protease factor XII, followed by sequential activation of FXI and FIX. The original name "intrinsic" came from the mistaken perception that coagulation of freshly let blood occurred without the addition of any external factors (Kitchens, 2005). It is now understood that an external factor is involved in the initiation of coagulation. Activation of the intrinsic pathway occurs following contact of plasma with artificial surfaces, such as metal or glass (Lister, 1863). It has been established that when blood comes into contact with negatively charged surfaces, a series of proteolytic reactions are initiated that result in the activation of the plasma proteins- FXII, FXI, prekallikrein (PK) and the cleavage of high molecular weight kininogen (HK) (Cochrane and Griffin, 1982, Colman and Schmaier, 1997, Kaplan *et al.*, 1997, Kaplan and Silverberg, 1987). Collectively, these events are known as the contact activation system and are thought to activate intrinsic coagulation, as well as several other host defence systems, which include fibrinolysis, inflammation, complement and angiogenesis (Colman, 2001) (Figure 2).

1.3.1 *Components of the Contact System*

Factor XII, or Hageman factor, is synthesized in the liver as an 80 kDa single-chain glycoprotein that circulates as an inactive zymogen at a physiological concentration of approximately 300-400 nM (Fuhrer *et al.*, 1990, Tans and Rosing, 1987). FXII is composed of 6 structural domains that make up the heavy and light chain of the molecule.

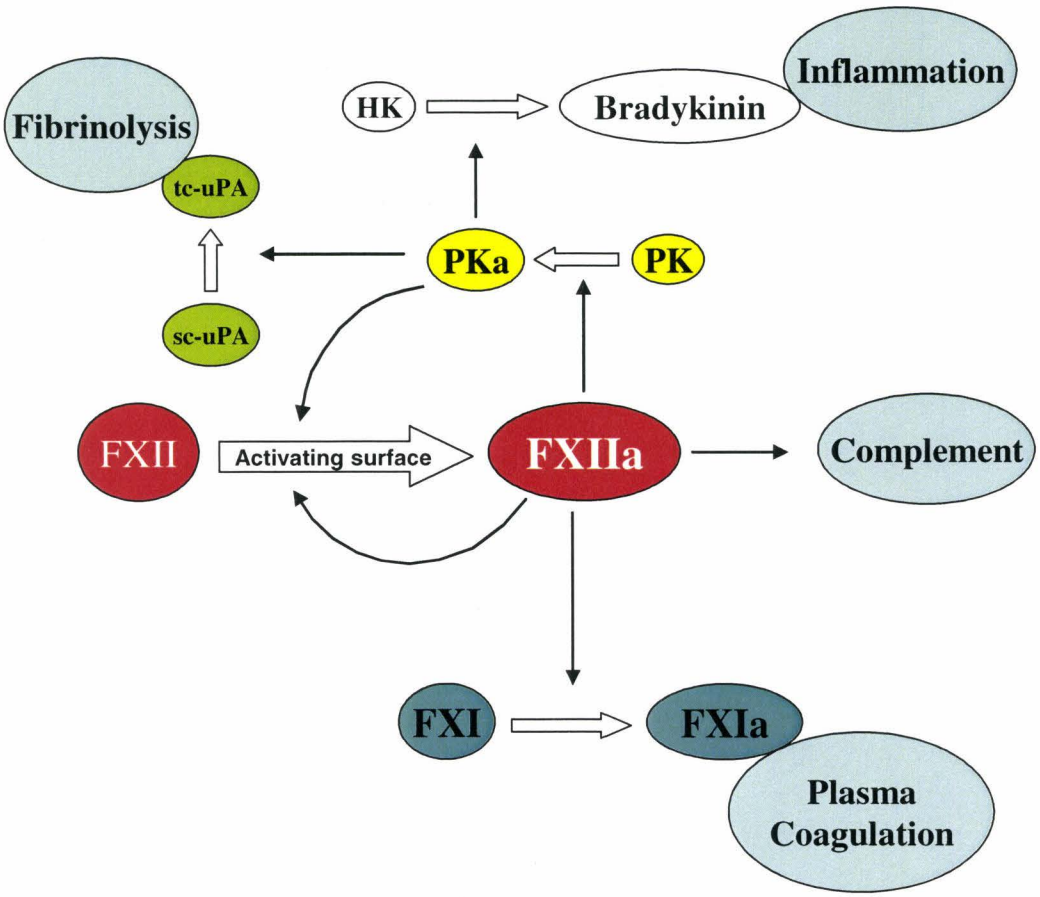


Figure 2. Contact activation reactions. When plasma is exposed to an exogenous negatively charged surface, contact activation is initiated through FXII-mediated auto-activation. FXIIa activates prekallikrein to kallikrein, which reciprocally activates FXII to amplify contact activation. FXIIa indirectly initiates fibrinolytic activation though kallikrein cleavage of sc-uPA to the active two-chain form (tc-uPA). Kallikrein also cleaves HK to liberate bradykinin, which participates in inflammatory responses. FXIIa activates C1 of the complement pathway, as well as initiates coagulation by converting FXI to FXIa on a negatively charged surface.

The heavy chain consists of the fibronectin type I & type II domains, two growth factor domains and a kringle domain, whereas the light chain contains the catalytic domain (Bhoola *et al.*, 1992, Colman and Scott, 1996, Fujikawa and McMullen, 1983, McMullen and Fujikawa, 1985, Schmaier *et al.*, 1987). FXII binding to negatively charged surfaces is mediated by the fibronectin type II domain (Citarella *et al.*, 2000). The functions of other heavy chain domains are not fully known and can only be inferred from the function of homologous domains of other serine proteases. Based on homology with similar domains of plasminogen and tPA (McMullen and Fujikawa, 1985, Tans and Rosing, 1987), it is likely that the kringle domain of FXII mediates interactions with proteins containing lysine residues.

Activation of FXII, the initial step in contact activation, occurs when FXII encounters a negatively charged surface and undergoes autolytic activation. Initially, exogenous surfaces such as glass, kaolin, dextran sulfate, and ellagic acid were identified as contact activators (Cochrane *et al.*, 1973). More recently, biological components have been attributed with triggering FXII activation, including negatively charged glycosaminoglycans (GAGS), some types of collagens, endotoxins, sulfatides, nucleic acids and polyphosphates (Cochrane and Griffin, 1982, Herwald *et al.*, 1998b, Hojima *et al.*, 1984, Smith *et al.*, 2006). FXII binds to these polyanionic compounds in the presence of Zn^{2+} via the NH_2-terminal region of its heavy chain (Citarella *et al.*, 1996). This binding induces a conformational change in the zymogen, which allows activated FXII (FXIIa) to cleave the zymogen at a single bond between Arg353-Val354, which produces αFXIIa by exposing the catalytic site of the serine protease (Fujikawa and

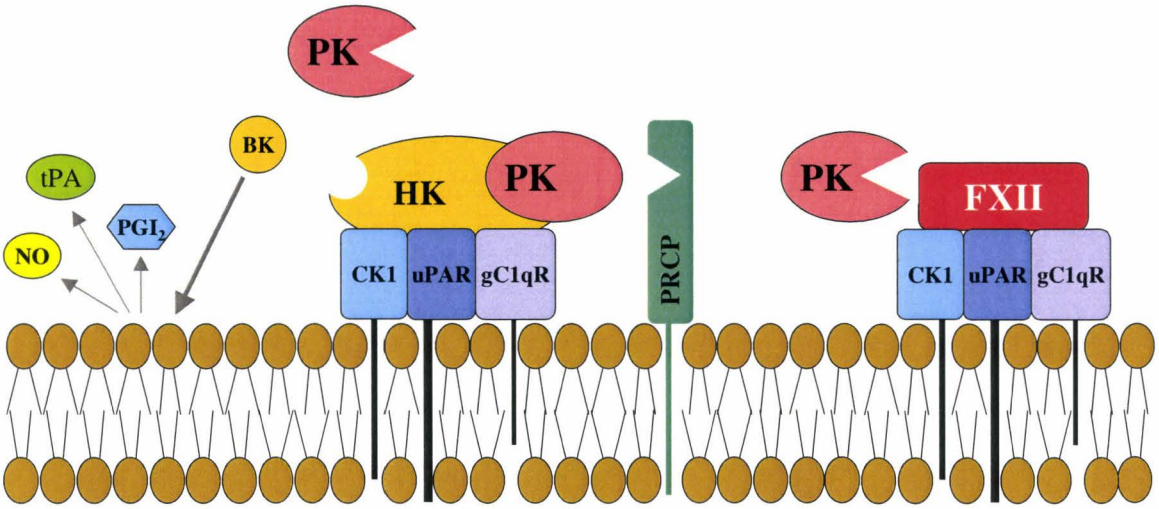
Davie, 1981). However, there is still the question as to the origin of the initial FXIIa (Citarella *et al.*, 1997). Small amounts of FXIIa may be present in plasma due to on going, but basal activation of coagulation factors. Alternatively, FXII may undergo random autolytic cleavage that generates the small amount of FXIIa required to drive FXII activation. Regardless of the mechanism of FXII autoactivation, this event results in the generation of small amounts of αFXIIa, in which the heavy and light chains are linked by a disulfide bound (Fujikawa and Davie, 1981).

Other components of the contact system include PK and HK. PK circulates in complex with HK and binds to negatively charged surfaces, where it is cleaved by FXIIa to generate kallikrein (PKa). PKa in turn activates additional surface-bound FXII, giving rise to the two active forms, αFXIIa and βFXIIa. The βFXIIa form is generated after two additional peptide bond cleavages between Arg334-Asn335 and Arg343-Lue344 of the αFXIIa form. βFXIIa contains the light chain and only a small fragment of the heavy chain (DeLa Cadena *et al.*, 1994, Dunn and Kaplan, 1982, Tans and Rosing, 1987) and lacks surface binding properties. βFXIIa is a more potent soluble-phase activator of PK (Revak *et al.*, 1978, Tans and Rosing, 1987), Pg (Goldsmith, Jr. *et al.*, 1978) and C1 of the complement pathway (Revak *et al.*, 1978). However, βFXIIa exhibits minimal clot-promoting activity (Colman *et al.*, 2001, Revak *et al.*, 1978).

Positive feedback by FXIIa in FXII autoactivation generates sufficient FXIIa to overcome inhibition by its natural inhibitor, C1 inhibitor (Forbes *et al.*, 1970, Pixley *et al.*, 1985). This enables FXIIa to initiate FXI activation, and the subsequent activation of FIX by FXIa. FXI is synthesized by the liver and circulates in plasma as an inactive

dimer at a concentration of approximately 30 nM (Busby *et al.*, 1985). Like PK, FXI also circulates in plasma in complex with HK. The FXI/HK complex can bind to negatively charged surface adjacent to surface bound FXIIa. αFXIIa is the more potent activator of FXI and hydrolyses the Arg369-Ile370 bond to generate FXIa (Shearer, 1995, Yarovaya *et al.*, 2002). The final stages of the intrinsic coagulation pathway conclude with the activation of FIX by FXIa, which takes place on a phospholipid surface and requires calcium. FIXa then assembles into the intrinsic tenase complex, which leads to propagation of coagulation.

A second model of surface-independent contact system activation has been proposed (Figure 3). This takes place on endothelial cells and is believed to be independent of FXII autoactivation. In this model, FXII and PK/HK assemble on the endothelium through interactions with heparan and chondroitin sulfate proteoglycans (Reddigari *et al.*, 1993, Renne *et al.*, 2005). A multiprotein receptor complex found on the surface of endothelial cells containing urokinase receptor, cytokeratin-1 and gC1qR/p33 maybe also be a docking site for FXII and PK/HK (Herwald *et al.*, 1996, Joseph *et al.*, 1996, Mahdi *et al.*, 2002, Schmaier *et al.*, 1999). The binding of contact proteins to the multireceptor complex is Zn^{2+}-dependent and FXIIa binding requires a 30-fold higher free Zn^{2+} concentration than HK (Mahdi *et al.*, 2002). Hence, this series of events most likely occurs during vessel injury where local Zn^{2+} concentrations are elevated due to its release from activated platelets. PK/HK is rapidly converted to PKa on the endothelial cell surface by the enzymes Hsp90 (Joseph *et al.*, 2002) and prolylcarboxypeptidase (Shariat-Madar *et al.*, 2004), which are constitutively active on



Modified from: Schmaier, AH. The plasma kallikrein-kinin system counterbalances the renin-antiotensin system. J. Clin. Invest. 2002; 109: 1007-1009.

Figure 3. Assembly and activation of contact proteins on endothelial cells. PK circulates in complex with HK. In the presence of Zn^{2+}, the PK/HK complex binds to a multi-receptor complex localized to the surface of endothelial cells. The multi-receptor complex consists of cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR) and gC1qR proteins. PK, localized at the endothelial cell surface, is converted to kallikrein (PKa) by prolylcarboxypeptidase (PRCP), which is a membrane protein constitutively active on the endothelial surface. HK is digested by the newly formed PKa, which generates bradykinin. Consequently, bradykinin stimulates the release of tPA, nitric oxide (NO) and prostacyclin (PGI_2) from endothelial cells, which has antithrombotic and inflammatory effects. In this revised model of contact activation, FXII is activated subsequent to PKa. FXII also binds to the multi-complex receptor, whereby it is activated by PKa.

endothelial cell membranes. Once PKa is activated, it can autodigest its cofactor, HK, to liberate bradykinin (BK). Consequently, activation of FXII occurs subsequent to PK activation. In the absence of HK, FXII can bind to the same multiprotein receptor complex on the endothelium, where it is activated by PKa (Schmaier, 2000). This alternative model of contact pathway activation identifies a biologically relevant surface for contact protein assembly and activation, where PK activation is FXIIa-independent and is independent of an artificial activating surface.

Over the past 50 years, the plasma contact proteins have been studied extensively to explore protein-protein interactions, as well as structure-function relationships (Gailani and Renne, 2007). However, the physiological relevance of contact activation and the intrinsic pathway of coagulation remains unclear. A compelling argument can be made that fibrin formation initiated through the contact pathway is insignificant because a physiologic FXII activator has yet to be definitively identified *in vivo*. To strengthen this argument, deficiency of any of the contact proteins (FXII, PK and HK) is not associated with hemorrhagic tendency. Only deficiency of FXI is associated with a mild bleeding disorder (Gailani *et al.*, 1997). This may reflect the fact that thrombin can activate FXI on the surface of platelets in vitro, and is a more potent activator of FXI than FXIIa (Oliver *et al.*, 1999)

The physiological role of FXII has been re-examined based on the results of studies in FXII-deficient mice. Intravital fluorescence microscopy and blood flow measurements in these mice revealed defective formation and stabilization of platelet-rich thrombi after arterial injury (Renne *et al.*, 2006). Contact activation and intrinsic

coagulation appear to play a role in clot formation by maintaining thrombus integrity over time, when clot formation is induced by TF or thrombin in the presence of a fibrinolytic activator (Von dem Borne *et al.*, 1995). In fact, FXI and FXII were found to be involved in the formation of occlusive thrombi in a rodent model by playing a critical role in the three-dimensional propagation of thrombus growth outward from the vessel wall (Wang *et al.*, 2005). Because contact activation and intrinsic coagulation are not essential for arrest of bleeding, yet appear to contribute to pathologic thrombus formation *in vivo*, FXIIa may be an attractive target for new anticoagulants. Inhibitors of FXIIa are likely to have little or no effects on haemostasis, yet should attenuate thrombosis. However, a better understanding of the physiologic and pathologic role of FXIIa in haemostasis is needed to rationalize the development of FXIIa targeted drugs.

1.3.2 *Contact Activation in Inflammation*

Another well known function of the contact system is in regulating blood pressure through the cleavage of HK and the release of BK. HK is a 120 kDa single-chain glycoprotein that circulates in plasma at approximately 670 nM in complex with either FIX or PK (Schmaier *et al.*, 1986). After FXIIa-mediated PK activation, PKa hydrolyses two peptide bonds in the fourth domain of HK, which leads to the production of the inflammatory mediator BK (Yarovaya *et al.*, 2002). BK is a potent vasodilator that activates the bradykinin B_2 receptor on endothelial cells and stimulates the generation of nitric oxide (NO) and prostacyclin (PGI_2), potent vasodilators (Zhao *et al.*, 2001). BK also stimulates the release of tissue-type plasminogen activation (tPA) from endothelial

cells and, together, these components initiate antithrombotic and vasodilating events to increase vascular permeability (D'Orleans-Juste *et al.*, 1989, Schmaier, 2002). This demonstrates the importance of the contact system and its contribution not only to BK formation and coagulation, but also to the modulating other host defence systems, including inflammation (Chien *et al.*, 1988, Schapira *et al.*, 1982, Toossi *et al.*, 1992), complement activation (Ghebrehiwet *et al.*, 1983) and fibrinolysis (Braat *et al.*, 1999). Because of its important role in hemostasis and its link to the contact system, the fibrinolytic system will be described in more detail.

1.4 Fibrinolysis

Fibrinolysis is the process that counteracts and limits clot formation by dissolving the fibrin clot by means of the potent protease, plasmin (Pm). Pm is the major fibrinolytic protease in blood and is generated after conversion of the zymogen, Pg, by plasma- or tissue-activators. The two predominant Pg activators are tPA and urinary-type Pg activator (uPA) (Collen and Lijnen, 2005, Taran, 2005). FXIIa has also been identified as a plasminogen activator *in vitro* (Braat *et al.*, 1999) (Figure 4).

NH_2-terminal glutamic acid plasminogen (Glu-Pg) is synthesized by the liver and circulates in the blood at a concentration of approximately 2.4 μM (Collen and Lijnen, 1991). Glu-Pg has a molecular weight of 88 kDa and is readily converted to Lys-Pg (83 kDa) by Pm hydrolysis of the Lys76-Lys77 peptide bind. Glu-Pg is composed of an activation peptide at its NH_2-terminus, five consecutive kringle domains, and a serine

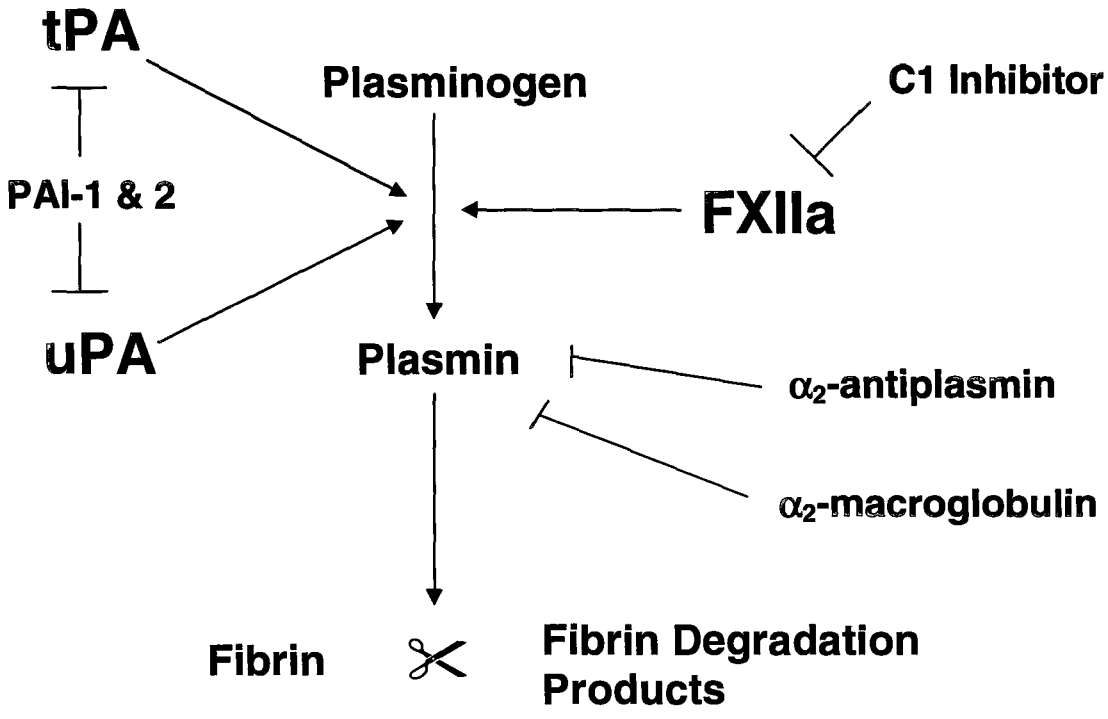


Figure 4. Fibrinolytic activation by various physiological Pg activators. The fibrinolytic system consists of various Pg activators and inhibitors that regulate the conversion of Pg to Pm. Pg can be activated by tPA, uPA, and FXIIa. Once Pm is generated, it can cleave fibrin into fibrin degradation products to initiate clot dissolution. Plasminogen activator inhibitor 1 & 2 (PAI-1 & 2) regulate tPA and uPA in plasma. C1 inhibitor regulates the plasminogen activator (PA) activity of FXIIa. The inhibitors, α_2-antiplasmin or α_2-macroglobulin, inhibit Pm released from the clot to prevent its vascular dissemination.

protease domain. Pg binding to various activators, inhibitors, and other physiological substrates (e.g. fibrin, cell surfaces) is facilitated through the lysine-binding kringle domains (Castellino and Ploplis, 2005, Smith *et al.*, 1984). As a result of binding to lysine residues on fibrin, Pg activation is facilitated (Lucas *et al.*, 1983, Thorsen, 1975).

1.4.1 *Plasminogen Activation*

Conversion of human Pg to Pm requires cleavage at the Arg561-Val562 peptide bond by a Pg activator. The physiological Pg activators, tPA, uPA and FXIIa are synthesized by the liver, endothelial cells (Levin and Loskutoff, 1982) or monocytes/macrophages (Grau and Moroz, 1989). Synthesis of tPA and its release into the circulation is stimulated by thrombin (Kitaguchi *et al.*, 1979, Levin *et al.*, 1984), states of hypoxia and acidosis (Tappy *et al.*, 1984), hormones (Liu *et al.*, 1991, Ny *et al.*, 1987, O'Connell *et al.*, 1987) and mediators of inflammation (Bevilacqua *et al.*, 1986, Minai *et al.*, 2001, Witherow *et al.*, 2003). Urokinase is primarily found in connective tissue and not in the blood. However, endothelial cells can synthesize uPA in response to endotoxin or tumor necrosis factor (Niedbala and Stein-Picarella, 1992). Both tPA and uPA are secreted as single-chain polypeptides. tPA is proteolytically active in its single form, but sc-uPA has little or no activity until it is cleaved by Pm at a single peptide bond to yield its two chain form (Ichinose *et al.*, 1986, Verde *et al.*, 1984). FXII circulates in the blood in zymogen form and becomes activated when it comes in contact with dextran sulfate, and possibly other negatively charged surfaces that trigger activation of the contact system. FXIIa can also indirectly stimulate uPA and tPA activation (Fuhrer *et*

al., 1990, Ichinose *et al.*, 1986). As a regulatory mechanism, circulating Pg activators are rapidly bound by their physiological inhibitors; the principal regulator of tPA and uPA in plasma is type 1 plasminogen activator inhibitor (PAI) (Andreasen *et al.*, 1994, Booth *et al.*, 1987) and FXIIa is inhibited by C1 inhibitor (Davis, III, 2004). These enzyme-inhibitor complexes are then cleared from the circulation by the liver (Andreasen *et al.*, 1994). PAI-1 is released from platelets and endothelial cells during the initial stages of clot formation to permit fibrin formation (Plow and Collen, 1981). However, if released in concentrations that are high enough to overwhelm PAI-1, tPA or uPA rapidly convert Pg to Pm on the surface of the fibrin clot (Lijnen and Collen, 1982). FXIIa has a slightly different mechanism of Pg activation that will be discussed later.

Pm that is generated on the surface of the fibrin clot is initially protected from inhibition by its natural inhibitor, α_2-antiplasmin and exerts positive feedback by converting Glu-Pg to the Lys-Pg form, which brings about an extensive conformational change (Ponting *et al.*, 1992). Glu-Pg has a compact structure that binds weakly to Fg and fibrin. Lys-Pg has a more open structure and demonstrates enhanced affinity for fibrin, compared with Glu-Pg, and is more readily activated by tPA or uPA (Cederholm-Williams, 1977, Thorsen, 1975). Although only a small proportion of circulating Pg is bound to fibrin, it is sufficient to initiate a fibrinolytic response (Alkjaersig *et al.*, 1959). Cessation of fibrinolysis occurs by at least three mechanisms. The first involves regulation by PAI-1, as described above. The second involves inhibition of Pm by α_2-antiplasmin. FXIIIa cross-linking of adjacent fibrin fibers and covalently attaching α_2-antiplasmin to the fibrin makes the fibrin clot more resistant to Pm degradation (Cleary

and Maurer, 2006, Sakata and Aoki, 1982). The third level of fibrinolytic regulation involves the enzyme, thrombin activatable fibrinolysis inhibitor (TAFI). TAFI is activated by thrombin and is responsible for inhibiting Pm-mediated feedback by cleaving COOH-terminal lysine residues from partially degraded fibrin (Mosnier and Bouma, 2006). Abrogating the fibrin cofactor function inhibits the conversion of Glu- to Lys-Pg, as well as Pm formation from Glu-Pg (Wang *et al.*, 1998). However, Lys-Pg generated at the fibrin surface no longer has the stringent requirement for partially degraded fibrin as a cofactor for activation by tPA and is less susceptible to down-regulation by TAFI (Miles *et al.*, 2003, Wang *et al.*, 1998). Ultimately, it is the level of Lys-Pg, Pg activators and Pm inhibitor molecules bound to the clot and the position of these components that influences the timing and degree of clot dissolution.

1.5 Additional Modulators of Haemostasis

1.5.1 *Histidine-rich Glycoprotein*

Various plasma proteins function to modulate fibrinolytic activity in blood, including fibrin, which binds tPA and Pg, PAI-1, α_2-antiplasmin, and histidine-rich glycoprotein (HRG). HRG is a 67 kDa, non-enzymatic protein found at a relatively high concentration of 2 μM in human plasma. HRG, which has a half life of approximately 3 days in humans (Lijnen *et al.*, 1981), is found in the plasma of many vertebrate species, including mammals and birds, and in invertebrate species, such as shell fish (Jones *et al.*, 2005, Nair and Robinson, 1999). HRG has been reported to bind to numerous ligands, which include heme, divalent metal ions, heparin (Heimburger *et al.*, 1972), heparan

sulfate (Brown and Parish, 1994), thrombospondin (Silverstein *et al.*, 1985a), fibrin/fibrinogen (Leung, 1986), plasmin/plasminogen (Borza and Morgan, 1997), complement proteins (Chang *et al.*, 1992, Gorgani *et al.*, 1997), tropomysin (Guan *et al.*, 2004), erythrocytes (Parish *et al.*, 1984), monocytes and macrophages (Chang *et al.*, 1994), T & B cells (Saigo *et al.*, 1989) and fibroblasts (Hennis *et al.*, 1995b). The capacity of HRG to bind to numerous plasma proteins and its ability to interact with a variety of cell types suggest that HRG has diverse biological functions and participates in physiological processes that include wound healing (Tsuchida-Straeten *et al.*, 2005), angiogenesis (Juarez *et al.*, 2002, Simantov *et al.*, 2001) immune complex clearance (Gorgani *et al.*, 1997, Gorgani *et al.*, 1999, Gorgani *et al.*, 2002), tumour metastasis (Olsson *et al.*, 2004) and fibrinolysis (Tsuchida-Straeten *et al.*, 2005). Congenital HRG deficiency has been associated with a prothrombotic phenotype, suggesting HRG has an anticoagulant function *in vivo* (Shigekiyo *et al.*, 1998). Studies in HRG knockout mice suggest that HRG deficiency is associated with accelerated clotting times and clot lysis times compared to wild type mice (Tsuchida-Straeten *et al.*, 2005). This would suggest that HRG acts as both an anticoagulant and antifibrinolytic agent. Yet, its major physiological role is still unclear.

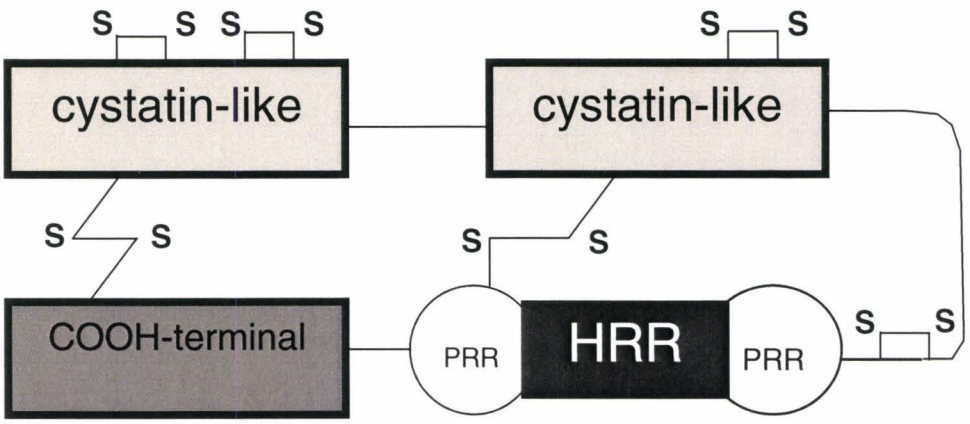
HRG is mainly synthesized in the liver (Koide *et al.*, 1986), but has also been detected in monocytes and macrophages (Sia *et al.*, 1982) and in the α-granules of platelets and megakaryocytes (Leung *et al.*, 1983). HRG is released from platelet granules after thrombin stimulation (Leung *et al.*, 1989). Circulating plasma levels of HRG can vary depending on age, environmental, and genetic factors (Hennis *et al.*,

1995a, Morgan *et al.*, 1978). Despite differences in plasma HRG concentration within the population, the levels of circulating HRG at birth are always low at approximately 20-30% of the adult HRG concentration (Morgan *et al.*, 1978). The levels steadily increase during the first year of life to reach adult levels. HRG levels continue to increase thereafter at a rate of 0.41% per year in adults (Corrigan Jr and Jeter, 1990, Corrigan Jr. *et al.*, 1990, Drasin and Sahud, 1996). This would suggest that HRG is an important biological regulator because higher levels are required with aging.

1.5.1.1 *HRG Structure*

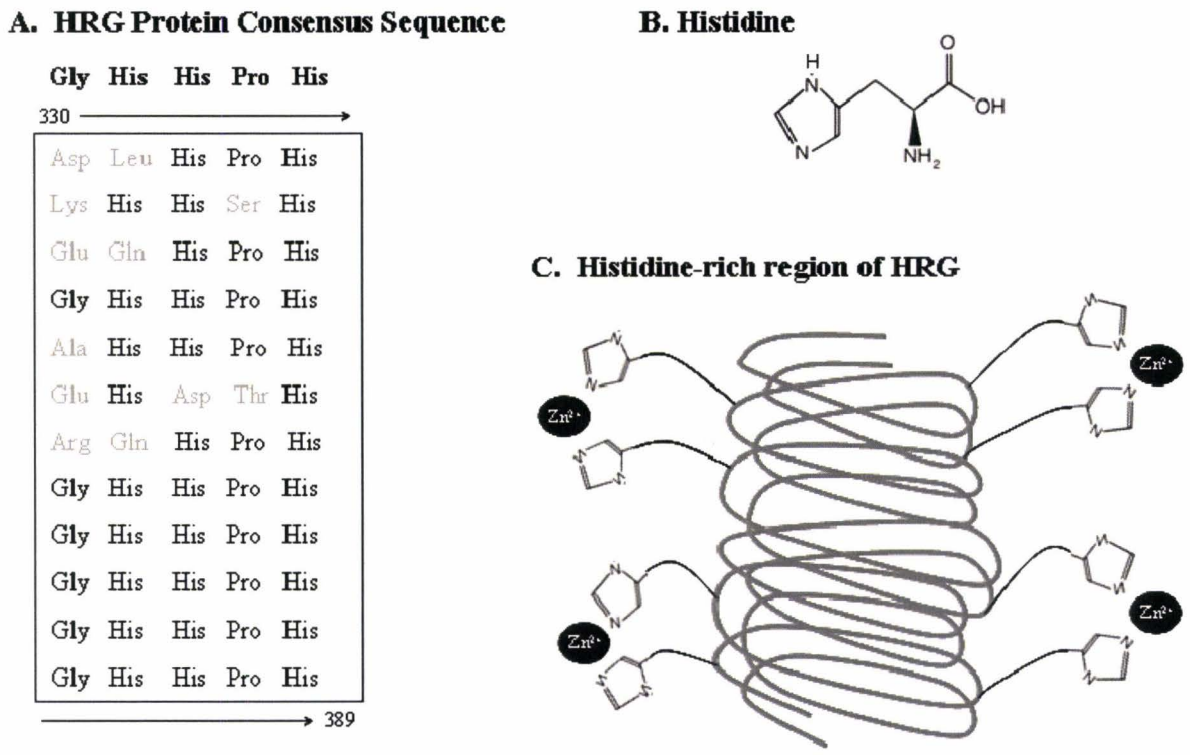
HRG, which was first isolated in 1972 (Haupt and Heimburger, 1972, Heimburger *et al.*, 1972), has an unusually high content of histidine and proline residues (Koide *et al.*, 1986). The unique multidomain structure of HRG allows it to bind numerous ligands simultaneously, bringing them into proximity. The NH_2-terminal region of HRG is composed of two cystatin-like domains (Koide and Odani, 1987), which bind to a variety of ligands such as Fg, fibrin, heparin and Pg (Figure 5). Interestingly, the NH_2-terminus of HRG also has sequence homology with antithrombin (Koide *et al.*, 1982, Koide *et al.*, 1986).

The central portion of the molecule is composed of a histidine-rich region (HRR) that contains 12 tandem repeats of the sequence GlyHisHisProHis flanked by two proline-rich regions (PRR) (Koide *et al.*, 1986). The HRR plays an important role in regulating the conformation of the molecule and is critical to its biological activity in the



Modified from: Jones AL, Hulett MD, Parish CR. Histidine-rich glycoprotein: A novel adaptor protein in plasma that moduates the immune, vascular and coagulation systems. Immunology and Cell Biology. 2005; 83: 106-118.

Figure 5. Schematic diagram of the domain structure of human HRG. HRG has a multidomain structure that is composed of two cystatin-like domains at the NH_2-terminus, a histidine-rich region (HRR) flanked by two proline rich regions (PRR) and a COOH-terminal domain. The S symbols indicate the disulfide bonds between cysteine residues within and between structural domains. The NH_2-terminal and COOH-terminal domains contain conserved lysine residues that allow it to bind the kringle domains of Pg. HRG also bind Zn^{2+} and heparin via the HRR domain.



Modified from: Jones AL, Hulett MD, Parish CR. Histidine-rich glycoprotein: A novel adaptor protein in plasma that moduates the immune, vascular and coagulation systems. Immunology and Cell Biology. 2005; 83: 106-118.

Figure 6. Structure of the histidine-rich region of HRG. Panel A represents the amino acid sequence of the HRR of human HRG, highlighting the conserved residues within the tandem repeated sequence. Panel B depicts the molecular structure of histidine that contains an imidazole side chain. Panel C displays the helical structure of the HRR core and the imidazole ring of each histidine residue protrudes outward of the twisted and elongated HRR core. Two imidazole rings of adjacent histidine residues form the Zn^{2+}-binding portion of the molecule.

presence of Zn^{2+} (Jones *et al.*, 2004a, Vanwildemeersch *et al.*, 2006). Each histidine residue found in the helical HRR contains an imidazole ring, and two adjacent imidazole rings form the metal binding site of the molecule (Koide *et al.*, 1986) (Figure 6). Zn^{2+} binds to the histidine residues in the HRR and induces a structural change that increases affinity of HRG for other ligands.

The histidine residues in the HRR can also become protonated under slightly acidic conditions (pH< 6.0) and this is believed to induce the same structural changes associated with Zn^{2+} binding (Jones *et al.*, 2004a). The mechanism by which the ligand affinity of HRG is increased in the presence of Zn^{2+} or acidic pH is not currently known. It is possible that protonation or Zn^{2+} binding to histidine residues of the HRR causes HRG to undergo a conformational change, exposing a new binding site. Alternatively, the introduction of positively charged residues at the HRR may create a unique binding site that allows HRG to bind to a negatively charged region of another molecule through electrostatic interactions. Regardless of the mechanism of ligand interaction, the HRR mediates the interaction of HRG with cell surfaces via negatively charged heparin sulfate or other heparin-like molecules found on the surface of endothelial cells or in the subendothelium (Jones *et al.*, 2004b).

The COOH-terminal domain contains lysine residues that are responsible for the interaction of HRG with the high-affinity lysine binding sites of Pg and Pm (Jones *et al.*, 2004a). The NH_2-terminus has also been shown to contain a conserved lysine residue responsible for Pg binding (Lijnen *et al.*, 1980). These two domains may cooperatively bind the high affinity lysine-binding kringles 1-3 of Pg, providing a strong interaction

between Pg and HRG. It is also possible that HRG interacts with tPA via the same lysine residues, because tPA readily binds to exposed lysine residues on fibrin (Christensen, 1985).

1.5.1.2 *Effect of HRG on Fibrinolysis*

The ability of HRG to bind Pg (Jones *et al.*, 2004a, Lijnen *et al.*, 1980, Saez *et al.*, 1995), heparin (Jones *et al.*, 2004b), and fibrin (Leung, 1986) suggests that it plays a role in the modulation of fibrinolysis. However, existing evidence in the literature surrounding HRG modulation of Pg activation is contradictory. Some reports suggest that HRG attenuates fibrinolysis by inhibiting Pm activation and Pg/Pm-fibrin interactions (Goodnough *et al.*, 1985) or by inhibiting Pm-dependent fibrinolysis (Chu and Mathews, 2003). Conversely, other investigators have suggested that HRG has profibrinolytic activities because it enhances Pg activation by tPA in the presence of Zn^{2+} or under acidic conditions (Borza *et al.*, 2004).

Borza et al. proposed a theory in an attempt to clarify the role of HRG in fibrinolysis and explain its anti- and pro-fibrinolytic activities (Borza *et al.*, 2004). They suggested that HRG is a modulator of Pg activation, but is not essential for activation. HRG can either promote or retard Pg activation in response to the surrounding conditions. At physiological pH, Pg remains bound to HRG, impairing the normal stimulatory effect of fibrin and Fg. Under conditions of vascular injury or thrombotic events where platelets release Zn^{2+} and HRG, or conditions of hypoxia where there is a decrease in pH, the histidine residues in the HRR of HRG become positively charged.

Under these circumstances, HRG interacts with negatively charged GAGS on cell surfaces through its HRR and stimulates tPA-mediated conversion of Pg to Pm (Borza *et al.*, 2004). Therefore, the physiological significance of this interaction is that Pg is localized to the endothelial cell surface by HRG, where it is positioned closer to tPA released from endothelial cells in response to vessel injury, suggesting a cell-based model of Pm regulation (Borza *et al.*, 2004).

1.5.2 *Factor XIIa as a Plasminogen Activator*

Another mechanism by which HRG may influence fibrinolysis is through a secondary Pg activation pathway. Factor XIIa, the initiator of the contact system of coagulation, has also been implicated in the activation of the fibrinolytic pathway. However, this has only been demonstrated in purified systems, not in plasma (Braat *et al.*, 1999, Lenich *et al.*, 1995). Structurally, FXIIa resembles Pg as well as its physiological activators, tPA and uPA, because they have a similar organization of structural domains (McMullen and Fujikawa, 1985, Tans and Rosing, 1987). The specific PA activity of FXIIa is approximately two orders of magnitude less than that of uPA (Schousboe *et al.*, 1999), and tPA in the presence of fibrin (Silverstein *et al.*, 1985b). However, it is believed that *in vivo,* FXIIa (375 nM) (Fuhrer *et al.*, 1990, Tans and Rosing, 1987) may be as important as uPA in Pg activation due to the low physiological concentration of 45 pM uPA in the blood (Braat *et al.*, 1999, Darras *et al.*, 1986, Schousboe *et al.*, 1999). The potential contribution of FXIIa to initiation of fibrinolysis becomes significant in the presence of Zn^{2+} and a potentiating surface, such as dextran sulfate or other negatively

charged surfaces, which increases PA activity approximately 6-fold (Braat *et al.*, 1999). FXIIa also indirectly initiates Pg activation by activating PK, which in turn cleaves prourokinase into active two-chain uPA (Binnema *et al.*, 1991, Ichinose *et al.*, 1986). PKa also liberates BK from HK and both PK and BK initiate the release of tPA from endothelial cells *in vivo* (Fuhrer *et al.*, 1990). The ability of FXIIa to directly, as well as indirectly, initiate Pg activation makes it likely to play a part in the regulation of fibrinolysis. However, the quantitative role of FXIIa as a physiological Pg activator is not known, nor is why FXIIa PA activity is seen in purified systems, but not in plasma.

It is likely that HRG is physiologically important because of its abundance in plasma and its capacity to bind to numerous ligands. The interaction of HRG with Pg must be important, since it is estimated that 50% of circulating Pg is bound to HRG (Borza and Morgan, 1997). The role of HRG in modulating the activity or activation of Pg needs to be further investigated to clarify the function of HRG and the significance of its interaction with Pg.

1.6 Proposal

The purpose of this research was to investigate the role of HRG in fibrinolysis by focusing on the significance of the interaction of HRG with Pg. We first tested the hypothesis that HRG modulates Pg activation by tPA. Contrary to one report (Borza and Morgan, 1997), but in agreement with another (Horne, III *et al.*, 2000), we found that HRG had no significant effect on Pg activation by tPA once contaminating Pg was eliminated from our HRG preparations.

We then went on to examine the role of HRG on Pg activation by other Pg activators, namely uPA and FXIIa. In preliminary work, we showed that HRG delayed FXIIa-mediated Pg activation. Next, we examined the effect of Zn^{2+} on these reactions because, in many situations, HRG function is modulated by the presence of Zn^{2+}. Binding studies were conducted to assess the affinity of HRG for Pg, tPA, uPA, FXII, FXIIa and heparin to confirm published data, as well as to generate new conclusions about how these interactions occur. HRG was found to have a high affinity for FXIIa and modulate its activity, so the effect of HRG was further assessed on FXIIa-mediated reactions in contact activation. Specifically, the effect of HRG on FXII activation was determined in FXII autoactivation and PKa-mediated FXII activation experiments. Next, the effect of HRG was assessed on FXIIa-mediated reactions, including FXI and PK activation by FXIIa. Binding studies measuring the affinity of HRG for FXI, FXIa and PKa were also conducted by surface plasmon resonance. Overall, these studies will help to further define the role of HRG in coagulation and fibrinolysis.

2 EXPERIMENTAL PROCEDURES

2.1 MATERIALS

2.1.1 *Reagents*

Glu-Pg was isolated by passing fresh frozen human plasma (Canadian Blood Services, Ottawa, ON, CA) through a lysine-Sepharose column (Stewart *et al.*, 1998). The column (50 ml) was washed with 2 volumes of 0.1 M sodium phosphate, pH 8.0, 3 volumes of 0.3 M sodium phosphate, pH 8.0, followed by 3 volumes of 0.1 M sodium phosphate, pH 8.0. Glu-Pg was eluted with 10 mM of the lysine analog, ε-amino-n-caproic acid (EACA), in 0.1 M sodium phosphate, pH 8.0. The Pg peak was concentrated on a SVC 200 H SpeedVac® Concentrator (Savant Instruments Inc., Farmingdale, NY) and EACA was removed by dialysis against 20 mM Tris-HCl & 150 mM NaCl pH 7.4 (TBS). The purity and integrity were determined by SDS polyacrylamide gel electrophoresis analysis (below) and Pg concentration was determined by measuring the absorbance at 280 nm on a Beckman DU 7400 spectrophotometer (Beckman Coulter, Inc.) and using a molecular weight of 90 kDa and an extinction coefficient of 1.7 $M^{-1}cm^{-1}$ (Castellino and Powell, 1981). Absorbance values were corrected for light scattering at 320 nm using the equation $A_{corr280} = A_{280} - (1.7 \times A_{320})$ (Bloom *et al.*, 1979).

Recombinant Pg was a generous gift from the Dr. Nesheim laboratory. Human Pm was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Deaminated heparin (MW 6,700) was purchased from Sigma Chemical Co. (St. Louis, MO) and 500 kDa dextran sulfate was from GE Healthcare (Baie-d'Urfé).

Sulfosuccinimidyl-6-biotinamindo (Sulfo-NHS-LC-biotin) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Biotinamindohexanoic acid hydrazide (biotin-hydrazide) was purchased from Sigma Chemical Co. D-Val-Phe-Lys chloromethyl ketone (VFKck) was obtained from EMD Chemicals Inc. (San Diego, CA). Single-chain recombinant tPA (Activase) was obtained from Genentech Inc. (South Francisco, CA). Two-chain recombinant uPA (Abbokinase) was purchased from Abbott Laboratories (Abbott Park, IL). Factor XII, factor XIIa, factor XI, factor XIa, PKa, thrombin and Fg were purchased from Enzyme Research Laboratories (South Bend, IN). Polyacrylamide gradient Ready-Gels were from Bio-Rad Laboratories, Inc. (Mississauga, ON). The chromogenic substrates S-2251, S-2222, S-2444, S-2366 were from DiaPharma Group Inc. (West Chester, OH). The chromogenic substrates Pefachrome VIIa and Pefachrome XIIa were supplied by Pentapharm Ltd. (Basel, Switzerland) and the fluorescent substrate, Pro-Phe-Arg 4-Methylcoumarin (PFR-AMC) was from Sigma. All chemicals used were of the highest purity available.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) was performed under reducing and non-reducing conditions in order to assess the integrity of the proteins used in the various experiments. Samples were electrophoresed on 4-15% polyacrylamide gradient, Tris-HCl gel. Samples were electrophoresed at 180 V for 1 hour in 1x running buffer (25 mM Tris, pH 7.5, 190 mM glycine & 0.1% SDS) and then placed in a fixative solution of 10% acetic acid, 40% methanol for 30 minutes. The gel was stained with Fast Stain (Zoion Research, St. Allston, MA) for 30 minutes and placed in a destain solution of 10% acedic acid

overnight. The gel was washed with a 3% glycerol solution and dried in GelWrap (Biodesign Inc. Carmel, NY).

Fg was further processed to remove factor XIII by Beverly Leslie, as previously described (Stewart *et al.*, 1998). Briefly, the Fg was diluted to 10 mg/ml with TBS, pH 7.4. Fg was rendered FXIII-free by affinity chromatography (sheep anti-FXIII IgG, Affinity Biologicals Inc., Hamilton, ON, CA) linked to cyanogen bromide-Sepharose 4B beads (GE Healthcare). Fg was eluted from the column with TBS and 10 ml fractions were collected. The flow through material was pooled together and concentrated by 50% ammonium sulfate precipitation in dialysis tubing overnight and pelleted at 2800 x *g* for 15 min at 23°C. The Fg pellet was resuspended in 39 mM Tris-HCl, pH 8.8 & 0.02% azide and then dialyzed twice against 1 L TBS.

2.1.2 *Purification of HRG*

The purification of HRG was modified from a previously published method (Borza and Morgan, 1997). HRG was isolated from 2 units of fresh frozen plasma (300-400 mL) supplemented with 1 μM VFKck and approximately 10,000 KIU aprotinin (Bayer, Berkley, CA) to minimize proteolytic activity. Plasma was centrifuged at 10,000 x *g* for 30 minutes at 4°C. Any visible lipid layer was removed and solid imidazole was added to plasma to give a final concentration of 5 mM. HRG was purified by metal-chelate chromatography by passing the plasma over a 50 ml nickel-NTA agarose column (Qiagen, Mississauga, ON). The column was washed with 5 volumes of TBS-Tw20 containing 5 mM imidazole, followed by another 5 volumes of 5 mM imidazole

containing 10 mM EACA, followed by 80 mM imidazole and 100 mM imidazole buffers, at pH 7.5. Aprotinin was added to the 80 mM and 100 mM imidazole wash buffers at 10 KIU/mL. HRG was eluted with TBS-Tw20 containing 250 mM imidazole, pH 7.5. 5 mL fractions were collected and protein concentration was determined by measuring absorbance at 280 nm. Before HRG-containing fractions were pooled and dialyzed, 4-12 μg of protein from peak fractions were analyzed by SDS-PAGE to assess protein purity. HRG-containing fractions were pooled together and concentrated using a Centriprep YM-30 (Millipore Corporation, Bedford, MA). Imidazole was removed by dialysis against HBS-Tw20. The concentration of HRG was calculated by measuring the absorbances at 280 and 320 nm and using the a molecular weight of 67 kDa and an extinction coefficient of 0.39 mL/mg/cm (Borza *et al.*, 2004). Each HRG preparation was tested for plasminogen contamination, as described below. Final preparations were assessed for protein purity and integrity by SDS-PAGE analysis.

2.2 METHODS

2.2.1 *Western Blotting Analysis*

An SDS-PAGE gel containing HRG, Pg, recombinant Pg and FXIIa samples was washed in trans-blot buffer (25 mM Tris, 192 mM Glycine and 10% v/v methanol, pH 8.3) for 30 minutes to remove SDS. The protein was transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (BioRad) in the Criterion blotting apparatus (BioRad) in ice-cold trans-blot buffer for 1 hour at 90 V. Non-specific binding sites were blocked by placing the membrane in 50 ml of blocking buffer (TBS and 5% milk

powder) for 1 hour at room temperature on an orbital shaker. The membrane was briefly washed with two changes of TBS-Tw20 and incubated for 1-2 hours with a rabbit anti-human HRG antibody (Diagnostica Stago, Asnières, France), diluted 1:2000 in blocking buffer. The membrane was briefly rinsed with two changes of wash buffer, followed by four 5-minute washes with wash buffer. A second incubation was done with an anti-rabbit IgG horseradish peroxidase antibody (Sigma), also diluted 1:2000 in blocking buffer, for 1 hour at room temperature on an orbital shaker. The membrane was again washed with wash buffer for 20 minutes and proteins were detected using the ECL plus Western Blotting Detection System (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). Two methods of detection were used to visualize the results. The first method used was chemiluminescent detection, which was done on Kodak BioMax XAR scientific imaging film with exposure times of 30 seconds to 5 minutes. The film was developed in the Kodak X-OMAT 1000A Processor. The second method was detected by chemifluorescence on a Typhoon 9410 Variable Mode Imager (Amersham BioSciences). The image was scanned in channel 1 at 450 V and 457 nm for medium sensitivity.

2.2.2 *NH_2-terminal Amino Acid Sequencing Analysis*

Samples from HRG preparations were subjected to SDS-PAGE. The gel was immersed in 10 mM 3-cyclohexylamino-1-propane-sulfonic acid (CAPS), 10% v/v methanol, pH 11 (CAPS buffer) for 30 minutes. The proteins were transferred from the gel to a PVDF membrane in a Criterion blotter in cold CAPS buffer (4°C) at 100 volts for

90 minutes. Once the transfer was complete, the PVDF membrane was removed from the apparatus and left to dry in the dark in a container overnight. The membrane was rinsed with 100% methanol until the membrane became water-soluble (~5 minutes). The membrane was then rinsed with distilled water for 10 minutes, stained with Ponceau S (Sigma) for 5 min, rinsed with distilled water 3-4 times and left in the dark to dry. The protein-stained PVDF membrane was sent to the Hospital for Sick Children: Advanced Protein Technology Centre Peptide Sequencing Facility for NH_2-terminal sequencing.

2.2.3 *Protein Identification Analysis*

A subsequent HRG preparation (2 μg) was subjected to SDS-PAGE on a 15% polyacrylamide gel to separate and identify two closely migrating bands, each assumed to be HRG. The two bands of approximately 59 and 66 kDa, stained with FAST Stain, were excised from the gel with an exacto-knife and placed in a well, containing 200 μl of distilled water, of a 96-well plate. The 96-well plate was sent to VWR BioSciences Inc. for protein analysis (Alphalyse Canada, Montreal, Quebec). Each sample was digested in the gel with trypsin to generate a mixture of tryptic fragments. The tryptic fragments were sequenced by mass spectroscopy and compared against a database containing over a million protein sequences. Proteins were scored based on the sequence homology with the unknown peptide sequences and the protein sequences in the database.

2.2.4 *Analysis of HRG Preparation of Pg Contamination*

To confirm its purity, 2 μM HRG was tested for contaminating Pg by adding tPA and measuring plasmin chromogenic activity by the hydrolysis of 200 μM of the plasmin substrate, S-2251. All reactions were initiated by the addition of 100 nM tPA and the absorbance was measured at 405 nm at 9 second intervals for 45minutes at 23°C with a kinetic plate reader (SpectraMAX 340, Molecular Devices Inc.). HRG and tPA were also tested separately for chromogenic activity as negative controls and 1.2 μM Glu-Pg was activated by tPA as a positive control. If Pg was detected in HRG preparations, it was passed through the 50 ml NTA-Ni column for a second time, washed with 5mM imidazole and 20mM EACA and re-isolated as described above.

2.2.5 *Analysis of Glu-Pg and FXIIa Samples for HRG Contamination*

Western blot analysis was used examine Pg and FXIIa stocks for HRG contamination. Pg and FXIIa samples were separated by SDS-PAGE and transferred to a PVDF membrane. ASSERA® HRGP rabbit anti-human serum (Diagnostica Stago, Asnières, France) (1:2000), followed by a horse-radish peroxidase-conjugated secondary antibody (1:4000) was used to detect the presence of HRG in 500 ng of Glu-Pg and FXIIa. 500 ng of recombinant Pg and 50 ng of HRG were included as controls. Following ECL-Plus blot detection, the presence of HRG was visualized by chemifluorescence on the Typhoon Imager. The image was scanned in channel 1 at 450 V and 457 nm for medium sensitivity.

2.2.6 *Effect of HRG on Enzyme Chromogenic Activity*

$ZnCl_2$, $CaCl_2$ and dextran sulfate were each tested for their effect on Pm chromogenic activity in the absence and presence of HRG. Pm activity was monitored by recording the hydrolysis of S-2251 in a kinetic plate reader. The reaction was initiated by the addition of Pm (final concentration 50 nM) to a mixture of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$, 2 μg/ml dextran sulfate and 200 μM S-2251 in 10 mM Hepes-OH, 150 mM NaCl, pH 7.4 containing Tween20 0.005% (HBS-Tw20), in a final volume of 100 μl. Absorbance at 405 nm was recorded every 9 seconds over a period of 15 minutes at 23°C. The same conditions were maintained for testing the effect of 2 μM HRG on Pm activity. Milli optical density/ minute (mOD/min) values were determined by the plate reader. The same experimental system was used to assess whether HRG has an effect on the chromogenic activity of tPA, uPA, FXIIa, FXIa or PKa. Activity of 50 nM tPA, 4 U/ml uPA, 50 nM FXIIa, 50nM FXIa, or 50 nM PKa was monitored with the chromogenic substrates Pefachrome XIIa (200 μM), S-2444 (600 μM), S-2222 (200 μM), S-2366 (200 μM) or Pefachrome VIIa (400 μM), respectively.

2.2.7 *Influence of HRG on Glu-Pg Activation by tPA*

Pg activation by tPA was quantified in the absence and presence of HRG. The reaction was initiated in a microtiter plate by the addition of 100 nM tPA to each well that contained 1.2 μM Glu-Pg, 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 0-2 μM HRG. Pm activity was measured by the hydrolysis of S-2251 in a plate reader. Absorbance was measured at 405 nm every 9 seconds for 45 minutes. OD values obtained were plotted

against time squared in order to observe the activation phase of the reaction. The slopes (OD/s^2) of the linear portion were converted to activation rate values, nM/second, by dividing slopes by the specific activity of 50 nM Pm, 2.29x 10^{-5} OD/s/nM. Rates of activation were plotted against HRG concentration and the data were analyzed in TableCurve 2D (Version 4, Jandel Scientific Software, SPSS Inc., Chicago, IL) using a rectangular hyperbola equation to determine the concentration of HRG that is required for 50% inhibition of Pg activation (IC_{50}).

2.2.8 *Influence of HRG on Glu-Pg Activation by uPA*

The effect of HRG on Pg activation by uPA was quantified in a similar fashion. The reaction was initiated in a 96-well plate by the addition of 4 U/ml of uPA to each well that contained 1.2 μM Glu-Pg, 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 0-5 μM of HRG. Rates of Pg activation were determined as described above.

2.2.9 *Influence of HRG on Glu-Pg Activation by FXIIa*

FXIIa activation of Glu-Pg was quantified in the presence of HRG. The reaction was initiated in a 96-well plate by the addition of 100 nM FXIIa to each well that contained 1.2 μM Glu-Pg, 2 mM $CaCl_2$ and 0-2 μM of HRG in the presence 12.5 μM $ZnCl_2$ or 2 mM EDTA in HBS. The rate of Pg activation and effect of HRG were calculated as described above.

2.2.10 *Effect of HRG on FXIIa Chromogenic Activity*

To ensure that HRG does not affect the active site of FXIIa, the effect of HRG on FXIIa chromogenic activity was assessed using three different chromogenic substrates. The reaction was initiated by the addition of FXII (final concentration 150 nM) to a mixture of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$, 2 μg/ml dextran sulfate ± 2 μM HRG in HBS. FXIIa activity was monitored by measuring hydrolysis of Pefachrome XIIa or S-2222 in a kinetic plate reader. FXIIa activity was also measured by hydrolysis of a fluorescent substrate PFR-AMC, which was monitored in a fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices Inc., Sunnyvale CA). In the fluorescence plate reader, fluorescence intensity was measured at λ_{ex} = 380 nm, λ_{em} = 440 nm with a 420 nm cut-off filter. Fluorescence intensities (FI) were plotted for each chromogenic substrate in the absence or presence of HRG.

2.2.11 *Influence of HRG on FXII(a)-Mediated Clot Lysis*

The role of HRG in FXII(a)-dependent fibrin degradation was analyzed in a clot lysis assay. A 150 μl mixture of 2 μM FXII-free fibrinogen, 1.2 μM Glu-Pg, 10 μg/ml dextran sulfate, 2 mM $CaCl_2$, 10 μM $ZnCl_2$ and 0-3 μM HRG were added to each well of a 96-well plate. Clot formation was initiated by adding a 50 μl stock solution that yielded final concentrations of 20 nM thrombin and 100 nM FXII or FXIIa. The thrombin/ FXII(a) mixture was prepared in an adjacent well. Absorbance was measured at 340 nm every 20 seconds for 6 hours at room temperature in a kinetic plate reader. Clot lysis

times were determined by finding the time at which the turbidity decreased to a half maximal OD value.

2.2.12 *Influence of HRG on Pm-Mediated Clot Lysis*

The effect of HRG on Pm-mediated clot lysis was analyzed in pre-formed 100 µl fibrin clots. 20 nM thrombin was added to a mixture of 2 µM FXII-free fibrinogen, 10 µg/ml dextran sulfate, 2 mM $CaCl_2$, 10 µM $ZnCl_2$. Clots were allowed to form for 30 min at room temperature. A 50 µl mixture of 150 nM Pm and 0-3 µM HRG was gently placed over the solid clot and absorbance was measured at 340 nm every 20 seconds for 6 hours in a kinetic plate reader. As a control, 50 µl of 150 nM Pm and 2 µM HRG were added to the 150 µl mixture that did not contain thrombin to determine the baseline OD. Clot lysis times were determined as described above.

2.2.13 *Influence of HRG on FXII Autoactivation Stimulated by Dextran Sulfate*

FXII autoactivation, stimulated by dextran sulfate, $CaCl_2$ and $ZnCl_2$ was evaluated in the absence and presence of HRG. The rate of FXII activation was measured by FXIIa hydrolysis of S-2222 in a plate reader. The reaction was initiated by the addition of 100 nM FXII to a mixture of 2 µg/ml of dextran sulfate, 12.5 µM $ZnCl_2$, 2 mM $CaCl_2$, 0-1.6 µM HRG and 200 µM S-2222 in HBS. Absorbance was measured at 405 nm every 9 seconds for 2 hours and 30 minutes. Rates of activation were calculated as described.

2.2.14 *Influence of HRG on FXII Activation by PKa*

FXII activation was stimulated by 100 nM PKa in a mixture of 2 μg/ml dextran sulfate, 12.5 μM corn trypsin inhibitor (CTI), 2 mM $CaCl_2$ and 12.5 μM $ZnCl_2$ in the absence and presence of HRG and evaluated by SDS-PAGE. Aliquots of 10 μl containing 2 μg FXII per sample were added to 10 μl of sample buffer at nine different time points ranging from 0-2 hrs and electrophoresed under reduced conditions. Each gel was stained with Fast Stain and imaging and protein density was analyzed using the Pharmacia Biotech ImageMaster™ VDS software. The rate of FXII activation was determined by analyzing the integrated optical density (IOD) of each protein band: 80 kDa FXII, 52 kDa FXIIa heavy chain and 28 kDa FXIIa light chain. At each time point, the percent of each band was calculated by dividing the IOD of the band by the sum of the total density within the lane and plotted %IOD vs. time.

2.2.15 *Influence of HRG on FXI Activation by FXIIa*

FXI activation was initiated by FXIIa in the presence of 2 μg/ml dextran sulfate, 2 mM $CaCl_2$, and increasing concentrations of HRG. A mixture of 2 nM FXIIa, 12.5 μM $ZnCl_2$ and 0-2 μM HRG was incubated for 5 min in HBS before 60 nM FXI was added. The rate of FXI activation was measured by FXIa hydrolysis of 200 μM S-2366. Absorbance was measured at 405 nm every 9 seconds for 1 hour and 30 minutes. Rates of activation were calculated as described.

2.2.16 *Influence of HRG on PK Activation by FXIIa*

PK activation was initiated by FXIIa in the presence of 2 μg/ml dextran sulfate, 2 mM $CaCl_2$, and increasing concentrations of HRG. A mixture of 0.05 nM FXIIa, 12.5 μM $ZnCl_2$ and 0-3 μM HRG was incubated for 10 min in HBS before 400 nM PK was added. The rate of PK activation was measured by kallikrein hydrolysis of 400 μM Pefachrome VIIa. Absorbance was measured at 405 nm every 9 seconds for 30 minutes. Rates of activation were calculated as described.

2.2.17 *Protein Biotinylation*

HRG and heparin (MW 6,700) preparations were biotin-labelled for immobilization purposes in surface plasmon resonance (SPR) experiments. Protein preparations were dialyzed in 0.1 M phosphate buffer, pH 7.5. Sulfo-NHS-LC-biotin was incubated with HRG and biotin-hydrazide with deaminated heparin at 10-fold excess over HRG/heparin concentrations for 1.5 hours, at pH 7.5. To stop the reaction, 16 μM glycine, pH 8.0 was added to the reaction mixture. The remaining free biotin was removed from biotin-labelled protein by passing the sample mixture through a PD10 column (GE Healthcare) equilibrated with HBS. 500 μl fractions were collected and absorbance was measured at 280 nm to determine protein concentration of biotinylated-HRG. Biotin-labelled heparin samples were lyophilized and protein concentration was determined by weight.

2.2.18 *Surface Plasmon Resonance*

Biomolecular interaction analysis was performed on the BIAcore 1000 biosensor system (BIAcore AB, Uppsala, Sweden). Biotinylated proteins were absorbed to a BIAcore SA streptavidin chip at a flow rate of 5 µl/ minute at 25°C. Approximately 800-900 response units (RU) of biotinylated HRG or 200 RU of biotinylated heparin were immobilized to the SA sensor chip surface. Prior to immobilization, biotinylated proteins were diluted in HBS-Tw20 buffer containing 12.5 µM $ZnCl_2$ and 2 mM $CaCl_2$, or 2 mM EDTA, at pH 7.4. All buffers were filtered and degassed before use. A three-minute injection of regenerating buffer was passed over the flow cell to remove any unbound protein following immobilization. Regeneration buffers containing 250 mM imidazole or 20 mM EACA in HBS-Tw20 were used. Analyte proteins were injected at 35 µL/ minute for approximately 4 minutes followed by a 4 minute injection of running buffer to measure the dissociation phase. After each cycle, the chip was regenerated with either 250 mM imidazole or 20 mM EACA for 3 minutes. This removed any bound analyte molecules from the chip before the next concentration of analyte was injected. Increasing concentrations of analyte (0-2000 nM) were injected over the flow cell containing an immobilized ligand as well as over a blank streptavidin-coated flow cell. The change in the mass concentration on the sensor chip was monitored as a resonance signal using the program supplied by the manufacturer and was recorded as a sensorgram. Coinjection experiments took place in a similar manner. The first protein analyte was injected for two minutes, followed by the injection of a second protein analyte for 2 minutes with no dissociation phase. Each flow cell containing an immobilized ligand was used for

approximately 60-100 runs. Sensorgrams were analyzed with BIAevaluation™ software version 3.2 or Scrubber2 software version 2.0a (BioLogic Software Pty Ltd, Campbell, Australia). The binding response generated on control surfaces (blank streptavidin-coated flow cell) was subtracted from the response generated on immobilized ligand surfaces.

In the Scrubber2 program, the K_d value reported was generated by analyzing each sensorgram using a 1:1 Langmuir equation and taking the average k_d and k_a values, which were used to calculate the K_d ($K_d = k_d/k_a$). This program was also able to correction for a mass transport effect, if present.

3 RESULTS

3.1 Isolation and Characterization of HRG

Metal chelate affinity chromatography using a Ni-NTA agarose column was used to isolate HRG from human fresh frozen plasma. Increasing concentrations of imidazole in a TBS-Tw20 buffer were used to separate HRG from other non-specific or Ni-binding proteins found in plasma. The elution profile observed by measuring the protein absorbance at 280 nm reveals four distinct peaks (Figure 7). The first peak that appears after elution with 5 mM imidazole and 10 mM EACA represents HRG-bound Pg, which was verified in a chromogenic assay with a Pm substrate. This additional wash step is a modification of a published protocol (Borza and Morgan, 1997) and is used to remove contaminating Pg in HRG preparations. Pg was detected in HRG preparations by monitoring Pm generation after the addition of tPA to 2 μM HRG (Figure 8). The second and third peaks eluting at 80 mM and 100mM imidazole, respectively, represent weaker affinity Ni-binding proteins. The peak that eluted at 250 mM imidazole was identified as HRG by NH_2-terminal sequencing. The integrity of HRG was assessed by SDS-PAGE analysis on 4-15% polyacrylamide gels under non-reducing conditions (Figure 9). When 2 μg HRG was electrophoresed, two closely migrating bands were observed. Both bands were confirmed as HRG by protein identification by mass spectrometry (discussed below). An overloaded sample of HRG (20 μg) was also run to exclude the presence of contaminating proteins. No other proteins were observed in HRG preparations and our isolation procedures yielded HRG preparations with over 99% purity based on gel analysis and limits of protein detection by gel staining.

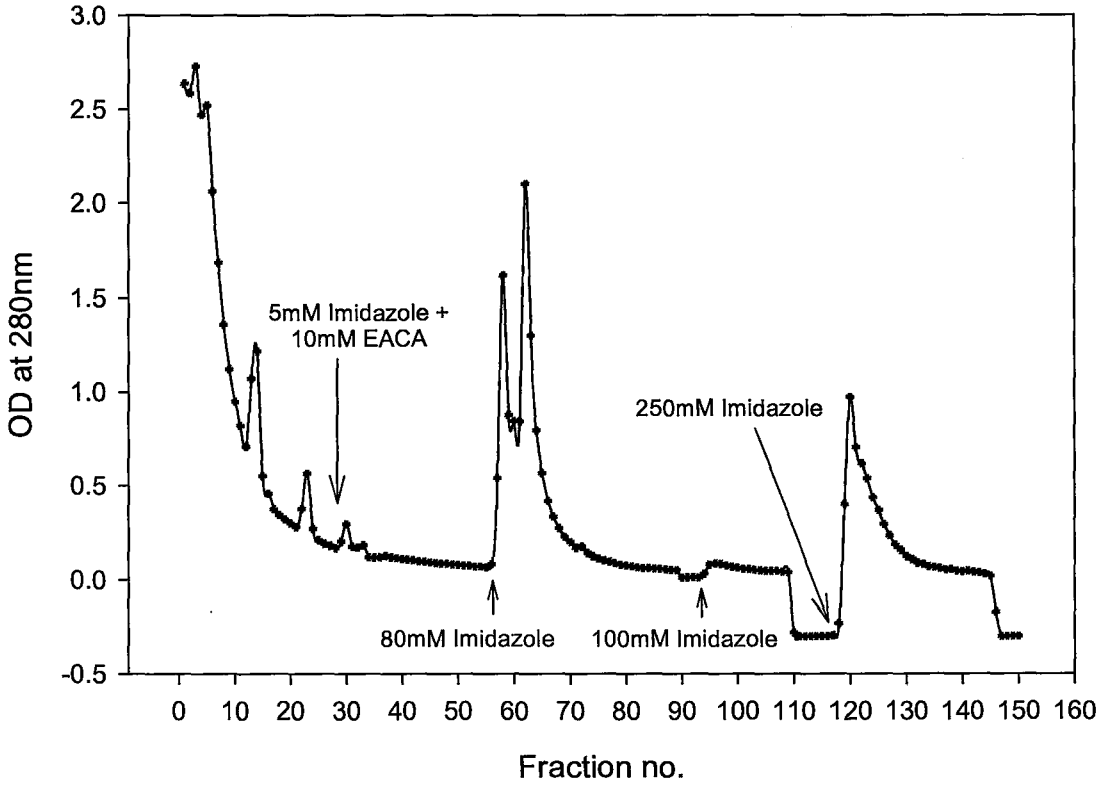


Figure 7. Elution profile of plasma adsorbed Ni-NTA agarose. Human plasma (300-500 mL) was supplemented with 5 mM imidzaole and was applied to the Ni-NTA column (2.8 x 10 cm) at a flow rate of 5 mL/min. The column was first washed with 5 mM imidazole to remove any remaining plasma from the column (fractions 1-28). Additional wash buffers, including 5 mM imidazole containing 10 mM EACA, 80 mM and 100 mM imidazole, were then passed over the column in order to elute other weak affinity Ni-binding proteins. The last peak represents HRG elution with 250 mM imidazole. Absorbance at 280 nm was monitored using a Beckman Gold 168 Detector.

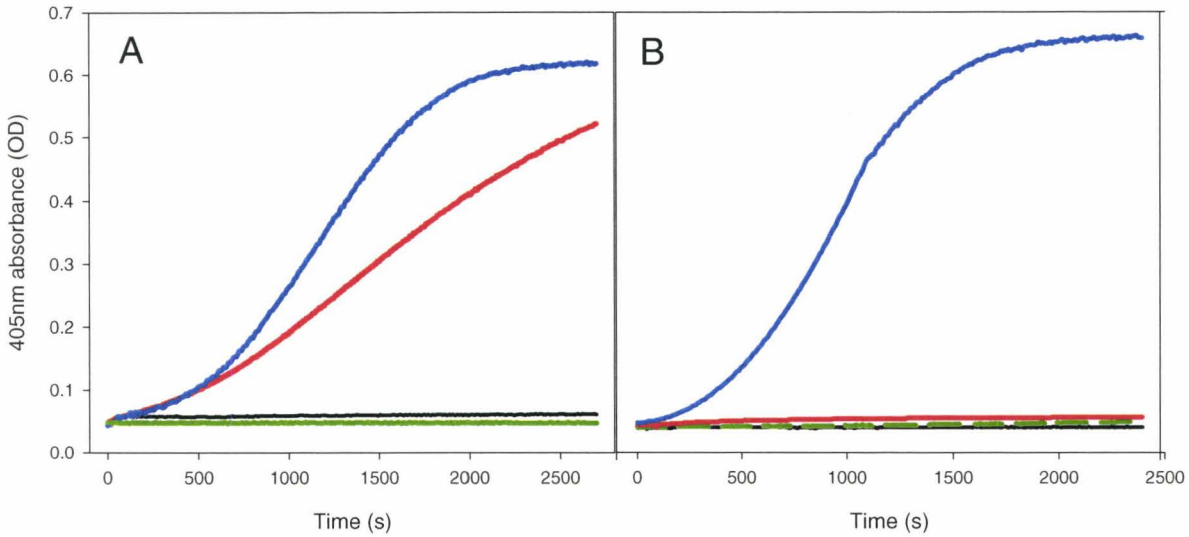


Figure 8. Assessment of HRG preparation for the presence of Pg. Pg activation was initiated by the addition of 100 nM tPA to 2 μM HRG and Pm activity was measured by hydrolysis of 200 μM S-2251 (red). tPA alone (black) and HRG alone (green) were also assessed and absorbance was read at 405 nm. As a positive control, 1.2 μM Glu-Pg was activated by 100 nM tPA (blue) in the presence of S-2251. Panels A and B represent preparations obtained before and after adoption of the EACA wash step, respectively.

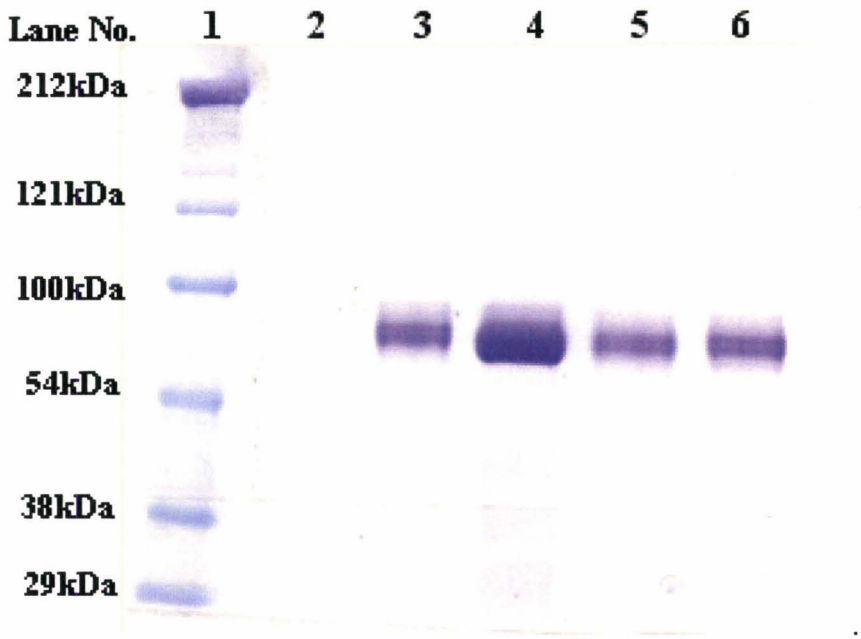


Figure 9. SDS-PAGE analysis of various HRG preparations. Three preparations of purified HRG samples isolated from human plasma were subjected to SDS-PAGE analysis under non-reducing conditions using 4-15% polyacrylamide gradient gels and stained with Fast-Stain. Lanes 3, 5 & 6 contain 2 µg of protein and lane 4 contains 20 µg of protein from the same preparation as lane 3. Lane 1 contains the molecular weight markers with molecular weights as indicated on the left.

3.3.1 *NH_2-terminal Sequencing*

Prior to making up imidazole wash buffers in TBS, instead of distilled water, two high molecular weight contaminants of approximately 190 kDa and 180 kDa were present in HRG preparations, as observed by SDS-PAGE analysis (not shown). To identify the high molecular weigh contaminants and confirm the identity of HRG, 34 μg of HRG was subjected to SDS-PAGE analysis and separated proteins were transferred to a PVDF membrane and stained with Ponceau S. Bands were cut from the membrane and subjected to NH_2-terminal sequence analysis by Edman reactions. The sequence, Val-Ser-Pro-Thr-Asp-Pro-Ser-Ala, was obtained for the 67kDa band and was positively identified as HRG by protein sequence analysis on the ExPASy proteomics server (http://br.expasy.org/). The unknown 190 kDa and 180 kDa proteins could not be positively identified. However, since these proteins were removed with modified isolation procedures, identifying these proteins was unnecessary.

3.1.2 *Protein Identification*

Although HRG had been positively identified by NH_2-terminal sequencing, electrophoresis of HRG at a lower concentration (2 μg) on a gradient polyacrylamide gel revealed two closely migrating bands of approximately 59 and 66 kDa (Figure 9). To more effectively separate and identify each band, HRG was electrophoresed on a 15% polyacrylamide SDS gel. The two bands were excised and the gel fragments were subjected to identification by mass spectroscopy. Briefly, the protein identification analysis involves trypsin digestion, peptide extraction, peptide sequencing and a database

search. After the protein samples were digested, 15 tryptic fragments were isolated and sequenced. Of the set, 10 fragments ranging from 8 to 21 amino acids, matched the human HRG sequence, exhibiting 100% sequence identity to human HRG. Altogether, the fragments from the 59 and the 66 kDa bands covered 32% and 29% of the HRG sequence, respectively. Therefore, both the 59 and the 66 kDa bands were identified as human HRG.

3.2 Analysis of Glu-Pg and FXIIa preparations for HRG contamination

Since Pg was found as a contaminant in early HRG preparations (Figure 8), it was important to confirm that HRG was not present in Pg or FXIIa stocks before conducting functional assays with these protein preparations. To examine this, Glu-Pg and FXIIa preparations were subjected to Western blot analysis using a commercially available anti-HRG polyclonal antibody (Figure 10). Recombinant Pg was included as a control. Positive staining of HRG by the antibody was confirmed at a molecular weight of approximately 66 kDa. However, positive staining was also observed in lanes that contained recombinant Pg, bovine serum albumin (BSA), FXIIa or Glu-Pg preparations, at the known molecular weights of these proteins. These results demonstrate that the polyclonal anti-human HRG antibody is not specific for HRG. This may reflect the use of impure HRG preparations for immunization.

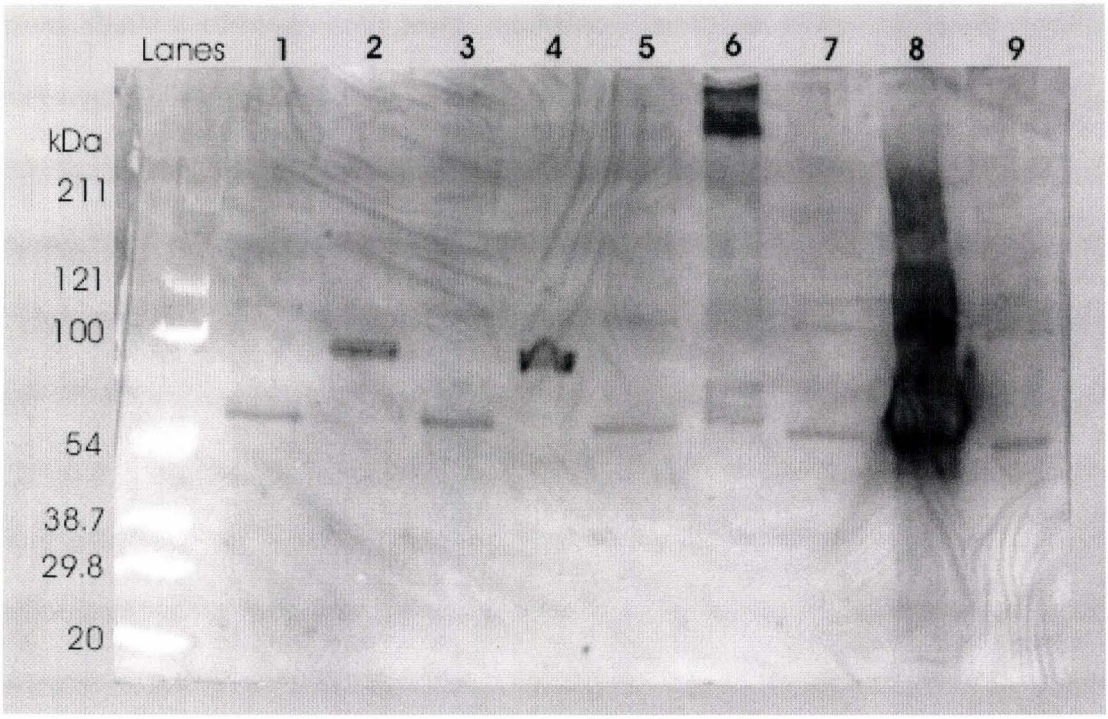


Figure 10. Western Blot detection of HRG in Pg and FXIIa preparations. Plasma-derived and recombinant Pg, FXIIa, and HRG were subject to SDS-PAGE under non-reducing conditions. Proteins were transferred from the gel to nitrocellulose and probed with a HRG-directed polyclonal antibody and a goat anti-rabbit horse radish peroxidase antibody detection. Lanes 1, 3, 5, 7 & 9 contain bovine albumin; lane 2 contains 500 ng of Glu-Pg; lane 4 contains 500 ng of recombinant Pg; lane 6 contains 500 ng FXIIa; and lane 8 contains 50 ng of human HRG.

3.3 Influence of HRG on Glu-Pg Activation

Initial experiments were undertaken to verify previous reports that HRG potentates tPA-mediated Pg activation (Borza and Morgan, 1997). Because Zn^{2+} plays an important role in modulating the activity of HRG in plasma and $CaCl_2$ also is important in coagulation, these cations were included in all functional assays of HRG. Pg activation by tPA was quantified in the presence of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and varying concentrations of 4 different HRG preparations (0-8 μM). Cleavage of the Pn-specific chromogenic substrate S-2251 was monitored to assess the conversion of Pg to Pn.

With the first HRG preparation, rates of Pg activation by tPA demonstrated a dose-dependent increase of approximately 6-fold with increasing concentrations of HRG (Figure 11). This was the first indication that this HRG preparation contained contaminating Pg. Subsequent HRG preparations isolated using procedures designed to exclude Pg, had much less of an effect on Pg activation by tPA, enhancing Pg activation by only 1.5-2 fold. When tested at physiological concentrations (2 μM), the most recent HRG preparation had no effect on tPA-mediated Pg activation (data not shown). Control experiments showed that 2 μM HRG does not affect the chromogenic activity of tPA with the chromogenic substrate Pefachrome XIIa, nor does it affect Pm activity with S-2251 (Figure 11, Inset). These results demonstrate that HRG does not affect Pg activation by tPA, contrary to what is reported by other investigators (Borza *et al.*, 2004). All subsequent experiments were done with HRG preparations lacking Pg.

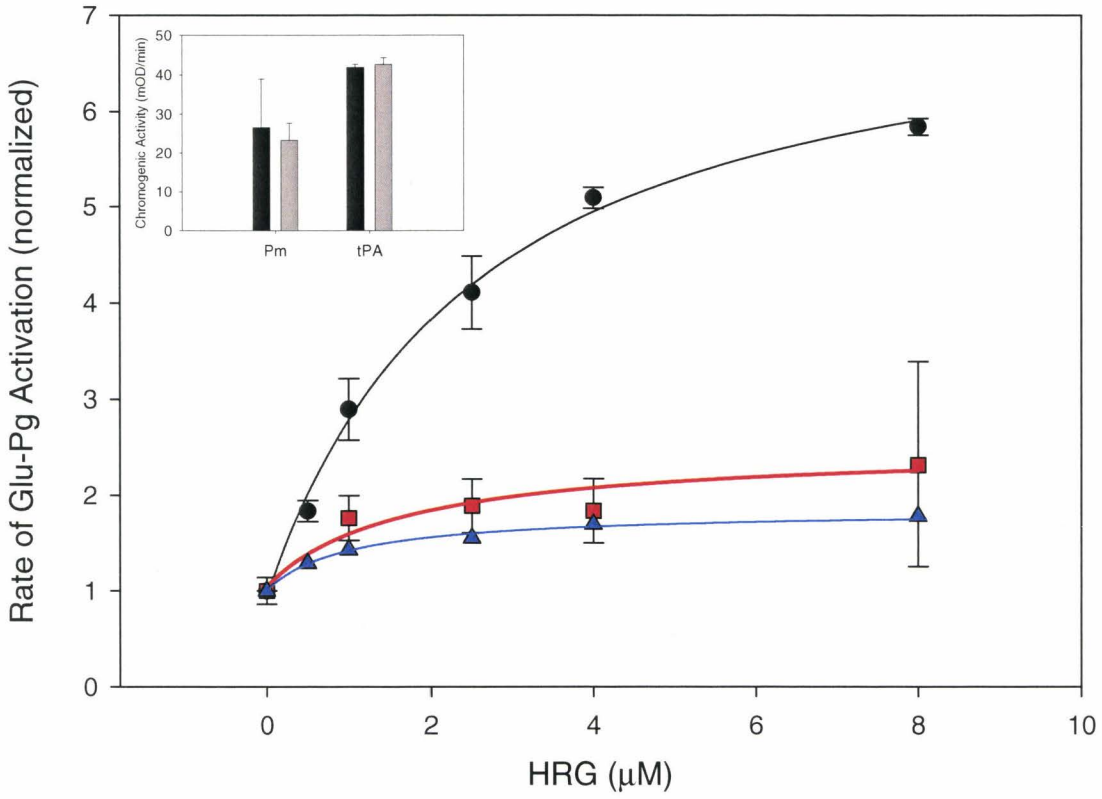


Figure 11. Influence of HRG on Glu-Pg activation by tPA. Pg activation was initiated by the addition of 100 nM tPA to a mixture of 1.2 μM Glu-Pg, 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 0-8 μM HRG. Pm activity was detected by hydrolysis of 200 μM S-2251. Three different HRG preparations were assessed for their effect on tPA mediated Pg activation, Feb 3/06 (●), May 30/06 (■) and HRG July12/06 (▲). Rates of Pg activation were determined using linear regression of mOD vs time squared plots. The data represent the mean of three duplicate experiments and standard error is shown for each. The inset shows the effect of HRG on 50 nM Pm and 50 nM tPA chromogenic activity assessed by measuring the hydrolysis of S-2251 and Pefachrome XIIa, respectively, in the absence (black bar) and presence of 2 μM HRG (grey bar).

We then went on to examine whether HRG modulates Glu-Pg activation by other Pg activators. The effect of HRG on Pg activation by uPA or FXIIa was assessed in a chromogenic assay in the presence of 12.5 μM $ZnCl_2$, 2 mM CaCl2 and increasing concentrations of HRG. HRG caused a dose-dependent decrease in the rate of uPA-mediated Pg activation, with half maximal inhibition (IC_{50}) observed at 662 ± 74.8 nM HRG (Figure 12). Maximum inhibition of Pg activation was greater than 95% at 5 μM HRG. HRG, at a concentration of 2 μM, did not affect uPA chromogenic activity with S-2444 (Figure 12, Inset).

Factor XIIa is believed to play a key role in fibrinolysis through the activation of Pg (Braat *et al.*, 1999). In order to evaluate the efficiency of FXIIa activation of Pg, the PA activity of both tPA and FXIIa were compared at 100 nM in a kinetic assay. Rates of Pg activation were determined by measuring the hydrolysis of the chromogenic substrate S-2251 (not shown). The rate of Pg activation by FXIIa was approximately 2.4-fold higher than that by an equal molar concentration of tPA. Because the plasma concentration of FXII is about 100-fold higher than tPA, these results raise the possibility that FXIIa may play a role in Pg activation in the absence of fibrin.

The ability of HRG to modulate FXIIa activation of Glu-Pg was subsequently investigated in more detail. Activation of 1.2 μM Glu-Pg by 100 nM FXIIa was monitored in the presence of 12.5 μM $ZnCl_2$ and 0-2 μM HRG (Figure 13). The effect of HRG on the rate of Pg activation was dose-dependent and half maximal inhibition was observed at 125 ± 32.6 nM HRG. The effect of HRG on FXIIa-mediated Pg activation

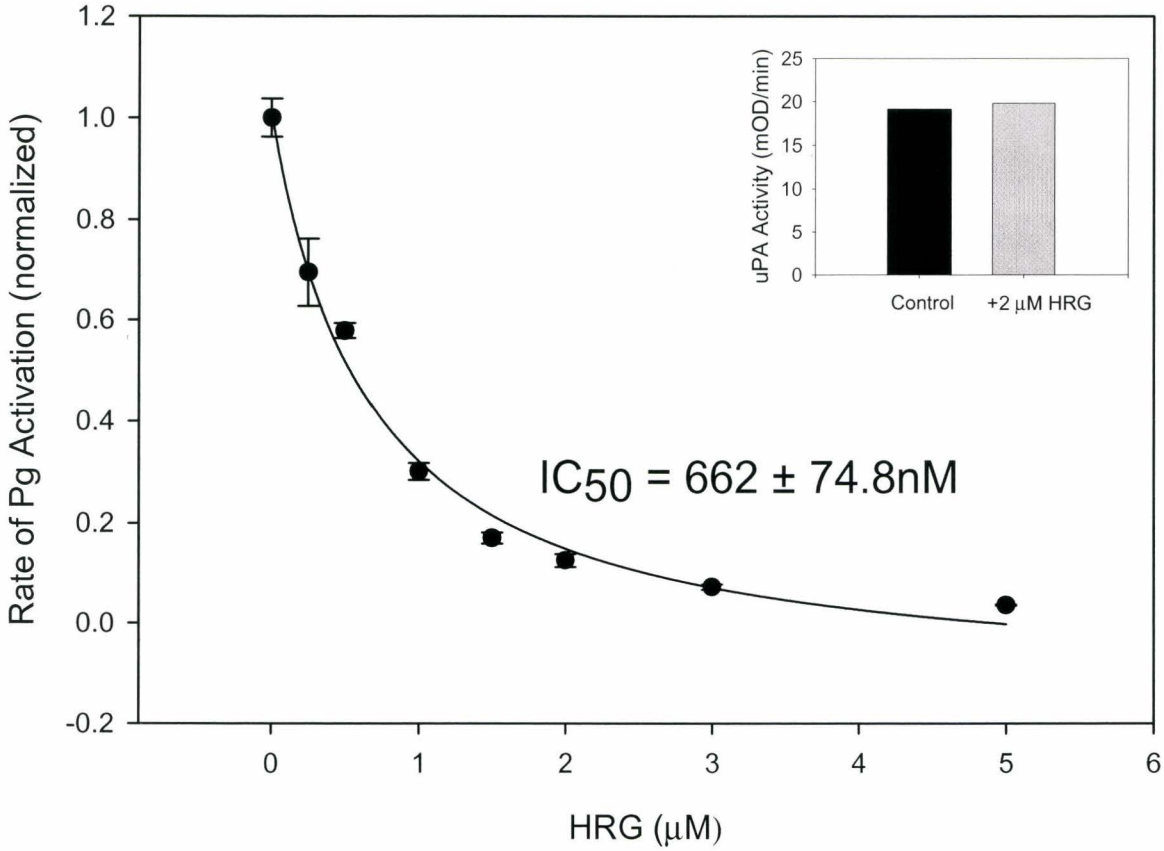


Figure 12. Influence of HRG on Glu-Pg activation by uPA. Pg (1.2 μM) was activated by 4 U/ml uPA in the presence of 12.5 μM $ZnCl_2$ and increasing concentrations of HRG. Pm activity was quantified by hydrolysis of 200 μM S-2251 and rates of activation were determined as previously described. The data were fit to a rectangular hyperbola equation to determine the concentration of HRG that causes half maximal inhibition (IC_{50}). Data points represent the mean of at least three experiments and standard error is shown. The inset shows the effect 2 μM HRG on uPA chromogenic activity measured by the hydrolysis of 600 μM S-2366.

was also measured in the absence of $ZnCl_2$, which gave a similar of IC_{50} value of 160 ± 55.6 nM. However, the overall rate of Pg activation by FXIIa in the absence of $ZnCl_2$ was significantly lower (data not shown). With uPA, the effect of HRG on Pg activation demonstrated an IC_{50} value of 662 ± 74.8 nM (Figure 12). The lower IC_{50} value observed with FXIIa-mediated Pg activation could suggest that HRG interacts with both FXIIa and Pg, affecting FXIIa activity, as well as the activation of Pg. HRG inhibition of Pg activation by FXIIa appears to be saturable, achieving greater than 80% inhibition of Pg activation at 2 μM HRG in the absence or presence of $ZnCl_2$. HRG does not alter FXIIa chromogenic activity against S-2222 (Figure 13, Inset). In addition, dextran sulfate was not included in Pg activation assays, because contrary a previous reported (Braat *et al.*, 1999), it did not enhance Pg activation by FXIIa (data not shown). The ability of HRG to bind and inhibit FXIIa PA activity in a purified system is a novel finding. These results may help to explain the controversy that surrounds FXIIa as a plasminogen activator because HRG is present in plasma and may alter FXIIa function. We then went on to determine whether HRG inhibits FXIIa activation of Pg and subsequent lysis of a fibrin clot.

3.4 Influence of HRG on FXIIa-Mediated Lysis of Fibrin Clots

Having confirmed that HRG inhibits Pg activation by FXIIa (Figure 13), it was of interest to determine whether HRG had the same effect on FXII-mediated Pg activation in a clot lysis assay. Samples containing 2 μM FXIII-free Fg, 10 μM $ZnCl_2$, 2 mM $CaCl_2$, 1.2 μM Glu-Pg, 10 μg/ml dextran sulfate, 0-3 μM HRG and 100 nM FXII

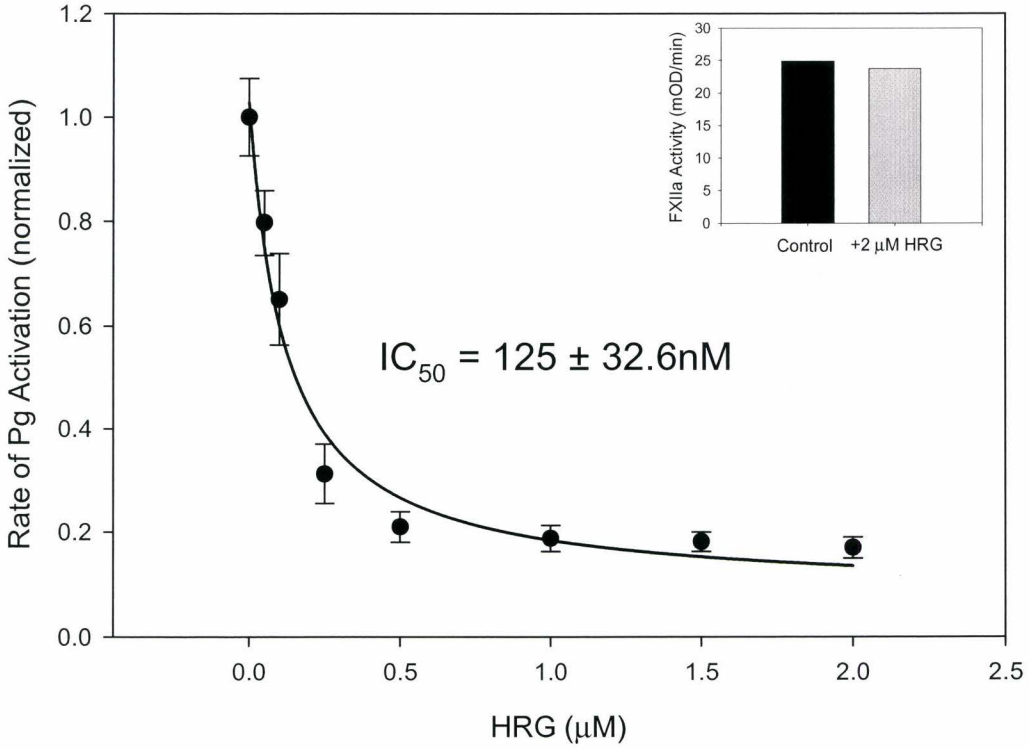


Figure 13. Influence of HRG on Glu-Pg activation by FXIIa. Pg (1.2 μM) was activated by 100 nM FXIIa in the presence of 12.5 μM $ZnCl_2$ and increasing concentrations of HRG (•). Pm activity was quantified by hydrolysis of 200 μM S-2251 in a continuous chromogenic assay. The data points represent the mean of at least three experiments. Data were analyzed by non-linear regression of a rectangular hyperbola equation to determine IC_{50} values. The inset shows the effect of 2 μM HRG on 50 nM FXIIa chromogenic activity by measuring the hydrolysis of 200 μM S-2222.

were clotted with 20 nM thrombin. Turbidity was monitored at 340 nm to observe clot formation and lysis (Figure 14). When clot formation occurs, the turbidity of the sample increases, which is detected as an increase in OD. When clot turbidity reaches a maximum, there is a plateau in the OD. As Pg is converted to Pm and the Pm degrades the fibrin, there is a decrease in turbidity as the fibrin undergoes lysis. Lysis time was defined as the time required to decrease the maximal absorbance by 50%. Lysis times were prolonged in a dose-dependent manner as the concentration of HRG was increased. At 2.5 μM HRG, clots did not lyse over the six hours that absorbance was monitored, suggesting that FXIIa-mediated Pg activation was completely inhibited. HRG was also able to inhibit clot lysis when initiated with FXIIa (Figure 15). HRG displayed similar inhibition of FXIIa-mediated clot lysis, with dose-dependent inhibition with increasing HRG concentrations and maximal inhibition observed at 1.5-2 μM HRG.

To ensure that the inhibitory effect on FXII/FXIIa mediated clot lysis is due to HRG inhibition of FXIIa activity and not due to Pm inhibition, the effect of HRG on lysis of fibrin clots by Pm also was assessed (Figure 16). Clots were preformed by incubation of 2 μM FXIII-free Fg with 20 nM thrombin for 30 min at 25°C in the presence of 2 mM $CaCl_2$, 10 μM $ZnCl_2$ and 2 μg/ml dextran sulfate. Lysis was then initiated by overlaying a mixture of 150 nM Pm and increasing concentrations (0-3 μM) of HRG on the preformed fibrin clots. Lysis was monitored by measuring turbidity. HRG promotes lysis in a dose-dependent and saturable fashion, with an EC_{50} of 66.7 ± 20.3 nM. Lysis times were reduced by approximately 30%. Since HRG does not inhibit clot lysis by Pm

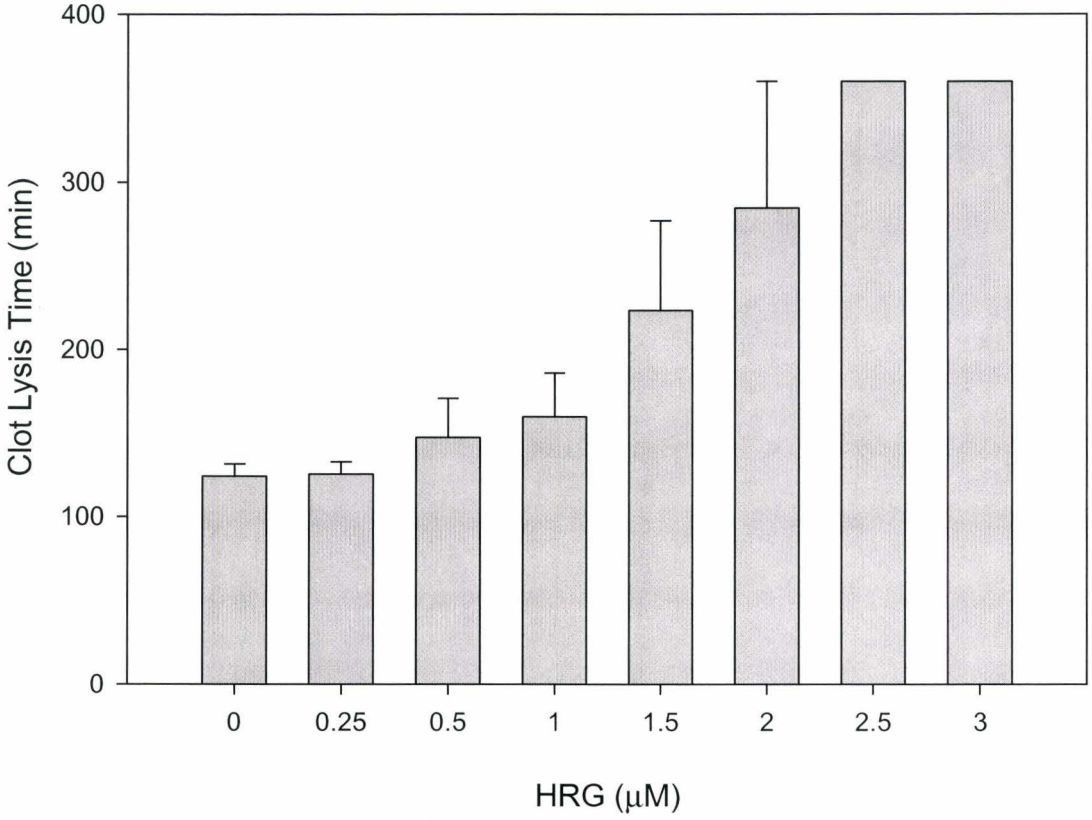


Figure 14. Effect of HRG on FXII-mediated clot lysis. Samples containing 1.2 μM Pg, 2 μg/ml dextran sulfate, 2 μM FXIII-free Fg, 2 mM $CaCl_2$, 10 μM $ZnCl_2$ and varying concentrations of HRG (0-3 μM) in HBS buffer were placed in individual wells of a microtiter plate. A mixture of 20 nM thrombin and 100 nM FXII was added to each well to initiate clotting and clot turbidity was monitored for 360 min at 340 nm in a plate reader. Clot lysis times were determined by calculating the time at which the maximum turbidity had decreased by half and were plotted against HRG concentration. Data points represent the average of two independent clot lysis experiments performed in duplicate and standard errors are shown for each condition.

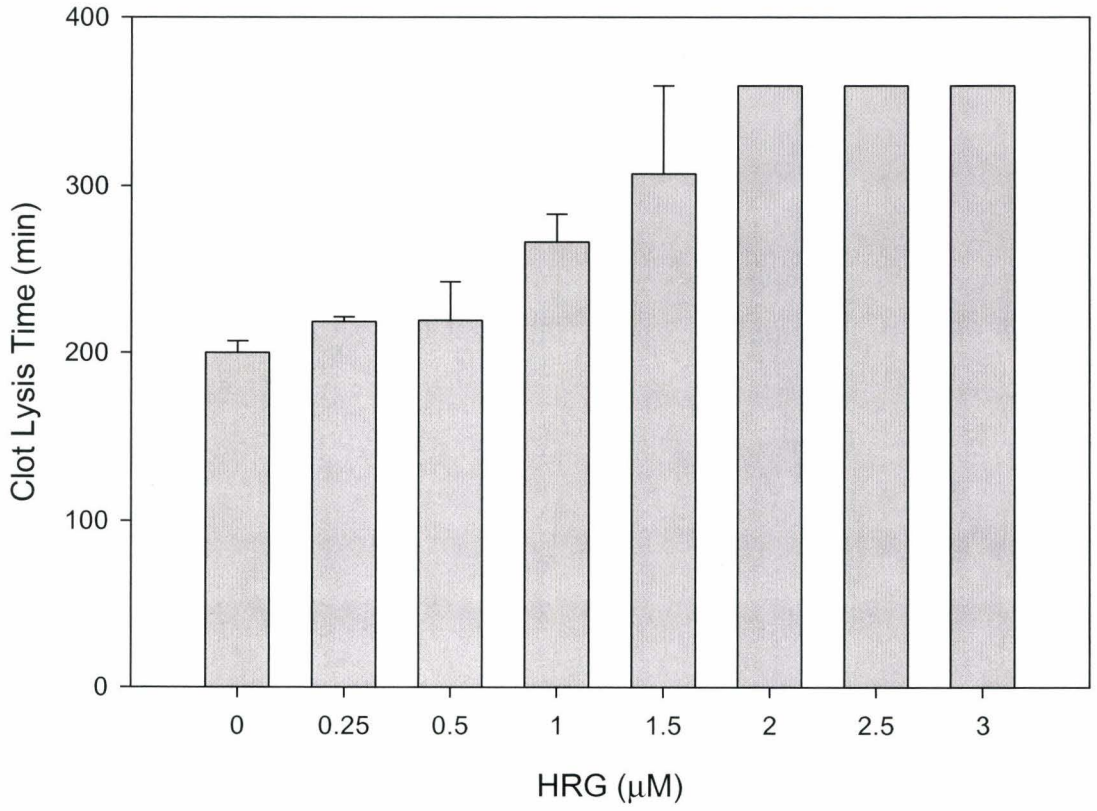


Figure 15. Effect of HRG on FXIIa-mediated clot lysis. Samples containing 1.2 μM Pg, 2 μg/ml dextran sulfate, 2 μM FXIII-free Fg, 2 mM $CaCl_2$, 10 μM $ZnCl_2$ and varying concentrations of HRG (0-3 μM) were placed in individual wells of a microtiter plate. A mixture of 20 nM IIa and 100 nM FXIIa was added to each well to initiate clotting and clot turbidity was monitored for 360 min at 340 nm in a plate reader. Clot lysis times were determined by calculating the time at which the maximum turbidity had decreased by half and were plotted against HRG concentration. Data points represent a single clot lysis experiment done in duplicate and standard error is shown.

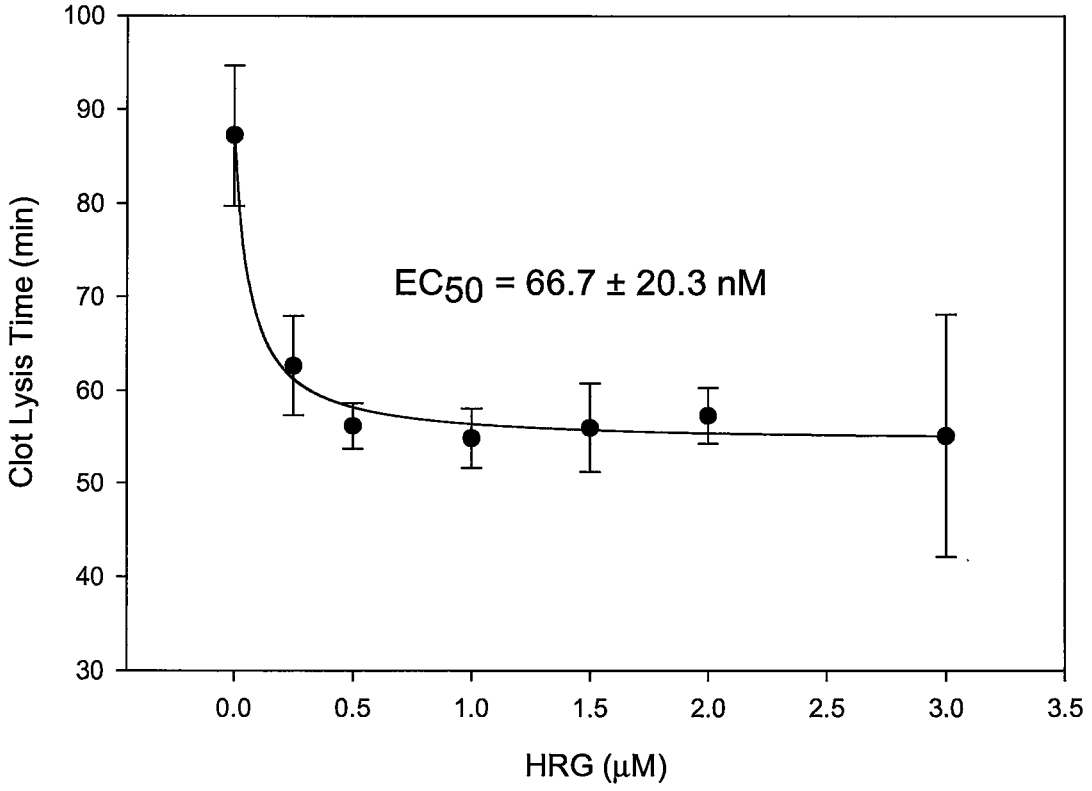


Figure 16. Effect of HRG on Pm-mediated clot lysis. Samples containing 2 μM FXIII-free Fg, 10 μM $ZnCl_2$, 2 mM $CaCl_2$, 2 μg/ml dextran sulfate in HBS buffer were placed in individual wells of a microplate and clotted with 20 nM IIa for 30 min at 37°C. Clot lysis was initiated by overlaying a mixture of 150 nM Pm containing increasing concentrations (0-3 μM) HRG in each well. Turbidity was monitored at 340 nm and clot lysis times were determined by calculating the time at which the turbidity had decreased by half. The data were fit to a rectangular hyperbola equation to determine the half maximal effective concentration of HRG (EC_{50}). The data points represent the average of two independent clot lysis experiments done in duplicate and SEM is shown for each concentration of HRG.

and in fact enhances this reaction, its inhibitory effect on FXII mediated clot lysis appears to be FXIIa-specific. Since it has been established that HRG does not affect Pm chromogenic activity (Figure 11, Inset), it may be concluded that HRG inhibits FXIIa PA activity as demonstrated in a Pg activation experiment and confirmed in this clot lysis experiment.

3.5 Binding of HRG to Glu-Pg and Its Physiological Activators

Previous research has demonstrated a strong interaction of HRG with Pg. HRG is reported to bind to the kringle domains of Pg with a K_d value of 72.4 ± 5.4 nM in the presence of $ZnCl_2$ through its NH_2- and COOH-terminal domains (Borza and Morgan, 1997). To confirm these findings, SPR was used to characterize the binding affinity of HRG with Glu-Pg. Since Zn^{2+} plays an important role in modulating HRG function, the HRG-Pg interaction was measured in the absence or presence of $ZnCl_2$. Biotinylated-HRG was immobilized on the strepdavidin-coated surface of a biosensor chip. Increasing concentrations of Pg (80-2560 nM) were injected across the HRG-immobilized surface. A blank streptavidin-coated cell served as a control. After each concentration of Pg was injected, regeneration buffer (HBS-Tw20 containing 20 mM EACA) was injected across the HRG-immobilized surface to remove any HRG-bound Pg. K_d values were determined by calculating the average rate of association and dissociation by using a global fit analysis of the sensorgrams to a 1:1 Langmuir interaction model for each Pg concentration (Figure 17). In the absence of $ZnCl_2$, HRG bound Pg with a K_d of 155 ±

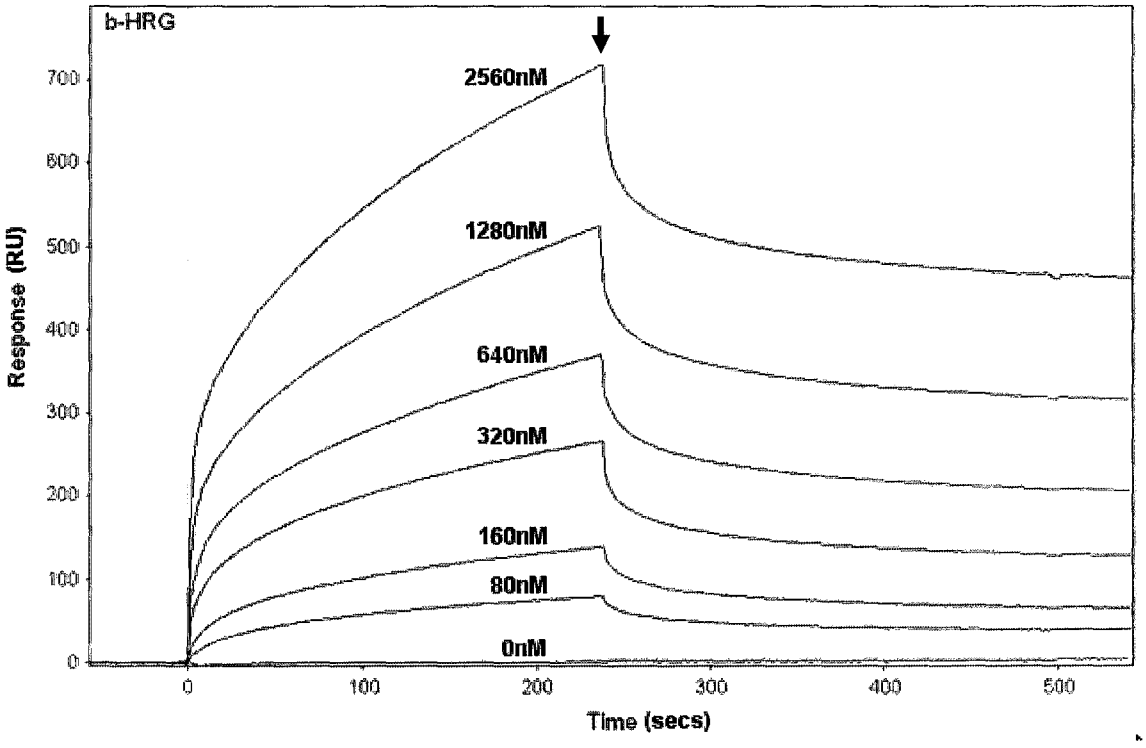


Figure 17. SPR analysis of Glu-Pg binding to immobilized-HRG in the presence of 12.5 μM $ZnCl_2$. Biotin-HRG (800-900 response units) was adsorbed onto a streptavidin-coated sensor chip. Increasing concentrations of Glu-Pg (0-2560 nM) were passed over the chip, which was followed by the injection of running buffer at 250 seconds to monitor dissociation (↓). A reference flow cell lacking biotin-HRG was used to correct for non-specific binding (background signal). Corrected response units (RU) are plotted versus time and K_d values were calculated by dividing the average rate of dissociation at each Pg concentration by the average rate of association.

46.8 nM (Table 1). In the presence of 12.5 μM $ZnCl_2$, HRG bound Pg with a K_d of 135 ± 54.5 nM, suggesting that $ZnCl_2$ does not affect the HRG-Pg interaction. The K_d values we determined are comparable to the published values (Borza and Morgan, 1997). HRG-bound Pg could be effectively displaced by exposure to a buffer containing 20mM EACA, suggesting that the interaction is likely mediated by lysine residues (Borza and Morgan, 1997).

3.5.1 *Interaction of HRG with tPA and uPA*

Binding interactions of HRG with other ligands and the role of $ZnCl_2$ in these interactions also were characterized by SPR, using the methods described above (Table 1). The interaction between HRG and uPA was assessed to determine whether the effect of HRG on Pg activation by uPA reflects an HRG-uPA interaction. HRG binding to tPA was also assessed and treated as a negative control, since HRG had no effect on tPA-mediated Pg activation. As expected, no binding to tPA was detected. HRG appears to bind uPA with relatively weak affinity, which was estimated to be in the micromolar range (data not shown). A K_d value could not be calculated for the interaction of uPA with HRG because the binding response units were very low. This could be due to an incorrect orientation of the immobilized biotinylated-HRG on the chip surface or the inability of the analyte protein to access its binding site on the ligand.

3.5.2 *Interaction of HRG with FXIIa*

The binding interaction of HRG to the third Pg activator, FXIIa, was assessed next. The interaction of increasing concentrations of FXIIa over a range of 0-1024 nM

Table 1: HRG Binding Interactions and Affinities for Various Ligands in the Absence and Presence of $ZnCl_2$[a]

Ligand	Region	K_d (μM)	
		0 μM $ZnCl_2$	12.5 μM $ZnCl_2$
Glu-Pg	COOH &/ NH_2-terminus	155 ± 46.8 nM	135 ± 54.5 nM
Heparin	HRR & NH_2-terminus	1.4 ± 0.15 μM	1.9 ± 0.15 nM
FXII	-----	NB	NB
FXIIa	HRR	14.5 ± 4.5 nM	56 ± 8.9 pM

[a] K_d values for binding to biotinylated-HRG were determined by SPR using a streptavidin-coated sensor chip. In each case, HRG was the immobilized protein except when using heparin. Biotin-labelled heparin was immobilized to the streptavidin-coated surface to determine the affinity of HRG for heparin. Based on the regeneration buffer used to disrupt ligand-analyte interactions, the binding region of HRG to its various ligands was also assessed. HBS-Tw20 containing 20 mM EACA was used to disrupt Glu-Pg and HRG interactions and HBS-Tw20 containing 250 mM imidazole was used to disrupt FXIIa-HRG interactions and HBS-Tw20 containing 20 mM EACA and 250 mM imidazole was used to disrupt heparin-HRG interactions. All K_d values given are the average of two or more experiments, including standard error. NB: no binding detected.

with the immobilized HRG surface was measured in the absence or presence of 12.5 μM $ZnCl_2$. HRG bound FXIIa with high affinity in the absence of $ZnCl_2$, with a calculated K_d of 14.5 ± 4.5 nM. In the presence of $ZnCl_2$, the affinity of HRG for FXIIa increased almost 1000-fold, yielding a K_d value of 56 ± 8.9 pM. Consistent with this high affinity interaction, binding occurred rapidly and there was almost no dissociation of FXIIa after the injection of buffer (Figure 18). This interaction was inhibited by 250 mM imidazole (data not shown), which suggests that FXIIa binds to the HRR of HRG.

To further characterize the binding interaction between HRG and FXIIa, a competition experiment was performed. In preliminary experiments designed to characterize HRG, HRG was observed to bind to immobilized heparin with K_d values of 1.4 ± 0.15 μM and 1.9 ± 0.3 nM in the absence and presence of $ZnCl_2$, respectively (Table 1). This interaction takes place through the HRR because binding was inhibited by 250 mM imidazole (data not shown). In the next experiment, biotin-heparin (~200 RU) was adsorbed onto a streptavidin-coated sensor chip. Samples containing 250 nM HRG and increasing concentrations of either (0-400 nM) FXIIa or FXII were passed over the heparin-surface and the binding of HRG to heparin was assessed (data not shown). Neither FXIIa nor FXII binds to heparin (data not shown). Therefore, it is only the binding of HRG to heparin that is measured. Competition experiments demonstrated that, at a concentration greater than 100 nM, FXIIa inhibits the interaction between HRG and heparin (data not shown). When this experiment was repeated with FXII, there was no inhibition of HRG binding to heparin (data not shown). Since heparin and FXIIa are

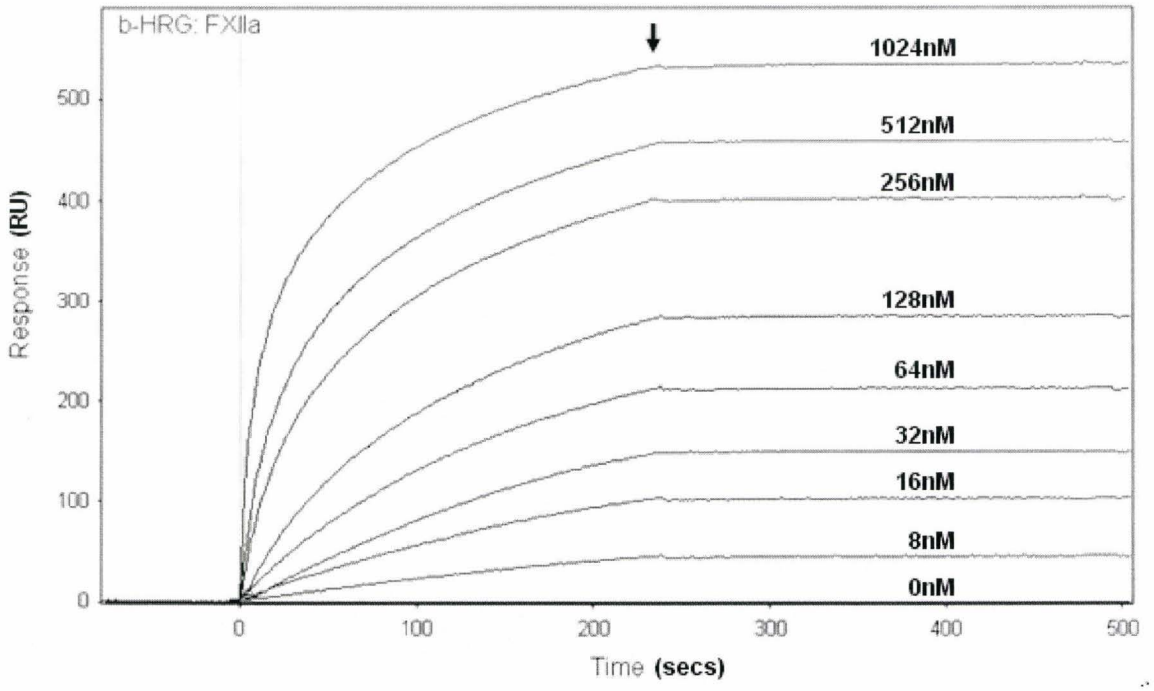


Figure 18. SPR Analysis of FXIIa binding to immobilized-HRG in the presence of 12.5 μM $ZnCl_2$. Biotin-HRG (800-900 response units) was adsorbed onto a streptavidin-coated sensor chip. Increasing concentrations of FXIIa, ranging from 0-1024nM, were passed over the HRG surface as well as over a blank reference flow cell. FXIIa association to the chip surface was monitored for 4 min followed by buffer (↓) to monitor FXIIa dissociation. Corrected response units are plotted versus time.

both believed to interact with the HRR of HRG, it appears that the binding of FXIIa to HRG blocks the interaction of HRG with heparin. This finding suggests that, in the presence of $ZnCl_2$, the mechanism of HRG inhibition of FXIIa activity is mediated by the HRR. This concept fits with the notion that $ZnCl_2$ binds to histidine residues in the HRR and induces the exposure of a unique site that enhances ligand binding.

SPR studies indicate that HRG interacts with FXIIa through its HRR, but it is unknown where HRG binds to FXIIa and why HRG does not bind to FXII. Chromogenic studies assessing the effect of HRG on FXIIa activity indicate that HRG does not influence FXIIa activity (Figure 13, Inset). Therefore, it does not appear to bind to the active site of FXIIa. To confirm the results observed with S-2222, another chromogenic and a fluorescent substrate were used. FXIIa activity was assessed in the presence of the chromogenic substrate, Pefachrome XIIa and the fluorescent substrate, PFR-AMC in the absence or presence of 2 μM HRG (Figure 19). HRG does not affect the hydrolysis of Pefachrome XIIa by FXIIa, but HRG does enhance cleavage of PFR-AMC by approximately 40%. Although HRG has an affect on the cleavage of PFR-AMC by FXIIa, it does not affect the cleavage of S-2222 or Pefachrome FXIIa. Therefore, we can be confident that HRG does not bind to the active site of FXIIa, since HRG did not inhibit FXIIa activity in a chromogenic assay using these two chromogenic substrates. HRG must bind to an alterative site on FXIIa, which subsequently becomes exposed when the zymogen becomes activated.

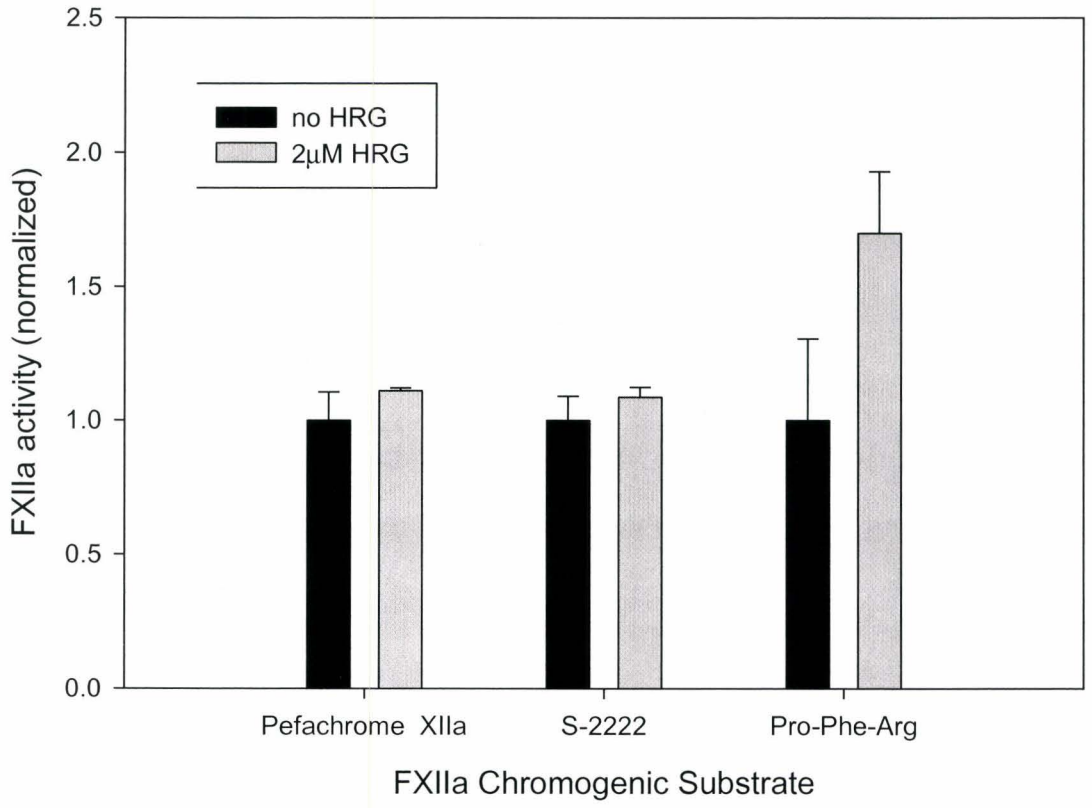


Figure 19. Effect of HRG on FXIIa Activity. FXIIa activity was assessed in the presence of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 2 μg/ml dextran sulfate in the absence (black bar) and presence (grey bar) of 2 μM HRG. FXIIa activity was quantified by the hydrolysis of 200 μM Pefachrome XIIa and S-2222 in a chromogenic assay and with PFR-AMC (Pro-Phe-Arg) in a fluorescence assay. The data represent the mean of two separate experiments and standard error is shown for each.

3.6 Effect of HRG on Reactions Involved in Contact Activation

3.6.1 *Influence of HRG on FXII Activation*

Because HRG was found to bind FXIIa and modify its activity, it was of interest to determine whether HRG has any influence on the contact pathway. Initially, the effect of HRG on FXII autoactivation was investigated. FXII autoactivation was stimulated by 2 μg/ml dextran sulfate in the presence of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 0-1.6 μM HRG. FXII activation was monitored by measuring the hydrolysis of a FXIIa-directed chromogenic substrate (S-2222) and the rate of autoactivation was determined by linear regression of a time squared plot. HRG inhibits FXII autoactivation by 50% from 50-200 nM and by 100% at concentrations greater than 400 nM (Figure 20). These results suggest that HRG modulates FXII autoactivation exhibiting an apparent threshold effect rather than a dose-dependent inhibitory effect. However, these results are inconsistent with the high affinity of HRG for FXIIa observed by SPR. It is possible that there is a contaminant in our HRG preparation that inhibits autoactivation of FXII when HRG is added at concentrations higher than 200 nM. Future studies could be conducted in the presence of an alternative potentiating surface to assess the effect of HRG on FXII autoactivation.

In order to further characterize the effect of HRG on FXII activation, FXII activation by PKa was analyzed. FXII activation by PKa was analyzed by gel analysis rather than in an activity assay. This was necessary because CTI was added to inhibit FXIIa-mediated autoactivation of FXII. FXII activation was initiated by 100 nM PKa in the presence of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 2 μg/ml dextran sulfate in the absence

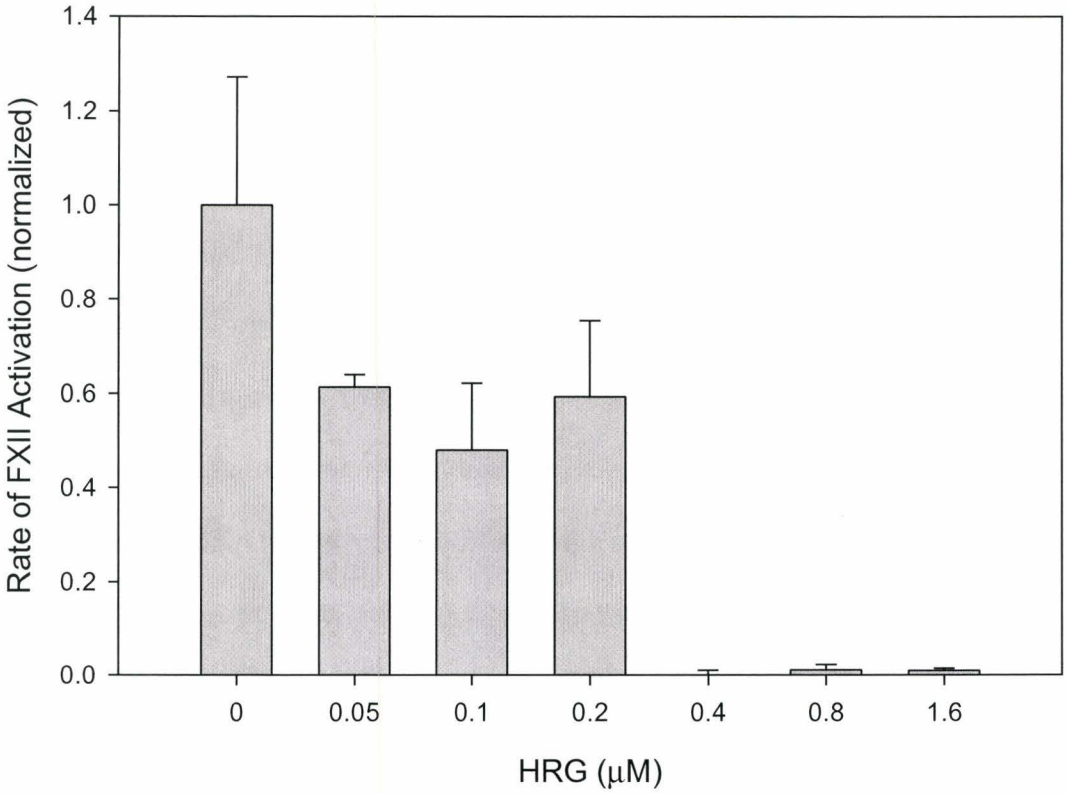


Figure 20. Influence of HRG on FXII autoactivation in the presence of dextran sulfate. FXII autoactivation was initiated by the addition of 100 nM FXII to a mixture of 2 μg/ml dextran sulfate (500 kDa), increasing concentrations (0-1.6 μM) of HRG, 2 mM $CaCl_2$, 12.5 μM $ZnCl_2$, and 200 μM S-2222 in HBS buffer. FXII activation was monitored by FXIIa hydrolysis of S-2222 and rates of activation were determined by linear regression of a time squared plot. Each bar represents the average of at least two separate experiments and the standard error is shown for each concentration of HRG.

or presence of 2 μM HRG. Aliquots were removed at intervals (0-2 hrs) and the extent of FXII activation was assessed by SDS-PAGE analysis. Activation was measured by determining the densities of individual bands by densitometry and calculated as a percentage of total integrated optical density (IOD) within each lane (Figure 21). In the absence of HRG, there was greater than 40% activation of FXII by PKa over a two-hour period, as measured by the disappearance of the 80 kDa band. However, in the presence of HRG, there was only about 20% activation of FXII over this time period. Appearance of the heavy and the light chains of FXIIa coincided with the disappearance of the FXII band (data not shown). These results suggest that HRG, at physiological concentrations, is able to inhibit the activation of FXII by PKa. This may limit the amplification of the contact pathway by inhibiting feedback activation of FXII by PKa and by attenuating FXII autoactivation.

3.6.2 *Influence of HRG on FXI Activation by FXIIa*

To further investigate HRG modulation of FXIIa activity, the effect of HRG on other aspects of the contact pathway was assessed. FXI is the downstream substrate of FXIIa in the coagulation cascade (Tans and Rosing, 1987). The effect of HRG on FXI activation by FXIIa was assessed in a continuous chromogenic assay using a FXIa-directed substrate. A mixture of 2 nM FXIIa, 12.5 μM $ZnCl_2$ and 0-2 μM HRG was incubated for 10 minutes before addition of 60 nM FXI in the presence of 2 μg/ml dextran sulfate and 2 mM $CaCl_2$. The rate of FXIa activation was determined by monitoring the hydrolysis of 200 μM S-2366. Background levels of FXIIa substrate

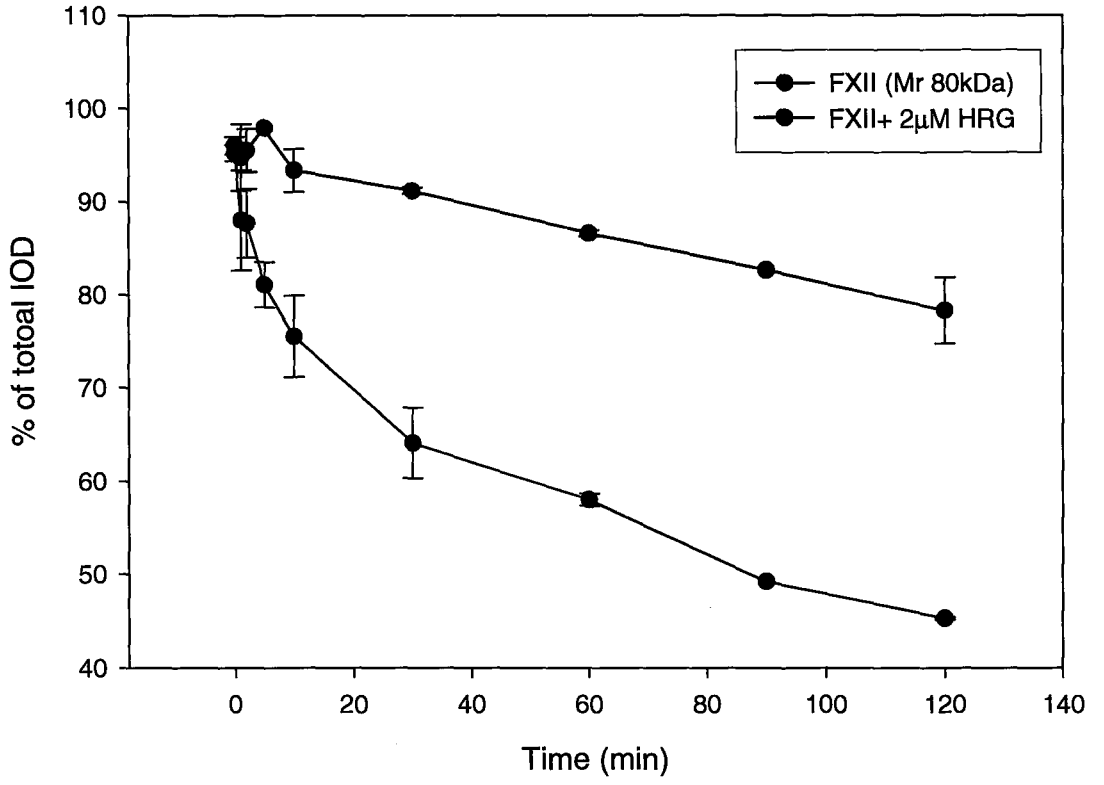


Figure 21. Influence of HRG on FXII activation by PKa. In samples containing 2.5 μM FXII, 12.5 μM CTI, 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$, FXII activation was initiated by the addition of 100 nM PKa and 2 μg/ml dextran sulfate in the absence (black) or presence (red) of 1 μM HRG in HBS. Aliquots of 10 μl were removed at each time point and subjected to electrophoresis in 4-15% polyacrylamide gels under reduced conditions. PAGE gels were stained with Fast Stain and protein concentrations of the FXII bands at each time point were determined by densitometry to determine the percentage of total integrated optical density (IOD) values. Each point represents the mean of two separate experiments and SEM as shown.

hydrolysis were subtracted. HRG had a potent inhibitory effect on FXI activation by FXIIa, with half maximal inhibition observed at 15 ± 1.2 nM HRG (Figure 22). At 500 nM HRG, FXI activation was inhibited by over 95%. In a control experiment, 2 μM HRG did not affect FXI chromogenic activity with S-2366 (Figure 22, Inset), demonstrating that HRG inhibition is FXIIa-specific. These results are consistent with the inhibitory effect of HRG on FXIIa activity. Given the potent inhibitory effect of HRG in this system it is possible that HRG interacts with both FXIIa and FXI to inhibit FXI activation by FXIIa. In addition, the IC_{50} value of HRG for this reaction may be even lower because the rate of FXI activation by FXIIa was not tested at concentrations less than 50 nM HRG. It will be important to assess rates of FXI activation at HRG concentrations lower than 50 nM to calculate an accurate IC_{50} value.

3.6.3 *Influence of HRG on PK activation by FXIIa*

Another downstream substrate of FXIIa in the contact activation pathway is PK (Tans and Rosing, 1987). The effect of HRG on FXIIa-mediated PK activation was analyzed in the presence of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$, 2 μg/ml dextran sulfate and 0-30 μM HRG (Figure 23). PKa activity was assessed by monitoring the hydrolysis of 400 μM Pefachrome VIIa in a continuous chromogenic assay. HRG increased the rate of PK activation by approximately 3.5 fold, but only at concentrations much higher than physiological levels. The effect of HRG appears to be saturable with an EC_{50} of 3.2 ± 0.28 μM. As a control, PK was tested for the presence of PKa by monitoring the

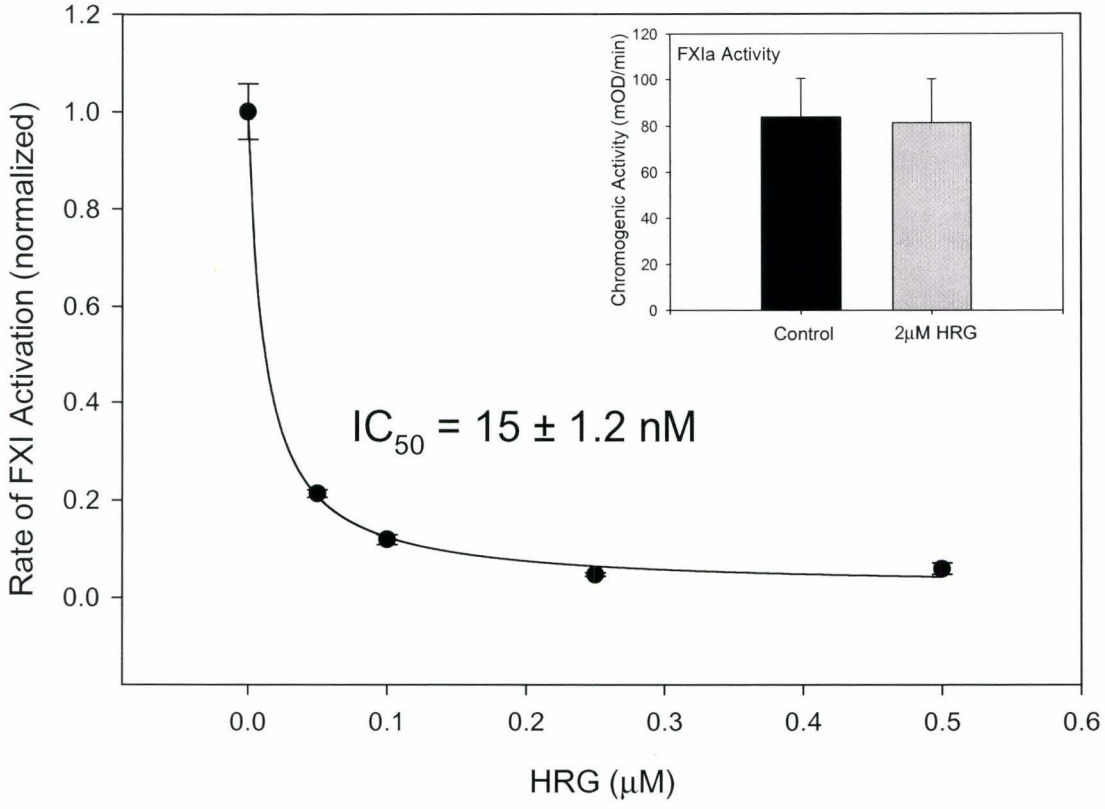


Figure 22. Influence of HRG on FXI activation by FXIIa. FXIIa (2 nM) was incubated with 12.5 μM $ZnCl_2$ and 0 to 500 nM HRG for 10 min and added to a mixture of 60 nM FXI, 2 mM $CaCl_2$, 2 μg/ml dextran and 200 μM S-2366. FXI activation was determined by FXIa hydrolysis of S-2366 and rates of activation were calculated by non-linear regression of a time squared plot and fit to a rectangular hyperbola equation to determine half-maximal inhibition, as described in the Methods section. The data points represent the mean of two duplicate experiments, whereas the error bars represent SEM. FXI chromogenic activity was assessed by measuring the hydrolysis of S-2366 in the presence of 2 μg/ml dextran sulfate, 2 mM $CaCl_2$, 12.5 μM $ZnCl_2$ in the absence (control) and presence of 2 μM HRG (inset).

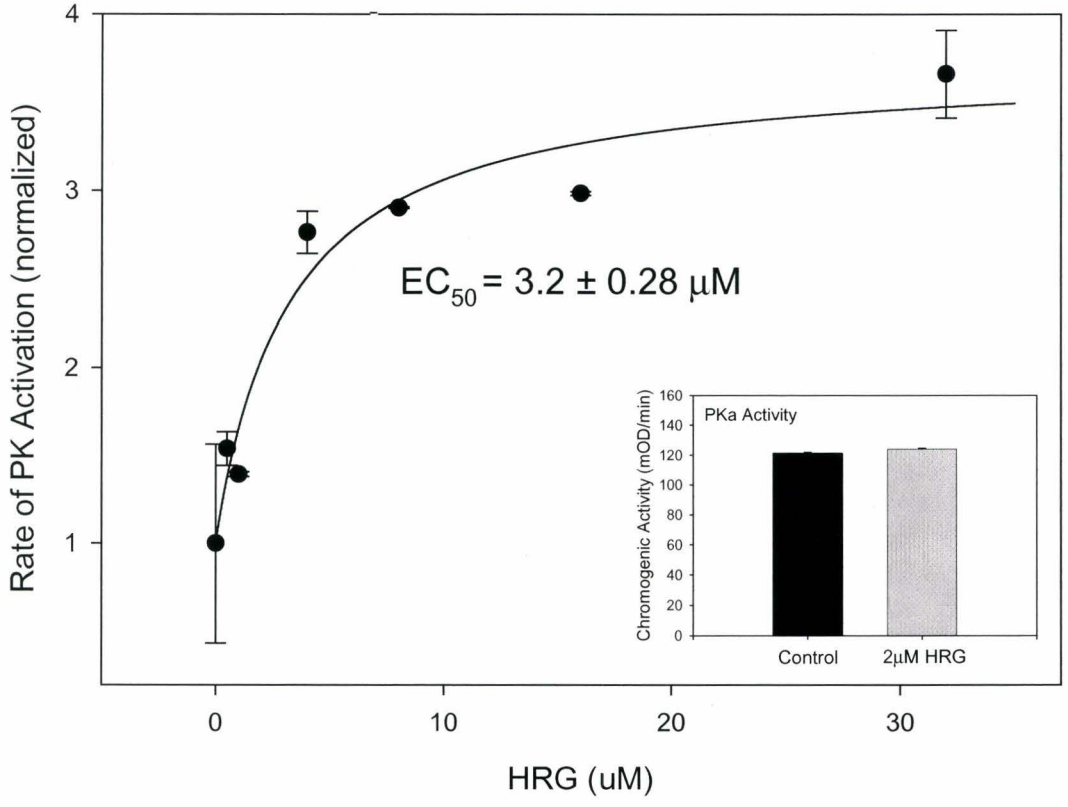


Figure 23. Influence of HRG on PK activation by FXIIa. FXIIa (0.05 nM) was incubated with 12.5 µM $ZnCl_2$ and 0 to 30 µM HRG for 10 min and added to a mixture of 4 µM PK, 2 mM $CaCl_2$, 2 µg/ml dextran and 400 µM Pefachrome VIIa. PK activation was measured by PKa hydrolysis of Pefachrome VIIa and rates of activation were calculated by non-linear regression of a time squared plot and fit to a rectangular hyperbola equation to determine an EC_{50} value, as described in the Methods section. The data points represent the mean of two duplicate experiments, whereas the error bars represent SEM. PKa chromogenic activity was assessed by measuring the hydrolysis of Pefachrome VIIa in the presence of 2µg/ml dextran sulfate, 2 mM $CaCl_2$, 12.5 µM $ZnCl_2$ in the absence (control) and presence of 2 µM HRG (inset).

hydrolysis of Pefachrome VIIa, which demonstrated chromogenic activity due to the presence of contaminating PKa. PKa activity observed in the PK preparation was subtracted from PKa activity in FXIIa-mediated PK activation experiments. HRG does not affect PKa chromogenic activity with Pefachrome VIIa (Figure 23, Inset). The effect of HRG on this FXIIa-mediated reaction is not consistent with the inhibitory effect of HRG on FXIIa activity in previous experiments.

3.6.4 *Binding of HRG to Contact Activation Proteins, FXI/FXIa and PKa*

Previous experiments showed that HRG interacts with FXIIa with a very high affinity in the presence of $ZnCl_2$ (Figure 17). Interestingly, HRG does not bind to FXII. HRG also affects FXIIa activity in both FXI and PK activation experiments. To further characterize the interaction of HRG with these proteins and its mechanism of inhibition, HRG binding to FXI/FXIa and PKa was assessed by SPR.

Increasing concentrations of either FXI or FXIa were passed over the HRG-immobilized surface and binding interactions were assessed in the absence or presence of 12.5 μM $ZnCl_2$. No binding of FXI or FXIa to HRG was detected either in the absence or presence of $ZnCl_2$ (data not shown). These results suggest that HRG does not bind to FXI or FXIa. Therefore, the inhibitory effect of HRG on FXIIa activation of FXI is likely through its interaction with FXIIa.

PKa binding to HRG was assessed in the same manner as FXI/FXIa, where HRG was immobilized to the chip surface and the binding of increasing concentrations of PKa was assessed in the absence or presence of 12.5 μM $ZnCl_2$. Again, no binding of PKa to

HRG could be detected either in the absence or presence of $ZnCl_2$ (data not shown). The lack of HRG binding to PKa or FXII does not explain why HRG is able to inhibit FXII activation by PKa. However, these results do suggest that the effect of HRG on enhancing PK activation by FXIIa is through its interaction with FXIIa.

4 DISCUSSION

Pg circulates in the plasma as an inactive zymogen and is converted to its active form, Pm on the surface of a fibrin clot or on cell surfaces by physiological activators, tPA or uPA. Pm is a key fibrinolytic component in plasma that participates in the degradation and solubilization of fibrin clots. Fibrinolysis is a critical process that maintains vascular patency by restricting fibrin clot formation to prevent occlusion of the blood vessel. FXIIa is another activator of Pg, but the role of FXIIa as a Pg activator *in vivo* is not well understood.

HRG is an abundant plasma glycoprotein that was discovered over 30 years ago (Heimburger *et al.*, 1972). HRG has been shown to regulate a number of important biological processes, including fibrinolysis. Approximately 50% of Pg in the blood circulates in complex with HRG. However, there are conflicting data as to the effect of HRG on Pg activation. Thus, one study demonstrated that HRG enhances Pg activation by tPA when bound to GAGS, in the presence of $ZnCl_2$ or under conditions of low pH (Borza *et al.*, 2004). In contrast, another study found that HRG bound to circulating Pg blocks the ability of Pg to bind to fibrin, subsequently inhibiting Pg activation (Goodnough *et al.*, 1985). Thus, it is unclear whether HRG is antifibrinolytic, or profibrinolytic or exhibits both properties, depending on local factors or conditions. The fact that HRG is an abundant plasma protein that has a number of distinct ligand binding domains suggests that it must serve an important physiological role, possibly as an accessory protein.

The overall goal of this project was to better elucidate the function of HRG in fibrinolysis. The first step was to optimize the isolation of HRG to remove contaminants that may influence functional assays. The effects of HRG on fibrinolytic enzymes were analyzed initially. These experiments also considered the influence of $ZnCl_2$. Plasma levels of free Zn^{2+} are relatively low because circulating Zn^{2+} is bound to other proteins in the blood (Mocchegiani *et al.*, 2000). However, after vascular injury, activated platelets release large amounts of Zn^{2+}, which increases the Zn^{2+} concentration in the local microenvironment (Foley *et al.*, 1968). Many proteins involved in haemostasis, including HRG, utilize Zn^{2+} to carry out various catalytic reactions. Therefore, the influence of $ZnCl_2$ was considered on HRG in the interactions and functional assays conducted in this study. Binding interactions between HRG and fibrinolytic proteins were then assessed to provide insight into how HRG might modulate fibrinolysis. The influence of HRG on extrinsic activators, tPA and uPA, and on FXIIa was then quantified. HRG had the greatest effect on FXIIa-mediated Pg activation. Because HRG influenced FXIIa activity and FXIIa is the initiator of the contact pathway, subsequent studies explored the effect of HRG on other FXIIa-mediated reactions. The ability of HRG to modulate this alternate fibrinolytic pathway and important interactions of the contact pathway shed new light on a role for HRG as a modulator of haemostasis.

4.1 HRG Isolation & Characterization

HRG was isolated from fresh frozen human plasma using Ni-chelate affinity chromatography because a high content of histidine residues endows strong Ni-binding

affinity. HRG was isolated from plasma at a high yield following optimization of the purification procedures. Utilizing a one-step purification procedure, protein recovery of HRG from human plasma was approximately 90%. High molecular weight contaminating proteins that co-purified with earlier preparations of HRG were removed by a modification of the procedure (Borza and Morgan, 1997). This included making up all imidazole wash buffers in TBS, instead of distilled water, and adding an EACA wash step to remove contaminating Pg. In order to assess the integrity of HRG preparations, 20 μg of protein was subjected to SDS-PAGE analysis. This analysis revealed that the sample was at least 99% pure since no contaminating bands were observed. Gel analysis of 2 μg HRG samples revealed that the HRG preparation was actually composed of two closely migrating bands present in approximately equal proportions (Figure 9). Protein identification, by mass spectrometry of tryptic fragments, determined that both proteins were HRG-derived. NH_2-terminal sequencing of this doublet was not performed as part of the protein identification process. However, the tryptic fragments that originated from these bands both contained the expected COOH-termini. It is possible that the smaller of the two proteins identified as HRG may be missing a portion of its NH_2-terminus. In addition, HRG has 5 potential N-linked glycosylation sites, which account for 14% of the molecular mass of the protein (Heimburger *et al.*, 1972). Therefore, the difference in the molecular mass between the two HRG variants may reflect different glycoforms. Consistent with these findings, another group found that under non-reducing conditions, HRG migrates as a closely-spaced doublet; although they did not provide an explanation (Vestergaard *et al.*, 1990). Additional NH_2-terminal sequencing and deglycosylation

experiments should reveal the nature of the heterogeneity. Activation experiments and binding interactions with Pg and FXIIa could be repeated with each of the HRG variants to explore whether these HRG variants are functionally different.

In this study, it was important to investigate the interaction between HRG and Pg to (a) confirm published results and (b) to characterize individual HRG preparations. SPR studies provided evidence that HRG interacts with Pg. However, this interaction is not affected by $ZnCl_2$, which is known to modulate the binding of HRG to heparin and heparan sulfate (Vanwildemeersch *et al.*, 2006). The K_d values for the binding of HRG to Glu-Pg in the absence and presence of $ZnCl_2$ are 155 ± 46.8 nM and 135 ± 54.5 nM, respectively. These results suggest that Pg and HRG have a strong association in plasma and the complex formed by HRG and Pg is maintained under physiological conditions where $ZnCl_2$ levels are relatively low. Disruption of the HRG-Pg interaction by the lysine analog EACA confirms published reports that lysine residues in the NH_2- and/or COOH-terminus of HRG are responsible for HRG binding to the kringle domains of Pg (Borza and Morgan, 1997, Silverstein *et al.*, 1985b). SPR analysis of binding of various HRG preparations to Pg revealed comparable K_d values, which suggests that procedures used to isolate HRG yielded consistent preparations.

4.2 HRG Detection by Western Blot Analysis

Since Pg was identified as a contaminant of early HRG preparations and a strong interaction between HRG and Pg was found by SPR, it was necessary to confirm that HRG was not present in Pg preparations. FXIIa also was analyzed because HRG has a

strong influence on FXIIa activity in Pg activation assays. Western blot analysis revealed cross-reactivity of the commercial rabbit anti-human HRG antibody with Pg, recombinant Pg, and FXIIa. Albumin was electrophoresed in every other lane of the western blot to separate Pg and FXIIa samples. Surprisingly the rabbit anti-human HRG antibody also cross-reacted with albumin. HRG has been shown to bind Pg, but no previous studies have shown that HRG binds to albumin (Borza and Morgan, 1997). Cross-reactivity of the HRG antibody with albumin and the fact that HRG and albumin are both heme- and Zn^{2+}-binding proteins (Guthans and Morgan, 1982, Morgan, 1978), may imply an association between these two proteins in plasma. However, SPR analysis would need to be performed to confirm this hypothesis. These results suggest that all three of these proteins were present in the HRG used for antibody induction. These results highlight the importance of detailed characterization of isolated plasma proteins and commercial reagents.

4.3 Effect of HRG on Enzyme Chromogenic Activity

$ZnCl_2$ and $CaCl_2$ are important divalent cations found in plasma. These cations play important roles in many enzymatic reactions and they may modulate the activity of HRG by enhancing ligand binding. It was important to first determine the influence of these effectors and HRG on the chromogenic activity of enzymes used in this study. Dextran sulfate was also included in FXII and FXI chromogenic assays since it was used as an activating surface in FXII and FXI activation experiments. Physiological concentrations of 12.5 μM $ZnCl_2$, 2 mM $CaCl_2$ and 2 μg/ml dextran sulfate were tested

for their effect on tPA, uPA, Pm, FXIIa, FXIa and PKa chromogenic activity in the absence and presence of 2 μM HRG. On their own, neither dextran sulfate nor $CaCl_2$ affected the chromogenic activity of any of the enzymes used in this study. However, $ZnCl_2$ affected tPA and Pm activity, causing a 37% decrease in tPA activity and a 22% decrease in Pm activity. $ZnCl_2$ has previously been reported to inhibit Pm activity through the formation of a precipitate (Kay, 1950). $ZnCl_2$ has also been reported to bind tPA and inhibit its activity in a dose-dependent fashion (Borza and Morgan, 1997). However, when the effect of $ZnCl_2$ was assessed in the presence of all experimental components, including HRG, $CaCl_2$ and dextran sulfate, it no longer had an effect on tPA. This was also true for uPA, Pm, FXIIa, FXIa and PKa chromogenic activity. The absence of an effect under these conditions likely reflects the binding of $ZnCl_2$ by HRG and/or FXII/FXIIa (Morgan, 1978, Rojkaer and Schousboe, 1997).

At a physiological concentration of 2 μM, HRG on its own or in the presence of $ZnCl_2$ and $CaCl_2$ had no significant effect on the chromogenic activity of tPA and uPA. Nor did HRG affect the chromogenic activity of FXIIa, FXIa or PKa in the presence of 12.5 μM $ZnCl_2$, 2mM $CaCl_2$ and 2 μg/ml dextran sulfate. These results confirm that HRG, $ZnCl_2$, $CaCl_2$ and dextran sulfate do not affect the chromogenic activity of any of the enzymes used in the functional assays conducted in this study. Therefore, we can be confident that effects on activation assays involving HRG are due to a modulatory effect of HRG and not an alteration in chromogenic substrate hydrolysis.

4.4 The Influence of HRG on Glu-Pg Activation by tPA & uPA

Contamination of Pg in HRG preparations, although undetectable by SDS-PAGE analysis, was confirmed in a kinetic assay. Pg was removed during HRG isolation by washing the Ni-NTA column with EACA-containing buffer. Consequently, the stimulatory effect HRG had on enhancing tPA-mediated Pg activation decreased from 6-fold to less than 2-fold. These findings are similar to those previously reported by Horne *et al*, who also found that HRG had no detectable effect on tPA-mediated conversion of Pg to Pm (Horne, III *et al.*, 2000). HRG also did not affect the lysis of fibrin clots mediated by Pg and tPA. Horne *et al* proposed that the HRG isolated by earlier investigators was partially degraded (Horne, III *et al.*, 2000). Proteins cleaved by plasmin contain a COOH-terminal lysine that, like fibrin, can stimulate the activity of tPA in a similar manner to fibrin (Christensen, 1985, Kost *et al.*, 1996). Therefore, unlike intact HRG, partially degraded HRG may enhance Pg activation by tPA through exposure of COOH-terminal lysine residues. The interaction of HRG with tPA was characterized by SPR and revealed that tPA did not bind to HRG in the absence or presence of $ZnCl_2$. These results are consistent with the fact that HRG does not effect Pg activation by tPA.

In order to determine the importance of the interaction of HRG with Pg, we examined the effect of HRG on Pg activation by uPA. Although one previous study reported that HRG had no effect on Pg activation by uPA (Chu and Mathews, 2003), it was important to assess this reaction with our pure HRG preparation. Surprisingly, HRG had a significant inhibitory effect on Pg activation by uPA, with an IC_{50} value of 662 ±

74.8 nM. At a concentration of 3 μM HRG, which is slightly higher than the physiological concentration, HRG inhibited Pg activation by uPA by over 95%. uPA plays an important role in generating Pm on cell surfaces. This Pm can then degrade protein barriers and allows cells to migrate during the early stages of wound healing (Castellino and Ploplis, 2005). HRG may regulate this process by inhibiting cell migration until the late stages of coagulation following clot formation. To further investigate this inhibitory effect, HRG was immobilized to a streptavidin-coated surface and uPA binding was assessed by SPR. However, a K_d value could not be determined. A second experiment was done where active-site biotinylated-uPA was immobilized, but again little binding was detected. Because HRG inhibits uPA activation of Pg, it was anticipated that HRG would bind to uPA. It is possible that the immobilized ligand is in an orientation inaccessible to the analyte. Additional SPR studies, where uPA is biotinylated at its amino terminus instead of at its active site may improve the orientation of the molecule. Alternatively, an ELISA could be used to quantify HRG-uPA binding. It is also possible that HRG binds to a region of Pg that blocks the binding of uPA but does not affect tPA binding.

Overall, HRG appears to have a significant inhibitory effect on Pg activation by uPA. The effect of HRG on uPA should be confirmed in a clot lysis experiment. If HRG inhibits uPA-mediated clot lysis, uPA-mediated Pg activation should be examined in HRG-deficient plasma to assess uPA PA activity in the absence of HRG. tPA-mediated clot lysis should also be assessed in HRG-deficient plasma as a negative control, since HRG had no effect on tPA-mediated Pg activation in this study. Since uPA is found in a

number of systems throughout the body, including urine, blood and the extracellular matrix, uncontrolled uPA activity can have significant effects that can lead to the development of vascular diseases, cell migration and metastasis in cancer, or impaired wound healing and in fertility (Sidenius and Blasi, 2003, Yamamoto *et al.*, 1994). uPA activity is generally controlled by PAI-1 and PAI-2. However, HRG may also play a role in regulating the Pg activator activity of uPA.

4.5 Influence of HRG on Glu-Pg Activation by FXIIa

FXIIa has been shown to activate Pg in a purified system (Schousboe *et al.*, 1999), but its role as a Pg activator *in vivo* is unclear. Compared with tPA and uPA, β-FXIIa is not a potent Pg activator (Miles *et al.*, 1983). The reported low PA activity of FXIIa may be due to the absence of a highly negatively charged surface, such as dextran sulfate that initiates FXIIa generation through FXII autoactivation (Goldsmith, Jr. *et al.*, 1978, Schousboe, 1997). It is unclear what specific biological surfaces play a role in the activation of FXIIa and the contact system. Extracellular matrix components, including collagen and GAGS, endothelial cells, nucleic acids and polyphosphates released from activated platelets are currently viewed as the most plausible physiological promoters of contact activation (Cochrane and Griffin, 1982, Herwald *et al.*, 1998a, Hojima *et al.*, 1984, Smith *et al.*, 2006). However, these surfaces may not be present or exposed to FXIIa in a plasma-based assay, which would then underestimate its PA activity. The low PA activity of FXIIa observed in these assays may also be due to the absence of a cofactor released during vascular injury or due to the presence of an inhibitor in plasma.

At this point in the project, we found that our HRG preparation had no effect on tPA-mediated Pg activation (Figure 6) and had a significant inhibitory effect on uPA-mediated Pg activation (Figure 7). However, uPA has only a limited role as a vascular Pg activator due to its low physiological concentration of 45 pM in the blood (Darras *et al.*, 1986). Therefore, we next focused on the effect of HRG on Pg activation by another Pg activator, FXIIa. Pg activation by FXIIa in the presence of 12.5 μM $ZnCl_2$ and 0-2 μM HRG was decreased by greater than 80% with an IC_{50} of 125 ± 32.6 nM HRG. Since HRG and FXII are both Zn^{2+} binding proteins, additional experiments were performed at higher $ZnCl_2$ concentrations (up to 100 μM $ZnCl_2$) to examine the $ZnCl_2$ dose response. Results showed that HRG had essentially the same inhibitory effect (data not shown). Even in the absence of $ZnCl_2$, HRG showed greater than 80% inhibition, with an IC_{50} value of 160 ± 55.6 nM. These results suggest that HRG inhibition reflects its interaction with FXIIa and is independent of Zn^{2+}. Contrary to a previous study that found dextran sulfate enhanced FXIIa-mediated Pg activation 6-fold (Braat *et al.*, 1999), in our experiments Pg activation by FXIIa was not affected by dextran sulfate. These results suggest that a negatively charged surface is not essential for FXIIa-PA activity, even though it enhances the conversion of FXII to FXIIa. These results also show that HRG directly affects FXIIa-PA activity. Therefore, the physiological concentration of 2 μM HRG in plasma is likely sufficient to inhibit FXIIa from activating Pg and may explain why FXIIa PA activity is observed in a purified system, but not in plasma.

SPR analysis revealed that HRG has an unusually high affinity for FXIIa (K_d = 14.5 ± 4.5 nM) and $ZnCl_2$ enhances binding by 260-fold to a K_d value of 56 ± 8.9 pM.

Almost no dissociation was observed between FXIIa bound to HRG following the injection of buffer across the flow cell, which is consistent with the high affinity of this interaction. The FXIIa-HRG interaction was inhibited by 250 mM imidazole, revealing that FXIIa interacts with the HRR of HRG. Zn^{2+} binds to the histidine residues in the HRR of HRG (Jones *et al.*, 2004b). Therefore, $ZnCl_2$ may cause a conformational change at the HRR that alters binding-site specificity and increases ligand affinity. In this case, Zn^{2+} bound at the HRR increases FXIIa binding affinity. Interestingly, no binding was observed between HRG and zymogen FXII either in the absence or presence of $ZnCl_2$. This finding suggests that activation of FXII exposes an HRG binding site. This region on FXIIa may interact with HRG indirectly via $ZnCl_2$ bound at the HRR of HRG or directly with the HRR. The K_d values obtained by SPR should be confirmed by using a different method for measuring affinity. For example, a fluorescence experiment could be done.

Competition experiments provided further insight into the interaction of HRG with FXIIa. Like FXIIa, heparin binds very tightly (K_d = 1.9 ± 0.3 nM) to the HRR of HRG and this interaction is mediated by Zn^{2+}. $ZnCl_2$ heightens the affinity of HRG for FXIIa and heparin, and the interactions are sensitive to imidazole. SPR revealed that preincubation of increasing concentrations (0-100 nM) of FXIIa, but not FXII, with HRG prevented the binding of HRG to immobilized heparin. Control experiments confirmed that FXIIa or FXII do not bind to immobilized heparin, indicating that the binding of FXIIa observed by SPR was to HRG alone. Thus, FXIIa and heparin compete for the same binding site on HRG. As previously mentioned, it appears that FXIIa interaction

with the HRR of HRG is enhanced by $ZnCl_2$. This interaction is unique to FXIIa since FXII does not bind to HRG in the absence of $ZnCl_2$. The physiological significance of FXIIa and heparin (or heparan sulfate *in vivo*) binding to HRG remains to be determined. However, heparin is a useful tool to further characterize the interaction between HRG and FXIIa.

FXIIa chromogenic experiments also provide insight into the binding interaction between HRG and FXIIa. HRG does not affect FXIIa chromogenic activity, confirmed with three different chromogenic substrates. This suggests that HRG does not bind at the active site of FXIIa and that HRG must have an alternative mechanism of inhibition. Alternatively, HRG may bind to an exosite on FXIIa, which is distinct from the catalytic site (Figure 24). Exosites on some zymogens exist in a low affinity proexosite state and are expressed with the conformational changes that accompany catalytic site activation; such a phenomenon can be observed upon prothrombin (Anderson *et al.*, 2003, Wu *et al.*, 1994), FX (Camire, 2002), FIX (Ahmad *et al.*, 1989) and FVII (Bajaj *et al.*, 2006, Eigenbrot *et al.*, 2001) activation. The concept of a proexosite on FXII may help to explain why HRG binds to FXIIa with very high affinity, but does not bind to FXII.

4.6 Influence of HRG on FXII(a)-Mediated Clot Lysis

The inhibitory effect of HRG on FXIIa-mediated Pg activation was also observed in a clot lysis experiment. At physiological concentrations, HRG completely inhibited FXIIa-mediated lysis of fibrin clots by Pg. In fact, HRG had comparable effects whether the experiment was initiated with FXII or FXIIa. Since HRG does not bind to FXII, as

Figure 24. Possible mechanism of HRG inhibition of FXIIa activity. HRG binds to an exosite on FXIIa, which may cause a conformational change at the active site of FXIIa that impairs its activation of Pg, FXI and FXII.

determined by SPR, FXII must first be activated to FXIIa before HRG can bind to it and inhibit Pg activation. These results are consistent with the effect of HRG on FXIIa-mediated Pg activation and further strengthen the idea that the mechanism of HRG inhibition of Pg activation is through its interaction with FXIIa, which inhibits FXIIa PA activity.

To ensure that HRG was not affecting the process of fibrin degradation by Pm, HRG was assessed in a Pm-clot lysis assay. Pm-mediated lysis was enhanced by approximately 35% as shown by a shortening of the clot lysis time (Figure 16). The effect of HRG was saturable at 500 nM. Therefore, HRG enhances fibrin degradation by Pm, but inhibits FXIIa-mediated clot lysis. Thus, the inhibition of FXII-mediated clot lysis by HRG is FXIIa-specific and independent of Pm activity. These results are consistent with the concept of why FXIIa is observed as a Pg activator in a purified system (Goldsmith, Jr. *et al.*, 1978), but not in a plasma based assay (Braat *et al.*, 1999) due to the presence of HRG, which suppresses FXIIa-mediated Pg activation. The next step will be to examine FXIIa PA activity in HRG-deficient plasma to assess FXIIa activity in the absence of HRG. Assuming that FXIIa PA activity is much higher in HRG-deficient plasma, HRG can then be supplemented back into the system to confirm its inhibition of FXIIa activity. These experiments will provide greater insight into the PA activity of FXIIa. The next step will be to quantify the rate of Pg activation by FXIIa and the rate of clot lysis by Pg and FXIIa in HRG depleted or deficient plasma and compare with FXIIa PA activity in normal plasma to further assess the PA activity of FXIIa and its contribution to fibrinolysis.

4.7 The Contact Pathway of Coagulation

4.7.1 *HRG Effect on FXII Activation*

The exceptionally strong affinity of HRG for FXIIa suggests that HRG may modulate contact activation, a role for HRG that has not been explored. Traditionally, the contact system was believed to activate the intrinsic pathway of coagulation through a series of proteolytic reactions that take place on a negatively charge surface. This was thought of as a necessary step in activation of FXIIa, FXIa and PKa. FXIa then activates FIX to FIXa, which complexes with FVIIIa and leads to the activation FX in the final common pathway of coagulation. Newer concepts in haemostasis suggest that intrinsic coagulation is activated by the extrinsic pathway, involving VIIa/TF activation of FXI and FIX (Butenas *et al.*, 1997, Lawson *et al.*, 1994, Lawson and Mann, 1991) and the positive feedback of thrombin mediated FXI activation (Oliver *et al.*, 1999). These concepts questioned the importance of the contact system in haemostasis. However, a recent study in FXIIa-deficient mice suggest that FXIIa plays a role in clot stabilization (Renne *et al.*, 2006). Therefore, the contact proteins may not be involved in the initial stages of clot formation, explaining why a deficiency of these proteins is not associated with bleeding (Poon *et al.*, 1982). If contact proteins contribution to clot stabilization, their activity may be inhibited by HRG until their function is needed in later stages of haemostasis. Previous results in this study involving FXIIa suggest that HRG may regulate contact activation through FXIIa-specific inhibition, allowing the contact pathway to occur in a precisely timed and orchestrated manner.

The first event in the contact pathway involves the activation of FXII, which can occur through autoactivation of FXII on a negatively charged surface or activation by the plasma protein, PKa. PKa is generated following FXIIa-mediated PK activation, which then functions as a positive feedback mechanism to activate additional FXII. The mechanism of FXIIa activation determines the form of FXIIa that is generated at the negatively charged surface. FXII autoactivation yields the αFXIIa form and activation by PKa also yields αFXIIa, which is subsequently cleaved by PKa into the βFXIIa form (Dunn and Kaplan, 1982, Griep *et al.*, 1985, Wachtfogel *et al.*, 1993). The effect of HRG was first assessed on FXII autoactivation stimulated in the presence of $ZnCl_2$ and dextran sulfate. The rate of FXII activation was decreased by approximately 40% at 50-200 nM HRG. At concentrations greater than 200 nM HRG, FXII activation was almost completely inhibited (98% inhibition). Inhibition by HRG appears to occur in a threshold manner. However, we cannot rule out the possibility of a contaminant present in HRG preparations that may inhibit FXII autoactivation at a HRG concentration greater than 200 nM. In addition, these results are not consistent with the high affinity observed between FXIIa and HRG (56 ± 8.9 pM) by SPR. If HRG does inhibit FXIIa at a threshold concentration, HRG may form a complex with αFXIIa, which inhibits αFXIIa-dependent activation of FXII at the negatively charged surface. Additional SPR analyses of HRG interactions with αFXIIa and dextran sulfate will provide greater insight into the mechanism of HRG inhibition of FXII activation. These would include determining whether HRG blocks the ability of αFXIIa to activate FXII at the dextran surface or if HRG binds to αFXIIa and inhibits binding to dextran sulfate. The latter seems more

likely because competition assays suggest that heparin and FXIIa compete for the same binding site on the HRR of HRG. Deciphering how HRG affects FXIIa activity could be addressed in similar competition experiments by assessing FXIIa and dextran sulfate binding to HRG. Different forms of FXIIa, including βFXIIa, could also be used in binding studies to identify the complementary binding sites on HRG and FXIIa.

Intrinsic coagulation initiated by dextran sulfate or ellagic acid should also be assessed in HRG-deficient plasma. Based on our findings, we hypothesize that clot formation will occur faster in HRG-depleted plasma. HRG-deficient plasma will provide a true indication of FXII autoactivation, since the HRG in normal plasma likely inhibits these reactions. A group of investigators has already shown that HRG has the ability to attenuate contact activation through inhibition of FXII and PK activation, induced by an ellagic acid-phospholipid suspension (Cephotest) and sulfatides in human plasma (Vestergaard *et al.*, 1990). These studies will be done once an HRG-directed antibody has been generated using our own purified HRG preparations as the immunogen.

To determine whether HRG also plays a role in regulating FXII activation by PKa, the rate of FXII activation by PKa in the presence of $ZnCl_2$, dextran sulfate and 2 μM HRG was assessed by gel analysis. In this experiment, CTI was used to inhibit FXIIa activity in order to prevent feedback autoactivation of FXII activation. As a result, a functional assay could not be used to monitor activation. HRG (2 μM) significantly inhibited the cleavage of FXII by PKa, causing an approximately 75% reduction in FXII activation over a 2 hour period. The mechanism of HRG inhibition is unclear because SPR analysis revealed that HRG does not interact with either PKa or FXII in the presence

of $ZnCl_2$. HRG binds to αFXIIa in the presence of $ZnCl_2$ and may inhibit the conversion of αFXIIa to βFXIIa by PKa. Additional studies are needed to address the mechanism of HRG inhibition, including (a) confirming that HRG does not interact with PKa in the presence of $ZnCl_2$ by repeating SPR or by using fluorescence studies and (b) determining whether HRG inhibition of PKa-mediated FXII activation is mediated through αFXIIa binding by repeating this experiment in the presence of an HRR-directed antibody that would block the interaction of αFXIIa with HRG. Future experiments could assess the effect of HRG on other PKa substrates, such as the cleavage of HK by PKa and subsequent BK release. This could be performed by gel analysis, since HK is non-enzymatic, by measuring the disappearance of HK in the presence of HRG by densitometry. Alternatively, the effect of HRG could also be assessed on PKa-mediated HK cleavage in an assay involving the detection of BK, which is released flowing PKa digestion of HK.

4.7.2 *Influence of HRG on FXIIa-Mediated Activation of FXI and PK*

Autoactivation of FXII on a negatively charged surface, such as dextran sulfate, produces αFXIIa that remains bound to the surface where it can activate either FXI or PK. αFXIIa is known to be a better activator of FXI than PK (Tans and Rosing, 1987). However, once a small proportion of PKa has been generated, it can hydrolyze αFXIIa to βFXIIa, which becomes a better activator of PKa (Tans and Rosing, 1987). This sequence of events appears to initially drive FXI activation by FXIIa that is subsequently replaced by PK activation over time. Previous results obtained in this study have found

that HRG can modulate FXIIa activity in Pg activation, as well as modulate FXII activation. To investigate this effect further, we examined the role HRG might play in the contact pathway by looking at the effect of HRG on FXIIa activation of (a) FXI and (b) PK.

The rate of FXI activation by FXIIa was analyzed in the presence of $ZnCl_2$, dextran sulfate and 0-500 nM HRG in a kinetic assay. HRG had a very potent dose-dependent inhibitory effect on FXI activation by FXIIa. Approximately 90% inhibition was achieved with 250 nM HRG and half maximal inhibition was observed with 15 ± 1.2 nM HRG. SPR analysis revealed that HRG does not bind to FXI in the presence of $ZnCl_2$, but it does bind to αFXIIa with a K_d of 56 ± 8.9 pM. Therefore, the mechanism of inhibition appears to occur through the binding of HRG to αFXIIa, where αFXIIa is no longer able to activate FXI. The potent inhibitory effect of HRG on this reaction may suggest that HRG attenuates the activation of FXI until sufficient FXIIa is generated to promote intrinsic coagulation at later stages of haemostasis.

PKa, resulting from FXIIa-mediated PK activation, has a wide range of bioregulatory functions including the activation of fibrinolytic and inflammatory responses. PKa hydrolyses two peptide bonds in HK yielding the inflammatory mediator bradykinin, which regulates blood pressure by stimulating the release of tPA, NO and PGI_2 (Brown *et al.*, 1997, Hasan *et al.*, 1996, Hong, 1980, Zhao *et al.*, 2001). PKa is known to have additional roles in stimulating fibrinolysis by directly activating Pg, as well as sc-uPA (Ichinose *et al.*, 1986). Since FXIIa plays a key role in the generation of PKa and HRG modulates FXIIa activity, the effect of HRG was assessed on the rate of

PK activation by FXIIa in the presence of $ZnCl_2$, dextran sulfate and 0-30 μM HRG in a continuous chromogenic assay. HRG enhanced the rate of PKa activation by approximately 3.5-fold and half maximal effective concentration was observed at 3.2 ± 0.28 μM HRG. The effect of HRG was saturable but only at concentrations above physiological level. At a physiological concentration of 2 μM HRG, PK activation by FXIIa was increased by approximately 50%. This minimal effect of HRG may be significant in the positive feedback of PKa activation of FXII, where 50% enhancement of each feedback cycle would significantly enhance the amplification of FXII activation and subsequently PKa activation. These results are not consistent with the effect HRG has on most FXIIa-mediated reactions analyzed in this study, where HRG had an inhibitory role rather than enhancing the rate of activation as seen here. This may suggest that HRG strongly inhibits αFXIIa, but once PKa is generated, it hydrolyzes αFXIIa to βFXIIa and HRG can no longer inhibit. In future studies, it will be important to determine whether HRG binds as tightly to βFXIIa as it does to αFXIIa, using SPR analysis. Secondly, it will be important to determine whether HRG has the same potent effect on βFXIIa activity, which is missing the surface-binding portion of the molecule, as it does on αFXIIa activity in Pg and FXI activation. It will also be important to incorporate other components of the contact pathway, such as HK, into these functional assays because HK plays an important role in FXI and PK surface binding. It will also allow the effect of HRG to be assessed in a complete system that more closely resembles an *in vivo* response.

4.8 Summary and Perspectives

The biological significance of HRG interaction with Pg is unknown. HRG has been reported to modulate fibrinolysis, but discrepant results have been reported. Some studies suggested that HRG modulates Pg activation by tPA (Borza *et al.*, 2004, Borza and Morgan, 1997, Lijnen *et al.*, 1980), while others propose that HRG interacts with Pg, but argue that HRG does not play a significant role in regulating Pg activation (Horne, III *et al.*, 2000). Our findings differ from those originally reported, demonstrating that HRG has no effect on Pg activation by tPA. We attribute this discrepancy to the purity of our HRG preparation. When looking at alternative Pg activators, HRG affected Pg activation by uPA, but had the most potent inhibitory effect on Pg activation by FXIIa. When further investigating the interaction between HRG and FXIIa, we found an unusually high affinity between the two proteins ($K_d = 56 \pm 8.9$ pM) and this interaction affected a number of FXIIa-mediated processes. HRG significantly inhibited (a) Pg activation by FXIIa, (b) FXII- and FXIIa-mediated clot lysis, (c) FXII autoactivation (c) FXII activation by PKa and (e) FXI activation by FXIIa. Conversely, HRG enhanced PK activation by FXIIa.

These observations not only challenge the current hypothesis of HRG function in fibrinolysis, but also raise the possibility that HRG plays a central role in the regulation of contact activation. In vitro, HRG inhibits a number of FXIIa-mediated processes. However, most inhibitors become inactivated following interaction with their target molecule and are rapidly cleared from the circulation by the liver. Currently, it is unknown how HRG concentrations are modulated *in vivo*. However, HRG is an acute

phase protein and its concentration is decreased in response to inflammation (Saigo *et al.*, 1990). Negative acute phase proteins can be removed from the circulation for many reasons. A decrease in plasma levels may be due to a role in neutralization of proteolytic enzymes. FXIIa is involved in many host defence systems, including inflammation, coagulation and fibrinolysis and HRG may have a role in the neutralization of FXIIa enzyme activity during these physiological responses. FXIIa is thought to contribute to stabilization of the fibrin clot in the later stages of haemostasis, rather than participate in initial stages of clot formation. This hypothesis is consistent with results obtained in FXII-deficient mice, as well as the lack of bleeding observed in FXII-deficient humans. Therefore, HRG may regulate FXIIa-mediated processes by inhibiting FXIIa activity until initial extrinsic driven coagulation events have occurred. In parallel, HRG enhancement of PK activation by FXIIa may amplify FXII activation, as well as promote inflammatory responses early in haemostasis.

Alternatively, HRG may be a carrier protein that binds to FXIIa with high affinity so as to preserve FXIIa. Taking this concept further, HRG may bind specifically to αFXIIa and preserve its function by preventing PKa-mediated conversion of αFXIIa to βFXIIa, which loses its surface binding properties and becomes a poor promoter of coagulation. In addition, HRG may protect αFXIIa from inhibition by C1 inhibitor. Consequently, the interaction of HRG with FXIIa in these functional assays demonstrated inhibitory effects on FXIIa function. Furthermore, HRG bound to circulating FXIIa may have a very different role. If this is the case, the role of HRG in modulating FXIIa

activity must be assessed in plasma or in a mouse model where other effectors that might influence this reaction are present.

It is evident that HRG has an important role in regulating physiological processes that occur in plasma. This can be demonstrated by its evolutionary conservation among species and by its relative abundance in plasma. In addition, HRG has a wide range of ligands, which suggests its importance in a number of biological systems. The involvement of HRG with the various complex physiological processes of coagulation, fibrinolysis, complement, immune function and angiogenesis make solving the specific biological function of HRG a very difficult task. The fact that FXIIa also is involved in these same processes raises the possibility that HRG activity may be modulated by FXIIa.

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