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INITIATION OF COAGULATION ON HERPESVIRUSES

Michael R. Sutherland

Thesis submitted to the Department of Biochemistry Microbiology and
Immunology in partial fulfillment of the requirements for the degree of Doctorate
of Philosophy

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ABSTRACT

Herpesviruses are highly prevalent human pathogens that have the ability to form life-long latent infection in host cells. The persistent reinjury of vessels upon virus reactivation has been suggested to link infection to vascular disease. The goal of this study was to determine the mechanism and role of early initiating events in virus-mediated vasculopathy. We now report that these events are mediated through the expression of tissue factor (TF) and a previously unknown mechanism, that both function in the acceleration of factor VIIa (FVIIa)-dependent activation of factor X (FX) to FXa. The current study identifies herpes simplex virus type 1 (HSV1)-encoded glycoprotein C (gC) as a novel second independent FX activating pathway on the virus surface. Using specific chromogenic assays, an HSV1 gC-deficient virus invariably generated 5 fold less FXa per particle than either wild type or gC-rescued strains. The direct involvement of gC was confirmed using purified recombinant gC, which enhanced FXa production, and like TF, was dependent on FVIIa, Ca^{2+} and anionic phospholipid. Differential inhibition of gC-competent and -deficient strains by an anti-TF antibody confirmed simultaneous and independent TF- and gC-dependent FX activating mechanisms on the virus. Hypothesizing that cell signaling by thrombin, the final coagulation protease, may be advantageous to the virus, the effect on Herpesvirus infection was assessed. Using plaque formation assays, a thrombin specific inhibitor, hirudin, was shown to attenuate the serum-dependent increase in infection, demonstrating the importance of virus initiated

thrombin production. In agreement, the addition of purified thrombin resulted in an approximate 60-80% increase in infectious events. The same enhancement was facilitated by incubation with a protease activated receptor 1 (PAR1) agonist, TRAP, indicating the effect is mediated through at least PAR1 on the cell surface. Using western blot analysis and chromogenic assays, individual variations in thrombogenic potential associated with each Herpesvirus was also determined and correlated with well-documented clinical observations. Cumulatively, these observations illustrate that Herpesviruses have evolved strategies to mimic and exploit host proteins to generate haemostatic cell signaling enzymes that may ultimately lead to the increased susceptibility of cells to infection and perturbation of the vasculature.

Dedication

None but those who have experienced them can conceive of the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in a scientific pursuit there is continual food for discovery and wonder.

Mary Shelley, *Frankenstein*

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List of Abbreviations

Alphabetical List

AnII, annexin II

Ca²⁺, calcium

CMV, cytomegalovirus

FII, prothrombin

FIIa, thrombin

FV, coagulation factor V

FVa, activated coagulation factor V

FVII, coagulation factor VII

FVIIa, activated coagulation factor VII

FX, coagulation factor X

FXa, activated coagulation factor X

gC, glycoprotein C

HBS, hepes buffered saline

HFF, human foreskin fibroblast cells

HSP, heparan sulfate proteoglycan

HSV, herpes simplex virus

HSV-1, herpes simplex virus type 1

HSV-2, herpes simplex virus type 2

IE, immediate early gene product

IgG, immunoglobulin G

kDa, kilodaltons

mAb, monoclonal antibody

NS, HSV1 clinical isolate

ns-1, HSV1 glycoprotein C-deficient strain

PAR, protease activated receptor

proPL, procoagulant phospholipid

PS, phosphatidylserine

rns, HSV1 glycoprotein C-restored strain

S-2238, H-D-Phe-Pip-Arg-p-nitroaniline dichloride

S-2765, Z-D-Arg-Gly-Arg-p-nitroaniline dichloride

sgC, soluble glycoprotein C

TF, tissue factor

TRAP, thrombin receptor activator peptide

Vero, African green monkey kidney cells

vp, virus particle

1. INTRODUCTION

1.1 OVERVIEW

Blood coagulation involves a biological amplification system in which there is sequential activation by proteolysis of circulating precursor enzymes and cofactors. The ultimate result is the local generation of thrombin, which is responsible for the deposition of an insoluble fibrin network and modulation of cells^{92,133}. Operation of this enzyme cascade requires the recruitment and local concentration of circulating coagulation factors. Blood contains all the necessary ingredients to form a clot, lacking only the triggering mechanism that is supplied by initiator molecules at the site of vascular injury. Coagulation is initiated when the cells of the sub-endothelium become exposed, allowing plasma clotting factors to come into contact with procoagulant phospholipid (proPL) and tissue factor (TF). Once available, the former provides a surface on which the activation of coagulation enzymes occurs, while the latter is an initiating cofactor. The accessibility of both proPL and TF is highly regulated and occurs only at sites of vascular damage, preventing the possible development of occlusive thrombi. This also ensures that the haemostatic balance between coagulation and the converse process, fibrinolysis, is maintained.

Herpesviruses such as herpes simplex virus type 1 (HSV1) and type 2 (HSV2) and cytomegalovirus (CMV) are highly prevalent human pathogens that have the ability to form life-long infections. These viruses are known to transform endothelial cells, from a resting noncoagulant to a procoagulant form and consequently these viruses have been linked to vascular disease. Reactivation of

the latent virus and expression of viral gene products have been postulated to be initiating factors in persistent vessel reinjury leading to atherosclerosis. Interestingly, many of the changes to the cell caused by these viruses can be attributed to the production of thrombin at the cell surface.

While the ability of Herpesviruses to alter the thrombogenic and atherogenic state of host cells has been well documented, the initial participation of virus in these processes prior to host cell entry has not been studied. The purpose of this thesis is to investigate surface constituents of HSV1 that may participate in the cell-independent initiation of coagulation and determine the contribution of subsequent thrombin production in Herpesvirus infection and vascular disease.

1.2 NORMAL THROMBIN PRODUCTION

1.2.1 Procoagulant Phospholipid

The plasma membrane that forms a physical barrier between the intra- and extracellular space plays an important role in maintaining the integrity of the cell. Most biological membranes actively maintain an asymmetric distribution of the phospholipid by shuttling specific phospholipid between the two leaflets of the membrane bilayer. This tight regulation is facilitated by the concerted actions of three different enzyme systems: an aminophospholipid translocase that directs the inward movement of amino-containing phospholipid; a floppase that controls the outward transport of phospholipid with little specificity; and a lipid scramblase, which facilitates the bi-directional movement of all phospholipid classes across the bilayer

^{18,25}. Since this is a highly controlled energy-requiring cellular process, the loss of membrane asymmetry is an important physiological trigger that functions in haemostasis and other important processes ^{18,25}.

Once a cell becomes damaged or is activated, the enzymatic control of phospholipid asymmetry is lost. Vascular cells then become procoagulant through the surface expression of procoagulant phospholipid (proPL), of which phosphatidylserine (PS) is considered to be the most important ^{48,149,163,223}. Under normal resting conditions, approximately 5% of the total PS content is present in the outer monolayer ²³⁹, and is increased when cellular perturbation occurs. Platelets represent a significant source of proPL that upon activation, express approximately 20% of the total PS directly on their surfaces ²³⁹. The primary function of proPL when it becomes exposed is to increase the reaction rate of coagulation enzymes. This is achieved by: 1) concentration of the enzyme, cofactor and substrate by recruitment of clotting proteins from the circulating blood ^{127,136,165}; 2) correctly assembling each protein component in an adequate orientation for these reactions to occur ^{98,237}; 3) stabilizing the interaction with non-enzymatic cofactors ^{4,5,128,182}; and 4) preventing the inactivation of enzymes by circulating anticoagulants ¹⁴⁹.

Another important function of proPL is the localization of thrombin production to sites of vascular injury. Under normal circumstances, uncontrolled thrombin generation is prevented by the intracellular sequestration of proPL. When damage occurs, the exposure of the subendothelium or the release of cell agonists induces a rearrangement of cell membrane phospholipids that results in a transbilayer "flip"

of proPL from the inner to the outer face of cells adjacent to the site of injury^{9,19,20,136,159,172,221,256}. Endothelial cells, activated platelets, and monocytes are the most important physiological sources of proPL^{141,222,245}.

1.2.2 Tissue Factor

1.2.2.1 Structure

Tissue factor (TF) consists of a 295 amino acid single polypeptide chain that was originally cloned in 1987. The 47 kDa type 1 transmembrane glycoprotein produced from the TF gene contains a 219 residue extended extracellular portion. A distinguishing feature of this region is its arrangement into two fibronectin-like domains that show structural and sequence homology to the cytokine receptor family^{175,224}. A membrane spanning region and a short cytoplasmic tail domain¹⁹⁴ follows this large cell surface domain.

1.2.2.2 Expression

Immunohistochemical studies have identified a selective distribution of TF in normal tissues and constitutive expression of the protein has been shown in several different cell types. In normal vessels, TF is synthesized at the subendothelial level by smooth muscle cells and predominantly by fibroblasts that surround the vessel. Therefore, disruption of the endothelial layer exposes TF to the circulating blood. Most importantly, peripheral blood cells and those that line the endothelium do not express TF activity under normal haemostatic conditions, permitting an anatomical

separation of TF from the blood and its circulating proteins. However, in response to stimulation such as cytokines, endotoxins or injury, endothelial cells, monocytes, smooth muscle cells and fibroblasts can express TF ^{11,172}. The organization of the TF promoter permits the gene to be regulated in an inducible (endothelial cell and monocyte) and a constitutive (fibroblast and epithelial cell) manner, allowing for both the basal and enhanced expression of TF following stimulation. This enhancement of TF expression is mediated through AP-1, NF-κB and Egr-1 signaling pathways with maximally levels in human umbilical vein endothelial cells seen four hours following stimulation ^{16,153}. The induction of surface bound active TF represents only about 20% of the total cellular TF ²⁰¹. The remainder of the TF was shown to be present in an intracellular pool or as encrypted surface TF that may become functional after damage ^{74,218}.

1.2.2.3 Function

The main function of TF is to orchestrate the induction of the blood clotting cascade, resulting from the initial activation of the closely homologous zymogen precursors factor VII (FVII), factor IX (FIX) and factor X (FX) by limited proteolysis. For this to occur, TF acts as the cellular receptor and cofactor for coagulation protein FVII and its activated form FVIIa and locks it in a position suitable for its subsequent enzymatic activity ¹⁷⁶. The formation of the TF-FVIIa complex allows TF to allosterically induce function to the FVIIa protease domain for full catalytic activity towards its substrates. TF also contributes to a remote macromolecular substrate

docking exosite created by an extended region of both TF and FVIIa that is important for subsequent FX binding and recognition ^{14,55,56}. Once FVIIa binds to TF it enhances the proteolytic activity of the bound protease several thousand fold due to an increase in the k_{cat} and, in some cases, a decrease in k_m . The TF-mediated acceleration of FVIIa substrate hydrolysis is achieved by enhancing the affinity of the substrate for FVIIa rather than by altering the rates of any of the subsequent catalytic steps ¹⁷³. Interestingly, previous studies have shown that all TF on the cell membrane can bind FVIIa, despite the evidence that only a subpopulation participates in the activation of coagulation FX ¹²³. A novel alternatively spliced soluble form of human tissue factor has recently been discovered that circulates in the blood and has been suggested to be involved in thrombus growth ²³.

1.2.3 Factor VII

1.2.3.1 Structure

Factor VII (FVII) is a 406 amino acid vitamin K-dependent glycoprotein synthesized in the liver. Similar to other coagulation proteins, the FVII amino acid sequence is arranged into discrete functional areas or domains. FVII contains ten glutamic acid residues located in the N-terminus of the molecule that are modified posttranslationally to gamma carboxyglutamic acid (Gla). Interaction of the Gla domain with Ca^{2+} causes a conformational change in the molecule that exposes novel epitopes important for its subsequent membrane binding activities. This area is followed by two epidermal growth factor like domains (EGF) that are involved in

protein recognition and binding events. The EGF domains also act as a spacer region to properly position the C-terminal trypsin-like serine protease domain²¹⁵.

Similar to the activation of other serine proteases, the conversion of the single- to two-chain form of FVII occurs by cleavage of a single peptide between Arg¹⁵² - Ile¹⁵³. Activation results in the insertion of the new N-terminus into the protease domain core, forming a salt bridge with an aspartate side chain adjacent to the active site. This interaction modifies the enzyme's substrate binding pocket for optimal substrate accommodation. However, there is evidence that the FVIIa N-terminal insertion does not take place, or there is only partial occupancy in the absence of TF⁹¹. It is hypothesized that the binding of FVIIa to TF further stabilizes this insertion and therefore promotes its protease activity. In the crystal structure of active site-inhibited FVIIa this N-terminal insertion is seen¹⁸¹, however it is most likely due to active site occupancy needed for crystallization.

In plasma, the vast majority of the 50 kDa FVII molecule is in the single chain inactive zymogen form while normal individuals have been found to have about 1% or less of the total FVII in the two chain activated FVIIa form¹⁶⁷. Activation of FVII results in the formation of a 20 kDa light chain and a 30 kDa heavy chain that contain the amino- and carboxy-terminal ends of the parent molecule respectively. A number of coagulation proteases have been shown *in vitro* to activate FVII including factor IXa (FIXa), factor Xa (FXa), factor XIIa, thrombin and the TF-FVIIa complex itself. However, the predominant physiological FVII activator has been shown to be membrane bound FXa³². Any plasma FVIIa that is not complexed to TF most likely

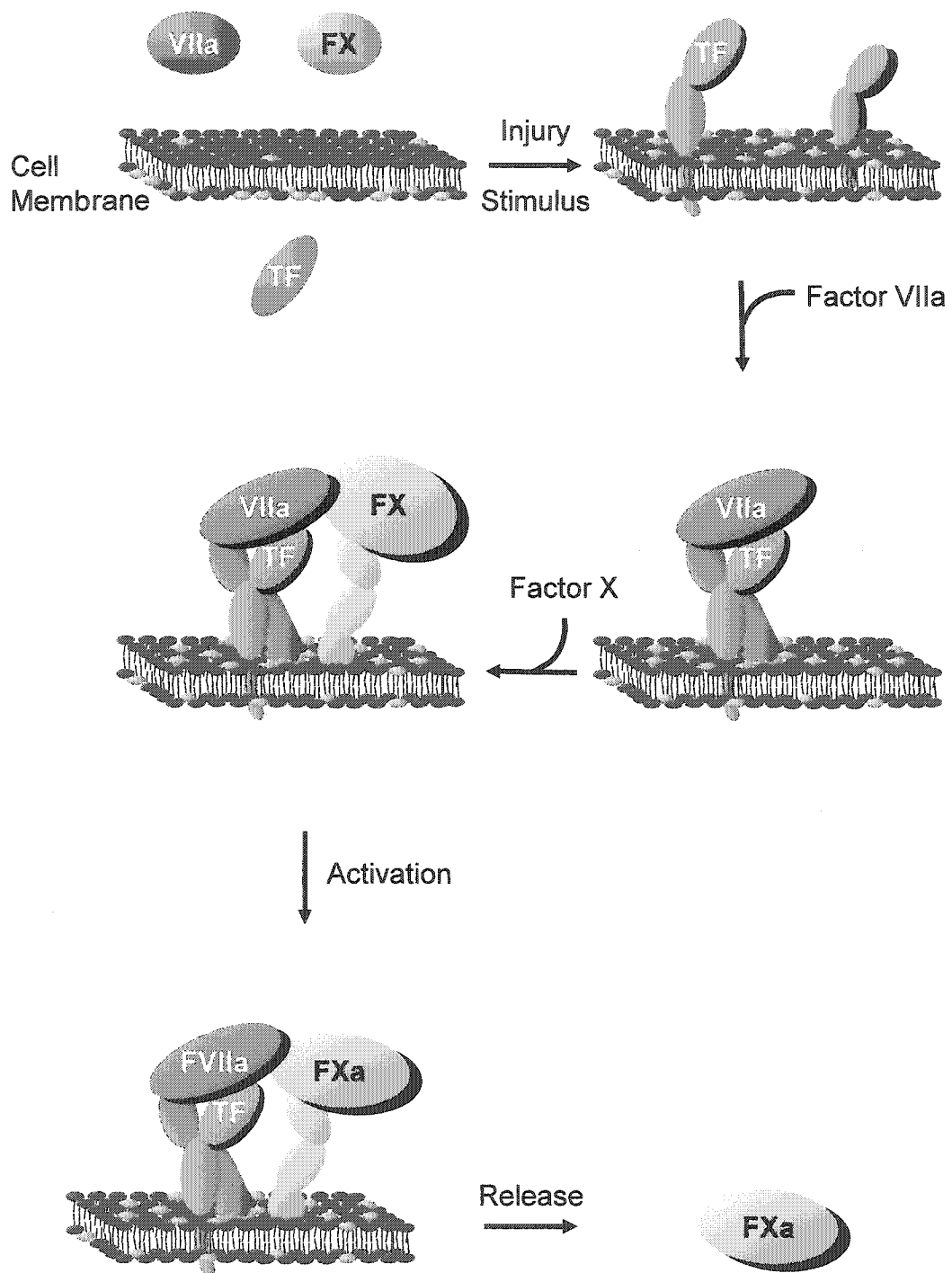
escapes inhibition by the natural inhibitors present in blood due its low enzymatic activity in the absence of TF.

1.2.3.2 Function

FVIIa is an extremely weak serine protease on its own since when not bound to TF it has unique sequence characteristics that retain its zymogen-like conformation¹⁷⁸. This is evident in the ability to chemically modify the N-terminal Ile, indicating that the salt-bridge needed for full enzymatic activity is not formed⁹¹. Additionally, a conformational sensitive antibody to the same N-terminus region has increased binding to free FVII and FVIIa compared to TF-FVIIa, suggesting residual zymogen-like features in FVIIa⁵¹. However, once bound to TF, its enzymatic activity is greatly enhanced. This occurs through changes in the surface and high-affinity Ca^{2+} binding loop regions of FVIIa that are rearranged upon TF binding as well as reregistration of a beta strand that runs between the TF binding region and the N-terminal insertion site^{179,219,220}. The movement that arises upon TF binding allows full insertion and salt-bridge formation of the N-terminus and completes the transition to the fully active form of FVIIa.

The major function of FVIIa is the proteolytic conversion of coagulation FX to its active form FXa, as depicted in Figure 1, resulting in the initiation of the coagulation cascade. Since TF is an integral membrane protein, the TF-VIIa complex is always tethered to the membrane surface and ensures that the

Figure 1 Initiation of coagulation. Under normal flow conditions, tissue factor (TF) and procoagulant phospholipid (proPL, yellow polar head groups) are sequestered inside the cell, away from circulating blood proteins. Upon injury or stimulus, these initiator molecules are exposed on the cell surface. TF can then act as the obligate receptor for factor FVIIa (FVIIa) and through interactions with proPL recruit factor X (FX) to the precise site where coagulation is to occur. This complex then enhances the FVIIa-dependent proteolytic cleavage of FX to its active form, FXa. Following activation, FXa stays associated with TF-FVIIa prior to its release and subsequent use in thrombin generation.



coagulation cascade is activated only where it is needed at sites of vascular injury. Recent evidence also suggests that FVIIa can participate in the activation of a number of intracellular pathways that result in cell proliferation and new gene expression. This may occur through either the mobilization of Ca^{2+} from intracellular stores¹⁹² or the induction of tyrosine phosphorylation of intracellular proteins^{139,213,234}. Also important is the emerging role of TF-FVIIa in angiogenesis, vascularization and tumor metastasis^{26,38,157}. Therefore the generation of the TF-FVIIa complex is an important link between the initiation of coagulation and cell signaling.

1.2.4 Factor X

1.2.4.1 Structure

Factor X (FX) circulates in plasma as an inactive zymogen composed of a 16 kDa light chain and a 42 kDa heavy chain that are linked by a disulfide bond. The light chain contains the Gla similar to FVII that functions in proPL binding while the heavy chain contains the catalytic domain and functions in substrate and cofactor binding. To be functional in coagulation, FX must be converted to an active form, factor Xa (FXa), through cleavage of the $\text{Arg}^{52}\text{-Ile}^{53}$ of the carboxy terminal heavy chain and subsequent release of a 52 amino acid activation fragment.

1.2.4.2 Activation

Several complexes that convert FX to FXa are known. The FX-activating

complex responsible for initiation of the coagulation pathway consists of the serine protease FVIIa bound to its specific cofactor TF^{10,162} (Figure 1). In complex with TF-FVIIa, the scissile bond of FX is presented to the active site of FVIIa and cleaved to generate the first FXa molecules. Once formed, the affinity of FXa for TF-FVIIa is reduced compared to FX¹⁵ and a ternary TF-FVIIa-FXa complex exists prior to the dissociation of FXa to participate in thrombin production. As with other coagulation zymogens, FX contains exosites that are away from the active site but required for the productive docking between enzyme and substrates and are responsible for its specific recognition by the TF-FVIIa complex^{219,252}. The Gla domain of FX has been shown to provide critical membrane binding activity in addition to interactions with an extended binding site in the C-terminal region of TF and the Gla domain of FVIIa^{50,51}. Additional interactions involving the protease domain of FX are with an exosite in the FVIIa protease domain. Studies using mutated TF and synthetic peptides towards a putative FX recognition site have shown that residues 157 to 185 participate in FX binding and are thought to be on the exposed surface of the TF molecule^{171,194}. The normal initiation of thrombin production is triggered by vascular damage and is strictly limited to neighboring cells where TF and proPL become accessible to plasma clotting factors. Based on work using model vesicles, the TF-FVIIa interaction and cofactor activity of TF were shown to be phospholipid independent¹⁹⁵. However, the association of FX with PS-containing vesicles was observed to enhance the overall $V_{\max}$ of the reaction with a modest decrease in K_m

The physiological regulator of TF is the 32 kDa TF pathway inhibitor (TFPI) that, once synthesized by megakaryocytes and endothelial cells, circulates in the plasma in association with lipoproteins. TFPI contains an acidic amino terminal region followed by three Kunitz inhibitor domains and a basic carboxy terminal. The presence of multiple Kunitz domains, two of which can simultaneously bind to the active site of FVIIa and FXa, is important for the regulation of coagulation. In order for TF inhibition to occur, TFPI first binds to FXa. Once the TFPI-FXa complex forms, it binds with higher affinity to TF-FVIIa than does the TFPI molecule alone, resulting in the formation of a fully inhibited tetramolecular complex comprised of TF-FVIIa-TFPI-FXa^{29,30}.

While the TF-FVIIa complex is considered the initiating tenase, a second enzyme complex is required for the amplification of the coagulation pathway. The second tenase is composed of the protease FIXa, its specific cofactor factor VIIIa (FVIIIa), and proPL. To become functional, FVIIIa must first be cleaved from its inactive precursor form, factor VIII, which results in its release from von Willebrand factor. The initial generation of FVIIIa is mediated by FXa, but is more efficiently generated by thrombin. The platelet membrane is the most important source of proPL and serves as a Ca^{2+} -dependent assembly point for tenase complexes⁶.

Important to the current research is that a tenase cofactor encoded by the HSV-1 genome has been identified^{8,62}. When expressed on the surface of infected endothelial cells, the 120 kDa HSV-1 encoded glycoprotein C (gC) has been shown to bind and activate FX through an unknown mechanism. gC also exists on the virus

surface and has been shown to participate in the initial HSV-host cell interaction²¹⁷ and in the evasion of complement-mediated immune clearance⁶⁹. It has also been speculated that gC is responsible for residual non-TF-induced thrombin generation directly on the surface of HSV²¹⁶.

1.2.5 Prothrombin

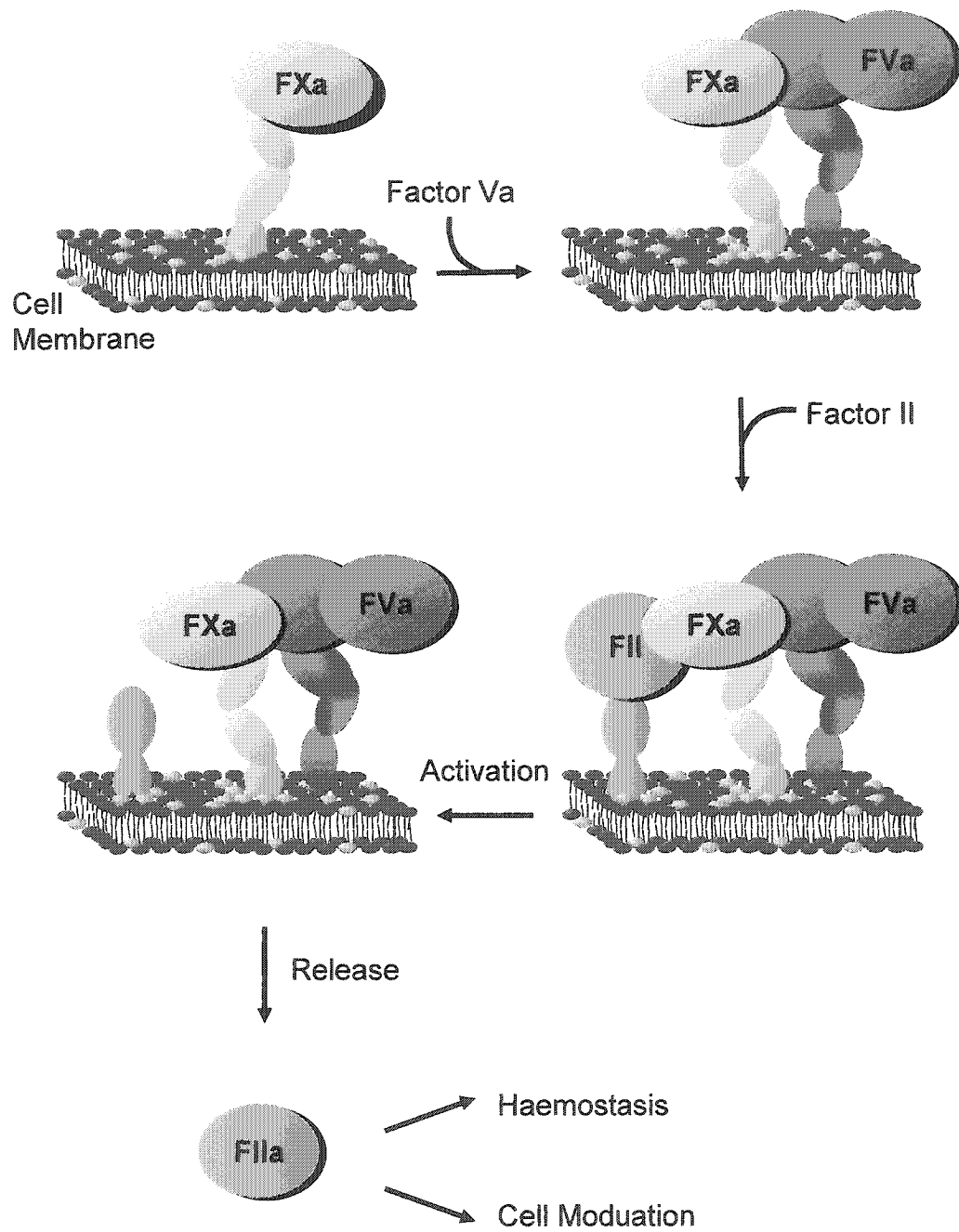
1.2.5.1 Structure

Prothrombin consists of a single polypeptide chain that upon proteolysis is activated to the potent cell modulator thrombin. During the activation by the prothrombinase enzyme complex, prothrombin is cleaved at Arg²⁷³-Thr²⁷⁴ and at Arg³²³-Ser³²⁴ to yield the proPL-binding Gla-domain pro fragment, and thrombin respectively. The active thrombin molecule that is released is composed of a covalently bound amino terminal "A" chain (6 kDa) and a carboxy terminal "B" chain (31 kDa) which contains the histidine, aspartic acid and serine residues that form the catalytic triad common to the active site of all serine proteases.

1.2.5.2 Activation

The principal role of FXa is to activate prothrombin to thrombin. For this to occur, FXa must first associate with the cofactor factor Va (FVa) and proPL in the presence of Ca²⁺ (Figure 2) to form the prothrombinase complex. Although FXa can activate prothrombin on its own, the catalytic efficiency for prothrombin activation is increased by approximately 300,000 fold when FXa is incorporated into the

Figure 2 Thrombin generation. The principle function of factor Xa (FXa) is the formation of the prothrombinase enzyme complex. This occurs when FXa combines with its cofactor, factor Va (FVa) through procoagulant phospholipid (yellow polar head groups) interactions on a cell surface. Prothrombin (FII) then binds and is proteolytically cleaved by FXa, resulting in the generation and release of the active thrombin molecule.



prothrombinase complex^{119,164}. The assembly of prothrombinase involves interactions between FXa and FVa^{105,182}; FVa and a proPL-containing membrane; and, FXa and a proPL-containing membrane^{118,163}, each having an element of Ca²⁺-dependence^{75,120,161,163,182}. Like FVIIIa, FVa circulates as the inactive precursor factor V (FV) and is proteolytically activated by thrombin or FXa¹³⁵.

Classically, the role of FVa in the prothrombinase complex was thought to be perturbation of the catalytic site of FXa that resulted in increased enzymatic activity. However, recent evidence suggests that FVa contributes to the enhanced catalytic activation of prothrombin by interacting with exosites on FXa to enhance the binding of the substrate to prothrombinase^{15,115}. The specific interactions between the enzyme and prothrombin are mediated by contacts between these exosites and substrate sites that are distinct from the structures that surround the scissile bond to be cleaved. Therefore, specificity between an enzyme and the substrate it cleaves are not merely due to interactions at the site of cleavage but are dominated by exosite interaction²⁴².

1.2.6 Thrombin

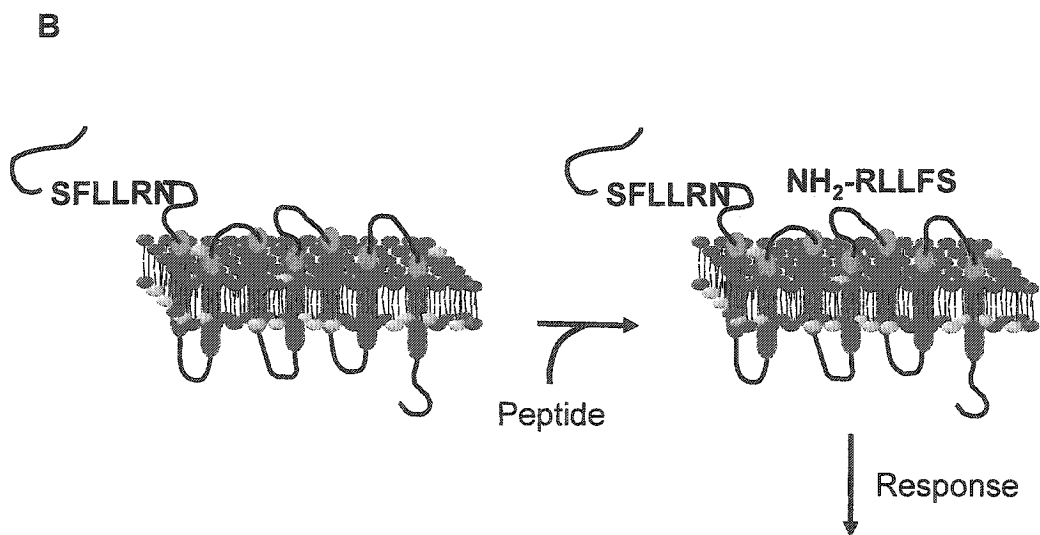
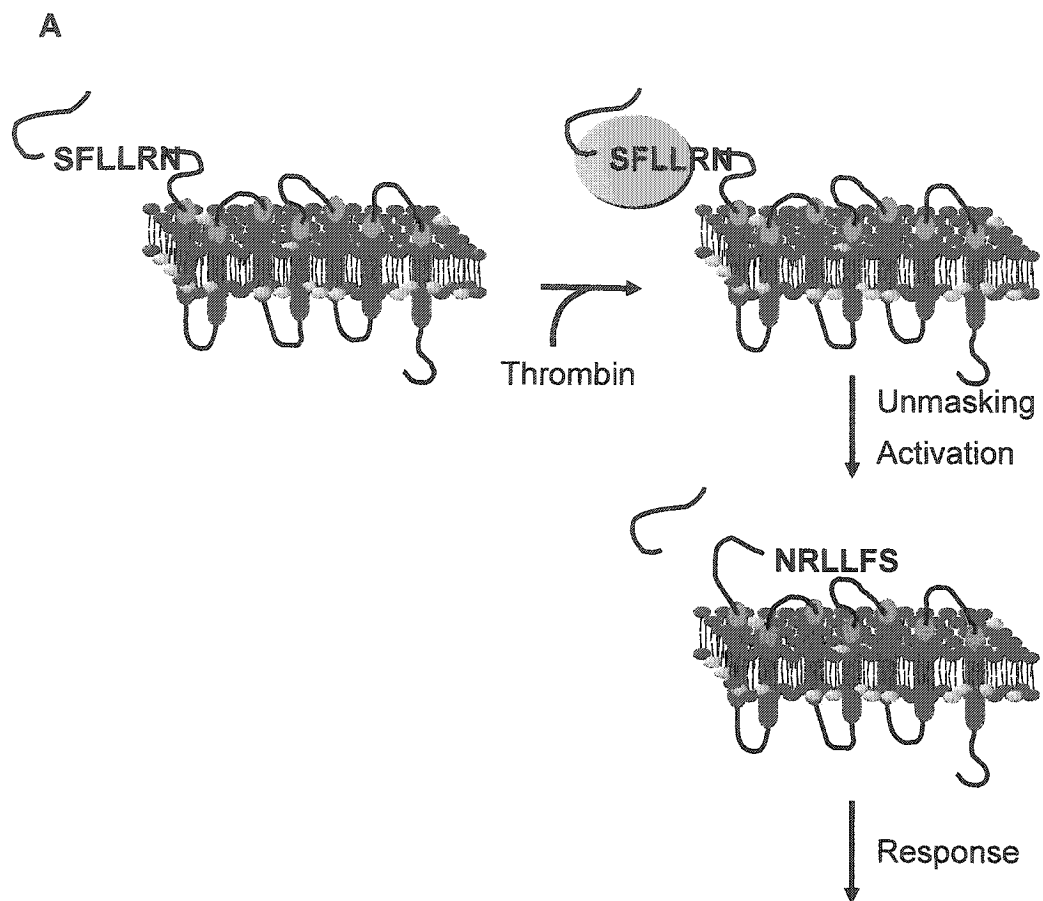
Thrombin is the final enzyme produced in the blood coagulation cascade. It is responsible for the generation of insoluble fibrin clot and feedback-amplification of coagulation enzyme activity. The latter is partly accomplished through rapid activation of the procofactors FVIII and FV. Of equal physiological significance is the ability for thrombin to function as a very potent cell modulator that can induce

important intracellular and extracellular regulatory processes ^{57,77,134}. Thrombin is able to stimulate 1) platelets to express proPL and secrete cell agonists ^{124,143}; 2) the endothelial cell exposure of proPL, TF and secrete platelet activating factor ^{62,81,140,174,203,229}; 3) the leucocyte induced expression of TF and proPL and to facilitate inflammation ^{103,104,154}; and 4) smooth muscle cells to express TF and proliferate after treatment at very low concentrations ^{85,143}. Thrombin also has an important role in the anticoagulant pathway by activating protein C ⁶⁰ and has been demonstrated to be involved in the regulation of fibrinolysis ¹².

1.2.7 Protease activated receptors

The main pathway of thrombin induced cell signaling is through protease-activated receptors (PARs). PARs belong to a unique group of G-protein-coupled receptors (GPCR) of which there is currently four known members, PAR-1, -2, -3 and -4. The primary function of PARs is the conversion of an extracellular proteolytic cleavage into a transmembrane signal. PARs are unique from other GPCRs in that they have an unusual mechanism of activation since these receptors carry their own activating ligands. Upon protease cleavage of the receptor, the newly unmasked N terminus acts as a tethered ligand that binds and activates the cleaved receptor molecule (Figure 3). The specificity of activation depends on the sequence of amino acids that are revealed upon receptor cleavage. Interestingly, short synthetic peptides corresponding to the decrypted N terminus can activate PARs without an initial proteolytic event. After activation there is a conformation change in the

Figure 3 Protease activated receptor activation. A) PAR1, the prototypical member of the G-protein-coupled-receptor family, is activated when thrombin cleaves its N-terminal domain at a specific sequence. This cleavage unmask a new N terminus that serves as a tethered ligand, binding intramolecularly to the receptor to cause a transmembrane signal. B) Synthetic peptides that mimic the tethered ligand can activate the receptor independent of protease and receptor cleavage.



receptor that is transmitted through the membrane and results in an interaction with heterotrimeric G proteins and the transduction of a signaling cascade^{45,49,93}. In humans, thrombin mediates its effects through cleavage of PAR 1, -3 and -4. Interestingly, TF-VIIa can activate the thrombin-insensitive PAR-2 while FXa has been shown to activate PAR-1 and -2^{35,187,188}. Once activated, PARs are rapidly uncoupled from signaling and internalized by phosphorylation-dependent mechanisms. New PARs are then delivered to the surface from a preformed intracellular pool or from new protein synthesis. Several lines of evidence suggest that thrombin activation of PAR1 has an important role in initiating vascular responses in thrombosis and atherosclerosis.

1.3 HERPESVIRUSES

1.3.1 General Characteristics

A typical Herpesvirus consists of a core containing the linear double-stranded DNA genome. The DNA is then surrounded by a characteristic icosahedral capsid that contains a highly ordered mixture of pentameric and hexameric protein arrangements. Surrounding the capsid is an amorphous variably proteinaceous layer known as the tegument that is finally enclosed by a phospholipid bilayer envelope, derived solely from the host cell membranes to form a mature virus particle. The envelope also contains virus-encoded glycoproteins as well as host cell proteins that are acquired by the virus during egress from the infected cells. Also common to each member of the Herpesviruses family is the observation that: 1) the

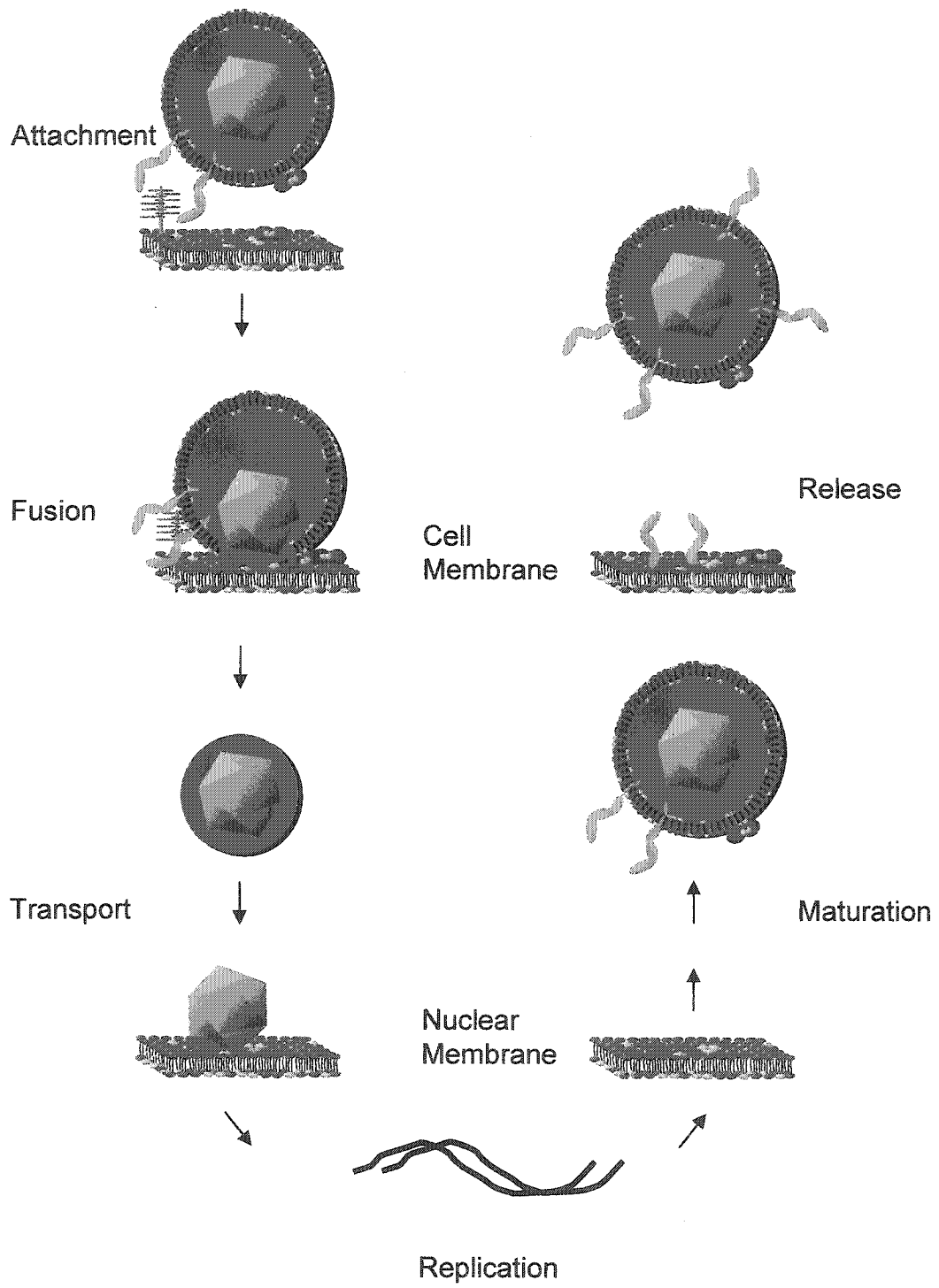
virus encodes enzymes that are involved in DNA synthesis, nucleic acid metabolism and processing of host and viral proteins; 2) progeny virus assembly occurs in the nucleus with subsequent envelopment through cellular membranes; 3) production of infectious virus ultimately results in destruction of the cell; and 4) the virus can form latent infections in their hosts ¹⁹⁰.

1.3.2 Herpes Simplex Virus

Herpes simplex virus types 1 (HSV1) and type 2 (HSV2) are members of the alpha herpesvirus family and have a short, rapid reproductive cycle (Figure 4) that results in complete destruction of infected cells ^{22,148}. The HSV genome has been estimated to encode approximately 75 gene products. During mature virion formation, three types of virus particles may be produced. Although structurally similar, viruses that differ in terms of 1) the presence of DNA; 2) the absence of DNA; and 3) the site of envelopment, may be produced. Therefore not all progeny virus produced are infectious.

Herpes simplex virus infections occur worldwide, with humans as the only natural reservoir. There are two distinct serotypes: HSV1, which is transmitted mainly by contact with oral secretions; and HSV2, which is transmitted by contact with genital secretions ²⁴⁹. After primary infection, the virus persists as an asymptomatic latent infection. Upon reactivation, fever blisters are the most common manifestation of HSV1, while recurrent genital lesions often follow HSV2 infection. HSV are highly prevalent human pathogens with 80-90% of the population

Figure 4 Herpesvirus lifecycle. To initiate infection, the viral glycoproteins interact with cell surface proteoglycans. This is followed by secondary interactions at the host cell surface that results in the eventual fusion of the virus to the host cell. Following entry into the cell, the virus is transported to the nucleus where DNA replication and encapsidation occur. The virus then travels through compartments in the cytoplasm where a series of envelopment and maturation steps take place. Finally, the progeny virus is released from the infected cell and carries viral encoded proteins as well as proteins and phospholipid of host cell origin.



expressing antibodies to HSV1 by the age of 50, while 20% are positive for antibodies to HSV2 by the same age²³⁶.

1.3.2.1 Morphology

1.3.2.1.1 Nucleocapsid

The Herpesvirus nucleocapsid is a highly ordered structure consisting of repeating pentameric and hexameric subunits^{254,255}. The assembly of the capsid proteins occurs in the nuclei of infected cells and coincides with replication of the progeny viral DNA. Prior to completion, the viral DNA is inserted into the capsid. The capsid then acquires an envelope by budding through the inner nuclear membrane; however, the subsequent route of virus maturation and egress remains controversial²¹⁰.

1.3.2.1.2 Tegument

The tegument is a non-structured protein layer located between the viral capsid and the envelope that contains several proteins⁷⁹ which are the first to interact with the host cell. These proteins are needed to initiate the production of new progeny viruses. In order to do so, tegument proteins control and shut off host cell protein synthesis²¹², induce transcription of the immediate early virus genes^{132,156} and are involved in capsid trafficking within the cell²³⁰.

1.3.2.1.3 Envelope

Important to the present study is the envelope that surrounds the mature virus particle. This outer layer of each Herpesvirus is composed of a phospholipid bilayer that is solely derived from the host cell membranes. The definitive cellular source of the viral membrane and sites of final envelopment remain unknown^{58,142,150,210}. However, the virus has been shown to preferentially acquire certain types of phospholipid²³¹ and expresses a higher level of proPL in its envelope than any cell membrane that it potentially arises from. Equally important is the protein component of the membrane that consists of glycoproteins encoded by the virus genome and host cell proteins obtained upon egress from the cell. The unique protein profile on the surface of Herpesviruses is important in virus-mediated interactions with the host cells.

1.3.3 Cytomegalovirus

Another well-characterized and highly prevalent member of the Herpesvirus family is cytomegalovirus (CMV). During the lytic cycle, this beta Herpesvirus¹⁹⁰ typically produces enlarged cells with intranuclear inclusions that are formed during a long reproductive cycle after which the virus may become latent^{101,209}. The virus contains a large genome that encodes greater than 200 gene products⁴¹. Morphologically, mature virions are the largest of the Herpesvirus family with an average diameter of approximately 150-200 nm. In addition to mature virus two non-infectious forms of CMV, which include dense bodies (DB) and non-infectious enveloped particles (NIEP) may also be produced⁹⁹.

Evidence for CMV exposure is detectable in at least 50% of adult North Americans. In normal individuals CMV infection is usually subclinical, although it has been found to account for some mononucleosis-like symptoms¹¹¹. Severe CMV-related pathology occurs in patients who are immunocompromised either therapeutically (e.g. transplant and radiotherapy recipients) or biologically (e.g. AIDS)^{54,87,145}. Disseminated CMV infection is a prevalent complication as a result of this extreme immunodeficiency and increases the morbidity and mortality with primary and recurrent infections.

1.3.4 Herpes Simplex Virus Glycoproteins

1.3.4.1 Function

The HSV1 genome encodes for several glycoproteins that are expressed on the virus envelope as well as the infected cell surface. These proteins serve a variety of functions that are important for productive virus infection, the roles of which have been determined through viral deletion mutants¹⁸⁹. Alternatively, production and purification or transfection of these proteins into target cells has lead to direct functional data. The glycoproteins are arranged independently on the virus surface but are closely packed so that cross-linking between them can occur^{82,83,189}. Individually, HSV1 surface glycoproteins are responsible for entry through the initial weak interactions of glycoprotein B (gB)^{42,122,205} with cell surface heparan sulfate proteoglycan (HSP) followed by the higher affinity binding of glycoprotein D (gD) to host cell surface receptor molecules^{121,152,204,240,244}. The glycoprotein H

(gH)/glycoprotein L (gL) complex is responsible for virus spread by functioning in cell-cell fusion, and with gB and gD, are absolutely required for HSV infectivity. Deletion of any one of these four glycoproteins is lethal and results in production of virus that binds but cannot penetrate into cells. Mechanisms that minimize viral clearance are also mediated by glycoproteins through the manipulation of the host cell immune response. This occurs through expression of gC, which protects the virus from complement mediated attack by binding complement protein C3b^{69,71,129,131}. Additionally, the formation of the glycoprotein E (gE)/glycoprotein I (gI) complex, which binds to the Fc portion of IgG, prevents the antibody mediated clearance of the virus^{130,160} and is also involved in virus spread¹⁹⁹. Many of the glycoproteins have overlapping functions and have been shown to be dispensable in culture.

1.3.5 Glycoprotein C

1.3.5.1 Structure

HSV1 encoded glycoprotein C (gC) is a 511 amino acid protein that is produced late in virus infection. When expressed on the virus or cell surface, the protein contains a large external N terminal domain followed by the transmembrane and short internal C terminus domains. The mature 120 kDa protein contains multiple potential N- and O-linked carbohydrate attachment sites near the N terminus⁹⁷. Although no definitive crystal structure exists, a model of the protein tertiary structure has been constructed through determination of the disulfide bond

arrangement and known discontinuous functional sites^{196,197,225}. A similar arrangement of HSV2 gC is postulated since the two proteins have a high degree of sequence homology and conserved spacing between critical functional residues⁶⁹.

1.3.5.2 Function

1.3.5.2.1 Attachment

gC is important in the infection process and has been implicated in the binding of the virion to the host cell surface. This results from the initial contact of gC and HSP that is mediated by a cluster of positively charged basic amino acids at the N terminus of gC interacting with the negatively charged HSP moieties located on the cell surface^{67,73,90,217}. Also, using antibody, peptide and mutagenesis data, additional residues critical for the gC-HSP interaction were localized in the central portion of the gC molecule²²⁵. These amino acids were mapped to one of the two discontinuous antigenic sites in the protein previously shown to be reactive towards host cell antibodies^{2,158}. Although gC deficient viruses have been shown to bind cells at a reduced capacity, this function is non essential *in vitro* and can be mediated solely by gB. Interestingly, differences in the HSP-binding capacity of gC from HSV1 and HSV2 have been shown and may partially explain differences in cell tropism^{42,73,226}.

1.3.5.2.2 Immune Evasion

Activation of the complement system serves as a major host defense

mechanism that can lead to virus neutralization, phagocytosis and lysis of infected cells. In order to minimize clearance, HSV1 has evolved a mechanism to evade this immune response that relies on the ability of gC to bind complement protein C3. The C3-binding region of gC that is responsible has been mapped to four noncontiguous regions within the central domain of gC^{97,196}. The binding of gC to C3 and its activation products, C3b, iC3b and C3c has been shown to result in the acceleration of the decay of the alternative complement pathway C3 convertase¹¹³. Alternatively, the N terminal region of gC has been shown to disrupt the ability of C3b to interact with properdin and C5^{70,129,130}. Both gC-mediated mechanisms result in the down-regulation of the complement cascade by preventing the formation of the membrane attack complex that eventually disrupts invading pathogens^{66,198}. Although gC negative mutants are readily selected *in vitro*, study of clinical isolates show that gC is conserved *in vivo*^{129,131}. A possible explanation is that gC is a virulence factor that offers a survival advantage for HSV1 by interfering with complement activation and subsequent clearance. Also important, HSV2 gC has been shown to differ from HSV1 gC in its interaction with C3b and C5^{96,196}, suggesting that although homologous, the two glycoproteins may have diverged in function.

1.3.5.2.3 FX Activation

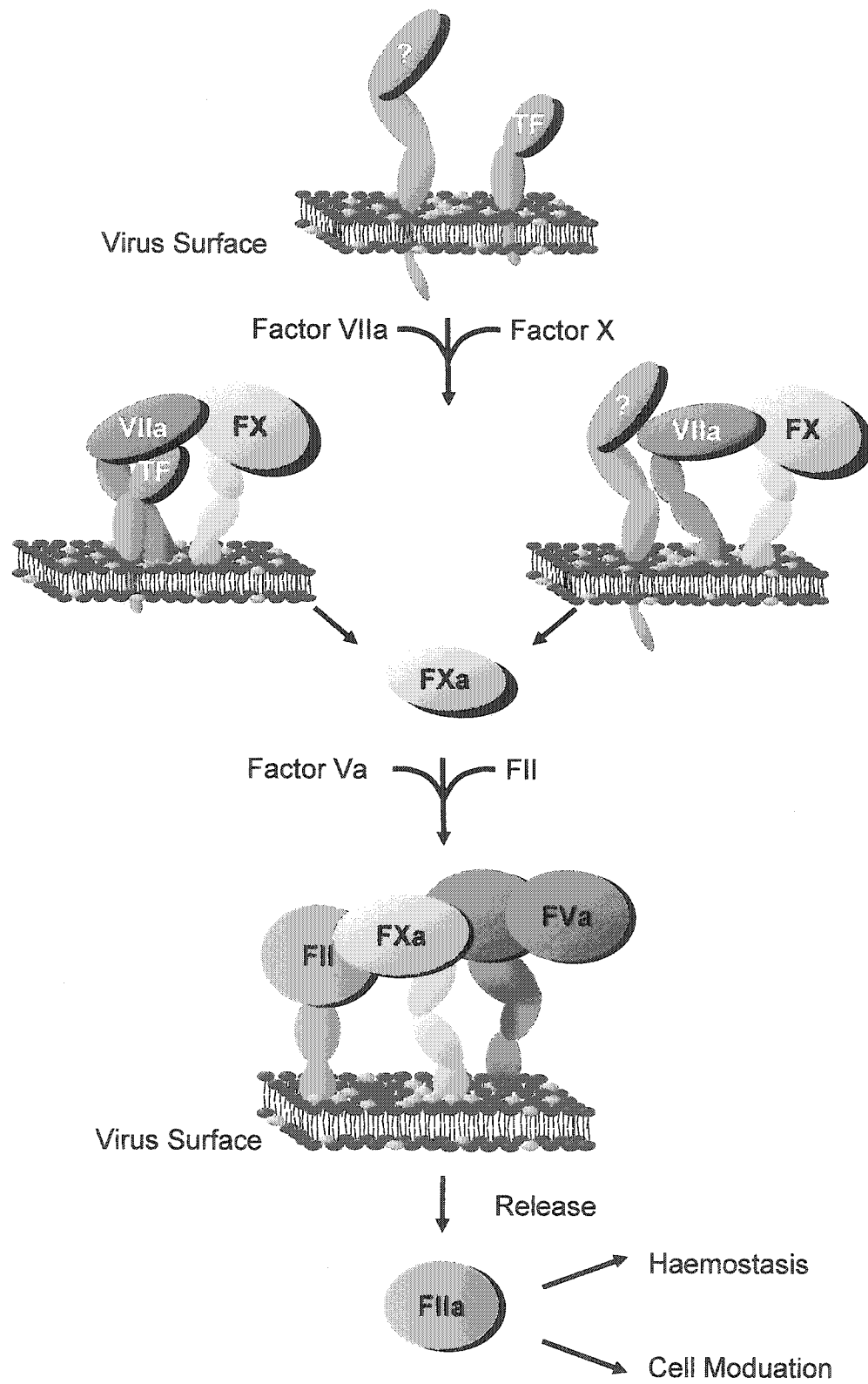
Important to the current investigation, previous reports have suggested that gC can function in FX activation. The original studies showed that when expressed

on the surface of infected cells, gC could participate in FXa generation by an unknown mechanism^{8,62}. Using a cross-linking reagent, this group was able to show that FX and gC are in close proximity on the cell surface and that FX could be converted to FXa. Also, increased binding of FX to cells induced to express gC and those infected with a gC-containing virus was seen when compared to non-induced or cells infected with a gC-deficient virus. Similarly, increased adherence of monocytes was noted in the same cell lines. In a subsequent study, the region of FX that is involved in the interaction with gC was mapped using a series of overlapping peptides and antibodies raised against these peptides. These results allowed the authors to propose a model in which gC acts as a FX binding protein with the subsequent conversion to FXa. The FXa that is produced can then function in the proteolytic conversion of prothrombin to thrombin, and result in procoagulant and atherosclerotic changes to the infected cell. However, no direct FX-gC binding data were given and the role of the virus itself was not determined. Previous chromogenic and antibody inhibition work from our lab showed that the virus itself could sustain FX activation directly on its surface and suggested that this was in part due to gC (Figure 5). The current work provides direct evidence for a role of HSV1 gC in FX activation.

1.4 HERPESVIRUSES IN VASCULAR DISEASE

1.4.1 Induction of Thrombogenic Changes to Host Cells

Figure 5 Thrombin generation on Herpesviruses. HSV1 expresses procoagulant phospholipid (proPL, yellow head groups) and tissue factor (TF) on its surface. Factor VIIa (FVIIa)-dependent activation of factor X (FX) to FXa can occur simultaneously and independently through either the TF- or a gC-postulated mechanism directly on the virus surface. FXa then combines with its cofactor, factor Va (FVa) through proPL interactions on a virus surface and results in the proteolytic cleavage of prothrombin (FII) to the active thrombin molecule.



HSV^{62,63,235} and CMV^{228,229} are known to convert resting vascular endothelial cells from a noncoagulant to a procoagulant state. Various thrombogenic changes have been identified in response to HSV that explain the viral influence on cellular procoagulant activity. These include: expression of TF²³²; expression of HSV-1 gC⁸; and rearrangement of the membrane phospholipid²³⁵. The latter may result in the exposure of proPL on the cell surface allowing the more efficient formation of coagulation enzyme complexes. Alternatively, HSV-induced cellular changes that reduce the inherent anticoagulant properties of cells have also been observed. Among these are: decreased prostacyclin secretion²³⁵, a platelet activation inhibitor; reduced expression of thrombomodulin²³², a cofactor within the protein C-dependent anticoagulant pathway; and reduced synthesis of HSP¹⁰⁶, a cofactor within the serpin-dependent anticoagulant pathway. Definitive molecular changes in response to CMV infection that result in a procoagulant phenotype have not been studied as extensively. Two possible changes due to CMV include the secretion of vWF³¹ and rearrangement of the membrane phospholipid has been reported²²⁹. These factors therefore cause the haemostatic balance between the thrombogenic and anticoagulant pathways associated with the cell to be altered by the virus. The ultimate result in all cases is to favor and perpetuate thrombin production.

1.4.2 Induction of Atherogenic Changes to Host Cells

Atherogenesis is believed to follow a response to endothelial injury, which

exposes the underlying arterial smooth muscle cells to stimuli causing them to proliferate excessively. Considerable advancement has been made in understanding circulating lipids and abnormal lipid metabolism, and their role in atherosclerosis. However, additional causative agents are being proposed with increasing attention is being focused on the cellular abnormalities of lesions in the vascular wall that can promote the development of an atherosclerotic phenotype.

There are several lines of evidence that support a HSV and CMV link to atherosclerosis: 1) HSV and CMV genetic material has been detected within the endothelial and smooth muscle cells of atherosclerotic tissue^{3,17,47,78,86,88,146,147} 2) a strong correlation of active CMV infection to an accelerated form of atherosclerosis in immunosuppressed organ transplant recipients has been documented^{68,94,95,248}; 3) CMV infection has been shown to be a strong risk factor for restenosis after angioplasty^{59,253}; and 4) CMV has been linked to vascular thickening¹⁶⁸.

Since aberrant smooth muscle cell proliferation and recruitment of inflammatory cells are well documented features of atherosclerotic plaque development^{100,147}, the ability to promote the generation of thrombin as a cell stimulant may link subclinical HSV or CMV infection to atherosclerosis^{144,145,147,232}. The virus-mediated generation of thrombin may also explain the observation of fibrin deposits in the microvasculature of mucosal lesions caused by HSV^{125,144} and the development of disseminated intravascular coagulation in neonates with severe HSV infection¹⁴⁴. While the clinical data are arguably circumstantial, a distinct cause-and-effect relationship was established in avian and rodent models where

viral infection induced atherosclerosis and thrombosis ^{64,65}.

Important advancements have recently been made in understanding the biochemical mechanisms connecting Herpesvirus exposure and the expression of genes that have been incorporated into the host genome (i.e. latent infection) to atherosclerosis. The first example involves the CMV gene product, IE84, which is capable of associating with the tumor suppressor protein, p53, and inducing smooth muscle cell proliferation ²¹⁴. Work from the same laboratories has also demonstrated that the CMV gene product, IE72, triggers the synthesis and expression of a "scavenger" receptor for oxidized low density lipoprotein (LDL) deposition, which is an early event in atherosclerosis ²⁸. The cumulative biochemical evidence provides a molecular explanation for the clinical studies.

1.5 RATIONALE

A previous study from our laboratory demonstrated accessible proPL and TF on the surface of purified HSV1, HSV2, and CMV ²¹⁶. Through the combined expression of these two cofactors molecules, the virus was able to circumvent the normal host cell regulation of coagulation initiation and generate thrombin directly on their surfaces (Figure 5). The ability of these viruses to bypass the normal control mechanisms imposed by cells that restricts thrombin generation to the sites of vascular injury provided initial biochemical evidence for the clinical observations of thrombosis associated with these viruses. However, this study also suggested that at least for HSV1, an additional virus-encoded protein might directly participate in

FX activation. The evidence that the Herpesvirus membrane has specifically developed as a procoagulant agent implies an advantage to the virus that is conferred by thrombin production.

The purpose of the current work was to identify the mechanism and role of Herpesvirus-mediated FX activation in order to provide a potential link between infection and vascular disease. The specific aims were to: 1) determine if HSV1 gC could participate in FX activation directly on the virus surface; 2) determine if the thrombogenic potential of HSV1, HSV2 and CMV correlates with specific clinical observations; and 3) demonstrate a role for thrombin in Herpesvirus infection.

2.0 MATERIALS AND METHODS

2.1 REAGENTS

Z-D-Arg-Gly-Arg-p-nitroaniline dichloride (S-2765) and H-D-Phe-Pip-Arg-p-nitroaniline dichloride (S-2238) were from Chromogenix. Ethylenediamine tetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), the thrombin receptor activating peptide (TRAP), low molecular weight heparin (3000 MW) and bovine serum albumin (BSA) were from Sigma. Purified recombinant hirudin from the medicinal leech was obtained from Calbiochem. All experiments were conducted in Hepes buffered saline (HBS; Hepes (20 mM), NaCl (150 mM) pH 7.2) plus BSA (0.1%).

2.2 VIRUS

2.2.1 Production

2.2.1.1 Herpes simplex virus

African green monkey kidney cells (Vero, ATCC CCL-81) were grown in Medium 199 supplemented with fetal calf serum (5%) and gentamycin (2 µg/ml) (Gibco). The cells were passaged onto Cytodex 2 microcarriers beads (Amersham) and allowed to attach for 4 hrs with occasional stirring in 500 ml Celstir flasks. Following attachment, the cell debris was removed and fresh media was added. Once complete monolayers were formed (4 to 6 days), the cells were inoculated with individual HSV1 (MacIntyre, NS, ns-1 or rns) and HSV2 (strain G) strains and the media was replaced with reduced serum (1%) media. Mature virus was

harvested from one half of the supernatant at 3 days post inoculation and replaced with fresh reduced serum media. At 6 days post inoculation, all the media was subjected to virus purification. The infected cell supernatant was clarified by centrifugation at 700 x g and the virus was pelleted at 23,000 x g. Virus pellets were then resuspended in HBS and subjected to ultracentrifugation at 200,000 x g through a 10-30-60% stepwise sucrose gradient. Once separated, the virus band was removed, diluted in HBS, and centrifuged at 200,000 x g. The resulting virus pellet was resuspended in HBS and frozen at -80°C.

Alternatively, human foreskin fibroblast cells (HFF, ATCC CRL-2056) were grown in basal medium eagle media (BME) supplemented with bovine calf serum (5%), glutamine (1.4 mM) and gentamycin (20 µg/ml). The cells were then passaged onto micocarrier beads. Once confluent the cells were inoculated with HSV1 MacIntyre and the media was replaced with reduced serum (2%) media. Virus was propagated and purified from the infected cell supernatant as described above.

2.2.1.2 Cytomegalovirus

HFF were grown in T-175 cm² tissue culture flasks. Once confluent, cells were inoculated with CMV AD169 for 1 hour at 37°C. The media was then removed and replaced with reduced serum (2%) media and the infection was allowed to proceed. After 7 days, the media was removed and replaced with fresh reduced serum media. Following another 7 days, the infected cell supernatant was harvested and clarified, and the cells were subjected to several freeze-thaw cycles to release

intracellular virus. Initially the virus pellets were resuspended in PBS followed by purification on a linear gradient of tartrate/glycerol (15/10 - 40/0) at 40,000 x g for 1 hour. The virus band was then removed, diluted in PBS, and centrifuged at 40,000 x g for 1 hour to pellet the virus. The purified virions were resuspended in PBS, aliquoted and frozen at -80°C.

2.2.2 Quantification

All purified virus preparations were evaluated for purity and quantified to derive particles numbers per ml by negative staining electron microscopy. Initially, viral stocks were diluted in water containing BSA (0.01%) to dilute the salt from the virus resuspension buffer. The virus was then further diluted in water plus BSA (0.01%) that contained a known concentration of standardized polystyrene latex spheres (Dako Scientific). A 5 µl drop of the virus/sphere mixture was placed on a formvar/carbon coated copper electron microscopy grid for 90 seconds. Following attachment, a phosphotungstic acid (2%, pH 6.5) plus BSA (0.01%) staining solution (10 µl) was placed onto the grid and the entire mixture was incubated for an additional minute after which the excess virus/sphere suspension was blotted off. The grids were stored at room temperature prior to quantification using a Hitachi series 7000 transmission electron microscope. All virus preparations were evaluated for intact virus particles as well as potential cell debris. The number of particles per ml was determined by $[(\# \text{ of particles} / \# \text{ of spheres}) \times [\text{spheres}] \times \text{virus dilution factor}]$. Typically 6-10 individual sections on 4 independent grids of different virus

dilutions were counted. All virus preparations used in the study contained more than 90% virus particles.

2.2.3 Infectivity

The infectivity of each virus stock produced was determined by standard plaque assays on Vero cells grown to confluence in 24 well plates. Prior to inoculation with each Vero-propagated virus strain, the cells were washed once with PBS and once with complete serum (5%) media. The virus was then 10 fold serially diluted in complete serum media and added to the cells (200 μ l/well). Infection was allowed to proceed for 90 minutes at 37°C with gentle rocking. The inoculum was then removed and the cells were washed once with PBS followed by reduced serum (1%) media and replaced with reduced serum media (1 ml/well). Alternatively, the initial cell wash and inoculation media was replaced with serum free (0%) media supplemented with BSA (1 mg/ml).

For HFF propagated HSV1 (MacIntyre), HFF were grown to confluency in 24 well plates. Prior to infection, cells were washed once with PBS followed by BME complete serum (5%) media. Serial 10-fold dilutions were made in complete serum media and added to the cells for 90 minutes. After infection, the virus was removed and the cells were washed with PBS followed by reduced serum (2%) media. The wash was removed and replaced with reduced serum media (1 ml/well) as above.

To quantify the infectivity of each virus HSV1 strain, 40-48 hours post infection the cells were stained to derive the number of productive infectious events

(plaques). The infected cell supernatant was removed and replaced with formaldehyde (20%) for 5 minutes. Following aspiration, the cells were further fixed by the addition of methanol and subsequently stained with crystal violet [crystal violet (0.4%), formaldehyde (10%), methanol (1%)] for 2 minutes. Excess stain was removed with water after which the cells were dried and plaques numbers were counted using an Olympus dissecting microscope. The number of plaque forming units per ml (pfu/ml) was determined by $[(\# \text{ plaques} \times \text{dilution factor}) / \text{volume of inoculum}]$. Typically the pfu/ml was determined using data from at least 3 separate dilutions of virus on multiple 24 well plates.

2.3 PROTEINS

2.3.1 Commercial

Purified human factor VIIa (FVIIa), factor X (FX), factor Xa (FXa), factor Va (FVa), prothrombin and thrombin were obtained commercially from Haematologic Technologies. Innovin (Dade Behring) was reconstituted according to the manufacturer's directions and used as a highly characterized source of purified recombinant tissue factor (TF). Cellular sources of TF activity were also prepared from HFF or Vero cells and used as controls for immunoinhibition experiments. To generate the cell sources, confluent monolayers were washed twice with HBS to remove residual growth media and detached from the flasks by scraping into HBS. The cell extract was then spun, resuspended in a small volume of HBS and stored -80°C.

2.3.2 Soluble glycoprotein C

2.3.2.1 Recombination

HSV1 glycoprotein C (gC) was recombined into the *Autographa californica* nuclear polyhedrosis virus (baculovirus) and expressed in infected *Spodoptera frugiperda* (Sf9, ATCC CRL-1711) cells. Prior to recombination, plasmid pCD14 was used as a PCR template to generate DNA fragments containing the gC gene that was truncated 18 residues prior to the transmembrane region at residue 457 of the native gC protein²¹⁷. The gene was cloned into the baculovirus transfer vector pVT-Bac to enable the insertion of the gene minus its natural signal sequence in frame with the signal peptide of honeybee melittin that allows more efficient secretion of the protein from the cell²⁰⁸. pVT-Bac also contains the promoter for the baculovirus polyhedrin gene and flanking sequences that permits recombination into the polyhedrin site of baculovirus to enhance the amount of recombined protein produced. To begin construction, the pVT-Bac vector was digested with *Bam*HI and *Kpn*I and the gC gene fragment was digested with *Bgl*II and *Kpn*I and ligated into pVT-Bac for 15 hours at 15°C using T4DNA ligase. The ligated plasmids were used to transform *Escherichia coli* XL-1 Blue competent cells and the ampicillin-resistant colonies were screened by restriction enzyme analysis²¹⁷. Once generated, the plasmids were recombined into baculovirus using Baculogold as the source of baculovirus DNA²⁰⁸. The primers were also designed to add three histidine residues to the two histidines already present at the carboxy-terminal end of gC which

provides a binding site for Ni^{2+} - nitriloacetic acid (NTA) agarose to facilitate purification of the secreted protein. The recombined virus was previously generated and kindly provided by Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania.

2.3.2.2 Purification

A previously established protocol for expression and purification of HSV glycoproteins was followed with modifications²⁴³. Confluent Sf9 cells grown in T-175 cm^2 tissue culture flasks were released by gentle tapping and subjected to a slow speed spin to pellet the cells. The supernatant and cell debris was removed and the cells were placed into 500 ml Celstir flasks containing Sf900IIM serum free media supplemented with penicillin/streptomycin (1%). Cells grown in suspension were passaged by the addition of fresh media when the concentration approached 1×10^6 cells/ml. Once all Celstir flasks reached a density of 4×10^6 cells/ml the cells were infected with the plaque purified recombinant baculovirus at a multiplicity of infection of 4 pfu per cell. Prior to and each day post infection, a 1 ml sample of the cell supernatant was removed to determine recombinant protein production. The sample was centrifuged and the supernatant was removed and frozen at -80°C . The spun cells were resuspended in HBS and also frozen at -80°C . An additional aliquot of cells was removed from each celstir and assessed for viability using the trypan blue exclusion method. The infection was allowed to proceed until the cell viability dropped below 70%, typically 72-96 hours.

For purification, the infected cell supernatant (2 litres) was centrifuged at 1500 rpm for 10 minutes in a Beckman benchtop centrifuge to pellet the cells. The clarified supernatant was then concentrated at 4°C through a YM (10,000 MWCO) filter to approximately 5% of the original volume, placed into Spectropor (12,000 MWCO) dialysis tubing, and exchanged overnight against 3 x 4 litres of PBS. Following dialysis, the sample was incubated overnight with 1 ml of nickel-NTA resin (Qiagen) at 4°C on an elliptical shaker to keep the resin in suspension. The resin was then loaded into a column and washed with 50 ml of PBS followed by 50 ml wash buffer (20 mM phosphate, 500 mM NaCl, 10 mM imidazole). Purified sgC was eluted in 1 ml fractions using a stepwise gradient of 10 - 500 mM imidazole in 20 mM phosphate, 500 mM NaCl. Each fractions as well as samples taken at the individual steps during purification were analyzed by Western blot using a conformation dependent antibody (1C8) to determine the presence of sgC. Those fractions containing the greatest amount of sgC were pooled and simultaneously concentrated and exchanged with PBS using Millipore centrifugal concentrators. Once concentrated, the purified protein was then aliquoted and frozen at -80°C. SDS PAGE was used to assess the protein purity and the concentration was determined by the BCA (Pierce) method.

2.4 GLYCOPROTEIN C-MEDIATED FACTOR X ACTIVATION

2.4.1 Viral glycoprotein C

2.4.1.1 Factor Xa-dependent chromogenic assays

To determine the constituents on HSV1 involved in coagulation initiation, an assay to detect the virus-dependent activation of FX to FXa was developed. Various sources of putative FX activators were incubated with FX, FVIIa and Ca^{2+} . At constant incubation times, FXa generation was stopped by EDTA (100 mM) - mediated chelation of Ca^{2+} . Production of activated FX was monitored by cleavage of a FXa-specific chromogenic substrate, S-2765 (200 μM in HBS/EDTA), in a kinetic plate reader (Vmax, Molecular Devices Inc). Each reagent concentration was optimized by extensive titration of individual components in the mixture to ensure that the amount of coagulation initiators in the system was rate limiting. Standard curves of S-2765 cleavage by known amounts of FXa were constructed and used to determine the concentration of FXa formed in the experimental system. Using this assay, picomolar concentrations of FXa generated on as few as 100 virus particles could be measured.

Initially, a well-characterized panel of HSVs was used to determine a role for gC in FX activation. The first virus studied was HSV1 NS, a low passage clinical isolate originally obtained from endothelial cells⁷¹. The second is ns-1, a naturally occurring gC deficient strain of the NS virus. ns-1 is a monoclonal antibody resistant mutant virus that escaped neutralization using a monoclonal anti gC antibody (1C8) and rabbit serum as a source of complement⁸⁴. Following isolation, the surviving virus was further purified in the presence of antibody and complement. Studies demonstrated that gC molecule produced by the ns-1 virus is smaller than the wild type with precursor and processed forms of 50 to 60 kDa, compared to 100 to 120

kDa for wild type gC⁸⁴. DNA analysis of ns-1 gC revealed the insertion of a single cytosine resulting in a frame shift mutation and the creation of a new stop codon. The end result is the generation of an altered gC that is truncated and not expressed in the purified virion or on the surface of infected cells⁸⁴. The final strain examined was rns. This gC-rescued strain was constructed using the entire *Bam*HI fragment containing the gC sequence of NS to rescue the gC defect in the ns-1 virus⁸⁴. Marker rescued virus was selected by using rabbit polyclonal anti-gC antibody in an immunoperoxidase assay. Therefore, the rns virus and infected cells both express full length gC on their surfaces⁶⁹. The HSV1 lab strain, MacIntyre, was also evaluated as a gC-competent wild type virus.

To systematically determine the importance of gC in FX activation each constituent in the system was evaluated. Initially, individual virus strains were titrated in the presence of FX (100 nM) and FVIIa (5 nM). Activation of FX was then initiated by addition of Ca²⁺ (5 mM). The amount of FXa generated after a 20 minute incubation at room temperature was monitored as above. The dependence of the reaction on FX and FVIIa was next evaluated. To ensure that the results were reflective of the amount of initiator molecules in the system, a low virus concentration from the previous experiment was chosen. This allowed an accurate reflection of FXa generation as well as a maximal difference between virus strains. In this case, FX was titrated in the presence of each virus strain (100 virus particles/ μ l) and a constant amount of FVIIa (5 nM) and Ca²⁺ (5 mM). Alternatively, the enzyme, FVIIa, was titrated with a constant amount of virus (100 virus

particles/ μ l), FX (100 nM) and Ca^{2+} (5 mM). In control experiments, each component of the reaction mixture was individually omitted and the amount of FXa generated was determined.

In order to explore potential differences in FX activation due to antigenic differences between species, purified HSV1 MacIntyre produced in both HFF and Vero cells were examined for their FXa generating ability. Each virus was titrated in the presence of FX (100 nM) and FVIIa (5 nM). Activation of FX was then initiated by the addition of Ca^{2+} (5 mM) and the amount of FXa generated after a 20 minute incubation at room temperature was monitored as above. The requirement for procoagulant phospholipid was evaluated by the addition of 25% phosphatidylserine / 75% phosphatidylcholine (PCPS, 300 μ M) small unilamellar vesicles to the system.

To determine if the region of gC that is responsible for FX activation is separate from known functional regions on the molecule a series of control experiments were performed. Since the areas of C3b binding to gC are highly characterized⁶⁹ we determined if C3b could affect gC-mediated FX activation. To generate C3b, human C3 (kindly provided by Dr. David Isenman, University of Toronto), (1 mg/ml) was incubated with trypsin (1% w/w) for 6 minutes at room temperature. To stop the reaction, soybean trypsin inhibitor (1%) was then added. Alternatively, a similar amount of BSA was subjected to the same activation conditions and used as a substitute for C3b in control experiments. SDS PAGE was used to confirm the complete conversion of C3 to C3b.

Using a constant number of virus particles (1.7×10^3), the number of gC

molecules in the system was estimated, assuming 5000 molecules of gC per virus⁸³. HSV1 strains MacIntyre, NS, ns-1 and rns were preincubated with a 10 or 1000 fold excess of C3/C3b particles over gC for 5 minutes. FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) were added and allowed to incubate at room temperature for an additional 20 minutes. Following termination, the amount of FXa generated was determined as above. In control experiments, equal volumes of mock activated BSA (1 mg/ml) or HBS were added in place of C3/C3b.

Since gC is also known to interact with cell surface HSP, the ability of an analogous molecule, heparin, to affect gC-mediated FX activation was also addressed. A constant amount of virus particle (4×10^3) was preincubated with an increasing amount of low molecule weight heparin (0 - 3.4 μM) for 5 minutes. FX (100 nM) and FVIIa (5 nM) were then added and FXa generation was initiated by the addition of Ca^{2+} . Following a 20 minute incubation, the reaction was stopped and the amount of FXa generated was determined.

2.4.2 Purified glycoprotein C

2.4.2.1 Factor Xa-dependent chromogenic assays

The direct participation of gC in FX activation was evaluated using a virus-free system in which sgC was added as the sole source of putative FVIIa-accelerating molecules. To begin, varying amounts of purified sgC was added to PCPS (100 μM), FX (100 nM) and FVIIa (5 nM). The reaction was initiated by the addition of Ca^{2+} (5 mM). After a 2 hour incubation at room temperature the amount

of FXa generated was determined. Individual components of the reaction mixture were systematically omitted to show dependence. The data were corrected for any chromogenic substrate turnover observed in identical experiments conducted in the absence of sgC.

To evaluate the possible involvement of an additional viral constituent in the gC-dependent acceleration of FX activation by FVIIa, sgC (1.5 μ M) was added to an increasing amount of each purified virus strain and incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 minutes. The data were corrected for the amount of virus-dependent FXa generation by subtracting the results of an identical experiment conducted in the absence of sgC. In similar experiments, sgC was titrated with a constant amount of virus, FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM). To maximize the enhancement of FXa generating activity by sgC, the virus concentration giving the highest activity observed when the virus was titrated with constant sgC were chosen (NS: 500 virus particles/ μ l; rns: 500 virus particles/ μ l; ns-1: 2500 virus particles/ μ l). The effect of virus on sgC-dependent FX activation was derived by correcting the data for the amount of FXa produced in the absence of sgC.

The possibility that exogenous sgC may directly affect the FX activating function of TF was investigated using Innovin as the source of purified TF. A concentration of Innovin that resulted in a comparable amount of FXa generation observed for the ns-1 strain in the previous experiment was incubated with increasing concentrations of sgC, FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM). The

specific sgC-mediated enhancement of Innovin TF activity was determined by subtracting the amount of FXa generated by Innovin alone.

2.4.2.2 Surface Plasmon Resonance

Surface plasmon resonance (SPR) was used to quantitatively evaluate a possible direct interaction between sgC and FVIIa or FX. In initial experiments, sgC was immobilized to the BIAchip matrix via amide coupling. Following blocking of the unreactive groups with ethanolamine, various analytes were flowed over the chip surface and the change in resonance units was monitored. Initially individual titrations of either FX or FVIIa (5 - 100 nM) were passed over the gC-coated surface. Alternatively, a mixture of FX (100 nM) and PCPS (100 μ M) was analyzed. In control experiments, BSA (1 mg/ml) and anti HSV1 gC (1C8) were substituted for FX or FVIIa. Sensorgrams over an uncoated flow cell were also evaluated. The possibility of the requirement for a macromolecular complex was also evaluated using a BIAchip that had been previously coated with the procoagulant phospholipids binding protein, annexin V. This surface was sequentially reacted with PCPS (100 μ M), sgC (100 nM), and either FX (100 nM) or FVIIa (50 nM). In each case, the sensorgrams were compared to those performed in the presence of BSA or an empty flow cell.

2.4.3 Immunoinhibition

In order to determine whether TF and gC act in parallel toward FXa

generation, MacIntyre, NS, rns or ns-1 virus strains (500 virus particles/ μ l) or Vero cell extracts (22.5 cells/ μ l) were preincubated with increasing concentrations of an inhibitory anti-TF monoclonal antibody (mAb) (American Diagnostica, product #4508) or an identical concentration of isotype-matched non-immune mouse IgG (Sigma) at room temperature for 2 hours. Virus strains or the Vero cell suspension were then incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 minutes at room temperature. FXa generation was stopped and measured chromogenically. The complete reaction mixture in the absence of mAb was taken as 100% FXa-generating activity. The specificity of inhibition by anti-TF was ascertained by correcting for the amount of FXa generated at an identical concentration of non-immune IgG.

To confirm the manufacturer's characterization of the anti-TF mAb (#4508) as being inhibitory to human TF and to ensure that it was equally effective against TF derived from the Vero cells used to propagate purified viruses, the TF activity associated with Innovin or HFF extracts was compared to Vero cell extracts. Alternatively, an identical concentration of a polyclonal sheep anti-TF antibody was titrated instead of #4508. As an additional control, #4508 was examined for its ability to inhibit HSV1 MacIntyre grown in either Vero or HFF cells. In each case, FXa generation was monitored as previously described. The possibility that gC may affect the efficacy of the inhibitory mAb was evaluated using a concentration of Innovin that resulted in a similar amount of FXa generation as with the highest concentration of virus. Additionally, the effect of the anti-TF mAb (100 μ g/ml) was

evaluated in the presence and absence of sgC (1.5 μ M). As a final control, the possibility that sgC could effect the cleavage of S-2765 by FXa (4.5 nM) was addressed.

2.5 THROMBOGENIC POTENTIAL

2.5.1 Factor Xa generation

To determine the thrombogenic potential associated with different members of the Herpesvirus family a series of well-characterized lab adapted strains were utilized. These included HSV1 MacIntyre strain, HSV2 strain G and CMV AD169. The FXa generating ability associated with each virus was ascertained by titration of individual viruses in the presence of FX (100 nM) and FVIIa (5 nM). Activation of FX was then initiated by addition of Ca^{2+} (5 mM) and the amount of FXa generated after a 20 minute incubation at room temperature was monitored as above. In control experiments, each component of the reaction mixture was individually omitted and the amount of FXa generated was determined.

2.5.2 Thrombin Generation

Similar to above, HSV1 MacIntyre strain, HSV2 strain G and CMV AD169 were titrated in the presence of FX (100 nM) and FVIIa (5 nM). After the addition of Ca^{2+} (5 mM) the reaction mixture was incubated for 5 minutes at room temperature. Factor Va (5 nM) and prothrombin (1.4 μ M) were then added and the reaction was allowed to proceed for an additional 20 minutes. The amount of thrombin generated

was monitored by the cleavage of the chromogenic substrate S-2238. Standard curves of substrate cleavage with known amounts of thrombin were constructed to accurately determine the amount of thrombin produced in the system. In control experiments, each component of the reaction mixture was individually omitted and the amount of thrombin generated was determined.

2.5.3 Immunoblots

2.5.3.1 Antigen Recognition

To determine the presence of gC on different virus strains, purified virus were subjected to SDS PAGE. Initially, similar virus particles or plaque forming units were diluted in HBS and 1X Laemmli sample buffer. The proteins were separated on an acrylamide (6%) gel under both reducing (2% β -mercaptoethanol) and non-reducing conditions. After electrophoresis, the proteins were transferred to polyvinyl difluoride (PVDF) membrane using the CAPS buffer system for 3 hours. Following transfer, the membranes were blocked with skim milk powder (5%) in CAPS for 1 hour and incubated with a panel of monoclonal antibodies raised against HSV1 gC in order to obtain one that could recognize gC on all virus preparations. The antibodies used included C8 and C13 (kind gifts of Dr. Glorioso, University of Pittsburg), 1C8 (kind gift of Dr. Harvey Friedman, University of Pennsylvania) and a commercially obtained anti HSV1 gC (Accurate Chemical Company). After rinsing, all membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti mouse IgG (Jackson Immunologics) for 1 hour. The immunoreactive bands were visualized by

enhanced chemiluminescence detection (ECL, Pierce).

In a similar manner the amount of TF on each virus preparation was ascertained. Identical numbers of virus particles were separated on an acrylamide gel (10%) under both reducing and non-reducing conditions. After transfer, the membranes were blocked with skim milk (5%) and probed for TF using a panel of anti human TF (American Diagnostica, product #4503 or #4508; Affinity Biologicals; or an in house rabbit anti TF) antibodies. After primary incubation, the membranes were washed and incubated with HRP conjugated goat anti mouse IgG or HRP conjugated goat anti rabbit IgG. Immunoreactive bands were visualized using an enhanced chemiluminescent substrate and exposure on Kodak X-OMAT film. For both TF and gC detection, specificity was determined by substitution of the primary antibody with an identical amount of isotype matched on immune IgG.

2.5.3.2 Quantitative

A quantitative Western was developed to evaluate the amount of putative coagulation initiators associated with each virus preparation studied. To detect gC, similar numbers of each virus were separated on an acrylamide gel (6%) under non-reducing conditions. A standard curve of 2 fold dilutions of sgC was also run on the same gel. This permitted both the virus and standard gC samples to be subjected to identical conditions. Following electrophoresis, the proteins were transferred to PVDF and probed for gC using anti HSV1 gC (1C8, 1.2 µg/ml). After washing, the membrane was incubated with HRP conjugated goat anti mouse IgG (1:10,000)

followed by additional washing. The immunoreactive bands were visualized using ECL. Several different exposure times were chosen to permit an accurate representation of the amount of antigen present and allowed the construction of a standard curve of the purified sgC. In order to accurately determine the standard curve and amount of antigen present, each exposed film was scanned and converted to a tagged image file format (.tiff) file. The scans were then analyzed using Northern Eclipse software to determine the intensity of each standard and viral band. Using the intensity and known concentration of each sgC standard, a standard curve was constructed and used to determine the amount of gC in each virus lane. Only those intensities within the linear portion of the sgC standard curve were used to determine the amount of gC associated with each virus preparation tested. Several individual gels were electrophoresed and the results pooled to determine the amount of sgC associated with each virus.

In a similar manner, the amount of TF associated with each virus prep was determined. TF antigen was detected by incubating the PVDF membrane with monoclonal anti human TF (#4503, American Diagnostica, 1.0 µg/ml). Initially, Innovin was used as the source of purified TF. However, during preparation, Innovin is stabilized with BSA, making the exact TF protein concentration difficult to determine. Therefore, a purified source of soluble TF (TF₁₋₂₁₈, kindly provided by Dr. Wolfrum Ruf, The Scripps Institute) was used to construct the standard curve. The immunoreactive bands were visualized by incubation with HRP conjugated goat anti- mouse IgG and detected by ECL. The exposed blots were processed as was

done for the determination of gC.

2.6 ROLE OF THROMBIN IN HERPESVIRUS INFECTION

2.6.1 Plaque Assays

2.6.1.1 Serum

HFF were grown to confluency in 24 well plates in complete serum media. The cells were then washed once with PBS and complete serum media. Prior to inoculation, hirudin was titrated in complete serum media and a constant amount of virus (HSV1 MacIntyre, NS, rns, ns-1; HSV2 Strain G) was added to each. The hirudin/virus mixture was incubated with the cells for 90 minutes at 37°C with gentle shaking. After infection, the cells were once again washed with PBS and reduced serum media, and replaced with reduced serum media. Following infection, 40 - 48 hours, the cells were stained and the number of plaques determined. In order to selectively evaluate the effect of hirudin, the data obtained were corrected for the number of plaques detected in the absence of added hirudin.

2.6.1.2 Thrombin/TRAP

To determine the effect of thrombin in HSV infection, HFF were grown to confluency in complete serum media. Prior to infection, the cells were washed once with PBS and once with serum free media supplemented with BSA (1 mg/ml) to remove any residual serum proteins. Thrombin was diluted in serum free media and a constant amount of virus particles (HSV1 MacIntyre, NS, rns, ns-1; HSV2 Strain

G) was added to each dilution prior to incubation with the cells for 90 minutes. Following infection the inoculation mixture was removed and the cells were washed once with PBS and reduced serum media. The cells were replaced with reduced serum media. Following 40 - 48 hours post infection the cells were stained to determine the number of plaques. Alternatively, TRAP was titrated in the place of thrombin.

2.7 STATISTICAL ANALYSIS

In order to determine the significance of the results obtained, each set of data were subjected to t-test analysis using the website: <http://home.clara.net/sisa/> . The p-values obtained are given in the figure legends of the individual experiments.

3.0 RESULTS

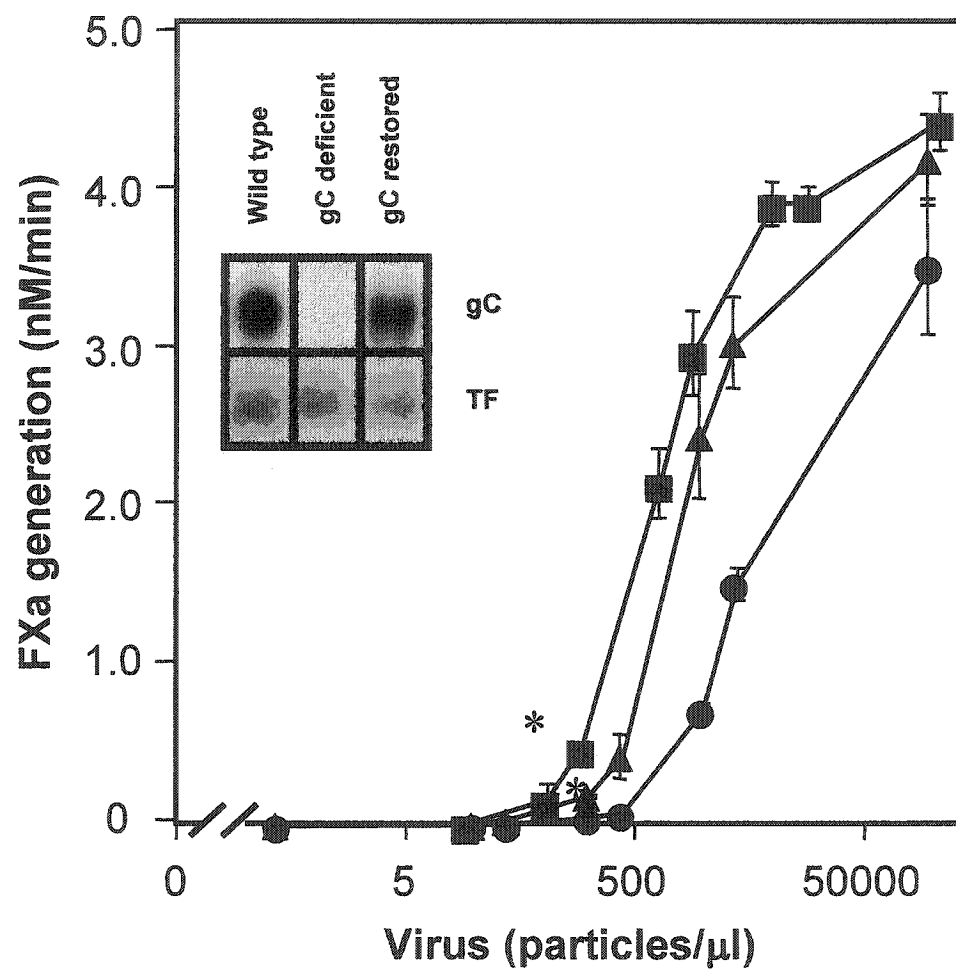
3.1 HSV1 GLYCOPROTEIN C MEDIATED FACTOR X ACTIVATION

3.1.1 Viral glycoprotein C

Previous work from our laboratory²¹⁶ demonstrated thrombin production by purified members of the Herpesvirus family. This resulted from the constitutive expression of the coagulation initiator, tissue factor (TF) and procoagulant phospholipid (proPL) on the surface of HSV1, herpes simplex virus type 2 (HSV2), and cytomegalovirus (CMV). Interestingly, this study revealed that the TF-specific thrombin production could be only partially inhibited by a TF-specific mAb, suggesting an additional mechanism of FX activation associated with these viruses. Since an earlier report using cross-linking techniques suggested that HSV1-infected cells could participate in FXa production through an interaction between gC and FX^{8,62}, we investigated the contribution of gC on the virus surface in cell-free FX activation by FVIIa.

A well-characterized panel of purified HSV1 consisting of wild type (MacIntyre or NS)⁷¹, gC-deficient (ns-1)⁸⁴ and gC-restored (rns)⁶⁹ strains were evaluated for their ability to generate FXa. To determine the importance of HSV1 surface gC as a FVIIa cofactor we initially varied the concentration of each virus (Figure 6). In the case of the gC-deficient virus, as much as 6-fold less FXa was generated per particle when compared to either of the gC-competent strains, indicating a role of gC in FX activation. The amount of FXa production by ns-1 was observed to

Figure 6 Loss of HSV1 glycoprotein C results in a reduced ability to generate factor Xa. Increasing amounts of the the naturally occurring HSV1 gC deficient ns1 strain (●), wild types (■, clinical isolate NS or MacIntyre) or the gC-restored rns strain (▲) were incubated with FX (100 nM), FVIIa (5 nM) and Ca²⁺ (5 mM) for 20 minutes. Following incubation, the amount of *in situ* virus-mediated FXa generated was determined by cleavage of the FXa-specific chromogenic substrate S-2765. (n=9) Inset: The presence of tissue factor (TF) and glycoprotein C (gC) on each virus was determined by Western blot analysis. (Wild type, ■, p = 0; gC-restored, ▲, p = 0.0002)



approach that of wild type and gC-restored viruses at high virus concentrations. This apparent convergence of FXa generating capabilities may be due to: 1) the abundance of both TF and gC initiator molecules in the system; 2) the binding of all available FVIIa or FX in the system; or 3) the sequestration of enzyme and substrate molecules on different viral particles. Therefore, to ensure that the results were dependent on the TF or gC associated with each virus, and to maximize the apparent differences caused by the presence or absence of gC on these viruses, subsequent experiments were conducted at a low virus concentration. The use of this rate-limiting number of virus particles also eliminated the possibility of substrate depletion in the system.

To illustrate the dependence of the novel gC function on FX, the amount of substrate molecules in the system was systematically varied. Consistent with a role in FX activation, the loss of gC in ns-1 invariably resulted in a vastly reduced capacity of this virus to generate FXa compared to wild type or gC-restored viruses (Figure 7). A similar result was realized when the amount of FVIIa was titrated with each virus in that ns-1 generated less FXa per particle than each of the gC-competent viruses (Figure 8). In all cases, either wild type virus generated the most FXa per particle followed by the gC-restored and gC-deficient viruses. Collectively, these data provide further support for the additional TF-like activity on HSV1 being mediated by gC. Under the experimental conditions we observed saturable activity as a function of either FX or FVIIa, qualitatively suggesting that like TF^{116,195}, gC may contribute to interactions with both enzyme and substrate. In control

Figure 7 HSV1 mediated factor Xa generation is dependent on the interaction of glycoprotein C with factor X. Increasing concentrations of FX were incubated with an equal amount of viral particles (Wild type, ■; g-C deficient, ●; and gC-restored, ▲; 100 virus particles/μl), FVIIa (5 nM) and Ca²⁺ (5mM) for 20 minutes and FXa generation was monitored by chromogenic substrate cleavage. (n=6) (Wild type, ■, p = 0; gC-restored, ▲, p = 0)

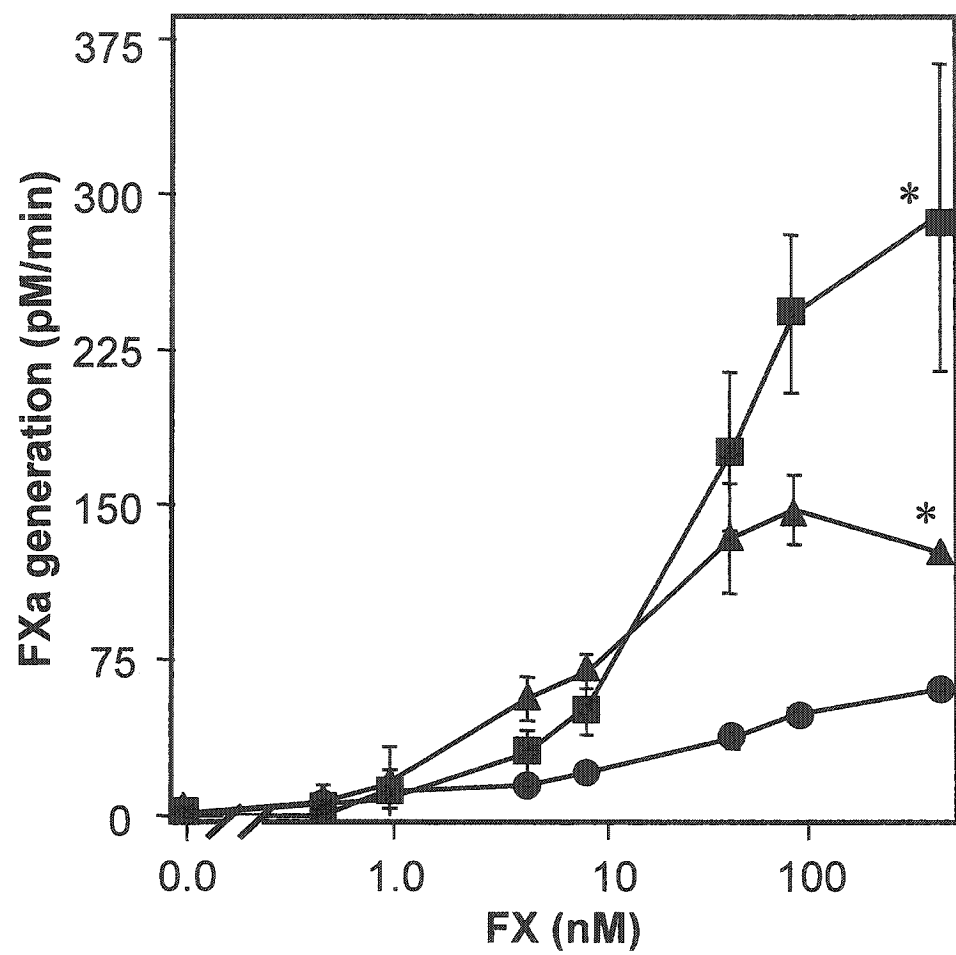
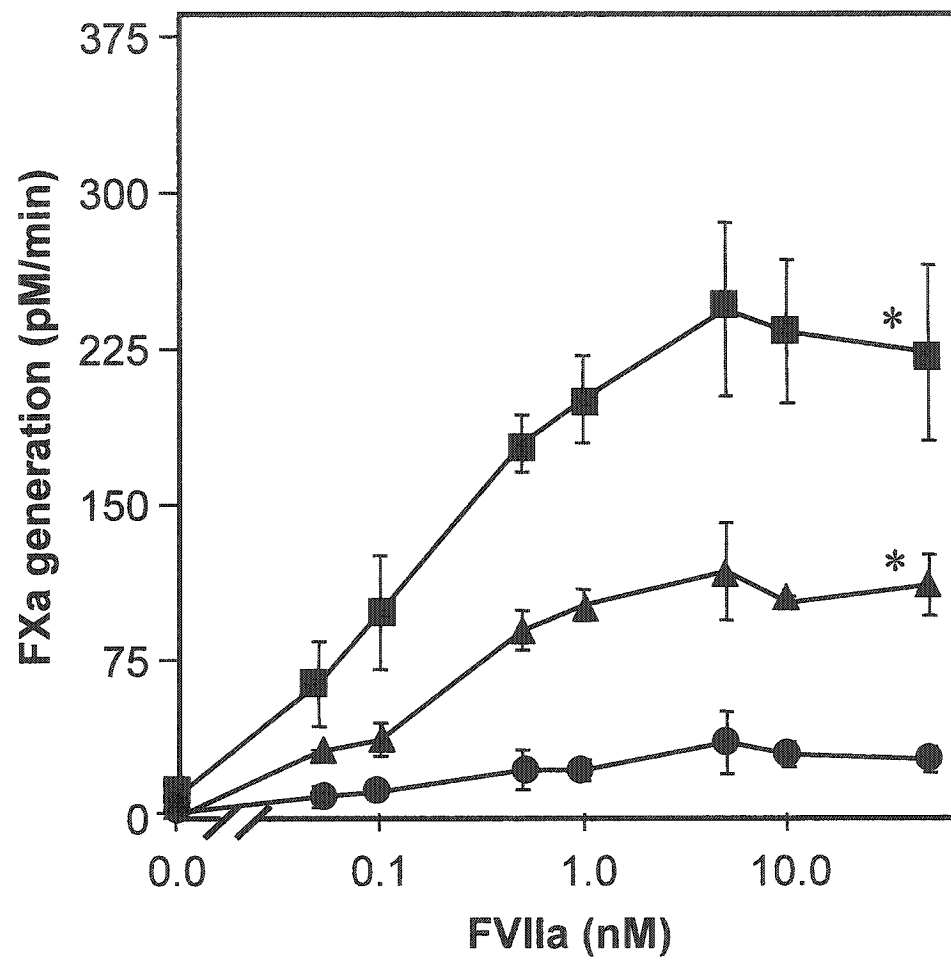
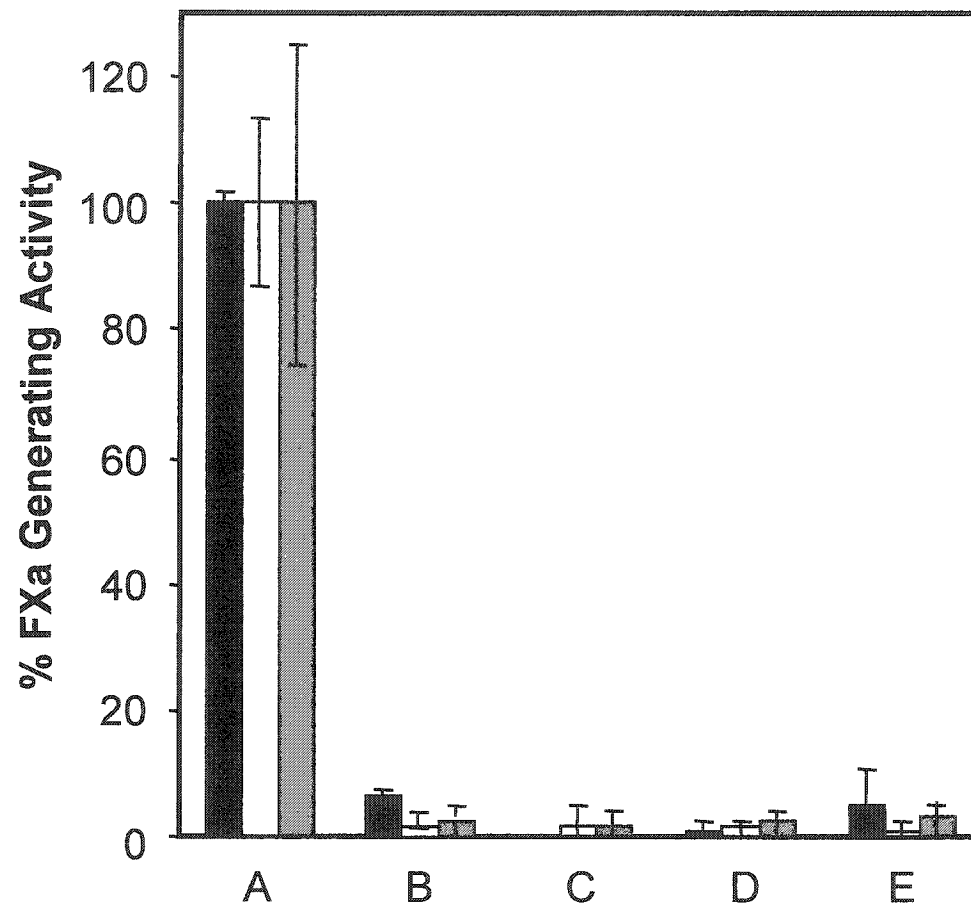


Figure 8 HSV1 glycoprotein C interactions with factor VIIa are important for factor Xa generation. An increasing amount of FVIIa was added to a constant amount of viral particles (Wild type, ■; g-C deficient, ●; and gC-restored, ▲; 100 virus particles/μl), excess FX (100 nM) and Ca²⁺ (5mM). The amount of FXa generated after a 20 minute incubation was determined chromogenically (n=6) (Wild type, ■, p = 0; gC-restored, ▲, p = 0)



experiments, negligible chromogenic activity was detected when virus, FX, FVIIa or Ca^{2+} were individually omitted from the reaction mixture (Figure 9). This indicated that each component in the system was important for the generation of FXa and the proteins used were free of possible contaminating coagulation proteins. These control experiments demonstrated the specificity of FXa generation in our assay and interestingly, showed that like TF, participation of gC in FX activation requires FVIIa. To ensure that the differences in FXa generation were reflective of the amount of putative initiator molecules in the system, Western blot analysis was utilized. Similar numbers of virus particles from each virus strain were separated by SDS PAGE and probed for either TF or gC antigen (Figure 6, inset). The exposed membranes indicated that the gC-restored virus, rns, expresses slightly more gC than the wild type NS virus. A similar result was seen with TF in that rns expressed slightly more TF antigen than wild type virus while wild type and the gC-deficient strain contained near identical levels of TF per virus particle. Therefore the reduced FXa generation by rns compared to NS is seemingly contradictory to the levels of TF and gC antigen on these viruses. The lower activity associated with the gC-restored virus may be a result of antigen presentation. Alternatively, a portion of the initiator molecules may be involved in multimeric complexes⁵² or encrypted¹²³ and therefore unable to function in FX activation. However, the probed membranes clearly show the loss of gC in the ns-1 strain and comparable levels of TF in this virus compared to the wild type preparations. Therefore, the reduced ability of the gC-deficient virus to generate FXa is not simply due to lower levels of TF associated

Figure 9 HSV1 glycoprotein C mediated factor Xa generation is dependent on each reaction constituents. A) Each virus (MacIntyre or NS, black; gC-deficient, white; gC-restored, grey) was incubated in the presence of FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) and was considered 100% FXa generating activity. The dependence of the reaction on each constituent (virus, B; FX, C; FVIIa, D; or Ca^{2+} , E) was also determined. (n=6)



with this virus.

A series of control experiments were conducted to determine additional specificity in our system. Since the virus panel studied was purified from infected Vero (monkey) cells, the possibility remained that the human coagulation proteins used would have a reduced affinity to the monkey-derived virus surface proteins. However, when HSV1 MacIntyre grown in Vero cells was compared to the same virus propagated in HFF (Figure 10) there was no difference in FXa generation between the two viruses. This indicated that the TF on both viruses was able to recognize FVIIa and effectively cleave FX. Also, low virus particle numbers were used in the FX and FVIIa titration experiments that may limit the reaction by the amount of available proPL in the system. This possibility was explored by monitoring FXa generation in the presence and absence of exogenous proPL (Figure 10). When proPL was added to the system, there was a reduction in the amount of FXa generated, most likely due to the partitioning of the substrate and enzyme to different virus or proPL particles³², and a reduction in FXa generated. Exogenous proPL was therefore not needed in the system and the experimental results were limited only by the amount of initiator molecules supplied by the virus surface.

Additional experiments were conducted to determine if overlap exists between the areas of known functionality in gC and those involved in FX activation. Initially, the ability of HSV1 gC binding to complement protein C3b or its inactive precursor, C3, on FX activation was explored. In support of distinct functional regions on the gC molecule, C3b or C3 (Figure 11) had a minimal effect on the

Figure 10 HSV1 mediated factor Xa generation is independent of virus propagating species and addition of exogenous proPL. (Top) HSV1 MacIntyre propagated in either Vero (◆) or HFF (◆) were incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 minutes. Following incubation, the amount of *in situ* virus-mediated FXa generated was determined. (n=3) (Bottom) Alternatively, the reaction was monitored in the presence (dashed line) or absence (solid line) of proPL (200 μM). (n=3)

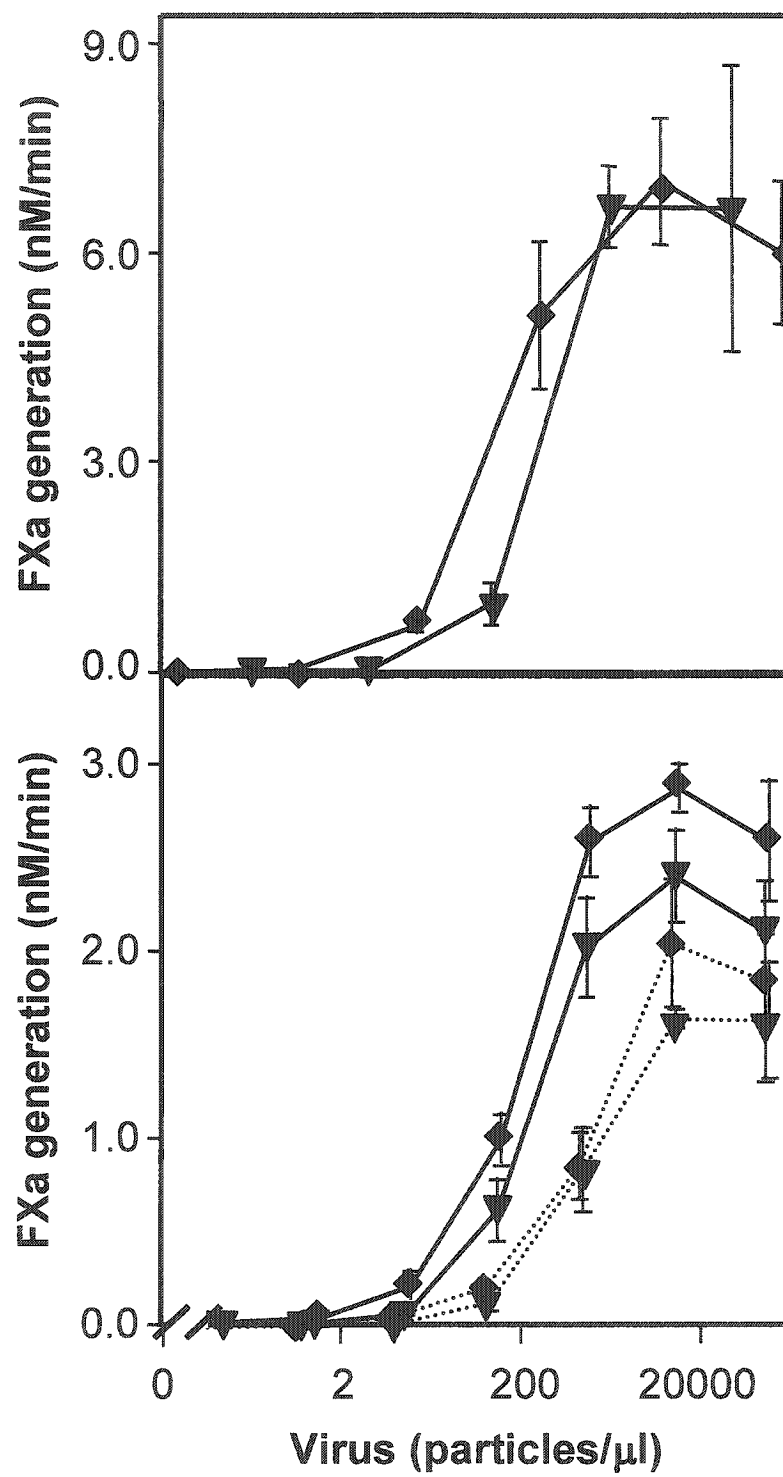
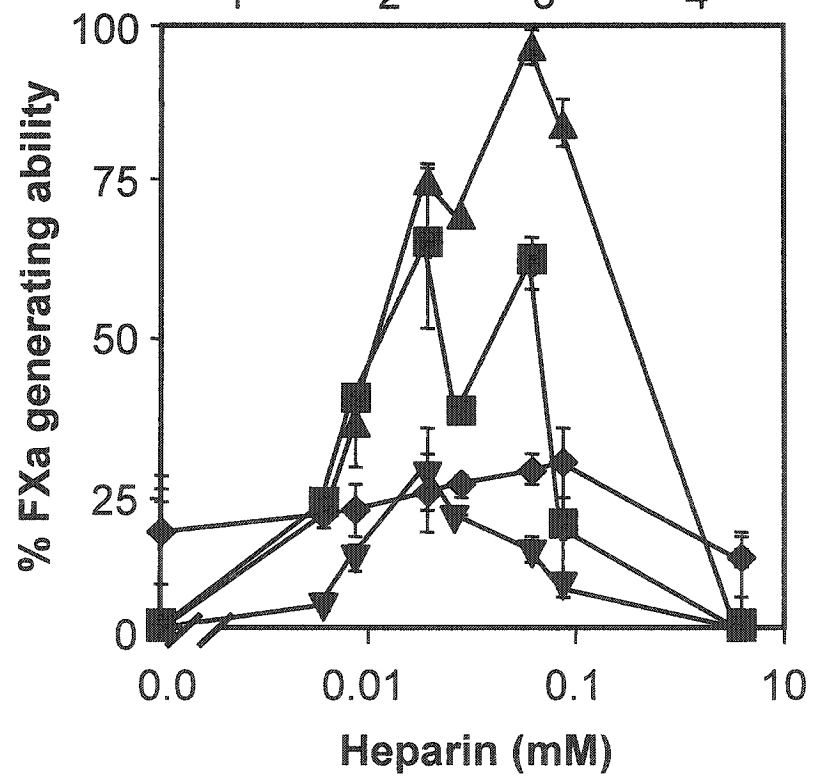
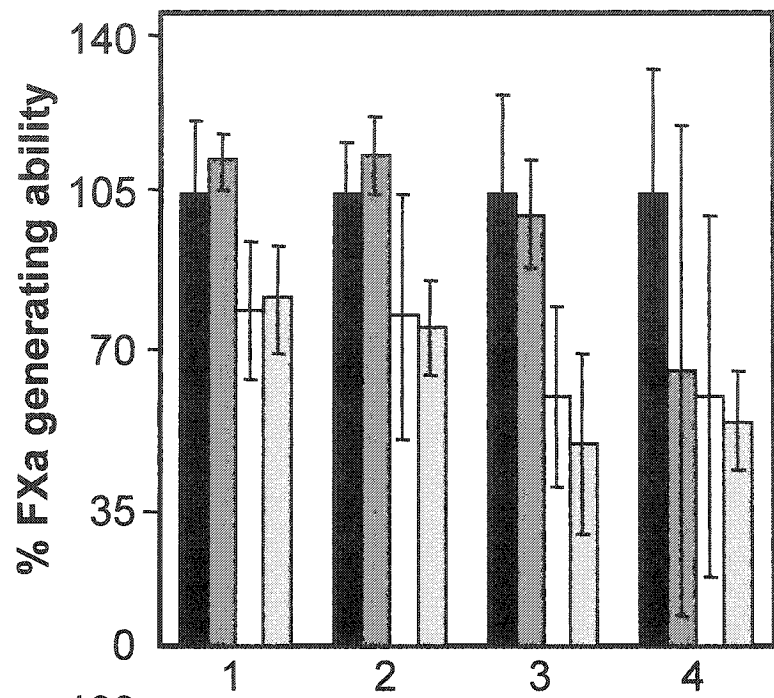


Figure 11 Independent functional regions exist on HSV1 glycoprotein C. HSV1 wild types, MacIntyre (1 or ■) or NS (2 or ♦); gC-restored (3 or ◆; or gC-deficient (4 or ◆) were incubated with FX (100 nM), FVIIa (5 nM) and Ca²⁺ (5 mM) for 20 minutes in the presence of (Top) HBS (black), complement protein C3 (dark grey), complement protein C3b (white) or bovine serum albumin (light grey) or (Bottom) increasing concentrations of heparin. Following incubation, the amount of *in situ* virus-mediated FXa generated was determined. (n=3)



ability of each virus in the panel to generate FX. However, when compared to a control protein, BSA, no significant change in FXa production was noted. The effect of heparin addition on the ability of each virus to generate FXa was also explored. When heparin was titrated with each virus (Figure 11) there was an increase in the amount of FXa generated. This occurs through an unknown mechanism and may not be specific to an interaction with gC since an alternative cell surface sulfated molecule was not explored in control experiments. However, the results give additional support to conservation of independent functional regions on gC for FX activation and C3b binding.

3.1.2 Purified HSV1 glycoprotein C

In order to selectively evaluate the novel TF-like gC function, a recombinant form of gC²¹⁷, produced in baculovirus was used. The protein produced excludes the hydrophobic transmembrane domain, making it soluble (sgC) and easily purified from the infected cell supernatant. Using extensive binding studies the protein has been previously highly characterized with respects to functional regions and compared to native viral gC. sgC has also been shown to retain conformation-dependent binding to antibodies. The protein produced in our lab was shown to be pure and have the correct molecular weight as determined by SDS PAGE and retained its ability to bind to the conformational dependent antibody 1C8.

Purified sgC was used in the development of a virus-free purified system to determine an independent role of gC in FX activation. In initial experiments, similar

to those used to show a role for soluble TF function in FX activation ¹⁹⁵, high concentrations of FX and FVIIa were used and sgC was added as the sole source of putative FVIIa cofactor. The results of this forced time course experiment were the first evidence that sgC could participate in FX activation and indicated that an initial lag phase in FXa generation existed. In subsequent experiments, lower concentrations of substrate and enzyme yielded similar results. Therefore, using identical FX and FVIIa concentrations as with viral mediated FX activation, sgC was observed to accelerate FX activation (Figure 12). However, when compared to the molar gC activity that was estimated for wild type virus strains (based on 5000 copies per virus ⁸³), sgC-enhanced FXa production was approximately 1000-fold less than the viral counterpart. In order to show the dependence of the components in the reaction mixture for FXa generation, each was independently omitted. Interestingly, the novel sgC-mediated FXa-generating ability emulated TF since each of FVIIa, FX, proPL and Ca^{2+} were required (Figure 13).

In preliminary experiments, an initial lag phase existed in the sgC-mediated generation of FXa. This suggested that the loss of the transmembrane domain in sgC may affect the formation of an effective enzyme complex to activate FX. Alternatively, the omission of the virus may result in the loss of an essential cofactor needed for gC to mediated FX activation. Therefore, to ensure proper complex formation, an extended preincubation step was undertaken to allow detectable levels of FXa to be generated.

To determine if the reduced ability of sgC to activate FX compared to viral gC

Figure 12 Purified soluble glycoprotein C can participate in factor X activation. An increasing amount of sgC was incubated with FX (100 nM), FVIIa (5 nM), Ca^{2+} (5 mM) and procoagulant phospholipid (100 μM) for 2 hours. The amount of FXa generated was monitored by cleavage of the chromogenic substrate, S-2765. All data were corrected for the amount of FXa generated in the absence of sgC. (n=6) (p = 0.0001)

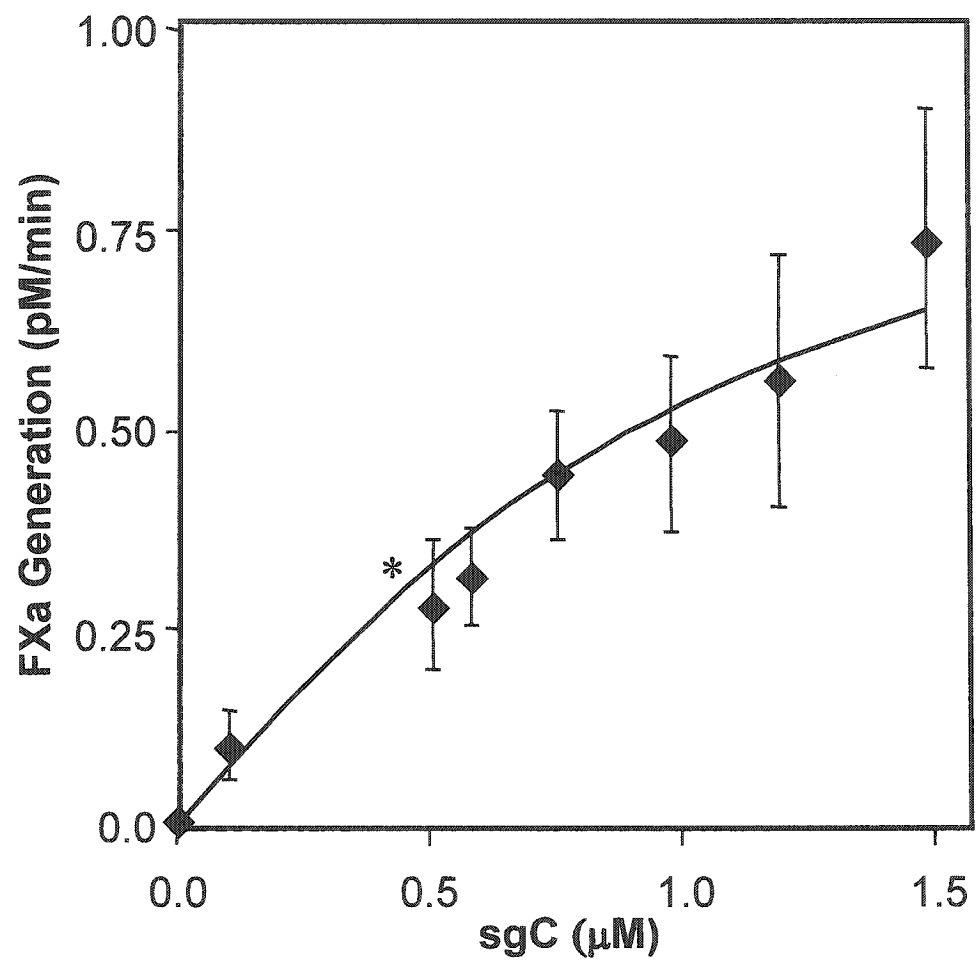
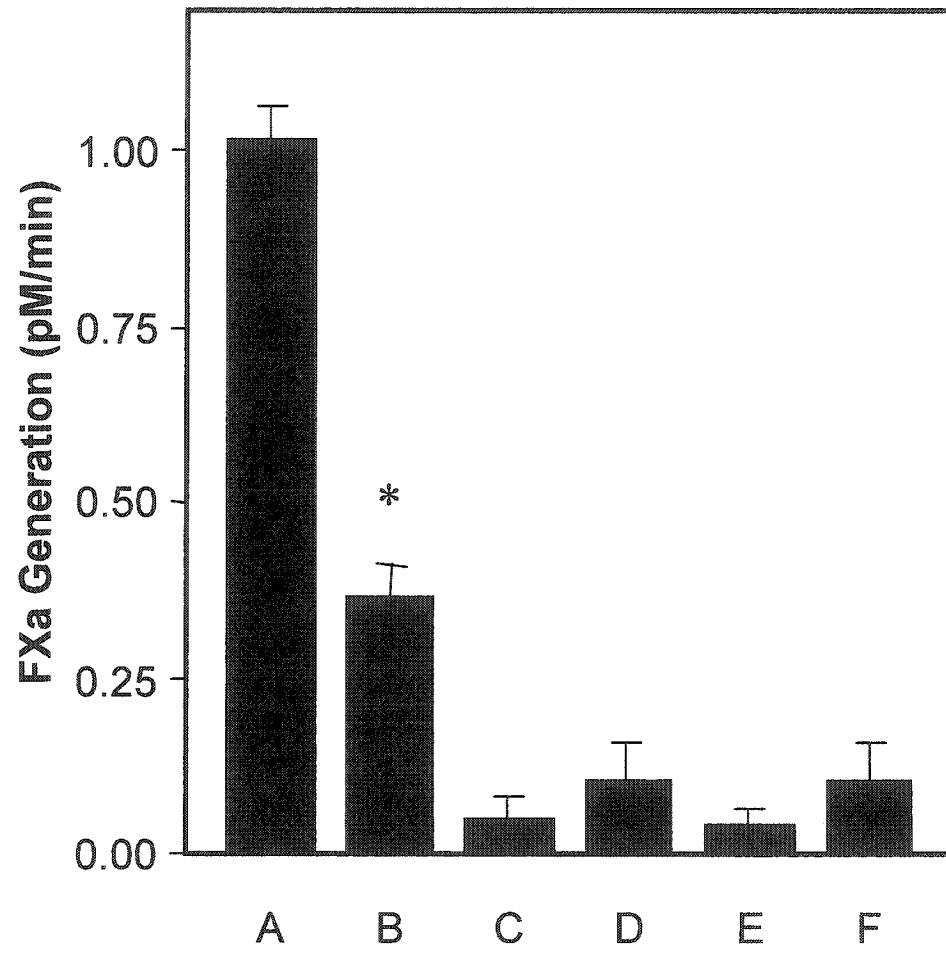


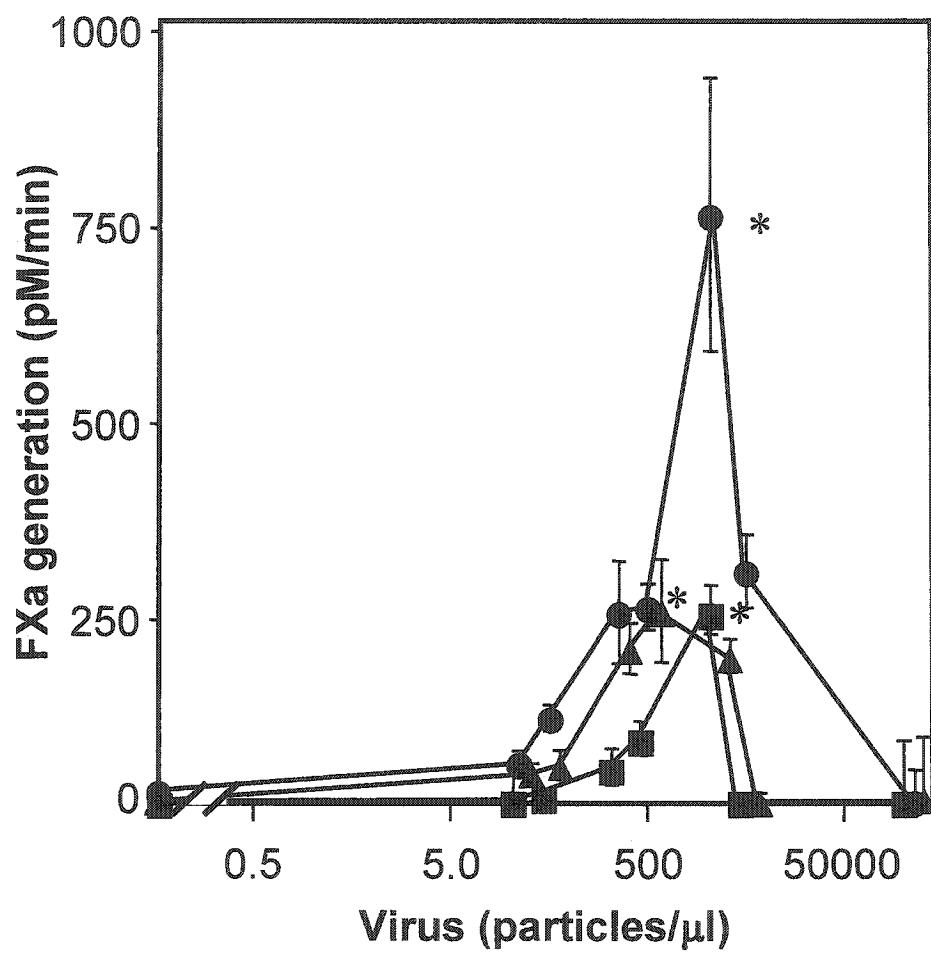
Figure 13 Purified soluble glycoprotein C-mediated factor X activation mimics tissue factor. (A) sgC (1.5 μ M) was incubated with FX (100 nM), FVIIa (5 nM), Ca^{2+} (5 mM) and proPL (100 μ M) for 2 hours and the amount of FXa generated was detected by chromogenic substrate cleavage. Each constituent was also omitted (sgC, B; FX, C; FVIIa, D; Ca^{2+} , E; or proPL, F) to show dependence. (n=6) (p =0)



was due to the requirement for other virus components, sgC was combined with each of the viruses in our panel and assayed for effects on FXa production. When the virus concentration was varied (Figure 14), the amount of sgC-dependent FXa production was enhanced by as much as three orders of magnitude, consistent with the predicted amount for intact viruses. The contribution of the virus on sgC function was selectively evaluated by correcting the data for FXa-generating activity due to virus in the absence of sgC. These data strongly imply that an additional virus constituent(s) associates with sgC to complement the FVIIa cofactor function identified for gC. In support of this concept, the greatest enhancing effect on sgC was observed for the gC-deficient virus. This occurs since the lack of endogenous gC may increase the number of unoccupied binding sites available for sgC on ns-1 that do not exist on either of the gC-containing viruses. An identical enhancement in FXa generation was observed with the addition of sgC to either wild type or gC-restored viruses. However, the maximal effect for the two viruses occurred at different particle numbers possibly due to differing amounts of gC associated with these viruses as indicated previously. As expected, in all cases when the virus was in vast excess, the sgC-specific enhancement decreased because of the overwhelming effects of endogenous virus FX activation at high particle numbers. Alternatively, the possibility existed that substrate and enzyme were partitioned onto different virus particles and were therefore unable to effectively cleave FX.

To further demonstrate the importance of gC in FX activation and the need for an additional virus constituent for maximal cofactor function, sgC was varied with

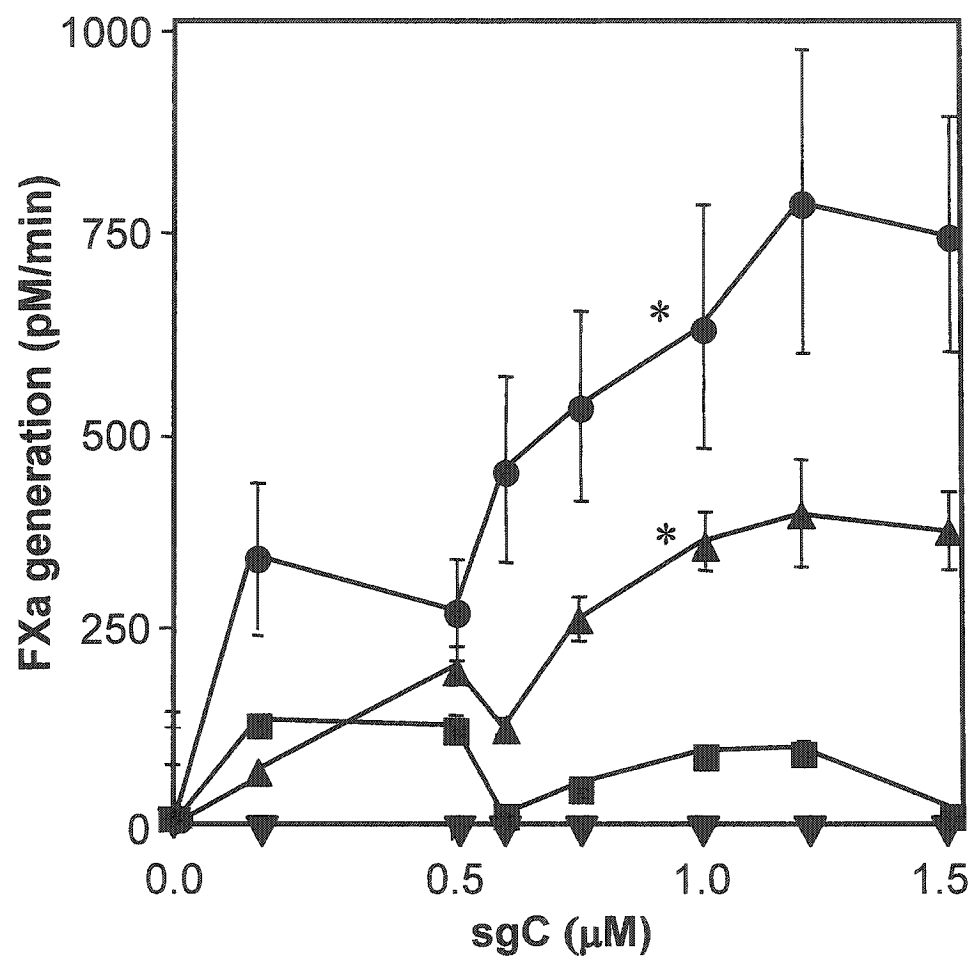
Figure 14 Soluble glycoprotein C-mediated factor Xa generation is enhanced in the presence of virus. A constant amount of sgC (1.5 μ M) was added to an increasing amount of HSV-1 strains (Wild type MacIntyre, ■; g-C deficient, ●; and gC-restored, ▲) and incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM). Following a 20 minute incubation the amount of FXa generated was determined. The data were corrected for the amount of FXa generated by virus alone. (n=5) (Wild type, ■, p = 0; g-C deficient, ●, p = 0.0007; gC-restored, ▲, p = 0.003)



a constant amount of virus (Figure 15). As in the virus-free system (Figure 12) the sgC-mediated enhancement of FXa generation was titratable. Once again, the gC-deficient strain resulted in 3 orders of magnitude more FXa generated than sgC alone, consistent with the data obtained when virus was titrated in the presence of constant amounts of sgC (Figure 14). The enhancement of FVIIa cofactor activity reached half-maximal saturation at approximately 0.7 μ M, which may be an indicator of the apparent dissociation constant between sgC and the species on the virus surface contributing to FVIIa cofactor function. In each case, the effect was specific to sgC as all data were corrected for any FXa generated in identical experiments conducted in the absence of sgC.

The possibility existed that the added sgC could directly affect either the cofactor function of TF, or the activity of FXa or FVIIa in our chromogenic assay. To ensure this was not the case a series of control experiments using Innovin were conducted. This reagent is advantageous in that it is a highly characterized source of TF activity and consists solely of recombinant human TF and proPL, allowing the investigation of possible TF and sgC interactions in a cell- or virus-free system. When Innovin was incubated with an identical titration of sgC as that used for the viruses (Figure 15), sgC was concluded to have no effect on the FXa generating cofactor function of Innovin after subtraction of an identical curve conducted in its absence. sgC also had no effect on the enzymatic activity of FVIIa since identical amounts of FXa were generated with and without added sgC. Finally, sgC did not affect the amidolytic activity of the FXa produced in our system since the ability of

Figure 15 Soluble glycoprotein C-mediated increase in HSV1-dependent factor Xa activation is saturable. An increasing amount of sgC was added to a constant amount of purified virions (Wild type MacIntyre, ■; g-C deficient, ●; and gC-restored, ▲) or Innovin (◆), FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 minutes. (n=6) The data were corrected for the amount of virus- or Innovin-dependent FXa generation alone. (Wild type, ■, not significant; g-C deficient, ●, $p = 0.005$; and gC-restored, ▲, $p = 0$)



purified FXa to cleave the chromogenic substrate was unaffected over the concentration range of sgC used in our assays.

3.1.3 Simultaneous pathways on HSV1 leading to Factor Xa generation

We previously demonstrated that approximately 50% of chromogenic activity initiated on the surface of HSV1 could be accounted for by the presence of TF using an inhibitory TF-specific mAb ²¹⁶. Since the current data suggests that gC may account for the remaining activity, we speculated that the gC-deficient HSV1 would be completely inhibited by the anti-TF mAb. Consistent with this hypothesis, the activation of FX by FVIIa on ns-1 was inhibited by anti-TF approximately 90%, whereas the gC-containing wild type (MacIntyre or NS) or the gC-restored (rns) HSV1 strains were maximally inhibited by 40% (Figure 16). This indicated that an additional TF-like mechanism exists on the wild type virus that was indeed facilitated by gC. Moreover, the results show that the two pathways of FVIIa-mediated FX activation can function both independently and simultaneously on the virus surface. The direct involvement of gC in FX activation was confirmed using the gC restored virus, which was affected by the TF inhibitory mAb comparably to the wild type strain.

In order to confirm the specificity of the inhibitory TF mAb used in the study, extensive control experiments were conducted (Figures 17-19). Human (HFF) and monkey (Vero) cell sources of TF activity were prepared and compared to the commercial (Innovin) source of TF with respect to FXa generation. Each source was

Figure 16 Incomplete inhibition of factor X activation by anti-tissue factor is due to HSV1 glycoprotein C. HSV1 strains (Wild types MacIntyre or NS, ■; gC- deficient, ●; and gC-restored, ▲; 500 particles/ul) or Vero Cells (◆, 22.5 cells/ul) were treated with increasing concentrations of an inhibitory anti TF antibody for 2 hours at room temperature. Purified FX (100 nM), FVIIa (5 nM) and Ca²⁺ (5 mM) were then added and FXa generation was monitored after 20 minutes by cleavage of a FXa-specific chromogenic substrate (n=6). The data were corrected for the amount of active enzyme generated in the presence of an identical amount of non-immune mouse IgG. (Wild type, ■, p = 0.02; gC- deficient, ●, p = 0.002; and gC-restored, ▲, p = .002)

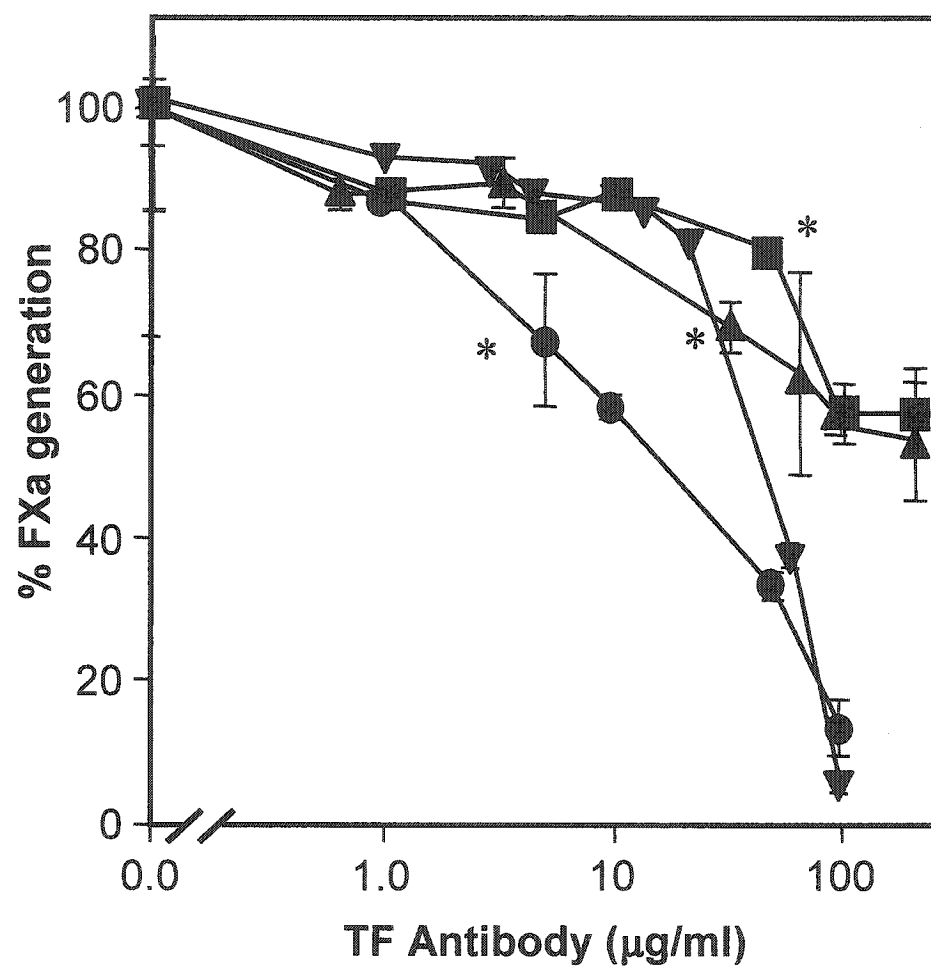


Figure 17 Tissue factor activity characterization - Innovin. (Top) Increasing concentrations of Innovin were incubated with purified FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 min and the amount of FXa generation was monitored. (Middle) The specificity of the reaction was demonstrated when the complete reaction mixture (D) was compared to the amount of FXa generated in the absence of Innovin (A), FX (B), FVIIa (C), or Ca^{2+} (E). (Bottom) Innovin was incubated with an increasing concentration of mouse anti-TF (solid) or sheep anti-TF (dashed) antibody for 2 hours. FX, FVIIa and Ca^{2+} were then added and the amount of FXa generated was determined. (n=3) The data were corrected for the amount of active enzyme (FXa) generated in the presence of an identical amount of non-immune mouse IgG.

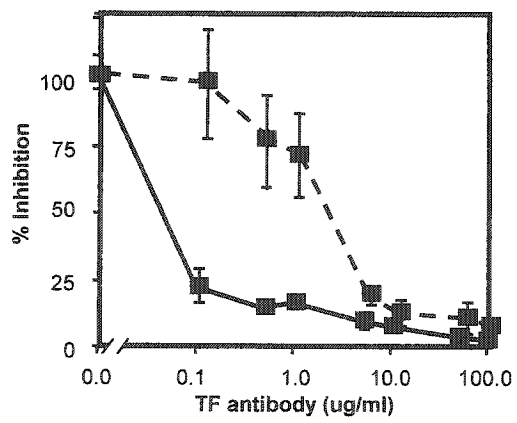
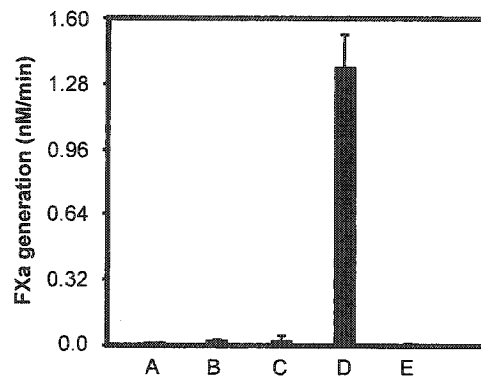
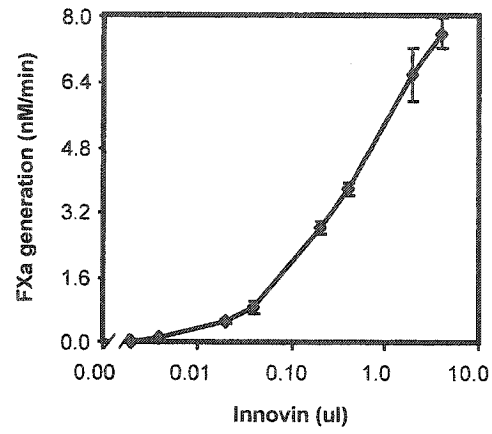


Figure 18 Tissue factor activity characterization - Veros. (Top) Increasing concentrations of a Vero cell suspension were incubated with purified FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 min and the amount of FXa generation was monitored. (Middle) The specificity of the reaction was demonstrated when the complete reaction mixture (D) was compared to the amount of FXa generated in the absence of Vero cells (A), FX (B), FVIIa (C), or Ca^{2+} (E). (Bottom) Veros cells were incubated with an increasing concentration of mouse anti-TF (solid) or sheep anti-TF (dashed) antibody for 2 hours. FX, FVIIa and Ca^{2+} were then added and the amount of FXa generated was determined. (n=3) The data were corrected for the amount of active enzyme (FXa) generated in the presence of an identical amount of non-immune mouse IgG.

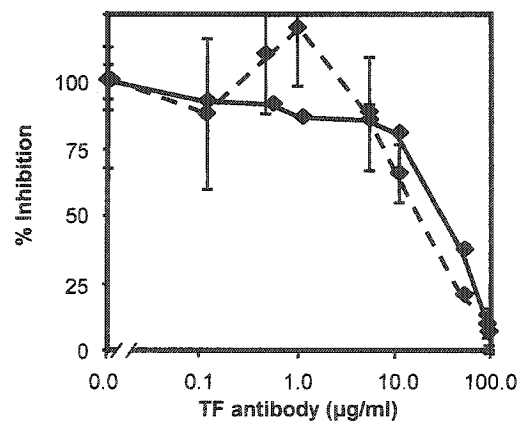
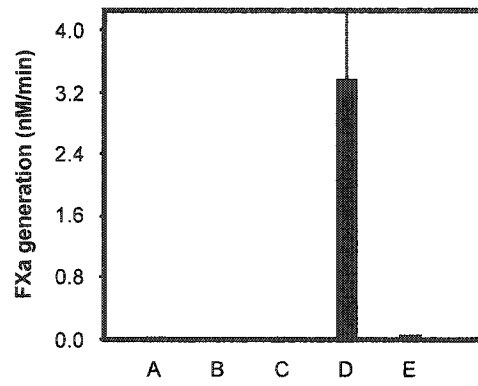
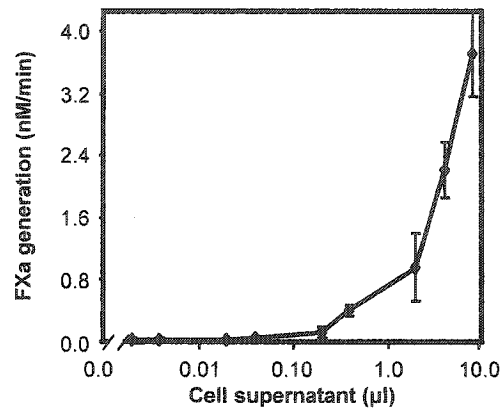
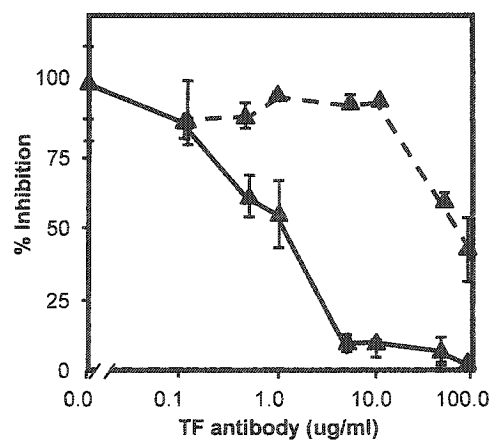
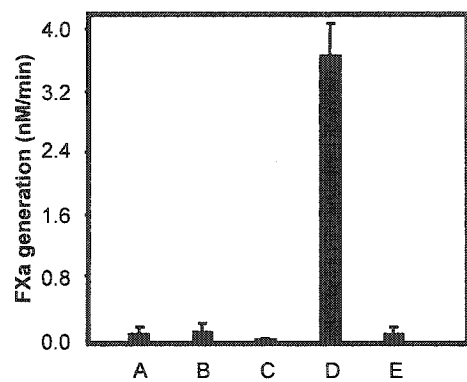
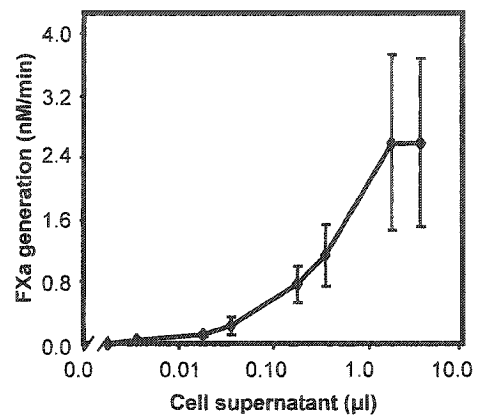


Figure 19 Tissue factor activity characterization - Fibroblasts. (Top) Increasing concentrations of an HFF cell suspension were incubated with purified FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 min and the amount of FXa generation was monitored. (Middle) The specificity of the reaction was demonstrated when the complete reaction mixture (D) was compared to the amount of FXa generated in the absence of HFF cells (A), FX (B), FVIIa (C), or Ca^{2+} (E). (Bottom) HFF cells were incubated with an increasing concentration of mouse anti-TF (solid) or sheep anti-TF (dashed) antibody for 2 hours. FX, FVIIa and Ca^{2+} were then added and the amount of FXa generated was determined. (n=3) The data were corrected for the amount of active enzyme (FXa) generated in the presence of an identical amount of non-immune mouse IgG.



shown to activate FX in a titratable manner. Additional experiments in which each component of the reaction mixture was individually omitted indicated that the activity was FVIIa-, Ca^{2+} -, and proPL-dependent, consistent with TF functioning. Using antibody concentrations that were the same as those used for the viruses (Figure 16), inhibition of 100% of the TF activity associated with the uninfected Vero cells used to propagate the gC-characterized virus strains, a standardized source of human recombinant TF, Innovin, or that of HFF origin were observed. A polyclonal anti-TF antibody also inhibited all sources of TF although less efficiently than #4508. Additionally, when the antibody was titrated with HSV1 MacIntyre grown in either Vero or HFF cells, similar amounts of FXa-generating inhibition was seen (Figure 20). The incomplete immunoinhibition seen previously and in this study was therefore not due to differences in the TF source since the antibody used could completely inhibit the TF activities of both purified and cell associated sources of monkey or human TF. Furthermore, gC had no effect on the TF-specific inhibitory antibody or function of other proteins in the system since inhibition of recombinant TF was independent of sgC over the antibody concentration range used here (Figure 21).

3.2 THROMBOGENIC POTENTIAL ASSOCIATED WITH HERPESVIRUSES

In a previous study we have shown that members of the Herpesvirus family differ in their ability to form an insoluble fibrin clot. These observations correlated with the ability of these viruses to induce clinical outcomes such as thrombosis or

Figure 20 Differential inhibition of factor X activation by HSV1 MacIntyre propagated in Vero or HFF cells. HSV1 MacIntyre grown in either Vero (◆) or HFF (◆) were incubated with increasing concentrations of a monoclonal antibody against human tissue factor (American Diagnostica, 4508) for 2 hours. FX (100 nM), FVIIa (5 nM) and Ca²⁺ (5 mM) were then added for 20 min and the amount of FXa generation was monitored chromogenically. (n=6)

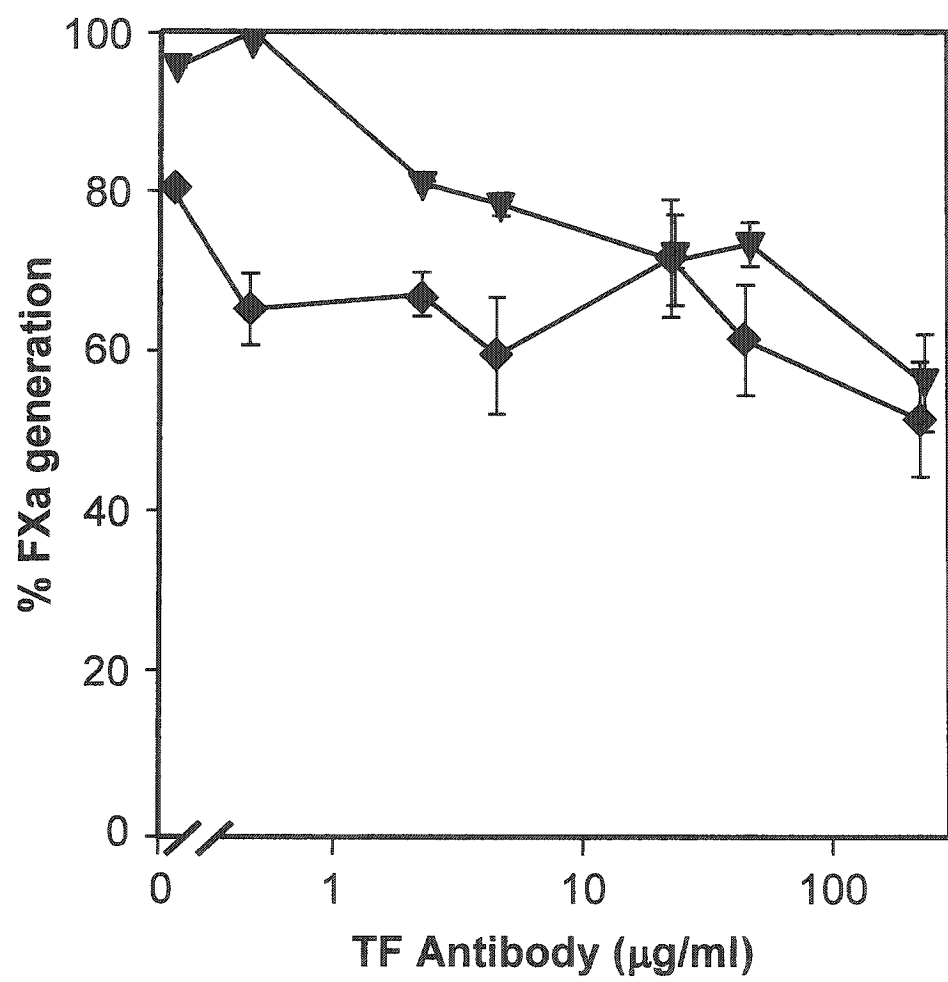
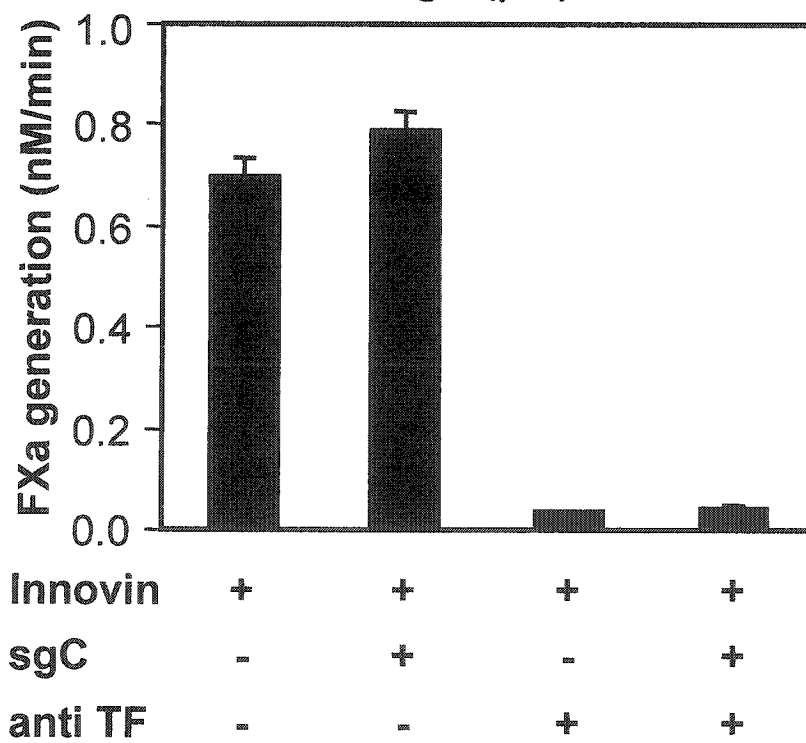
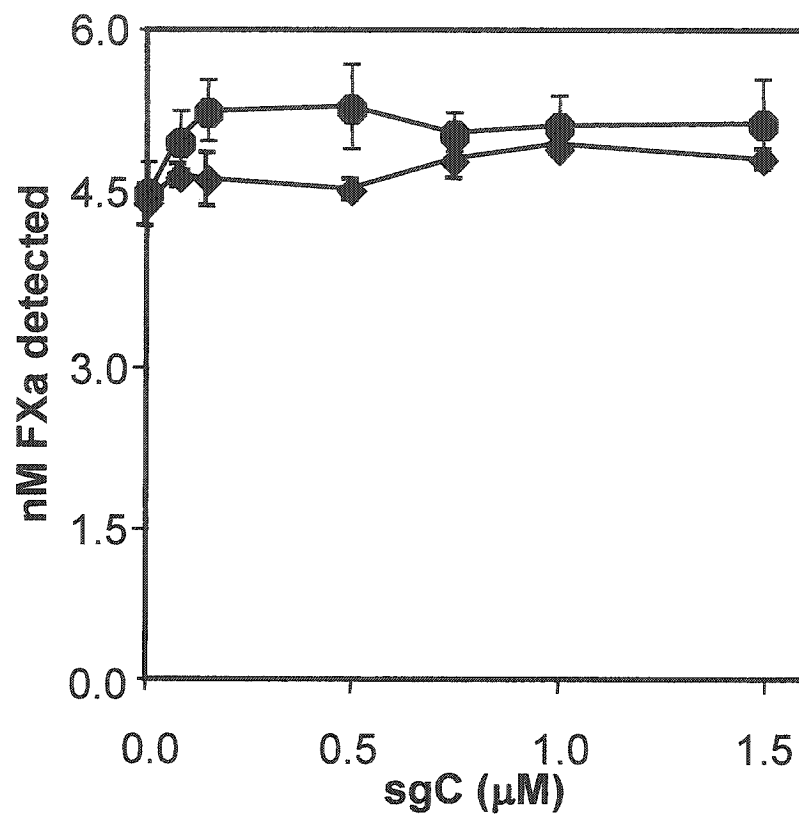


Figure 21 Effect of soluble glycoprotein C on reaction constituents. (Top) Purified FXa (5 nM) was incubated with increasing amounts of sgC (●) or an equivalent volume of HBS (◆) and the amidolytic activity of FXa was monitored. (Bottom) Innovin was incubated in the presence or absence of sgC (1.5 μ M) and anti-TF antibody (100 μ g/ml) and the amount of FXa generated was monitored. (n=3)



atherosclerosis. We therefore wanted to determine the ability of several members of this virus family to initiate thrombin generation directly on their surfaces in a purified system. The ability to sustain coagulation initiation events was examined by the generation of FXa on HSV1 MacIntyre, herpes simplex virus type 2 (HSV2) strain G and cytomegalovirus (CMV) AD169. Consistent with our previous clotting data showing a reduced ability for CMV to initiate fibrin clot formation ²¹⁶, CMV produced less FXa than either HSV1 or HSV2 in our FXa specific chromogenic assay (Figure 22).

In order to substantiate these data, a thrombin-specific chromogenic assay was developed. This involved a short FXa generating step followed by the addition of plasma concentrations of prothrombin and FVa. Initially an identical virus titration as for FX activation was used to generate thrombin. However, at these high virus numbers, large amounts of thrombin were produced that resulted in complete chromogenic substrate cleavage prior to reading. The amount of virus was therefore reduced to reflect the amount of initial activation in the system and accentuate any potential difference that existed between the viruses. Under the conditions used, each virus differed in ability to generate thrombin (Figure 23). These data reflected the results of the FXa generation in that CMV produced several fold less thrombin than either HSV1 or HSV2. However, in contrast to the FXa generation, HSV1 generated slightly more thrombin than did HSV2. This may indicate a more efficient assembly of the prothrombinase complex on the surface of this virus. Collectively, these observations provide a biochemical link for clinical observations in that

Figure 22 Herpesvirus-mediated factor Xa generation. To determine the thrombogenic potential of different members of the Herpesvirus, HSV1 (■), HSV2 (▲) and CMV (●) were assayed for their ability to activate FX. Increasing amounts of the lab strains MacIntyre (HSV1), strain G (HSV2) or AD169 (CMV) were incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 20 minutes. Following incubation, the amount of in situ virus-mediated FXa generated was determined by cleavage of the FXa-specific chromogenic substrate S-2765. (n=9) (HSV1 (■), $p = 0.00002$; HSV2 (▲), $p = 0$; CMV (●), $p = 0.005$)

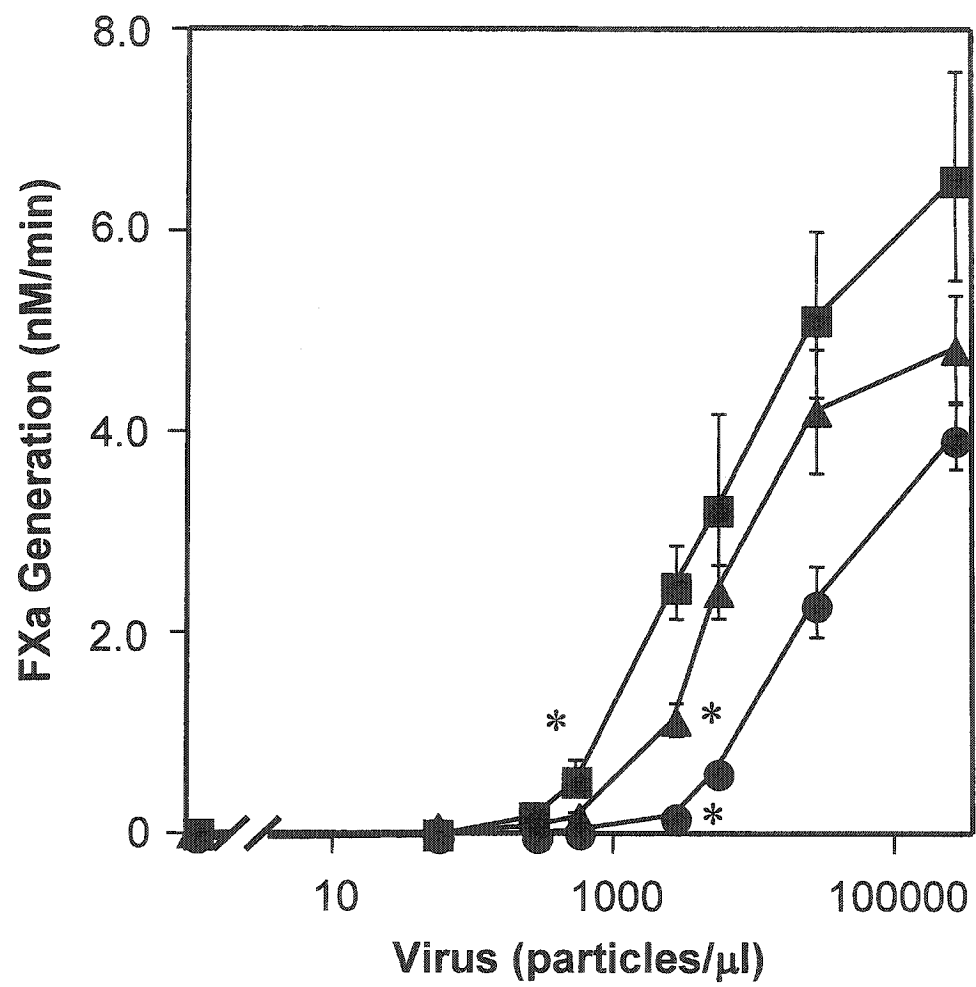
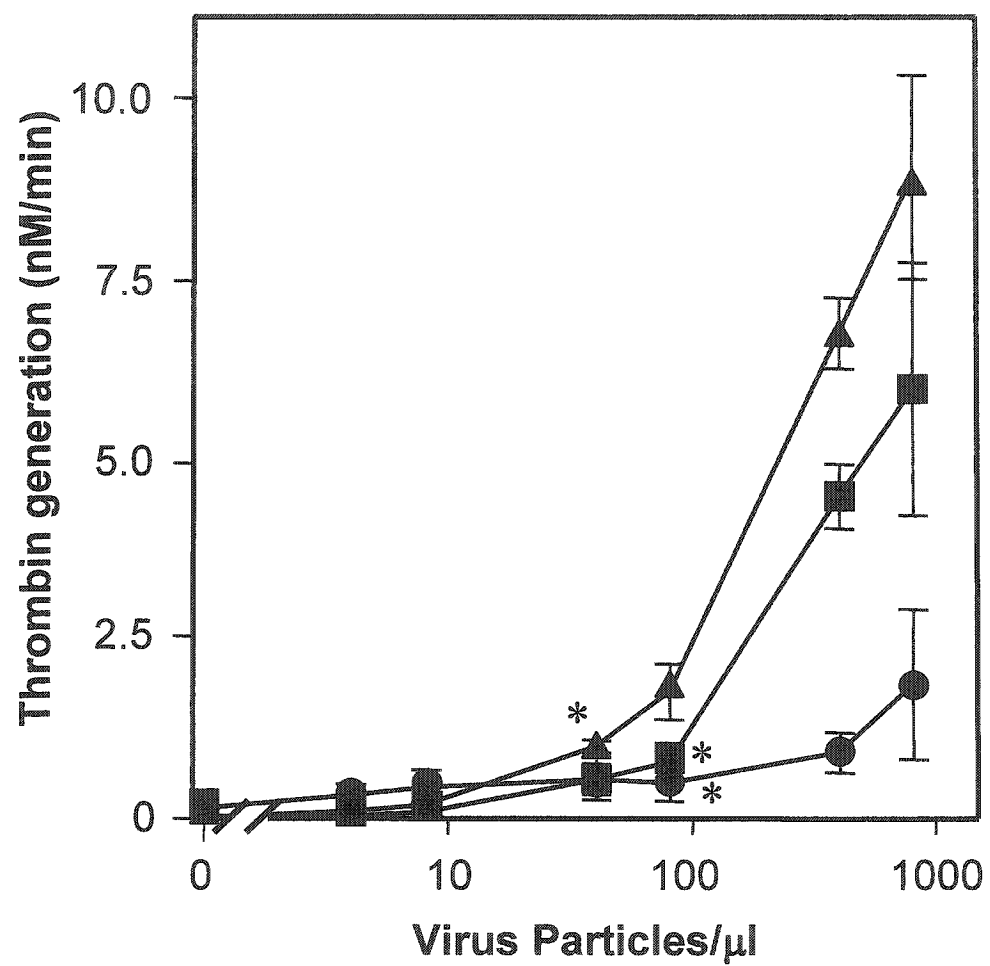


Figure 23 Herpesvirus-mediated thrombin production. HSV1 (■), HSV2 (▲) and CMV (●) lab strains were incubated with FX (100 nM), FVIIa (5 nM) and Ca^{2+} (5 mM) for 5 minutes. Prothrombin (1.4 μM) and FVa (5 nM) were then added and the mixture was further incubated for 20 minutes. Following incubation, the amount of thrombin generated was determined by cleavage of the thrombin-specific chromogenic substrate S-2238. (n=9) (HSV1 (■), p = 0; HSV2 (▲), p = 0; CMV (●), p = 0.0001)

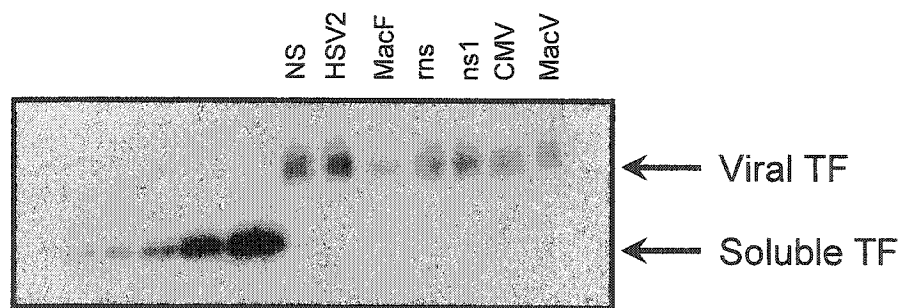
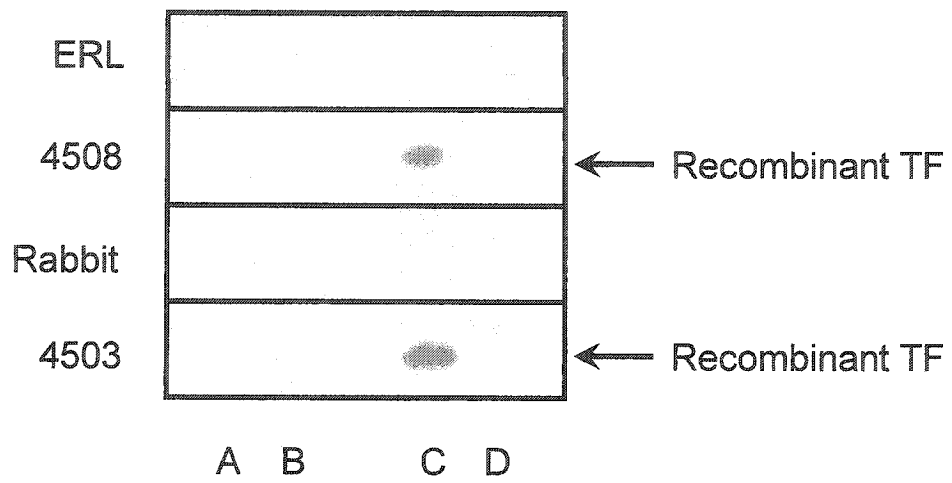


thrombotic complications occur with HSV1 and HSV2 infections and not for CMV. Clear dependence was seen for each constituent when each was individually omitted from the reaction mixture and also indicated that the FVa and prothrombin were free of possible contaminating FX or FVIIa due to the purification process.

Western blot analysis was used to provide a direct correlation between the FXa and thrombin activity generated and the number of initiator molecules associated with each virus. Initially the ability of a panel of TF-specific antibodies to detect various concentrations of Innovin as the source of TF was determined. The TF antigen was recognized by both American Diagnostica products and only under non-reducing conditions (Figure 24) with #4503 being chosen for subsequent experiments since it was able to recognize lower amounts of TF. In a similar manner, a series of HSV1 gC-specific were evaluated for their ability to recognize viral gC. The previously well-characterized 1C8 antibody was the most effective at detecting the antigen (Figure 25), however, none of the antibodies tested were able to recognize gC associated with the MacIntyre strain. The sequence comparison between the MacIntyre and NS gC, against which the antibody was raised, is not known and the current data suggests that they may differ antigenically. In both cases the individual antibodies chosen for detection of TF and gC was able to recognize both soluble and viral forms of the respective proteins (Figures 24 and 25).

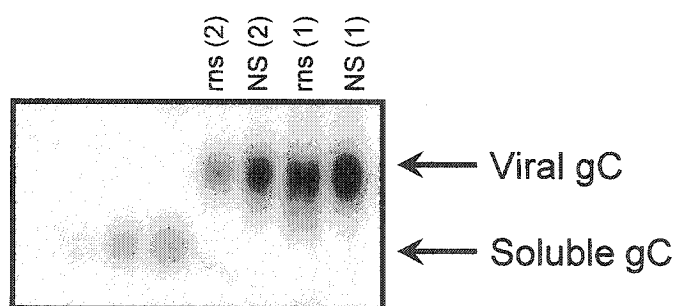
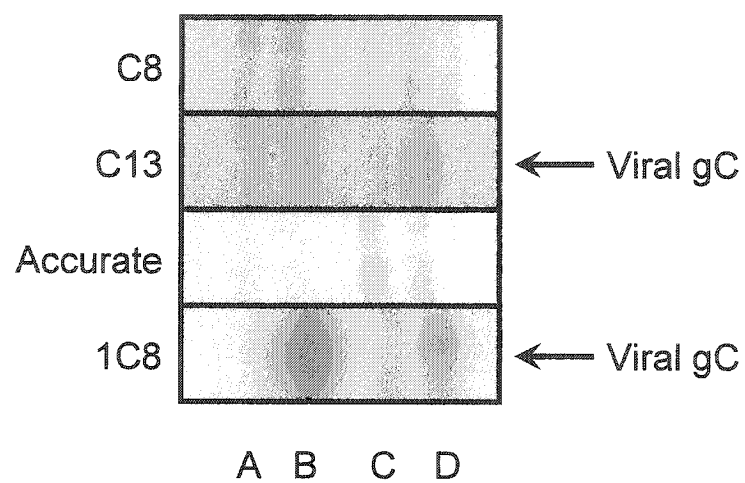
To accurately determine if the differential FXa generating ability associated with each virus was due to the amount of TF and gC on individual viral particles, a

Figure 24 Tissue factor antigenic determination. (Top) Innovin (10 μ l, A and C; 1 μ l, B and D) was separated by SDS PAGE under reducing (A and B) or non reducing (C and D) conditions. The transferred protein was then probed with a panel of anti-TF antibodies to determine antibody specificity. (Middle) Viral and recombinant sources of TF were separated by SDS PAGE under non reducing conditions and detected using anti-TF (4503). (Bottom) Representative amount of TF associated with each virus preparation.



Virus	TF (ng/10 ⁹ vp)
MacIntyre (Vero)	5.3 +/- 2.7
ns-1	3.1 +/- 1.9
rms	2.1 +/- 1.5
NS	0.3 +/- 0.29
HSV2	1.6 +/- 1.1
MacIntyre (HFF)	22.1 +/- 14.8
CMV	N/A

Figure 25 Glycoprotein C antigenic determination. (Top) Viral proteins (10 μ l HSV1 ns-1, A and C; 10 μ l NS, B and D) was separated by SDS PAGE under non-reducing (A and B) or reducing (C and D) conditions. The transferred protein was then probed with a panel of anti-gC antibodies to determine antibody specificity. (Middle) Viral and soluble sources of gC were separated by SDS PAGE under non reducing conditions and detected using anti-gC (1C8). (Bottom) Representative amount of gc associated with each virus preparation.



Virus	gC ($\mu\text{g}/10^9 \text{ vp}$)
NS (1)	3.9 $\pm$ 1.6
rns (1)	5.2 $\pm$ 2.5
NS (2)	0.4 $\pm$ 0.2
rns (2)	0.4 $\pm$ 0.2

quantitative western blot assay was developed. This assay was based on the construction of standard curves of known amounts of soluble TF or gC generated following detection with a chemiluminescent substrate. Viral antigen that had been separated and subjected to identical conditions was then compared to the standard curves generated. Only those values that fell within the linear portion of the standard curve generated on the same PVDF membrane were used for quantification. In this manner, the amount of TF and gC was determined on all virus strains presently used in the study. In almost every case, the amount of TF detected was within the same order of magnitude. Interestingly, NS was shown to have approximately 10 fold less TF per particle than the other viruses while the HFF propagated MacIntyre strain generated 10 fold more (Figure 24). This indicated that potential differences exist in either the cellular response to generate TF or the presentation of the antigen on the purified virus surface. Similarly, individual preparations of virus had minimal variations with respect to the amount of gC present (Figure 25). In all cases, the virus strains used in each individual experiment contained comparable levels of TF and gC per virus particle.

3.3 ROLE OF THROMBIN IN HERPESVIRUS INFECTION

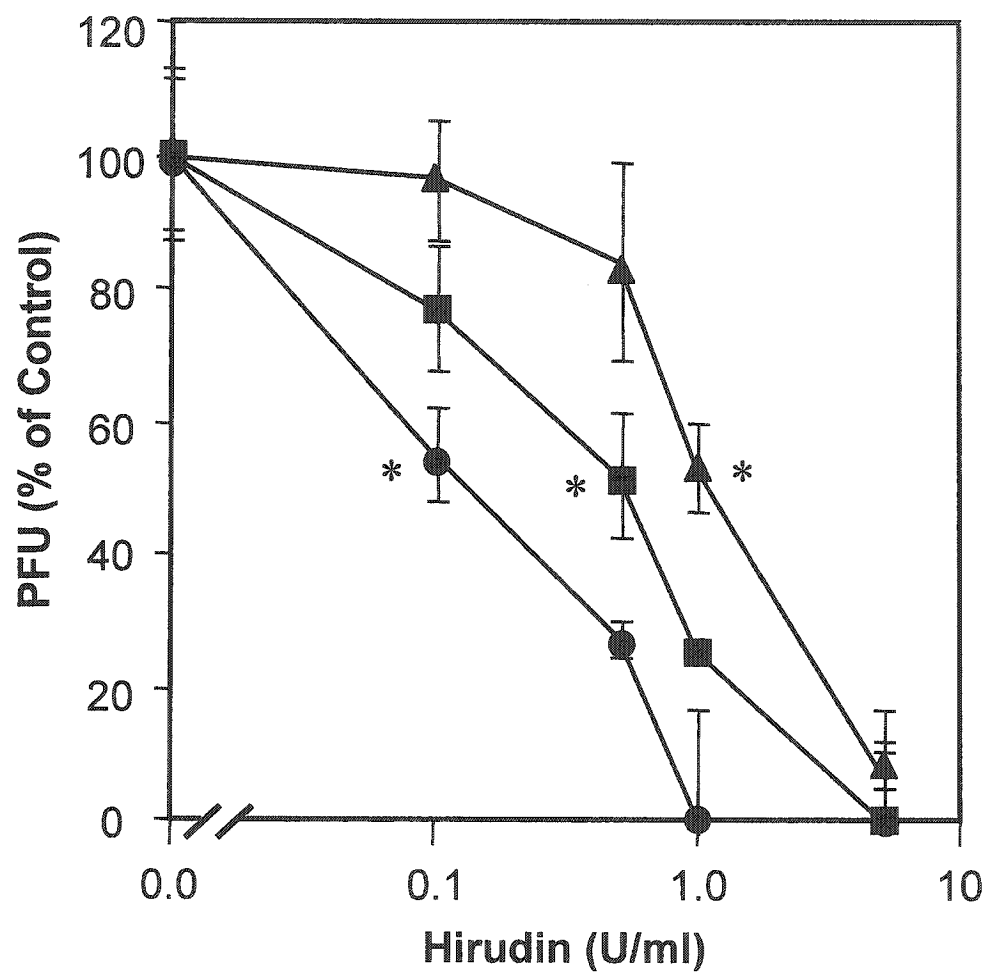
The evolution of the Herpesvirus surface as a procoagulant agent suggests a role for coagulation initiation in the viral lifecycle. We therefore used standard plaque assays to determine a possible advantage to the virus conferred by the eventual production of thrombin. Preliminary results obtained when determining the

infectivity of each virus indicated a potential role for serum in virus infection. This was realized when plaques conducted in the absence of serum resulted in approximately 50% less plaques compared to when serum was included in the inoculation media. We therefore explored the serum dependence of plaque formation.

Since thrombin generation was postulated to be important in the infection process, the infectivity of each virus was determined in the presence of the specific thrombin inhibitor, hirudin. Hirudin was chosen since it is a bivalent inhibitor that binds to sites near the active site and additional exosites on thrombin to prevent its subsequent proteolytic activity²³⁸. When titrated in the inoculation media that contained serum (Figure 26), hirudin resulted in a dose dependent decrease in the infectivity of each gC-characterized virus. The effect of thrombin inhibition was most pronounced with the gC-deficient virus since the half maximal hirudin concentration needed was 0.1 U/ml, while 1.0 U/ml was required for either of the wild type or gC-competent viruses. This correlates with our previous observation that the gC-deficient virus consistently generated less FXa than its gC-containing counterparts and should therefore generate less thrombin per particle as well. This also indicates that thrombin production mediated by the virus greatly enhances the ability of the virus to entry into the cell.

In order to specifically determine the role of thrombin in virus infection, a serum-free plaque assay was developed. This involved numerous cell washes to remove serum components from the cell propagation media and the use of serum-

Figure 26 Serum components in media are needed for optimal virus infection. Human foreskin fibroblasts were inoculated with HSV1 wild type (■), gC-deficient (●) or gC-restored (▲) strains in the presence of 5% bovine calf serum. Various concentrations of the thrombin-specific inhibitor, hirudin, were added simultaneously with the virus at 37°C for 90 minutes. 48 hours post infection, the cells were fixed and the number of infectious events (plaques) were counted. (n=10) (wild type (■), p = 0; gC-deficient (●), p = 0; or gC-restored (▲), p = 0)



free media for virus incubations to limit any virus-mediated thrombin production. In this system, the direct involvement of thrombin in HSV1 infection was illustrated with the addition of exogenous thrombin during the infection period (Figure 27). The simultaneous incubation of thrombin with the virus resulted in a 60 - 80% enhancement in the infection of all viruses compared to when thrombin was omitted. In this case, an identical enhancement was seen since thrombin was added exogenously and not generated on each virus. In order to determine the mechanism by which thrombin mediates the enhancement of Herpesvirus infection, the analogous signaling peptide, TRAP, was used. This peptide mimics the new N terminus of the PAR1 thrombin receptor that becomes unmasked upon receptor activation. The use of this peptide therefore eliminates other potential signaling pathways initiated by thrombin. The addition of TRAP (Figure 28) shows a 60 - 80% enhancement in infectious events for each of the gC-characterized viruses and provides further evidence that HSV1-mediated thrombin production enables the virus to better enter the cell through a PAR1 mediated process.

In similar experiments, the importance of thrombin in infection of Herpesvirus lab strains was determined. When HSV1 MacIntyre and HSV2 Strain G are compared there are differences in the amount of infection due to each of serum (Figure 29), thrombin (Figure 30) and TRAP (Figure 31). The differential response to these factors suggests that additional mechanisms to PAR1 activation are important in virus entry. Also, different response have been noted between active protease and soluble ligand signaling in platelets and receptor mutants, indicating that

different receptor activation sites and pathways exist between tethered and soluble ligand^{7,21,184}. However, in all cases, a role for virus mediated thrombin production in the infection of Herpesviruses is clearly indicated.

Figure 27 Exogenously added thrombin results in increased Herpesvirus infection. To illustrate a role for thrombin in Herpesvirus infection, HFF were simultaneously incubated with either HSV1 wild type (■), gC-deficient (●) or gC-restored (▲♦) strains and increasing concentrations of thrombin for 90 minutes at 37°C. To limit virus-mediated *in situ* thrombin production, all incubations were carried out in serum-free media. Forty eight hours post infection, the cells were fixed and the number of plaques counted. (n=10) (wild type (■), p = 0; gC-deficient (●), p = 0; or gC-restored (▲♦), p = 0)

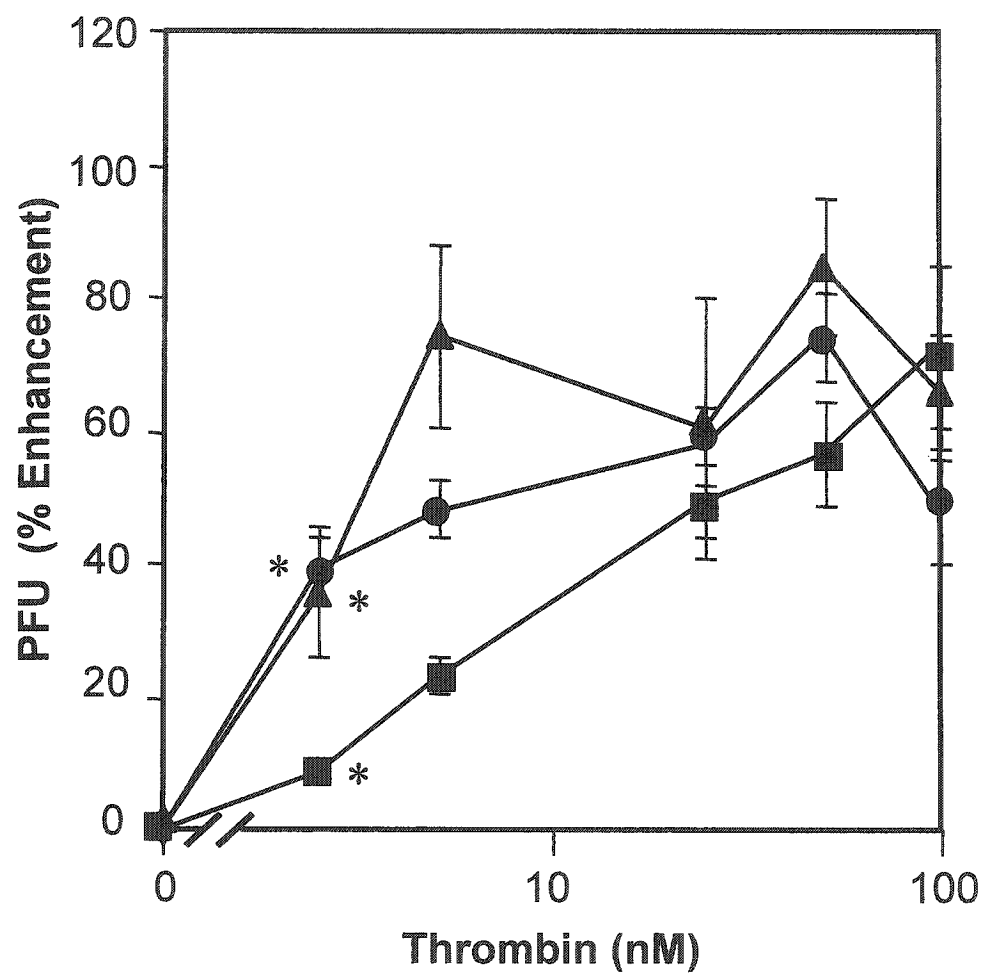


Figure 28 Thrombin-mediated increase in infection is through a mechanism that involves protease activated receptor 1. To determine the mechanism through which thrombin is functioning to increase virus infection, the PAR1-specific thrombin receptor activating peptide (TRAP) was used. HFF were inoculated with HSV1 wild type (■), gC-deficient (●) or gC-restored (▲) strains in serum free media in order to minimize virus-mediated thrombin production. Each virus was added simultaneously with TRAP for 90 minutes at 37°C. Following treatment, the cells were washed and 48 hours post infection were fixed and the number of plaques counted. (n=10) (wild type (■), p = 0; gC-deficient (●), p = 0; or gC-restored (▲), p = 0)

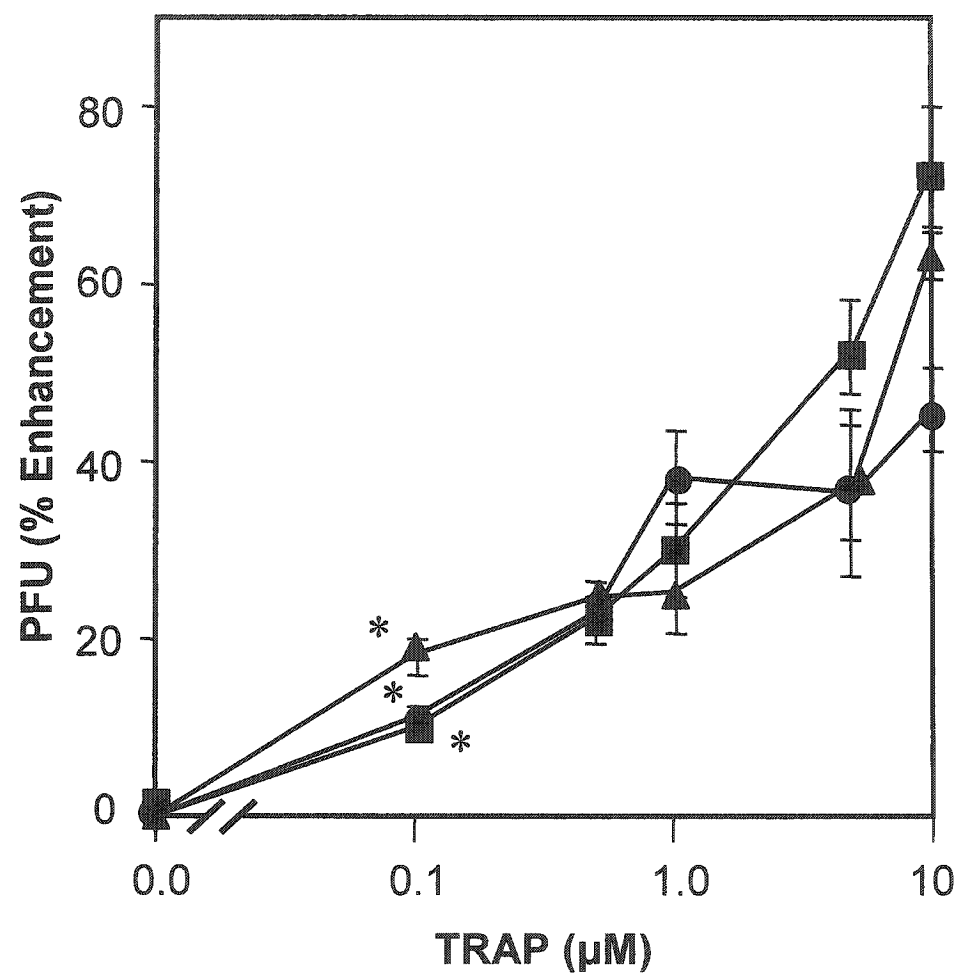


Figure 29 Differential dependence on serum component for Herpesvirus infection. HFF were simultaneously inoculated with either HSV1 (MacIntyre, ▲) or HSV2 (Strain G, ■) in the presence of 5% bovine calf serum and increasing concentrations of the thrombin-specific inhibitor, hirudin. Following incubation for 90 minutes the cells were washed and infection was allowed to proceed for 48 hours. The cells were then fixed and the number of infectious events (plaques) were counted. (n=10) (MacIntyre, ▲, $p = 0$; or Strain G, ■, $p = 0.00001$)

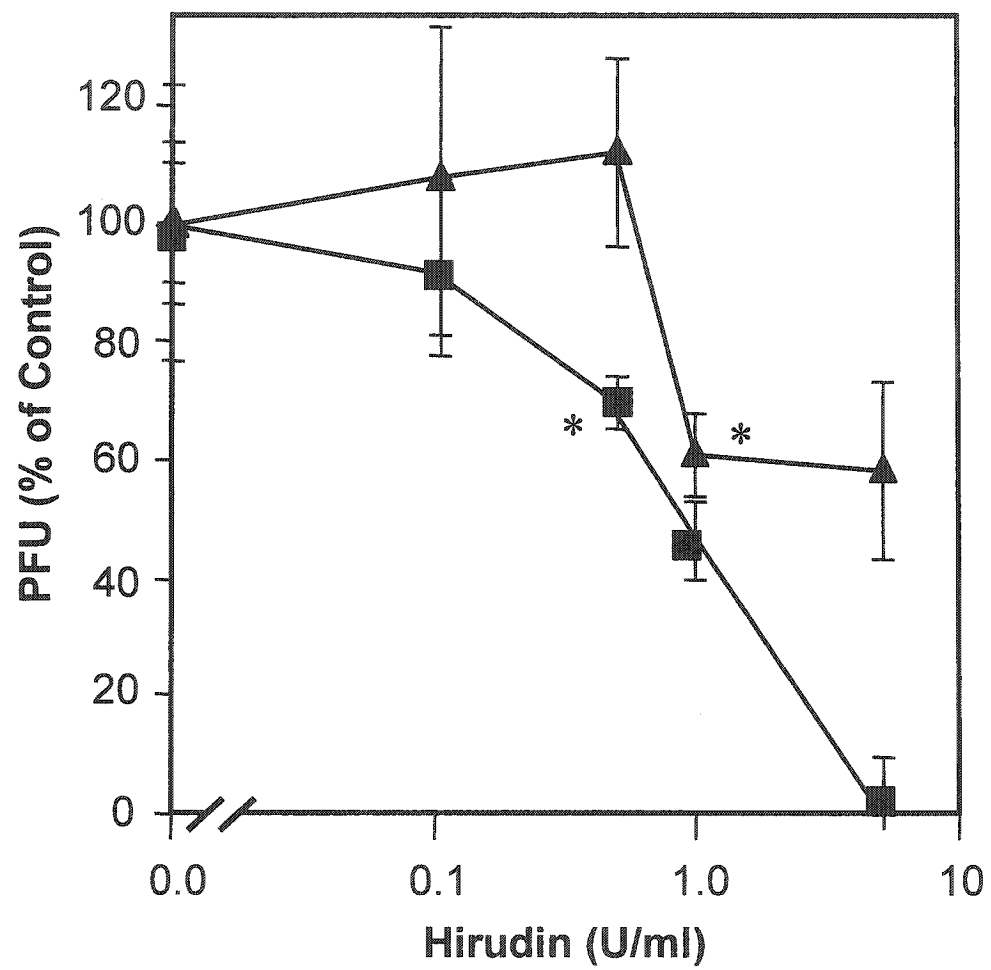


Figure 30 HSV1 and HSV2 differ in their response to exogenously added thrombin. To determine a role for thrombin in Herpesvirus infection, HFF were simultaneously incubated with a fixed number of HSV1 (MacIntyre, ▲) or HSV2 (Strain G, ■) virus particles and an increasing concentration of thrombin. In order to limit virus-mediated *in situ* thrombin production, all incubations were carried out in serum free media for 90 minutes at 37°C. Forty eight hours post infection, the cells were fixed and the number of plaques counted. (n=10) (MacIntyre, ▲, p = 0; or Strain G, ■, p = 0)

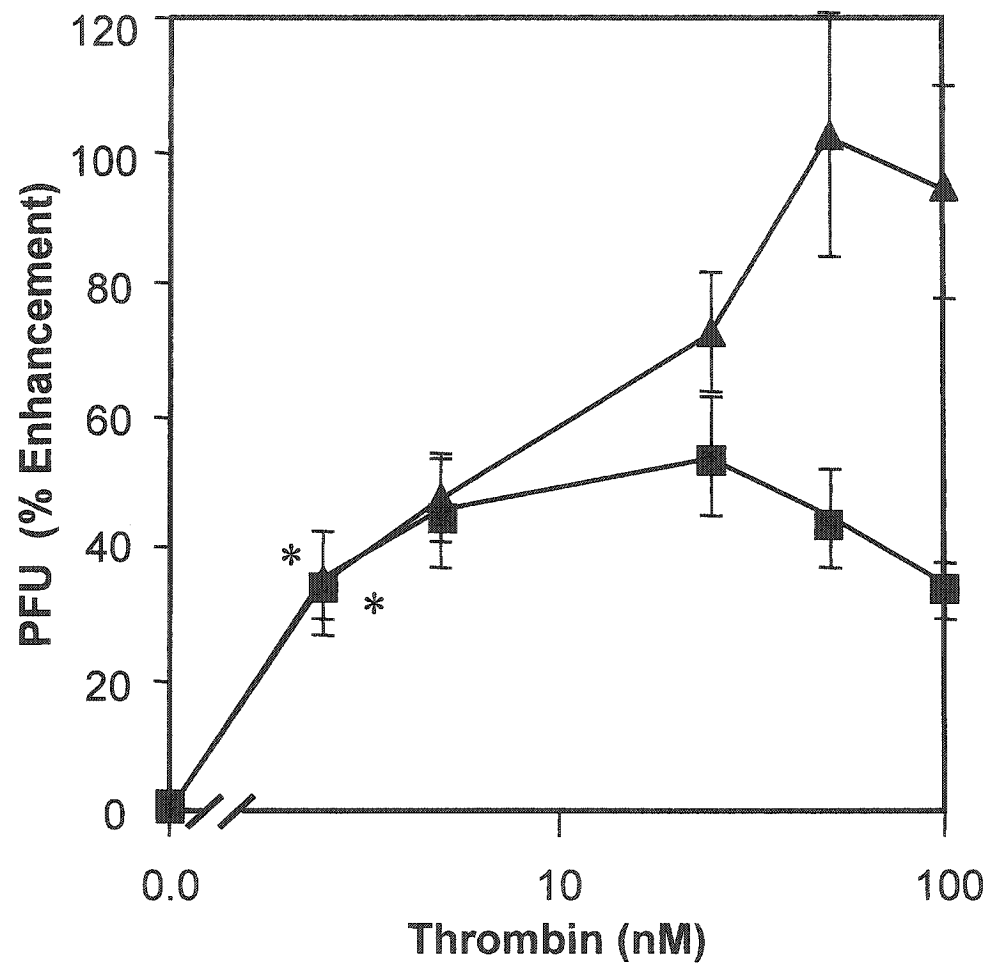
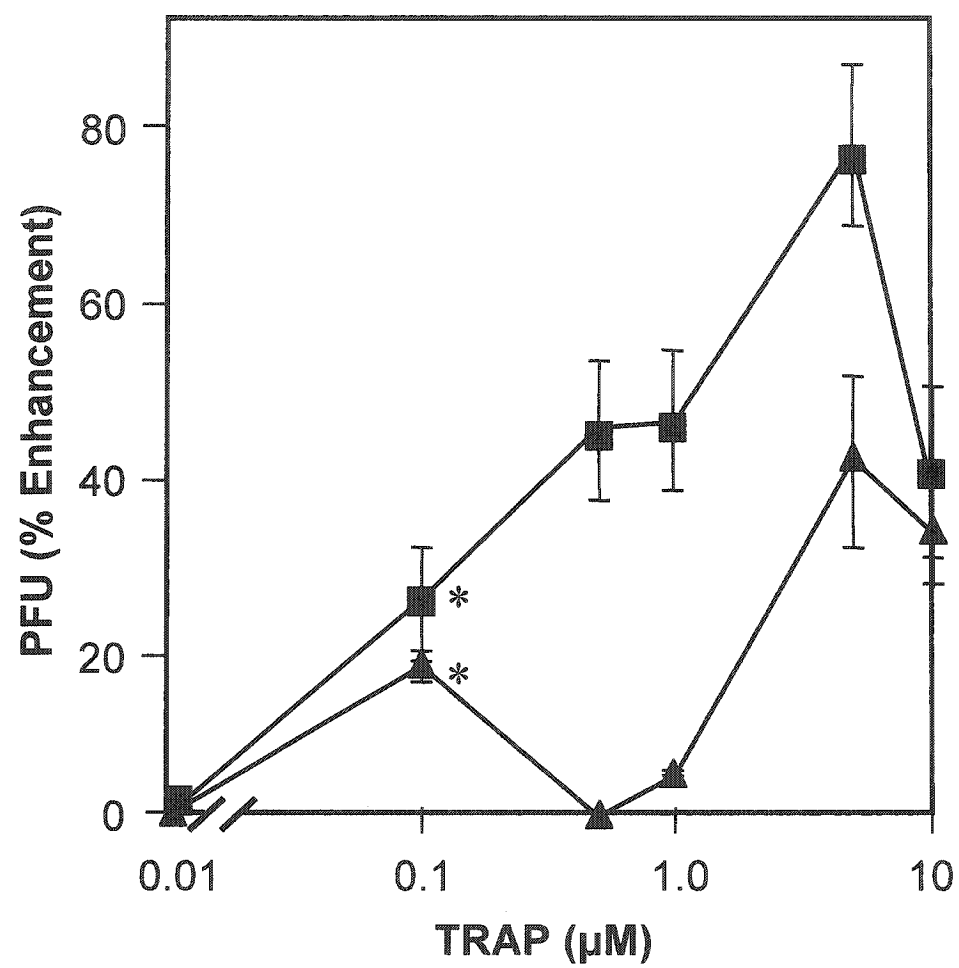


Figure 31 Thrombin-mediated increase in Herpesvirus infection is dependent on protease activated receptor 1 activation. To determine the mechanism through which thrombin is functioning to increase virus infection, the PAR1-specific thrombin receptor activating peptide (TRAP) was used. HFF were inoculated with HSV1 and HSV2 lab strains, (MacIntyre, ▲; or Strain G, ■; respectively) that contained increasing concentrations of TRAP. In order to minimize any virus-mediated thrombin production the incubations were conducted in serum free media. Each virus was added simultaneously with TRAP for 90 minutes at 37°C. Following treatment, the cells were washed and 48 hours post infection were fixed to determine the number of plaques. (n=9) (MacIntyre, ▲, p = 0; or Strain G, ■, p = .000001)



4.0 DISCUSSION

4.1 OVERVIEW

Numerous lines of evidence suggest that Herpesviruses contribute to the development of vasculopathy. These include: 1) clinical studies which demonstrate active viral infection is a risk factor for restenosis after angioplasty ^{59,253} and vascular thickening ¹⁶⁸ ; 2) the finding that herpes simplex virus type 1 (HSV1) infection results in an increased risk of death due to myocardial infarction and coronary heart disease ²⁰⁷; and 3) the frequent discovery of HSV1 and cytomegalovirus (CMV) genetic material within the endothelial and smooth muscle cells of some atherosclerotic tissue samples ^{17,47,78,86,146,147}, although discrepant reports exist concerning the latter ⁷⁶. These retrospective studies have been substantiated by direct animal models where viral infection induced atherosclerosis and thrombosis ^{64,65}. Herpesvirus infection furthermore converts resting vascular endothelial cells from a noncoagulant to a procoagulant state ^{62,63,123,229,232,235}. While these studies provide a clear connection between Herpesviruses and vascular disease, they do not answer the critical question, "What is the first vasculopathic event triggered by the virus?". The current report demonstrates that the initiating event in virus-mediated vasculopathy is the activation of FXa directly on its surface that can lead to important cellular changes even prior to infection by the virus.

4.2 FACTOR Xa PRODUCTION ON HSV1

A distinguishing feature of Herpesviruses is the presence of a surrounding

phospholipid bilayer membrane that functions in the early stages of infection by contributing components necessary for host cell entry ¹⁹⁰ and evasion of the immune system ⁶⁹. The envelope contains proteins that are encoded by both the virus and host genomes, while the phospholipid component is derived solely from the host cell. In previous studies we have shown that Herpesviruses can act as a procoagulant agent and sustain thrombin generation directly on their surfaces through the combined effects of TF and proPL ^{183,216}. Expression of these coagulation initiating molecules allows the viruses to circumvent an important cellular haemostatic control mechanism that limits thrombin generation to sites of vascular damage. However, this earlier work led to speculation of an additional initiating mechanism for FXa generation on the HSV1 surface since incomplete inhibition of thrombin generation was observed in the presence of an anti-TF antibody known to inhibit 100% of the TF activity in a clotting assay. Using a well-characterized panel of viruses and purified recombinant protein, the current study shows that in addition to TF, HSV1-encoded glycoprotein C (gC) can facilitate FXa generation directly on the virus surface.

4.2.1 Tissue Factor

In order to initiate thrombin production, proPL and a mechanism to convert FX to FXa must become available to circulating plasma coagulation proteins. Under normal physiological conditions the first FXa molecules are generated by the TF-FVIIa tenase complex ¹³⁷. Herpesvirus infection with live, UV inactivated or

replication-defective virus has been shown to enhance TF expression on host endothelial cells ^{110,232,233}. Therefore, it was speculated that TF produced in response to infection is routed to the virus particle when the envelope is formed. In agreement with this hypothesis, using immunogold electron microscopy, immunoinhibition and functional clotting and chromogenic assays, we have previously shown that TF antigen exists on the virus surface that can participate in thrombin generation ²¹⁶. In the present study, we specifically demonstrate that HSV1-mediated thrombin production is dependent on the initial generation of FXa by the virus that, consistent with the thrombin data ²¹⁶, is only partially inhibited with a TF specific monoclonal antibody.

In the case of both HSV1 wild type viruses assayed (MacIntyre and NS), the TF antibody used consistently resulted in a titratable inhibition of FXa and thrombin generation. The effect was saturable with a maximal inhibition of 40%, however near millimolar concentrations of the TF antibody were needed. Control experiments showed that the antibody was able to inhibit purified human recombinant TF, that of human and monkey cell origin as well as all viral sources of TF. In each case, the inhibition was TF antigen specific since the data were corrected for any effect caused by an identical concentration of isotype-matched non-immune IgG. Further demonstrating the inhibitory capacity of the chosen antibody, other monoclonal and polyclonal antibodies resulted in a reduced amount of TF activity inhibition when either FXa or thrombin generation was assayed.

Also important is the observation that the inhibition profile for each antibody

differed between recombinant and cell sources of human TF, and the inhibition of viral TF mirrored that of the corresponding cellular source. This suggests that the higher concentration of antibody needed to produce maximal inhibition may be due to changes in the antigenicity of TF as a result of the local cell- or virus-surface environments. This is supported by previous studies that showed near micromolar concentrations of TF antibodies were needed to inhibit TF activity in plasma, however complete inhibition of purified TF activity in a chromogenic assay was achieved in the nanomolar range^{39,193} as seen in the present study with Innovin. It was also suggested that FVIIa concentrations lower than those used in the current report may inhibit the TF mAb by competing for binding to FVIIa. Since the antibody used in the study is not fully characterized, it may also compete for FVIIa binding. These results indicate that the incomplete inhibition of HSV1-mediated FXa generation is not due to antibody specificity, but rather points to an additional mechanism of FX activation on the virus.

4.2.2 Glycoprotein C

The reason we targeted gC on the virus surface as a probable contributor to FX activation was based on two reports from the early 1990's suggesting that gC, when expressed on the HSV1-infected endothelial cell surface, could participate in thrombin generation. The first study by Etingin *et al*⁶² explored changes in endothelial cells due to HSV1 infection that may lead to vascular disease. Using radiolabelled cells, these investigators were able to show enhanced adhesion of

monocytes to the infected endothelium that was subsequently blocked by antibodies to gC. To corroborate the implication of gC in this process, the adhesion was reduced when the cells were infected with gC-deficient virus strains. An effect of gC-mediated thrombin production was suggested by observations that monocyte adhesion to endothelial cells was blocked either by using specific thrombin inhibitors or depletion of prothrombin from the serum. These investigators then went on to suggest that the procoagulant activity of the infected cells correlated with gC expression on the cell surface although no direct evidence of cell surface gC or expression levels was given. Also important to the interpretation of the results, the procoagulant contribution of progeny virus in the supernatant or cell lysates produced after the 24 hour incubation time period was not addressed. The observation that the gC-deficient viruses resulted in no appreciable procoagulant activity in the clotting data presented seems contradictory since changes upon HSV1 infection are known to promote TF expression¹¹⁰. Potential differences in replication efficiencies of the individual viruses that would affect viral gene and glycoprotein expression on the cell surface to promote a procoagulant phenotype were not addressed.

Using the data presented, the group then hypothesized that HSV gC may mediate thrombin generation by promoting FX binding and localized assembly of the prothrombinase complex on the endothelial cell surface. To this end, ¹²⁵I-FX bound to HSV infected human umbilical vein endothelial cells or murine L cells that had been transfected to express gC. Using an anti-gC antibody, a cross-linked species

was detected that contained FX which, by molecular weight, was presumed to be complexed to gC after infection or induction of the transfected cell line. An additional band was also detected in the cross-linking experiments and suggested to be FXa, however, FVIIa was not added to the system to promote FX activation. In each case, no direct contact between gC and FX was demonstrated and the results may be solely due to the proximity of gC and FX or FXa on the infected or transfected cell surface. An alternative explanation is that the increased binding and activation of FX may be due to the more general phenomenon of proPL and TF expression upon infection or induction of the murine cell line. The specific cell surface contribution of gC can therefore be addressed by the same experiments conducted in the presence of an inhibitory TF antibody.

In a follow up study using a panel of partially overlapping synthetic peptides to specific regions of the FX molecule, Altieri *et al*⁸ mapped regions on FX that were reported to be involved in the interaction with gC. Results indicated that three peptides from noncontiguous segments of the catalytic domain of FX as well as antibodies raised against these peptides were able to inhibit ¹²⁵I-FX binding to monocyte Mac-1, a known FX-binding protein. The same peptides were reported to inhibit FX binding to the gC on HSV infected endothelial cells at near millimolar concentrations, however, no direct evidence of a gC-FX interaction was given. The authors also postulate that FX contains a vascular cell-binding domain and through convergent evolution, Mac-1 and gC have developed similar functional properties, such as FX and C3b binding.

The two studies present evidence that HSV-infected cell surface gC functions in FX activation and implies a role for thrombin in virus-mediated vasculopathy. However, in these studies and those of Vercellotti, the participation of gC or TF directly on the virus surface and the effect of virus-mediated thrombin production were not considered. Initially the current study examined the ability of viral gC to function in an analogous manner in FX activation as was suggested for infected cell surface gC.

In order to selectively evaluate the role of viral gC in FXa generation, a well characterized panel of HSV1 strains was utilized. To begin, a lab strain, MacIntyre, and a low passage clinical isolate, NS, were chosen as wild type strains. These were chosen as gC-competent viruses since they represent a commonly studied, well-characterized virus (MacIntyre) and a less adapted, naturally occurring virus (NS). These were also used to determine if possible differences in FX activating properties are due to adaptation of the virus to cell culture. The second virus studied was a naturally occurring gC-deficient virus (ns-1) and is therefore devoid of gC on its surface. The final virus in the panel is a gC-competent virus (rms) in which the gC deficiency in ns-1 was restored by the specific reintroduction of the complete gC coding sequence from the NS virus. The use of ns-1 and rms allowed the direct function of gC to be determined since these viruses differ only in their gC content as the method used to restore the gC defect would minimize potential differences associated with using separate virus strains. An additional benefit of this panel is it allowed the delineation of the TF- and gC-dependent pathways of FX activation

mediated by HSV1.

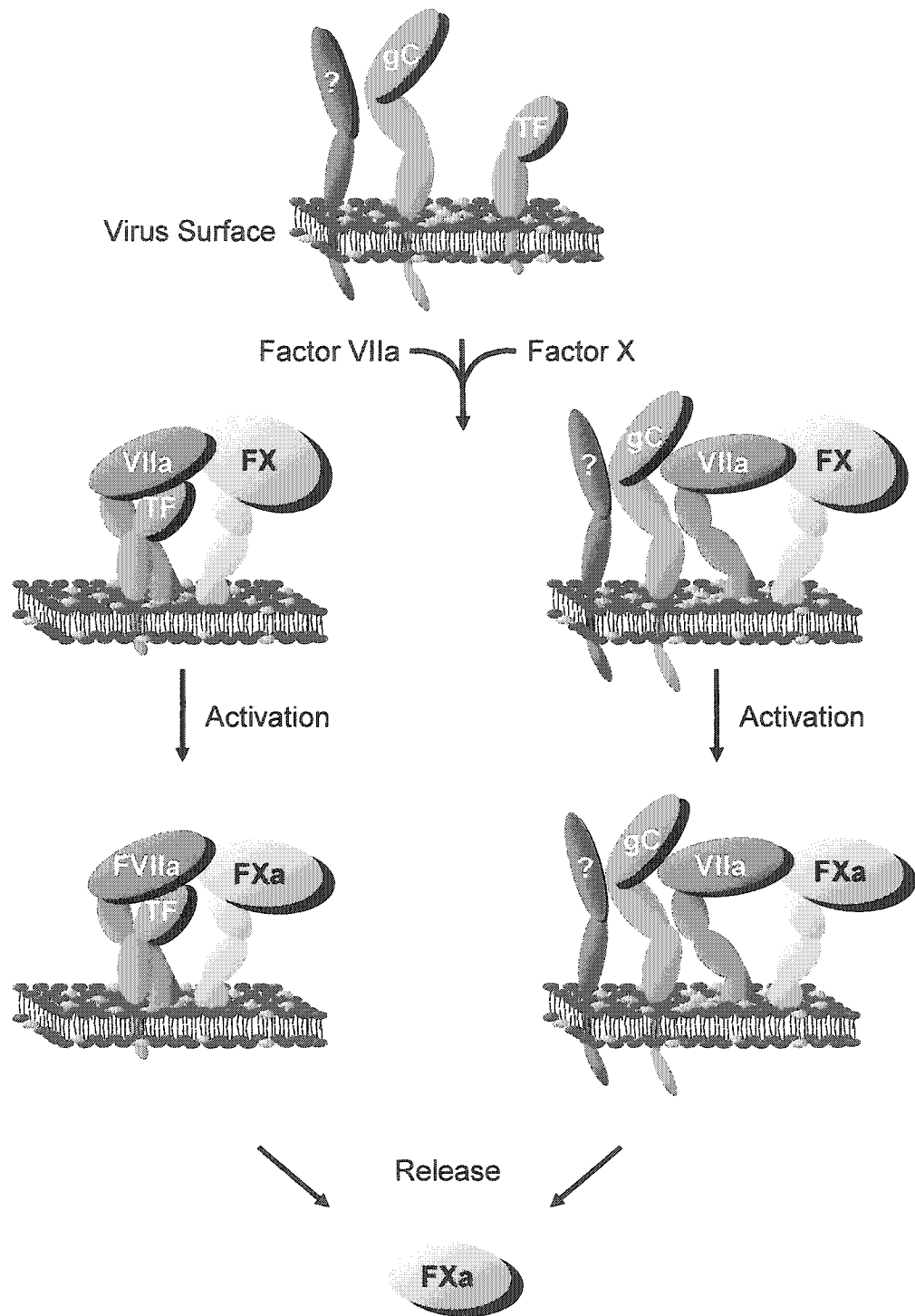
From the experimental results obtained, a clear role for viral surface gC is provided. In all cases, when compared to either wild type or the gC-restored viruses, the loss of gC in ns-1 resulted in a diminished ability of this virus to generate FXa. The data obtained indicate that the gC-dependent acceleration of FVIIa activity is mediated through interactions with both FX and FVIIa, although no direct gC binding is demonstrated. Importantly, the novel role of viral gC in FX activation is also dependent on Ca^{2+} and proPL, suggesting functional convergence with TF. We therefore extend the previous studies to demonstrate that gC generates FXa through the acceleration of FVIIa. As depicted in Figure 32, the virus can generate FXa and initiate thrombin production through the parallel effects of TF, a normal cellular protein, and gC, which is encoded by the virus genome. Thus cells are not required for gC-mediated FX activation, which can occur even before virus entry or expression of additional gC on the host cell surface.

An additional line of evidence that gC functions in FX activation was provided through the use of a soluble recombinant form of the protein (sgC). Like its viral counterpart, sgC emulated TF and was able to participate in the FVIIa-dependent acceleration of FX activation. However, a significant reduction in the molar capacity of sgC to generate FXa when compared to viral gC was realized. If gC functions in a similar cofactor manner as TF it is conceivable that correct protein orientation and presentation are important to subsequent cleavage events. The diminished FX activation may then result from deletion of the transmembrane domain needed to

make the protein soluble in the absence of detergents. The deletion of this anchoring structure may also result in the loss of important contact points for either FX or FVIIa. A similar effect was seen when a truncated form of FVII was unable to bind TF and consequently not accelerated effectively²⁰⁶. In agreement with our experiments monitoring sgC-mediated FX activation, relatively high concentrations (micromolar) of soluble TF were also required to generate FXa¹⁹⁵. In solution, sgC may also not localize to the procoagulant membrane where important FX interactions and cleavage is known to occur.

An additional explanation for the reduced capacity of sgC to generate FXa was investigated using virus "add-back" experiments. In these protocols, sgC was added to each of the viruses to determine if FXa generation could be further increased. The results showed that the reduced ability to generate FXa associated with the gC-deficient virus could be restored by the addition of sgC however, a lower enhancing effect was seen with either gC-competent strain. These observations provide evidence that gC may interact with an additional viral constituent (Figure 32) that is required for its TF-like activity. Since this component was not present in the virus-free conditions, the ability of sgC to activate FX was reduced. In support of an additional component needed for full gC-dependent FVIIa acceleration, sgC had the greatest effect when added to the gC-deficient virus since the number of potential unoccupied binding sites for sgC is greatest on this virus. The identity of the additional component is unknown, however prior studies have suggested multiple interactions between glycoproteins on the virus surface. Importantly, a cross-linking

Figure 32 Simultaneous pathways of factor X activation on HSV1. HSV1 expresses procoagulant phospholipid (yellow head groups), tissue factor (TF) and glycoprotein C (gC) on its surface. Factor VIIa (FVIIa)-dependent activation of factor X (FX) can then occur simultaneously and independently through either the TF- or gC-dependent mechanism directly on the virus surface. An additional component on the virus implicated in the FVIIa-accelerating capability of gC is also illustrated.



study that implied interactions between gC and HSV1 gB, gD and gH/gL^{82,83}, suggests these virus glycoproteins as plausible candidates. Studies are currently ongoing to determine the identity of this accessory protein.

In order to unambiguously show that the FXa generation data demonstrated a role for gC in FX activation, a series of control experiments were conducted. These were designed to investigate the possibility that gC could somehow effect the function of the purified coagulation proteins used. The results exclude a direct enhancing effect of gC on virus surface TF, since purified sgC accelerated FVIIa independent of TF and did not affect the FXa generating ability of a well-characterized source of TF. Additional assays showed that sgC did not alter the enzymatic activity of FVIIa as similar amounts of FXa were generated in its presence or absence. Lastly, sgC did not affect the amidolytic activity of purified FXa as judged by chromogenic substrate cleavage.

As with the early studies, the current work suggests that FX can somehow bind to gC and generate FXa, however, no evidence of direct binding was given. An attempt was therefore made to demonstrate an interaction between the two proteins using surface plasmon resonance. Initially, sgC was attached to the BIAchip surface and the individual binding of FX or FVIIa was monitored. This arrangement was chosen to emulate *in vivo* reactions in which FX and FVIIa would contact the virus surface. Subsequently, various combinations of FX, FVIIa, and proPL were flowed over the immobilized gC. In all cases, no significant association between the analytes was seen. While negative results are inconclusive, these data may be

indicating a possible requirement for a ternary sgC-FX-FVIIa complex formation or the need of the putative additional constituent on the virus for an sgC-FVIIa or -FX interaction to occur.

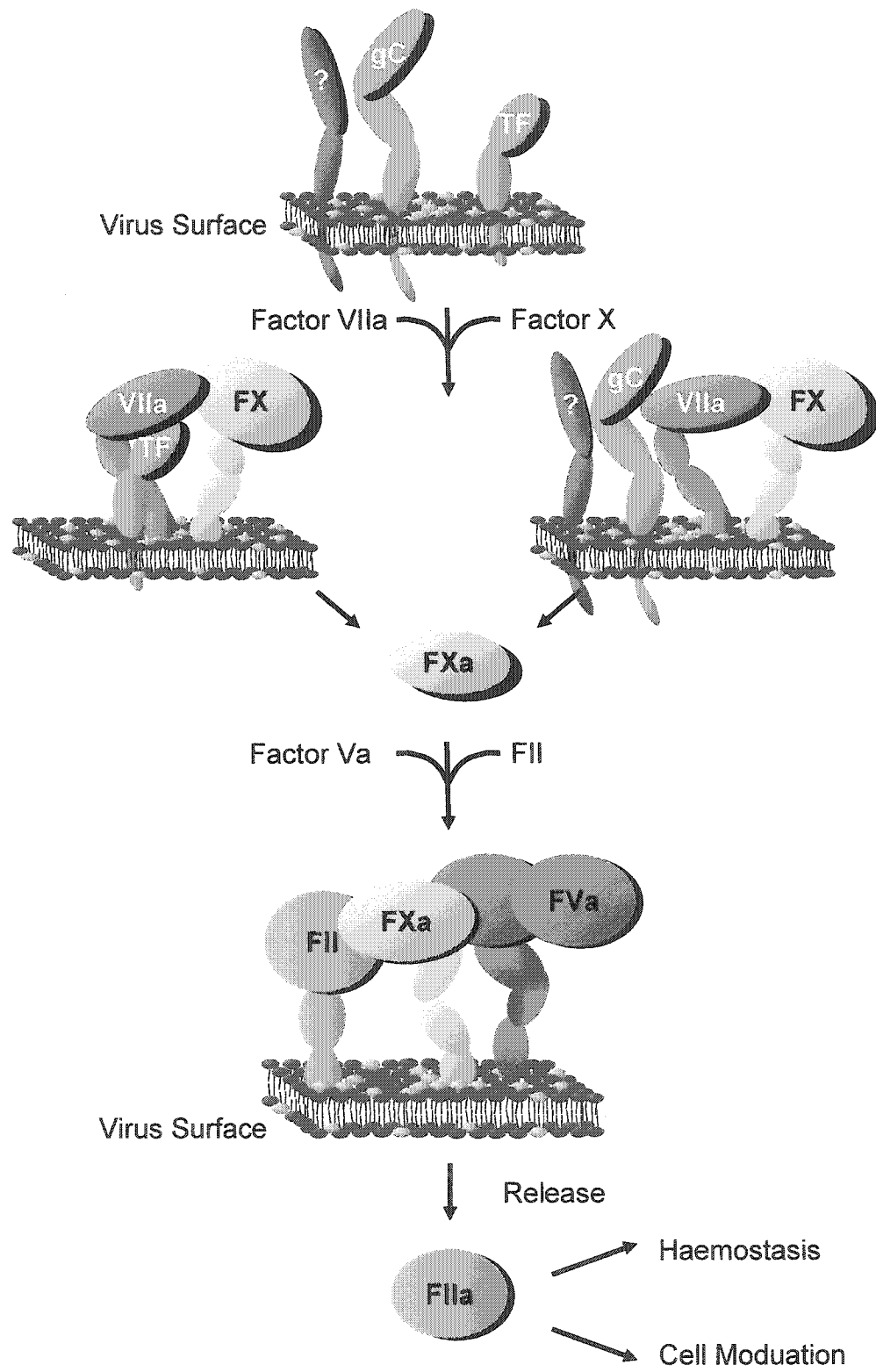
The inability to demonstrate an interaction using SPR may be due to a number of factors. The first drawback that is inherent to this technique is the initial immobilization to the chip surface via amide coupling. This may potentially lead to a functionally unfavorable orientation of sgC or chemical modification of important areas within the protein. The specific sequential addition of the proteins may also be important in that binding of one may be needed to generate or expose a binding region for another protein. Therefore, if the initial rate of binding is too slow, interaction needed for subsequent ternary complex formation may not occur over the time course of the analysis. The detection of interactions using SPR is also sensitive to alterations in concentration, pH and buffer that may render small changes upon binding undetectable. In order to adapt and improve on the conditions for the current project, the 5 histidine tag on the C-terminus of sgC can be utilized. The immobilization of an anti-histidine antibody followed by addition of sgC would allow a membrane-bound-like orientation in which the N terminus is pointed away from the surface. This too has the advantage in that sgC would not be subjected to chemical modification. Also, preassembled binary or tertiary complexes may be needed for interactions to occur and therefore can be formed prior to addition to the immobilized sgC. Alternatively, intact UV-inactivated viruses could be absorbed to the chip surface and differences in interaction between the gC-

competent and gC-deficient strains with respect to FX and FVIIa binding can be monitored.

From the work of Vercellotti, Hajjar and Altieri a model for cell-dependent viral activation of the coagulation cascade and subsequent vascular thrombotic changes has been proposed. In this scheme, after HSV enters the host cell, gC expressed on the infected cell surface acts as a binding site for FX. The combined effects of concomitant TF and gC expression then convert bound FX to FXa. The FXa generated can stay associated with the cell surface and form an active prothrombinase complex that results in thrombin formation. Thrombin can then trigger the cell and induces the expression of receptors for inflammatory cells and platelets that upon binding, release mediators that further amplify the cascade.

From the experimental evidence provided in the current work, we are able to add to this model and to the understanding of the contribution of HSV in vascular disease. As depicted in Figure 33, the virus itself can promote the activation of FXa directly on its surface through the simultaneous expression of gC and TF and clearly demonstrates that the virus can sustain an early and immediate response to initiate cell-signaling events. Therefore, these changes can occur even before host cell entry and productive infection of the cell. This is important since it had been previously thought that intracellular HSV was exclusively responsible for the atherosclerotic changes to the cell. Also of interest, the current findings show that in addition to the virus being able to over-ride the cellular control of coagulation initiating events, it can also generate potent cell-signaling molecules as it circulates.

Figure 33 Simultaneous pathways of thrombin generation on Herpesviruses. HSV1 expresses procoagulant phospholipid (yellow head groups), tissue factor (TF) and glycoprotein C (gC) on its surface. FVIIa (FVIIa)-dependent activation of factor X (FX) to FXa can occur simultaneously and independently through either the TF- or gC mechanism directly on the virus surface. FXa then combines with its cofactor, factor Va (FVa) through procoagulant phospholipid interactions on a virus surface and results in conversion of prothrombin (FII) to thrombin.



This becomes important when the virus is released from the cell during the initial lytic infection and subsequently after reactivation from latency.

4.2.3 Tissue factor/glycoprotein C comparison

The data presented here demonstrate that the participation of gC in FX activation is dependent on FVIIa, Ca^{2+} and proPL. This is interesting in that it functionally mimics TF. Using the GeneStream align Internet based protein sequence search program, an alignment between the TF and gC sequences was performed and is presented in Figure 34. When total complete sequences were compared, there was approximately 20% homology between the larger gC and TF protein. Of note, the alignment revealed three noncontiguous regions in gC that are homologous to TF when charged, hydrophobic or hydrophilic amino acids were compared. These areas lie mostly in the N terminal and membrane spanning regions, while sporadic regions within the center of the molecule show similarity. Due to the size difference between the proteins, multiple breaks were placed in the smaller TF molecule to generate the alignment.

Several intriguing observations arise from the sequence alignment presented. The first is that gC is predicted to contain 14 of the 21 TF amino acids known to facilitate contact with FVIIa in the TF-FVIIa crystal structure¹⁴. As shown in Figure 35, many of these homologous amino acids form an extended interface over one side of the TF molecule. Functionally, these residues are involved in contacts with the Gla and EGF domains of FVIIa that are important in its attraction to TF and

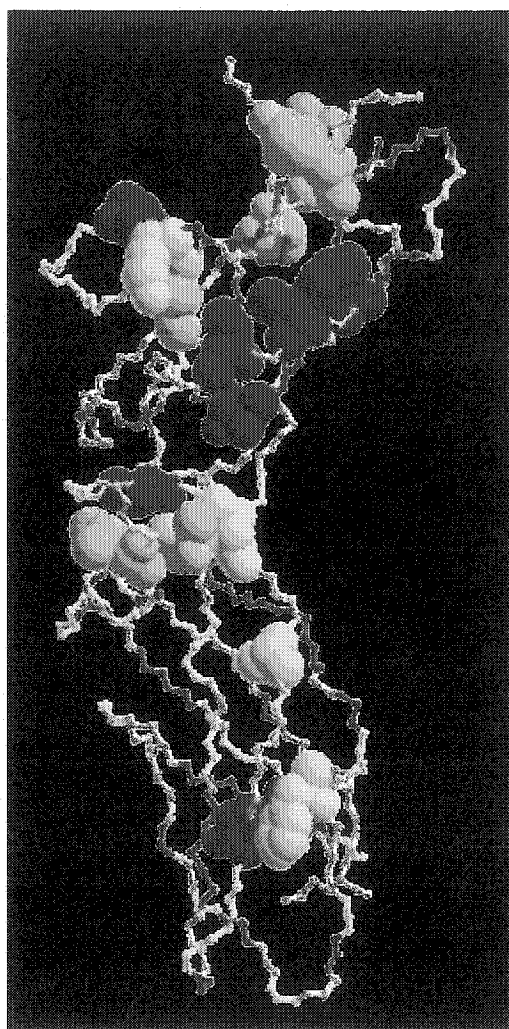
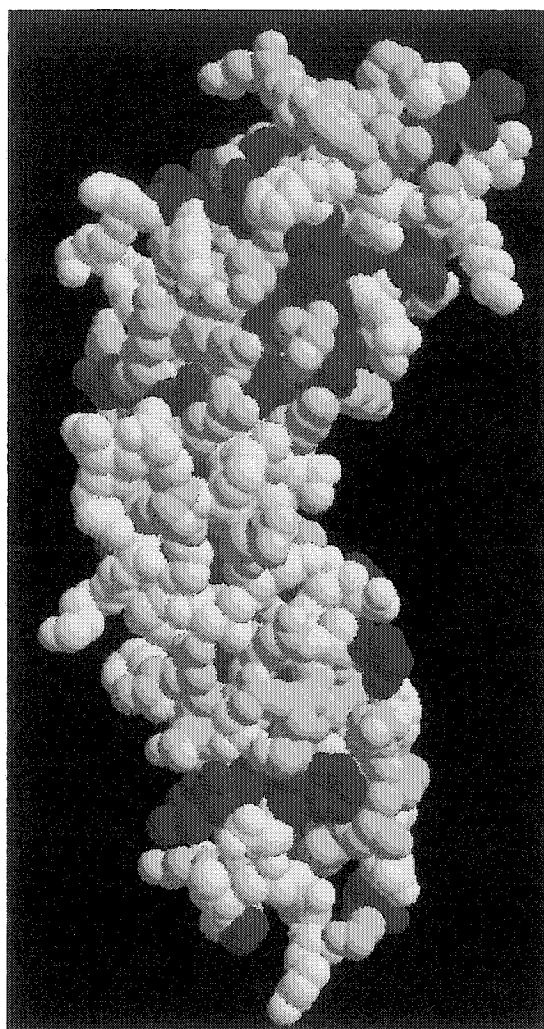
Figure 34 Sequence alignment of human tissue factor and HSV1 glycoprotein C. A sequence alignment inserted numerous breaks into the smaller TF sequence, but revealed three nearly contiguous TF regions of approximately 50% conservation only when general amino acid classes were compared (i.e. charged, hydrophobic or hydrophilic). The conserved (.), identical (:) or unique () amino acids are indicated. The residues in TF involved in factor VIIa contacts are in blue bold case. Areas of gC demonstrated to be important in C3b binding to gC are highlighted in yellow.

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TF  METPA-----WPRV-----PRPETA-VARTLLLGWVF-AQVAGASGTTNTVAAYN
gC  M-APGRVGLAVVLWSLLWLGAAGVAGGSETASTGPTITAGAVTNASEAPTSGSPGSAASPE
TF  LTWKST-NFKTILEWE-----PKPVNQVYTVQIST-----KSG---
gC  VTPTSTPNPNWNTQNKTTPTTEPASPTTPKPTSTPKSPPTSTPDKPKKNNTTPAKSGRPT
TF  -----DW-KSKCFYTTDTTCDLTDEIVKDVKT-----YL
gC  KPPGPVWCDRRDPLARYGSWVQIRCFERNSTMEFRLQIWRYSMGSPPIAPAPDLEEV
TF  ARVFSYPAG-----NV-----ESTG-SAGEPLYE-----
gC  TNITAPPGLLVYDSAPNLTDPHVLWAEGAGPGADPPLYSVTGPLEPTQRLIIGEVTPATQ
TF  -----NSP-EF-TPYLETNL-----GQPTIQS-----
gC  GMYYLAWGRMDSPEHYGTWVRVRFPPSLTLQPHAVMEGQPFKATCTAAAYYPRNPVEF
TF  --FE-----QVGTKVN-----VTVE-----DERTLV
gC  VWFEDDRQVFNPGQIDTQTHEHPDGFTTVSTVTSEAVGGQVPPRTFTCQMTWHRDSVTS
TF  RRNNTFLSL---RDV---FG-KDLIYT-----LYYW-----KSSSSGKKTA
gC  RRNATGLALVLPRTITMEFGVRHVVCITAGCVPEGVTFAWFLGDDPSPAKSAVTAQESC
TF  K-----TNTNEFLIDVDKGENYC--FSVQAVIPSRVTNKRSTDSPVECMGQEKGEFREIF
gC  DHPGLATVRSTLPISYDYSEYICRLTGYPAGIP--VLEHHGSHQPPPRDPTERQVIEAIE
TF  YI-IGAVVFVVIILVILLAISLHKCRKAGVGQSWKENSPLNVS
gC  WVGIGIGVLAAGVLVVTIAIVVVR-----TSQSRQRHR----R

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Figure 35 **Space filling model of tissue factor.** Those residues in tissue factor known to interact with factor VIIa that are identical (red), conserved (yellow), or unique (grey) compared to gC are indicated.



localization to the procoagulant membrane surface. However, since no 3D structure of gC is presently available, it is tempting to speculate that a similar interface exists in gC to facilitate an interaction with FVIIa. TF residues known to interact with the protease domain of FVIIa are not conserved in gC, providing a molecular basis for our conclusion that additional constituents on the virus are required to confer full TF-like functionality to sgC. The accessory molecule may supply the necessary contacts itself or orientate gC in such a way as to stabilize a FX-activating conformation of FVIIa.

The sequence alignment also presents insight into how gC may facilitate FX activation while maintaining its role in immune evasion. Using the TF molecule as a framework, Figure 35 shows that the TF regions having low homology with gC are on the TF surface and therefore make candidate sites for insertion of the additional amino acids from the larger gC structure. This would allow the creation of distinct functional areas within gC. To this end, the amino acid comparison showed that the four gC regions involved in complement component C3b binding^{97,196} were distinct from those having similarity to TF, suggesting the preservation of independent functional sites. In agreement with this hypothesis, purified C3b or its inactive precursor, C3, had no effect on HSV1-dependent FVIIa acceleration.

HSV1 gC may also be involved in cell attachment through the initial binding of the N terminus to HSP. To determine if this area is also removed from the FX activating region on gC, the effect of heparin on gC-mediated FX activation was explored. In this case, an increase in FXa generation was realized that might result

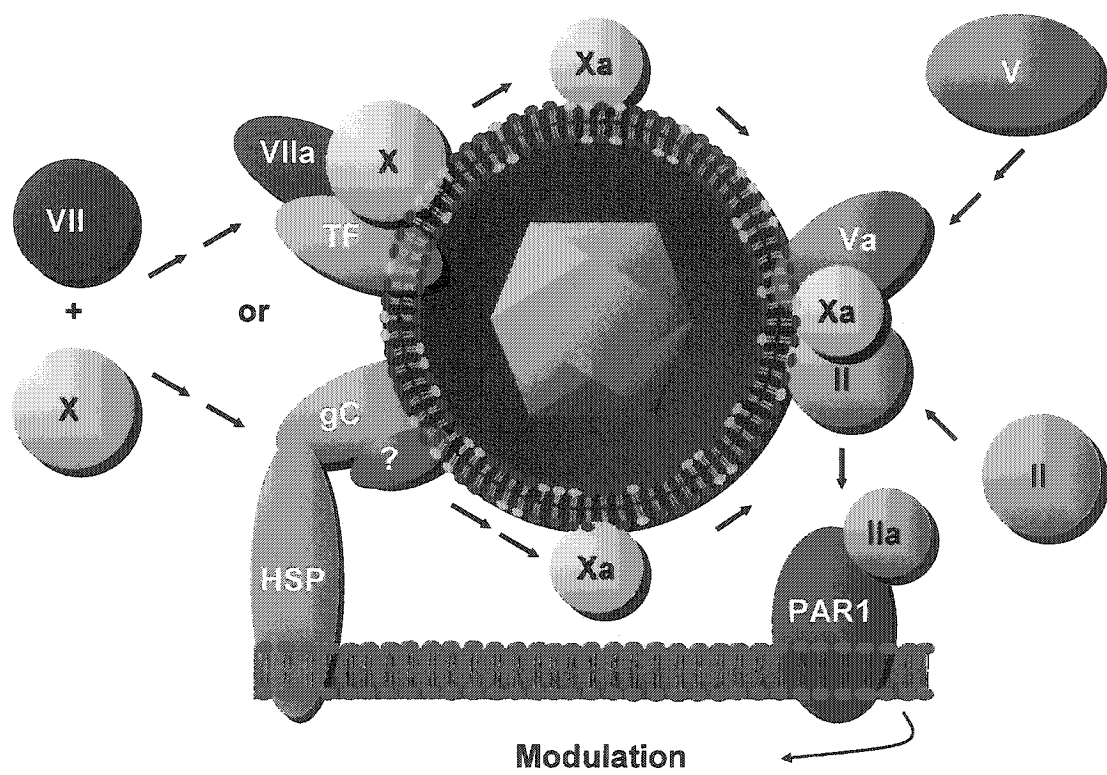
from a rearrangement in the N terminus of gC that facilitates a better FX activating conformation of the protein. Alternatively, the addition of a large concentration of negatively charged heparin molecules somehow resulted in an increase in FXa generation. However, unambiguous conclusions cannot be made from the heparin data since carbohydrate control molecules were not included in the experimental system. Cumulatively, the data suggest that gC can mimic the FXa generating ability of TF, while maintaining other independent and discrete functional regions. The generation of an X-ray crystal structure of viral or soluble gC as currently being attempted for gD³⁷ would provide definitive evidence as to the arrangement of important functional areas.

4.3 EFFECTS OF HSV PRODUCED FACTOR Xa

4.3.1 Thrombin generation

Results from our previous study suggested that FXa could be generated by several Herpesviruses through the action of TF on their surface culminating in thrombin production²¹⁶. The current report extends these findings to provide direct evidence of two independent and simultaneous pathways of FX activation on the HSV1 surface. As summarized in Figure 36, viral TF is expected to bind FVIIa in a manner analogous to cell surface TF, and activate plasma-derived FX to FXa. At the same time, gC may interact with FVIIa through an unknown mechanism and promote FXa generation. Assembly of these coagulation enzyme complexes is facilitated by the accessibility of proPL on the virus envelope as previously

Figure 36 Herpesvirus-mediated protease activated receptor activation. HSV1 expresses procoagulant phospholipid (green head groups), tissue factor (TF) and glycoprotein C (gC) on its surface that can simultaneously accelerate the activation of factor X (FX) by factor VIIa (FVIIa) through independent mechanisms. The FXa produced can interact with factor Va (Va) and participate in the activation of prothrombin (II) to thrombin (IIa). Thrombin can then interact with the cell surface PAR1 receptor and result in host cell modulation. An additional component on the virus implicated in the FVIIa-accelerating capability of gC is also depicted.



described^{183,216}. Although the *in vivo* source of activated FVII is unknown, it is likely that the first complexes are assembled using the small amount of FVIIa known to circulate in blood¹⁶⁶. Alternatively, the viral TF may substitute for cell surface TF, which has been shown to activate FVII when in complex with FVIIa, effectively generating its own enzymatic partner^{32,52}.

Our present results also provide evidence that an additional virus component helps facilitate the gC acceleration of FVIIa as shown in Figure 36. Currently there is no evidence of a direct interaction between gC and FX or FVIIa and is therefore not depicted. However, it is not known if the gC-accessory protein complex can also promote FVII activation as seen with TF. Regardless of the pathway of generation, the FXa that remains associated with, or released from the virus, can function in the prothrombinase complex to generate thrombin. Thrombin is short lived in the circulation due to inhibition by endogenous anticoagulants and is thought to generate its pleiotropic haemostatic and cell signaling effects near the site at which it is produced⁶¹. The data show that coagulation enzyme complex assembly and proteolytic events can occur directly on the virus and may cause immediate thrombogenic changes to the host cell even prior to infection and expression of viral gene products.

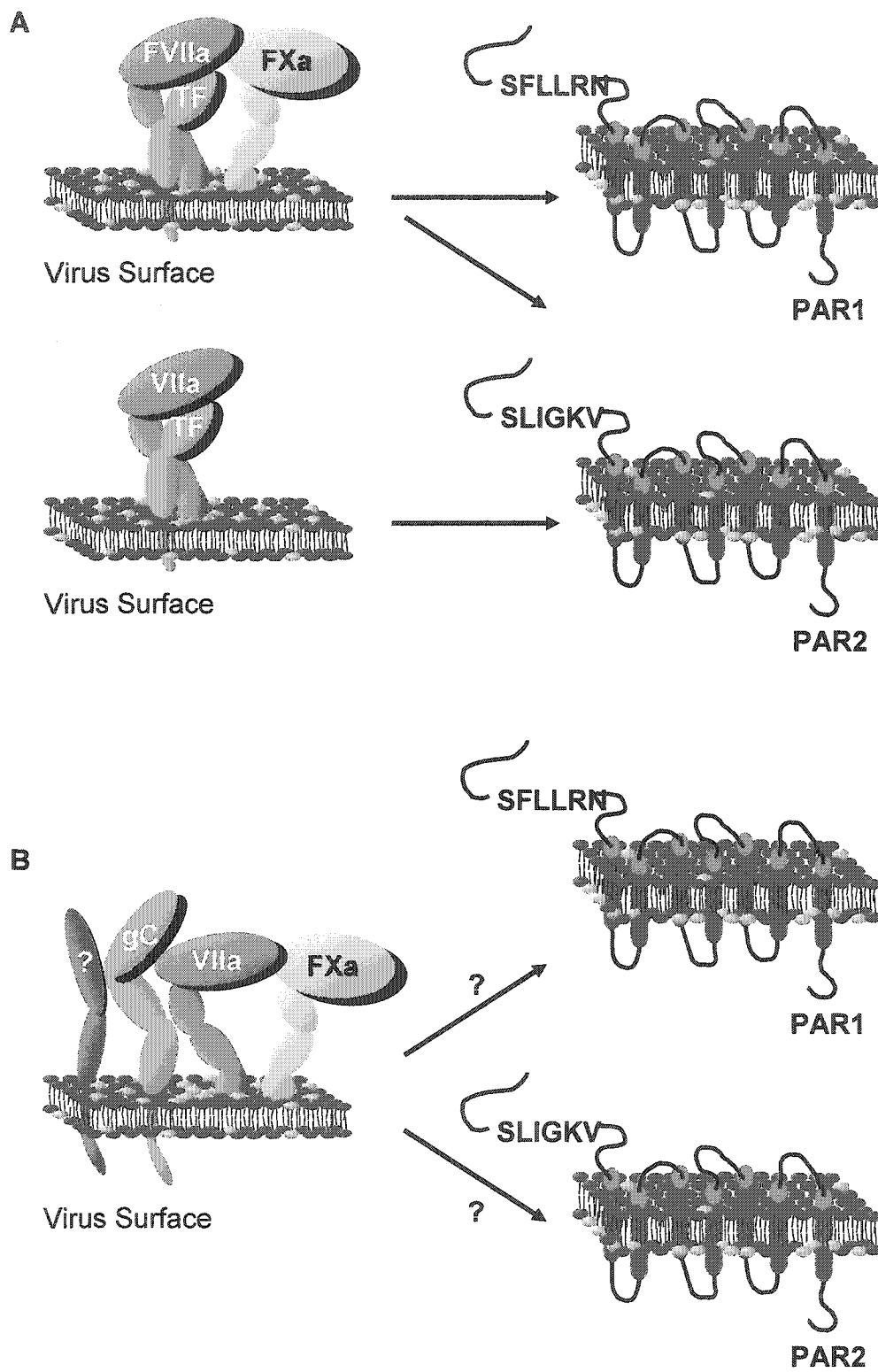
4.3.2 Cell Signaling

FXa and FVIIa are active serine proteases whose function in the early initiating events of coagulation has been well documented. However, increasing

evidence indicates that the activation of these clotting factors also elicits alterations in the cells where the enzymes are produced or bound. Using a variety of human cell types, FXa or TF-FVIIa signaling has been implicated in many thrombotic, atherogenic and inflammatory responses. Of importance to the current work, these changes include: induction of phosphorylation events that upregulate gene expression ³⁶; induction of endothelial growth factors ¹⁷⁰; smooth muscle cell proliferation ¹¹²; changes in intracellular Ca^{2+} levels ¹⁹²; and induction of synthesis of cytokines and expression of adhesion molecules ^{35,170}. Thus, FXa, FVIIa or thrombin that are concurrently produced on the HSV-1 surface may contribute to cell signaling.

Recent advances in cell and gene knock-out mouse models have provided evidence that FXa and TF-FVIIa signaling is mediated by PARs ^{1,34,35}. As depicted in Figure 37, TF-FVIIa has been shown to effectively cleave PAR2 whereas FXa can activate both PAR1 and PAR2. A major criticism in the relevance of FXa-mediated signaling is the relatively high concentrations needed to evoke a PAR response when only sub-nanomolar concentrations induce blood clotting ³⁴. This has been reconciled by showing the signaling efficacy of exogenous FXa is inefficient compared to equivalent concentrations generated by the TF-FVIIa complex ¹⁸⁷. Interestingly, the same group went on to show that the ternary TF-FVIIa-FXa complex appears to be most effective in PAR2 activation through the positioning of the active site of FXa in a more favorable orientation for the activation of PARs as compared to FVIIa or FXa alone. Kinetic analysis demonstrated that FXa signaling

Figure 37 HSV1 surface-associated protease activated receptor (PAR) activation. If assembled at close proximity to the host cell surface, HSV1 may mediate PAR activation. (A) Upon formation, the TF-FVIIa complex can activate PAR2. Also, prior to release of FXa, the TF-FVIIa-FXa complex may result in the activation of PAR1 and 2. (B) Similar to the TF-FVIIa-FXa complex, the gC-dependent FXa that is formed may potentially activate PAR1 and 2 on the cell surface. Not depicted, the virus-dependent thrombin that is produced may also result in PAR1, 3 and 4 activation at the host cell surface.



could occur at the low concentrations of FXa that are typical for the initiation phase of coagulation and therefore provides a mechanism to activate PAR1 and 2 prior to thrombin generation.

These findings have three important implications in the current work. First, each virus studied generated nanomolar concentrations of FXa during the reaction time frame. This suggests that in addition to clot formation as previous shown²¹⁶, the amount of FXa generated by these viruses could be sufficient to cause PAR activation. Second, the TF-FVIIa-FXa complex on the virus surface may potentially activate cell surface PAR and mediate proatherosclerotic changes to the cell prior to virus entry. It is also intriguing to speculate that if a gC-accessory protein-FVIIa-FXa complex exists as depicted in Figure 37, it may also mediate PAR2 signaling, analogous to TF-FVIIa-FXa. And third, PAR signaling may also occur through the preformed TF-FVIIa complex on each virus. PAR2 is expressed on endothelial and fibroblast cells, suggesting an early pathway in HSV1-mediated signaling. An extension of these findings is that non-infectious viruses that are sometimes in 1000 fold excess of infectious particles may also mediate signaling events prior to clearance. Therefore, productive infection may not be necessary for cellular changes to occur.

4.4 EFFECT OF HERPESVIRUS PRODUCED THROMBIN

The ability of viruses to convert resting cells from an anticoagulant to a procoagulant state is not unique to Herpesviruses. Measles virus, murine hepatitis

virus and avian hemangioma retrovirus also cause host cells to become thrombogenic^{72,138,186}. It is therefore possible that virus infection in general may cause certain procoagulant effects. Perhaps more importantly, the ability of thrombin to function as a potent cell modulator may be exploited by the viruses as a way to signal host cells during infection. Both Herpesvirus infection and thrombin have been shown to trigger similar intra- and extracellular events, many of which can be attributed to G protein-coupled receptor perturbation^{46,251}.

The finding that gC, which is encoded by the HSV1 genome, is able to initiate coagulation signifies that the capacity to generate thrombin is not merely circumstantial with consequences to the host, but may assist the virus during infection. To test this hypothesis, we followed HSV1 plaque formation in the presence of a thrombin specific inhibitor (hirudin), purified thrombin, or a thrombin receptor agonist peptide (TRAP). The combined data using the gC-characterized virus panel showed that thrombin production initiated by viral TF or gC *in situ* enhanced the susceptibility of host cells to Herpesvirus infection by a process that is reproduced by direct stimulation of the cell signaling receptor, protease activated receptor-1 (PAR1). HSV2 infection was also enhanced by thrombin in an analogous PAR1 dependent manner. A role for thrombin in the lifecycle of another clinically important Herpesvirus, CMV, is also proposed. The current data suggests that the production of thrombin on virus attached to the host cell surface may therefore be an initiating event in the infection process and vascular pathology mediated by Herpesviruses. There are several points along the pathway leading to productive

infection that may be enhanced by thrombin.

4.4.1. Attachment

The attachment of Herpesviruses to host cells is a sequential process that relies on initial contacts with cell surface HSP ¹⁰⁷. This involves interactions between the negatively charged moieties of HSP with known virus surface glycoproteins. For CMV, binding is dependent on virus-encoded glycoprotein C-II (gCII) ¹⁰⁷ and gB ⁴³, while gC ⁹⁰ and gB ⁸⁹ have been identified for HSV. This interaction, which is inhibited by soluble heparin, prepares the cell for a second attachment step. For HSV1, this subsequent attachment step is at least mediated in part by the interaction between gD and known Herpesvirus entry mediators. However, the identity of the additional molecule needed for CMV entry remains controversial. Following attachment, entry of the virus occurs through an unknown mechanism that may involve scrambling of the cell and viral membranes or crossing over of opposing membranes surfaces ²⁴¹.

Work from our lab suggests that the proPL binding protein, annexin II (AnII) may serve as a CMV-binding protein ^{27,246,247}. The discovery of a cell surface component with electrophoretic properties identical to AnII that correlated with viral tropism ¹⁶⁹ and a report that an antiserum raised against AnII inhibited infection ²⁴⁶ provide indirect support for the receptor role of AnII. There has also been direct evidence that purified AnII can independently function as a CMV receptor and enhance viral plaque formation and mediate proPL membrane fusion ¹⁸⁵. The

intracellular localization of AnII and lack of known redistribution pathways makes its function as a cell surface receptor speculative. Of relevance to the current study, we have recently identified that thrombin is able to induce the expression of AnII on the cell surface¹⁷⁷.

In conjunction with the current report, the inducible expression of AnII on the cell surface provides a direct function for thrombin in the initial attachment to the host cell. In this model, the virus can generate a similar amount of thrombin as added exogenously in the previous study¹⁷⁷. If produced in close proximity to the cell, the thrombin can then cause the redistribution of AnII to the host cell surface. Once on the surface, interactions between AnII and CMV gB or AnII already present on the virus surface are thought to mediate the final attachment and entry into the cell. This model can be transferred to HSV1 and HSV2 since results from our laboratory indicate that AnII is also found on the surface of these viruses, although an additional AnII-interacting glycoprotein is not known for these viruses.

Another interesting model is suggested by the thrombin inducible cell surface expression of AnII. Experimental evidence suggests that AnII can act as a profibrinolytic agent through acceleration of the tissue plasminogen activator (tPA)-mediated conversion of plasminogen to plasmin. This occurs through the binding of tPA to C-terminal lysines on the p11 subunit associated with AnII, ultimately resulting in the degradation of fibrin. The virus may therefore initiate the opposing pathways of coagulation and fibrinolysis. A possible advantage of plasmin generation by Herpesviruses is to limit fibrin deposition on the virus or host cell

surface that would potentially inhibit virus attachment and entry into the cell. This may explain why fibrin deposition and clot formation are relatively rare clinical manifestations of Herpesvirus infection.

4.4.2 Cell Signaling

In addition to its role in haemostasis, an important consequence of thrombin production by the virus is the aberrant generation of a potent cell modulator. The thrombin signal can be transmitted through the cleavage and activation of multiple PARs on the cell surface, the most important of which is PAR1. Upon activation, changes in the G proteins coupled to the receptor trigger phosphorylation events that can lead to enhanced transcription, mitogenesis and growth of the cell ⁴⁹. The current report indicates that both thrombin and PAR1 perturbation result in enhanced virus infection. These results therefore provide a direct link between the previous independent observations that thrombin and virus stimulation of the cell results in similar intracellular events. Also important, as seen with PAR activation by FXa and FVIIa, PAR1 activation can result in atherosclerotic changes to the cell.

4.4.3 Replication

After the initial contacts with the host cell have been made, pH-independent fusion of the Herpesvirus envelope with the cell membrane occurs. For CMV, this is believed to involve the virus-encoded gH and gB ¹⁰⁸. An anti-idiotypic antibody that mimics the cell-binding site on gH showed that a 92.5 kDa host cell cognate

receptor was involved in virus penetration¹³ and also functions in phosphorylation by protein-tyrosine kinases¹⁰⁹. PKC activity, which is increased by thrombin, has also been correlated to virus infection²¹¹. A number of HSV glycoproteins have been identified that are important for penetration of the host cell membrane and include gB, gD, and the gH/gL complex^{33,126,191,200}. A potential link between HSV-1 penetration and thrombin stimulation is implied by observations, like those for CMV, that PKC inhibitors attenuate the entry of virus into fibroblasts⁴⁴.

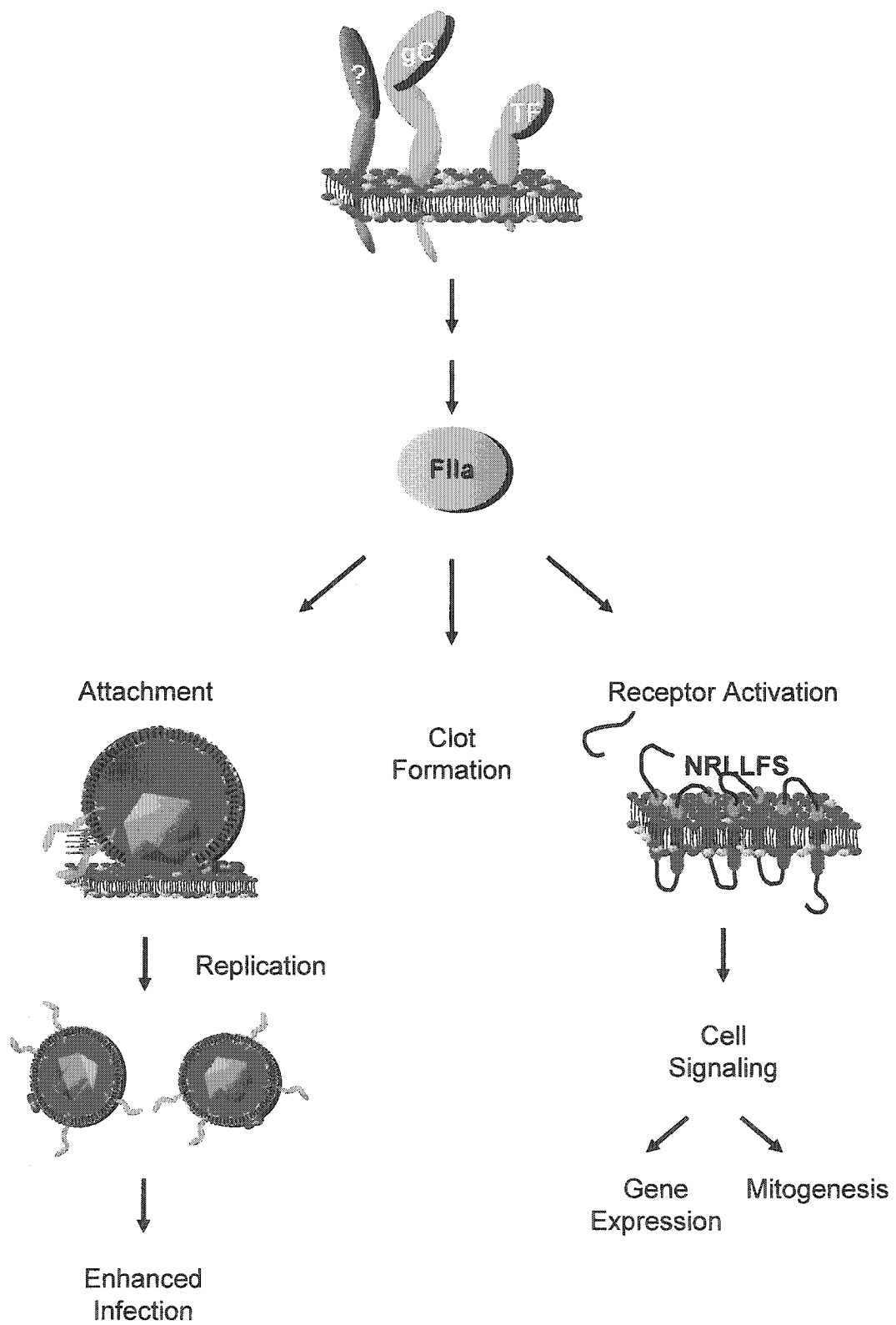
The transcription of HSV- and CMV-encoded genes is sequential with those designated "immediate early" (IE) being expressed first⁴⁰. These depend heavily on host transcription factors and do not require the prior expression of viral or host genes. The IE genes have the ability to function as transcription factors and are essential for the expression of later viral genes. The expression of the HSV-1 IE genes that function in the generation of early and late genes products requires the contribution of both virus and host cellular proteins. The transcription of the IE genes is initiated upon entry into the cell by the virion phosphoprotein, VP16, which targets specific upstream promoters of the HSV IE genes^{152,190}. In order to regulate transcription, VP16 directs the assembly of a multiprotein-DNA complex with the cellular factors Oct-1 and HCF¹³². Therefore, changes in the phosphorylation states of cellular and viral proteins are important catalysts in IE transcription and are mediated by exogenous cellular kinases such as PKC, PKA and cyclin-dependent kinases^{102,155,156}. Interestingly, thrombin receptor perturbation can also result in the activation of these kinases, linking thrombin generation on the virus to manipulation

of the host cell machinery that function in virus replication. Extracellular virus-mediated thrombin production may also prime adjacent cells for infection.

Host transcription factors have also been shown important in the regulation to the CMV major IE promoter (MIEP). There are several host cell proteins known to activate the MIEP. The functioning of NF- κ B in this process is considered to be fundamental¹⁵¹ and is important within the context of thrombin-mediated receptor activation. Prior to stimulation, NF- κ B is stored in a cytoplasmic pool primarily bound to the inhibitory protein, I- κ B. Several reports have demonstrated that CMV treatment of host cells enhances NF- κ B activity within 2 hours^{24,114}. This effect does not require protein synthesis and occurs through mobilization of the cytoplasmic store to initiate CMV IE gene transcription²⁵⁰. Thus, an additional involvement of thrombin in CMV infection may be at this point of regulation because thrombin is known to rapidly enhance NF- κ B activity²⁰⁰.

A model summarizing a possible mechanism for the thrombin-dependent enhancement of HSV1 infection is shown in Figure 38. In a previous study we reported that purified Herpesviruses could function effectively as the only source of coagulation initiator in a clotting assay and was therefore capable of activating factor VII and factor V²¹⁶, as depicted in the model. We now add to the understanding of direct initiation of coagulation on the virus surface by showing here that gC and TF simultaneously accelerate the activation of FX by FVIIa through independent pathways. The FXa that is generated can then assemble with the cofactor factor Va in association with proPL and convert prothrombin to thrombin¹⁸³.

Figure 38 Effects of Herpesvirus-mediated thrombin production. The thrombin that is produced on the virus surface can: 1) result in the enhancement of virus infection through increased virus attachment and replication; 2) promote fibrin clot formation at the site of infection; and 3) cause intracellular signaling events through protease activated receptor activation.



The thrombin that is produced can then interact with cell surface PAR1 and assist entry of the virus into the cell. Because the novel FVIIa accelerating activity of purified sgC was enhanced by 3 orders of magnitude when combined with HSV1, an additional contributing viral constituent is depicted in the model.

Based on the observation that thrombin can be generated on the HSV1 surface, a summary of the potential cell modulatory effects of virus-produced thrombin are shown in Figure 38. The amount of thrombin that can be produced on a single blood-borne virus particle may not be sufficient to overcome the anticoagulant threshold of plasma if the virus is free in the circulation. However, when HSV1 binds near thrombin receptors on a host cell surface or when a large local concentration of virus is released upon cell lysis, the probability of thrombin inhibition by normal physiological anticoagulant pathways is decreased. Thus, thrombin production on the virus is seen primarily as a cell-triggering event, which may have two roles in pathology. First, thrombin receptor perturbation may signal cellular events that lead to the expression of viral receptors or result in enhanced viral gene transcription, ultimately leading to increased infection. Second, Herpesviruses establish latent infection, which means that the host is exposed to numerous recurrent, subclinical infections. The resulting procoagulant and proinflammatory effects of small amounts of localized thrombin production would be cumulative and predispose to vascular disease^{80,147,232}. The induction of cellular thrombogenic activity by virus infection may furthermore propagate the effect and establish localized areas of vasculature that are especially susceptible to infection.

4.5 THROMBOGENIC POTENTIAL

The generation of a cellular procoagulant phenotype is a well-documented outcome of viral infections. In our previous studies, HSV1, HSV2 and CMV differed in their ability to generate thrombin²¹⁶. This is interesting since it implies that subtle differences exist between viruses that may be correlated to published effects in vascular pathology. Earlier work using clotting assays showed that HSV generated 100 times more thrombin per particle than CMV²¹⁶. In agreement, data from chromogenic assays in the current study shows that there is only a 10 fold increase in thrombin production when HSVs are compared to CMV. This may indicate that an additional component in serum gives rise to the increased thrombin generation seen in the clotting assay. The most plausible reason for the discrepancy is the FIXa-FVIIIa tenase pathway that can also augment thrombin generation in the clotting assay whereas it is not present in the purified chromogenic assays. A similar result was realized when initiating events were examined on HSV compared to CMV. As predicted, CMV generated less FXa than either HSV1 or HSV2 and therefore would explain the differential generation of thrombin. These findings provide direct evidence to explain why severe HSV infection leads to fibrin deposition in microvasculature^{125,144} and disseminated intravascular coagulation¹⁸⁰ in humans. Thrombotic complications due to CMV are rare; however, a correlation with restenosis has been suggested.

The higher thrombogenic potential of HSVs compared to CMV raises a

number of interesting hypotheses concerning the FXa and thrombin generating pathways associated with the Herpesvirus family. The first is based on the observation that CMV and the gC-deficient virus generates less FXa per particle than HSV1, HSV2, or the gC-competent strain and therefore leads to reduced thrombin generation. Incubation of the same viruses with inhibitory anti-TF antibody also showed reduced FXa generation when compared to HSV1, HSV2, or the gC-competent virus. This clearly shows that like the gC-deficient virus, the prominent FXa generating mechanism on CMV is TF. Second, HSV1 and HSV2 have only slight differences in FXa and thrombin generation as well as TF inhibition profiles of thrombin generation, indicating similar initiating mechanisms associated with these viruses. This is interesting since HSV1 and HSV2 gC share a low degree of sequence homology² and suggests that another glycoprotein on HSV2 can function in FXa generation. The FXa generating capability of HSV2 gC can also be determined.

The reduced infectivity of HSV2 compared to HSV1 in response to thrombin or the PAR1 agonist peptide indicates that multiple signaling pathways may be involved in the infection of the two viruses. The reported 2 fold difference in FXa and thrombin generation between HSV1 and HSV2, although slight, may have an effect on induced pathology. HSV2 was shown to generate more FXa than HSV1 and was less sensitive to TRAP treatment, suggesting that in addition to PAR1, PAR2 perturbation may be important for entry. The relative infectivity due to purified FXa treatment of cells as well as a PAR2 agonist peptide would illustrate the

importance of PAR2 in HSV1 and HSV2 infection.

The expression of TF was shown on all Herpesviruses preparations tested, however, differing amounts of the protein were associated with each. This may be due to several factors. The first is that since the TF is derived solely from the host cell membranes, the amount associated with the virus may vary due to cellular TF levels. This in turn may be due the synchronization of the cells at the time of initial infection with actively dividing cells being able to synthesize more protein. Alternatively, infection with a particular virus strain may result in a more robust infection and generation of TF while infection with the different viruses may result in alterations in transcription rates that would affect the amount of host cell and viral proteins within a cell. Therefore, the coordinated expression of TF on the cell surface with virus egress will ultimately determine the amount of TF associated with each individual virus.

In some cases, the TF activity produced on the virus does not reflect the amount of antigen detected. It is important to note that in each experiment, the inter virus variation in TF antigen was minimal, indicating that the results were reflective of two initiating pathways on the virus and not simply due to reduced levels of TF in the system. A possible explanation for the reduced TF activity is based on the local environment in which it is present on the virus. Since TF can traffic between areas of the membrane that are rich in proPL or glycosphingolipid, regulation of function may result from the localization of TF in different cell surface microdomains²⁰². The changes in the membrane phospholipid distribution that is responsible for the overall

enhancement of TF proteolytic function⁵² may also differ amongst the virus tested.

An additional explanation for differences in TF antigen levels and activity detected is suggested by previous reports that determined the availability of functional TF on the membrane surface. These studies identified a pool of cell surface encrypted TF that has a much lower procoagulant activity than cells that have been damaged, lysed or treated with calcium ionophore¹²³. Although TF is present on the surface of such cells, it only becomes fully active when membrane properties of the cell are altered^{11,53}. There are several potential explanations for this phenomenon of TF encryption. First, cells are known to restrict the distribution of aminophospholipid to the inner leaflet of the plasma membrane. Negatively charged phospholipids are required for substrate molecules to bind to the membrane, so their sequestration limits the activity of cell surface TF. When cells are activated the phospholipid asymmetry is lost and the TF would become functional. Secondly, in some cell types, TF may associate with caveolae, which are areas of the cell surface with altered lipid composition. This extracellular pool of TF was shown to bind VIIa normally, but is incapable of forming the ternary complex that leads to FX activation¹²³. Since the TF on the virus is acquired from the host cell membrane, a fraction may also be encrypted and unable to activate FX, accounting for the increased TF antigen associated with some virus preparations compared to the TF activity generated. In order to determine the proportion of potentially non-functional TF associated with each virus, the ability of FVIIa to cleave small peptidyl substrates, which is unchanged when TF is encrypted, can be

followed.

Also of potential importance to the current work are studies demonstrating the presence of a dimeric TF on the cell surface. It has been proposed that dimerization or oligomerization of TF in the membrane may reduce its activity, and that damage or lysis of cells may promote the formation of active monomers. However, it was subsequently shown that TF dimerization did not influence the amidolytic or FX activation activities of the TF-FVIIa complex⁵². It is currently not known whether TF dimers exist on the virus surface and if their formation has an effect on virus-mediated FX activation. Also, it is unknown if the formation of these dimers would affect the antigenicity of TF.

Another parameter to consider in the determination of functional cofactor molecules is the amount of proPL associated with each virus preparation. As previously mentioned, the availability of surface proPL is important for the recruitment of FX to the TF-FVIIa and potentially gC-FVIIa complexes. Therefore if a variation in the presence and availability of proPL exists on the virus, a change in the full function of initiator molecules will occur. In the present study, the amount of proPL associated with each virus was not determined. However, limiting of complex formation due to the available proPL on each virus seems unlikely since no increase in FXa generation was seen with the addition of exogenous proPL. In agreement, approximately 9% of the total phospholipid content of CMV was estimated to be proPL accessible on its surface and was not limiting with respect to FX or FXa binding¹⁸³.

Evidence from the current report suggests that 50% of the FXa generation by HSV1 is due to gC and should be considered as an important factor in the thrombogenic potential of this virus. This protein is encoded by the virus and therefore implies that a constant number of copies of gC should be present on the surface. This was the case as each HSV1 used in an individual experiment contained comparable levels of gC antigen. Also of interest, an antibody was not found that would recognize the gC antigen on both the lab strain and clinical isolate of HSV1 used in the study. This was surprising since gC was not found to be significantly variable between HSV1 strains²²⁷. The antibody used may be directed towards a highly antigenic region in the clinical isolate that is altered or not present in the lab adapted strain and therefore not recognized. From the functional data presented, the clinical isolate and lab strain are suggested to have comparable levels of gC since they had identical FXa generating and TF inhibition profiles. Currently, the glycoprotein in HSV2 that functions analogously to HSV1 gC in FX activation is not known. Once identified, this protein would also add to the thrombogenic potential of that virus.

4.6 VASCULAR DISEASE

Atherosclerosis is a complex disease that is induced by a combination of risk factors. Many of the early studies developed a clear involvement of lipid accumulation and smooth muscle cell proliferation in the development of the atheroma. Thrombin, the biological effector of the coagulation pathway^{57,134}, was

suggested to play a role through the stimulation of smooth muscle cell proliferation, recruitment of monocytes and macrophages, and the development of a procoagulant phenotype, all of which may lead to atherosclerosis. The involvement of thrombin in these changes is supported in the literature showing that hirudin, a specific thrombin inhibitor, reduced monocyte adhesion to infected vascular endothelial cells and similarly, adhesion was inhibited by depleting the inoculation media of prothrombin⁶². Since many of these same cellular changes are caused by Herpesvirus infection^{145,147,232} the thrombin produced on the virus surface is speculated to be the cause.

Research over the past decade has provided an inflammatory component to the development of vascular disease that may be mediated by coagulation protease signaling through PARs. Thrombin and TF-FVIIa activate endothelial cell PAR1 and PAR2 respectively to signal changes in vascular tone, stimulate permeability and increase the adhesion of inflammatory molecules⁴⁹. Therefore, Herpesvirus can potentially direct a similar chain of events by activating cell surface PAR1 and PAR2 through the TF-FVIIa and TF-FVIIa-FXa complexes directly on their surface as well as the generation and release of FXa and thrombin. Importantly, these events can occur without virus entry into the cell. This report adds to the previous work, providing a direct link between the ability of the Herpesvirus surface to initiate coagulation and the development of an atherosclerotic phenotype. This demonstrates the earliest known event linking subclinical Herpesvirus infection to atherogenesis. The data also suggest that a hypercoagulable state may lead to an

increased susceptibility to Herpesvirus infection.

4.7 FUTURE DIRECTIONS

4.7.1 *In situ* thrombin production

Results from this and our previous study have shown that members of the Herpesvirus family differ in their ability to generate FXa and thrombin. Using clotting and chromogenic assays, similar interpretations can be made regarding the virus-mediated initiation of coagulation. However, the results from clotting assays showed a greater difference between each virus with respect to FX activating ability. This implies that something in plasma influences the ability to initiate coagulation and may be a more *in vivo* like setting than the use of purified proteins. Therefore, the ability of each virus to produce a titratable effect of both FXa and thrombin generation will be examined. The gC-characterized viruses will also be examined to determine the importance of gC in plasma clotting events.

4.7.2 Further effects of coagulation proteases

The data from this report indicated that HSV infection of host cells is enhanced by simultaneous incubation of the cells with thrombin. It is conceivable that an augmentation in infection may be realized if the cells are stimulated prior to the addition of virus. Therefore the effect of thrombin pretreatment on infection should be determined. Also, the ability of thrombin generated at the virus surface to

trigger cellular events will be investigated. In order to distinguish effects induced by: a) thrombin produced on the virus; b) thrombin produced on the cell; and c) direct virus-cell interactions, the use of purified coagulation factors and a series of preincubation/washing steps will control where and how thrombin is being produced. The parameters that will be initially focused upon will include the effects on overall infection and virus receptor expression.

Current evidence suggests that PAR activation through FXa may also result in cellular changes that increase virus infection. Therefore, as with thrombin, the function of FXa in virus infection will be explored. This involves addition of exogenous FXa, use of specific inhibitors and the effect of PAR2 activation on infection. Again, the role on FXa preincubation as well as *in situ* FXa production on the virus should also be investigated. This would allow the direct comparison of PAR1 and PAR2 mediated events in virus infection.

4.7.3 Direct interactions of proteins

Preliminary experiments using SPR were unable to demonstrate an interaction between gC and either FX or FVIIa. Further experiments designed to explore direct virus surface interactions can be achieved by direct binding studies of FX or FVIIa to the gC-characterized virus panel through either SPR or high-speed ultracentrifugation. Also, the finding that an additional viral constituent is needed for the full function of gC in FVIIa acceleration implies synergy between the two molecules. Therefore, the identity of the accessory protein will be explored. To

begin other glycoproteins known to interact with gC will be examined by following FXa generation in the presence of specific antibodies. Alternatively, simultaneous incubation with different combinations of these glycoproteins in a purified system may identify possible candidates involved in FXa generation.

4.7.4 Animal Models

In order to demonstrate the clinical significance of the current results previously described animal models will be used. The development of a thrombotic state after injection with the different members of the Herpesvirus family into normal mice can determine by FXa and thrombin detection in plasma. These viruses will then be compared to the gC-characterized virus panel with the hypothesis that infection with the gC-deficient virus would result in less FXa and thrombin production. C3-deficient mice will also be monitored for any changes due to complement-mediated effects. To test the hypothesis that a prothrombotic condition may predispose to virus infection, the progression of infection in a hypercoaguable mouse model, such as active protein C resistance, can be examined.

5.0 REFERENCES

1. **Aasrum, M. and H. Prydz.** 2002. Gene targeting of tissue factor, factor X, and factor VII in mice: Their involvement in embryonic development. *Biochemistry-Moscow* **67**:25-32.
2. **Ackermann, G., F. Ackermann, H. J. Eggers, U. Wieland, and J. E. Kuhn.** 1998. Mapping of linear antigenic determinants on glycoprotein C of herpes simplex virus type 1 and type 2 recognized by human serum immunoglobulin G antibodies. *Journal of Medical Virology* **55**:281-287.
3. **Adam, E., J. L. Melnick, J. L. Probstfield, B. L. Petrie, J. Burek, K. R. Bailey, C. H. McCollum, and M. E. DeBakey.** 1987. High levels of cytomegalovirus antibody in patients requiring vascular surgery for atherosclerosis. *Lancet* **2**:291-293.
4. **Ahmad, S. S., R. Rawala-Sheikh, and P. N. Walsh.** 1989. Comparative interactions of factor IX and factor IXa with human platelets. *Journal of Biological Chemistry* **264**:3244-3251.
5. **Ahmad, S. S., R. Rawala-Sheikh, and P. N. Walsh.** 1989. Platelet receptor occupancy with factor IXa promotes factor X activation. *Journal of Biological Chemistry* **264**:20012-20016.
6. **Ahmed, A. A. and B. J. Holub.** 1984. Alteration and recovery of bleeding times, platelet aggregation and fatty acid composition of individual phospholipids in platelets of human subject receiving a supplement of cod-liver oil. *Lipids* **19** (8):617-624.
7. **Al Ani, B., M. Saifeddine, A. Kawabata, and M. D. Hollenberg.** 1999. Proteinase activated receptor 2: role of extracellular loop 2 for ligand-mediated activation. *British Journal of Pharmacology* **128**:1105-1113.
8. **Altieri, D. C., O. R. Ettinger, D. S. Fair, T. K. Brunck, J. E. Geltosky, D. P. Hajjar, and T. S. Edgington.** 1992. Structurally homologous ligand binding of integrin Mac-1 and viral glycoprotein C receptors. *Science* **254**:1200-1202.
9. **Auland, M. E., B. D. Roufogalis, P. F. Devaux, and A. Zachowski.** 1994. Reconstitution of ATP-dependent aminophospholipid translocation in proteoliposomes. *Proceedings of the National Academy of Science U. S. A.* **91**:10938-10942.
10. **Bach, R.** 1988. Initiation of coagulation by tissue factor. *CRC Critical Reviews in*

Biochemistry **23**:339-368.

11. **Bach, R. and D. B. Rifkin.** 1990. Expression of tissue factor procoagulant activity: Regulation by cytosolic calcium. *Proceedings of the National Academy of Science U. S. A.* **87**:6995-6999.
12. **Bajzar, L., R. Manuel, and M. E. Nesheim.** 1995. Purification and characterization of TAFI, a thrombin activable fibrinolysis inhibitor. *Journal of Biological Chemistry* **270**:14477-14484.
13. **Baldwin, B. R., M. Kleinberg, and S. Keay.** 1996. Molecular cloning and expression of receptor peptides that block human cytomegalovirus/cell fusion. *Biochemical and Biophysical Research Communication* **219**:668-673.
14. **Banner, D. W., A. D'Arcy, C. Chene, F. K. Winkler, A. Guha, W. H. Konigsberg, Y. Nemerson, and D. Kirchhofer.** 1996. The crystal structure of the complex of blood coagulation factor VIIa with soluble tissue factor. *Nature* **380**:41-46.
15. **Baugh, R. J., C. D. Dickinson, W. Ruf, and S. Krishnaswamy.** 2000. Exosite interactions determine the affinity of factor X for the extrinsic Xase complex. *Journal of Biological Chemistry* **275**:28826-28833.
16. **Bavendiek, U., P. Libby, M. Kilbride, R. Reynolds, N. Mackman, and U. Schonbeck.** 2002. Induction of tissue factor expression in human endothelial cells by CD40 ligand is mediated via activator protein 1, nuclear factor kappa B, and Egr-1. *Journal of Biological Chemistry* **277**:25032-25039.
17. **Benditt, E. P., T. Barrett, and J. K. McDougall.** 1983. Viruses in the etiology of atherosclerosis. *Proceedings of the National Academy of Science U. S. A.* **80**:6386-6389.
18. **Bevers, E. M., P. Comfurius, D. W. C. Dekkers, and R. F. A. Zwaal.** 1999. Lipid translocation across the plasma membrane of mammalian cells. *Biochimica et Biophysica Acta-Molecular and Cell Biology of Lipids* **1439**:317-330.
19. **Bevers, E. M., P. Comfurius, J. L. van Rijn, H. C. Hemker, and R. F. Zwaal.** 1982. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *European Journal of Biochemistry* **122**:429-436.
20. **Bevers, E. M., P. Comfurius, and R. F. A. Zwaal.** 1983. Changes in membrane phospholipid distribution during platelet activation. *Biochimica et Biophysica Acta* **736**:57-66.

21. **Blackhart, B. D., L. Ruslim-Litrus, C. C. Lu, V. L. Alves, W. Teng, R. M. Scarborough, E. E. Reynolds, and D. Oksenberg.** 2000. Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. *Molecular Pharmacology* **58**:1178-1187.
22. **Boehmer, P. E. and A. V. Nimonkar.** 2003. Herpes virus replication. *Journal of Virology* **55**:13-22.
23. **Bogdanov, V. Y., V. Balasubramanian, J. Hathcock, O. Vele, M. Lieb, and Y. Nemerson.** 2003. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nature Medicine* **9**:458-462.
24. **Boldogh, I., M. P. Fons, and T. Albrecht.** 1993. Increased levels of sequence specific DNA-binding proteins in human cytomegalovirus-infected cells. *Biochemical and Biophysical Research Communication* **197**:1505-1510.
25. **Boon, J. M. and B. D. Smith.** 2002. Chemical control of phospholipid distribution across bilayer membranes. *Medicinal Research Reviews* **22**:251-281.
26. **Bromberg, M. E., R. Sundaram, R. J. Homer, A. Garen, and W. H. Konigsberg.** 1999. Role of tissue factor in metastasis: Functions of the cytoplasmic and extracellular domains of the molecule. *Thrombosis and Haemostasis* **82**:88-92.
27. **Brooks, N. D., J. E. Grundy, N. Lavigne, M. C. Derry, C. M. Restall, C. R. Mackenzie, D. M. Waisman, and E. L. G. Prydzial.** 2002. Calcium-dependent and phospholipid-independent binding of annexin 2 to annexin 5. *Biochemical Journal*. **367**:895-900.
28. **Brown, M. S. and J. L. Goldstein.** 1990. Scavenging for receptors. *Nature* **343**:508-509.
29. **Broze, G. J.** 1995. Tissue factor pathway inhibitor and the current concept of blood coagulation. *Blood coagulation & fibrinolysis* **6**:S7-S13.
30. **Broze, G. J.** 1995. Tissue factor pathway inhibitor and the revised theory of coagulation. *Annual Review Medicine*. **46**:103-112.
31. **Bruggeman, C. A., W. H. M. Debie, A. D. Muller, B. Schutte, and M. C. E. van Dam-Mieras.** 1988. Cytomegalovirus alters the von Willebrand factor content of human endothelial cells. *Thrombosis and Haemostasis* **59**:264-268.
32. **Butenas, S. and K. G. Mann.** 1995. Kinetics of human factor VII activation.

Blood 86:297.

33. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus 1 in cell entry and fusion. *Journal of Virology* 62:2596-2604.
34. **Camerer, E., W. Huang, and S. R. Coughlin.** 2000. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proceedings of the National Academy of Sciences of the United States of America* 97:5255-5260.
35. **Camerer, E., H. Kataoka, M. Kahn, K. Lease, and S. R. Coughlin.** 2002. Genetic evidence that protease-activated receptors mediate factor Xa signaling in endothelial cells. *Journal of Biological Chemistry* 277:16081-16087.
36. **Camerer, E., J. A. Rottingen, E. Gjernes, K. Larsen, A. H. Skartlien, J. G. Iversen, and H. Prydz.** 1999. Coagulation factors VIIa and Xa induce cell signaling leading to up-regulation of the egr-1 gene. *Journal of Biological Chemistry* 274:32225-32233.
37. **Carfi, A., H. Y. Gong, H. Lou, S. H. Willis, G. H. Cohen, R. J. Eisenberg, and D. C. Wiley.** 2002. Crystallization and preliminary diffraction studies of the ectodomain of the envelope glycoprotein D from herpes simplex virus 1 alone and in complex with the ectodomain of the human receptor HveA. *Acta Crystallographica Section D-Biological Crystallography* 58:836-838.
38. **Carmeliet, P., N. Mackman, L. Moons, T. Luther, P. Gressens, I. VanVlaenderen, H. Demunck, M. Kasper, G. Breier, P. Evrard, M. Muller, W. Risau, T. Edgington, and D. Collen.** 1996. Role of tissue factor in embryonic blood vessel development. *Nature* 383:73-75.
39. **Carson, S. D., S. E. Ross, R. Bach, and A. Guha.** 1987. An inhibitory monoclonal antibody against human tissue factor. *Blood* 70:490-493.
40. **Castillo, J. P. and T. F. Kowalik.** 2002. Human cytomegalovirus immediate early proteins and cell growth control. *Gene* 290:19-34.
41. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Current Topics in Microbiology and Immunology* 154:125-169.
42. **Cheshenko, N. and B. C. Herold.** 2002. Glycoprotein B plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for

- entry and cell-to-cell spread. *Journal of General Virology* **83**:2247-2255.
43. **Compton, T., D. M. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell-surface heparan sulfate. *Virology* **193**:834-841.
 44. **Constantinescu, S. N., C. D. Cernescu, and L. M. Popescu.** 1991. Effects of protein kinase C inhibitors on viral entry and infectivity. *FEBS Lett.* **292**(1-2):31-33.
 45. **Coughlin, S. R.** 2001. Protease-activated receptors in vascular biology. *Thrombosis and Haemostasis* **86**:298-307.
 46. **Coughlin, S. R., T.-K. H. Vu, D. T. Hung, and V. I. Wheaton.** 1992. Expression cloning and characterization of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Seminars in Thrombosis and Hemostasis* **18**:161-166.
 47. **Cunningham, M. J. and R. C. Pasternak.** 1988. The potential role of viruses in the pathogenesis of atherosclerosis. *Circulation* **77**:964-966.
 48. **Dahlbach, B. and J. Stenflo.** 1978. Binding of bovine coagulation factor Xa to platelets. *Biochemistry* **17**:4938-4945.
 49. **Dery, O., C. U. Corvera, M. Steinhoff, and N. W. Bunnett.** 1998. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *American Journal of Physiology-Cell Physiology* **43**:C1429-C1452.
 50. **Dickinson, C. D., C. R. Kelly, and W. Ruf.** 1996. Identification of surface residues mediating tissue factor binding and catalytic function of the serine protease factor VIIa. *Proceedings of the National Academy of Sciences of the United States of America* **93**:14379-14384.
 51. **Dickinson, C. D., J. Shobe, and W. Ruf.** 1998. Influence of cofactor binding and active site occupancy on the conformation of the macromolecular substrate exosite of factor VIIa. *Journal of Molecular Biology* **277**:959-971.
 52. **Donate, F., C. R. Kelly, W. Ruf, and T. S. Edgington.** 2000. Dimerization of tissue factor supports solution-phase autoactivation of factor VII without influencing proteolytic activation of factor X. *Biochemistry* **39**:11467-11476.
 53. **Drake, T. A., W. Ruf, J. H. Morrissey, and T. S. Edgington.** 1989. Functional tissue factor is entirely cell surface expressed on lipopolysaccharide-stimulated human blood monocytes and a constitutively tissue factor-producing neoplastic cell line. *Journal of Cell Biology* **109**:389-395.

54. **Drew, W. L.** 1992. Cytomegalovirus infection in patients with AIDS. *Clinical Infectious Disease* **14**:608-615.
55. **Eigenbrot, C. and D. Kirchhofer.** 2002. New insight into how tissue factor allosterically regulates Factor VIIa. *Trends in Cardiovascular Medicine.* **12**:19-26.
56. **Eigenbrot, C., D. Kirchhofer, M. S. Dennis, L. Santell, R. A. Lazarus, J. Stamos, and M. H. Ultsch.** 2001. The factor VII zymogen structure reveals reregistration of beta strands during activation. *Structure* **9**:627-636.
57. **Elion, J., M. R. Downing, R. J. Butkowski, and K. G. Mann.** 1977. Structure of human thrombin: comparison with other serine proteases. pp. 97-111. In: Lundblad, R.L., et. al., ed. *Chemistry. and biology. of thrombin.* Ann. Arbor, Ann. Arbor. Science,.
58. **Elliott, G. and P. O'Hare.** 1999. Live-cell analysis of a green fluorescent protein-tagged herpes simplex virus infection. *Journal of Virology* **73**:4110-4119.
59. **Epstein, S. E., E. Speir, Y. F. Zhou, E. Guetta, M. Leon, and T. Finkel.** 1996. The role of infection in restenosis and atherosclerosis: Focus on cytomegalovirus. *Lancet* **348**:s13-s17.
60. **Esmon, C. T. and N. L. Esmon.** 1984. Protein C activation. *Seminars in Thrombosis and Haemostasis.* **10**:122-129.
61. **Esmon, C. T., J.-M. Gu, J. Xu, D. Qu, D. Stearns-Kurosawa, and S. Kurosawa.** 1999. Regulation and functions of the protein C anticoagulant pathway. *Haematologica* **84**:363-368.
62. **Etingin, O. R., R. L. Silverstein, H. M. Friedman, and D. P. Hajjar.** 1990. Viral activation of the coagulation cascade: Molecular interaction at the surface of infected endothelial cells. *Cell* **61**:657-662.
63. **Etingin, O. R., R. L. Silverstein, and D. P. Hajjar.** 1991. Identification of a monocyte receptor on herpesvirus-infected endothelial cells. *Proceedings of the National Academy of Science USA* **88**:7200-7203.
64. **Fabricant, C. G., J. Fabricant, M. M. Litrenta, and C. R. Minick.** 1978. Virus induced atherosclerosis. *Journal of Experimental Medicine* **148**:335-340.
65. **Fabricant, C. G., J. Fabricant, C. R. Minick, and M. M. Litrenta.** 1983. Herpesvirus induced atherosclerosis in chickens. *Federal Proceedings* **42**:2476-2479.

66. Favoreel, H. W., G. R. Van de Walle, H. J. Nauwynck, and M. B. Pensaert. 2003. Virus complement evasion strategies. *Journal of General Virology* **84**:1-15.
67. Feyzi, E., E. Trybala, T. Bergstrom, U. Lindahl, and D. Spillmann. 1997. Structural requirement of heparan sulfate for interaction with herpes simplex virus type 1 virions and isolated glycoprotein C. *Journal of Biological Chemistry* **272**:24850-24857.
68. Foegh, M. L. 1990. Chronic rejection - Graft arteriosclerosis. *Transplant. Proceedings* **22**:119-122.
69. Friedman, H. M., L. Wang, N. O. Fishman, J. D. Lambris, R. J. Eisenberg, G. H. Cohen, and J. Lubinski. 1996. Immune evasion of herpes simplex virus type 1 glycoprotein gC. *Journal of Virology* **70**:4253-4260.
70. Friedman, H. M., L. Y. Wang, M. K. Pangburn, J. D. Lambris, and J. Lubinski. 2000. Novel mechanism of antibody-independent complement neutralization of herpes simplex virus type 1. *Journal of Immunology* **165**:4528-4536.
71. Friedman, H. M., A. Yee, G. H. Cohen, R. J. Eisenberg, C. Seidel, and D. B. Cines. 1984. Glycoprotein C of herpes simplex virus type 1 acts as a receptor for the C3b complement component on infected cells. *Nature* **309**:633-635.
72. Fung, L. S., G. Neil, J. Leibowitz, E. H. Cole, S. Chung, A. Crow, and G. A. Levy. 1991. Monoclonal antibody analysis of a unique macrophage procoagulant activity induced by murine hepatitis virus strain 3 infection. *Journal of Biological Chemistry* **266**:1789-1795.
73. Gerber, S. I., B. J. Belval, and B. C. Herold. 1995. Differences in the Role of Glycoprotein-C of HSV-1 and HSV-2 in Viral Binding May Contribute to Serotype Differences in Cell Tropism. *Virology* **214**:29-39.
74. Giesen, P. L. A., U. Rauch, B. Bohrmann, D. Kling, M. Roque, J. T. Fallon, J. J. Badimon, J. Himber, M. A. Riederer, and Y. Nemerson. 1999. Blood-borne tissue factor: Another view of thrombosis. *Proceedings of the National Academy of Science USA* **96**:2311-2315.
75. Guinto, E. R. and C. T. Esmon. 1982. Formation of a calcium-binding site on bovine activated factor V following recombination of the isolated subunits. *Journal of Biological Chemistry* **257**:10038-10043.
76. Gulizia, J. M., R. Kandolf, T. J. Kendall, S. L. Thiesen, J. E. Wilson, S. J. Radio, M. R. Costanzo, G. L. Winters, L. L. Miller, and B. M. McManus.

1995. Infrequency of cytomegalovirus genome in coronary arteriopathy of human heart allografts. *American Journal of Pathology* **147**:461-475.
77. **Gurwitz, D. and D. D. Cunningham.** 1990. Neurite outgrowth activity of protease nexin-1 on neuroblastoma cells requires thrombin inhibition. *Journal of Cell Physiology* **142**:155-162.
78. **Gyorkey, F., J. L. Melnick, G. A. Guinn, P. Gyorkey, and M. E. DeBakey.** 1984. Herpesviridae in the endothelial and smooth muscle cells of the proximal aorta in arteriosclerotic patients. *Experimental Molecular Pathology* **40**:328-339.
79. **Haarr, L. and S. Skulstad.** 1994. The herpes simplex virus type-1 particle - Structure and molecular functions. *APMIS* **102**:321-346.
80. **Hajjar, D. P.** 1991. Viral pathogenesis of atherosclerosis: Impact of molecular mimicry and viral genes. *American Journal of Pathology* **139**:1195-1211.
81. **Hallam, T. J., J. D. Pearson, and L. A. Needham.** 1988. Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. *Biochemical Journal* **251**:243-249.
82. **Handler, C. G., G. H. Cohen, and R. J. Eisenberg.** 1996. Cross-linking of glycoprotein oligomers during herpes simplex virus type 1 entry. *Journal of Virology* **70**:6076-6082.
83. **Handler, C. G., R. J. Eisenberg, and G. H. Cohen.** 1996. Oligomeric structure of glycoproteins in herpes simplex virus 1. *Journal of Virology* **70**:6067-6075.
84. **Harris, S. L., I. Frank, G. H. Yee, H. Cohen, R. J. Eisenberg, and H. M. Friedman.** 1990. Glycoprotein gC of herpes simplex virus type 1 prevents complement-mediated cell lysis and virus neutralization. *Journal of Infectious Disease* **162**:331-337.
85. **Haver, V. M. and D. H. Namm.** 1984. Characterization of the thrombin-induced contraction of vascular smooth muscle. *Blood Vessels* **21**:53-63.
86. **Hendrix, M. G., M. Daemen, and C. A. Bruggeman.** 1991. Cytomegalovirus nucleic acid distribution within the human vascular tree. *American Journal of Pathology* **138**:563-567.
87. **Hendrix, M. G., M. M. Salimans, C. P. van Boven, and C. A. Bruggeman.** 1990. High prevalence of latently present cytomegalovirus in arterial walls of patients suffering from grade III atherosclerosis. *American Journal of*

88. Hendrix, M. R. G., P. H. J. Dormans, P. Kitslaar, F. Bosman, and C. A. Bruggeman. 1989. The presence of cytomegalovirus nucleic acids in arterial walls of atherosclerotic and nonatherosclerotic patients. *American Journal of Pathology* 134:1151.
89. Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulfate and glycoprotein B. *Journal of General Virology* 75:1211-1222.
90. Herold, B. C., D. Wudunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *Journal of Virology* 65:1090-1098.
91. Higashi, S., H. Nishimura, K. Aita, and S. Iwanaga. 1994. Identification of regions of bovine factor VII essential for binding to tissue factor. *Journal of Biological Chemistry* 269:18891-18898.
92. Hockin, M. F., K. C. Jones, S. J. Everse, and K. G. Mann. 2002. A model for the stoichiometric regulation of blood coagulation. *Journal of Biological Chemistry* 277:18322-18333.
93. Hollenberg, M. D. and S. J. Compton. 2002. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacological Reviews* 54:203-217.
94. Hruban, R. H., W. E. Beschoner, W. A. Baumgartner, S. M. Augustine, H. Ren, B. A. Reitz, and G. M. Hutchins. 1990. Accelerated atherosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endotheliitis. *American Journal of Pathology* 137:871.
95. Hruban, R. H., T.-C. Wu, W. E. Beschoner, D. E. Cameron, R. F. Ambinder, W. A. Baumgartner, B. A. Reitz, and G. M. Hutchins. 1990. Cytomegalovirus nucleic acids in allografted hearts. *Human Pathology* 21:981-983.
96. Hung, S. L., C. Peng, I. Kostavasili, H. M. Friedman, J. D. Lambris, R. J. Eisenberg, and G. H. Cohen. 1994. The interaction of glycoprotein-C of herpes simplex virus type-1 and type-2 with the alternative complement pathway. *Virology* 203:299-312.
97. Hung, S.-L., S. Srinivasan, H. M. Friedman, R. J. Eisenberg, and G. H. Cohen. 1992. Structural basis of C3b binding by glycoprotein C of herpes

simplex virus. *Journal of Virology* **66**:4013-4027.

98. **Husten, E. J., C. T. Esmon, and A. E. Johnson.** 1987. The active site of blood coagulation factor Xa. Its distance from the phospholipid surface and its conformational sensitivity to components of the prothrombinase complex. *Journal of Biological Chemistry* **262**:12953-12961.
99. **Irmiere, A. and W. Gibson.** 1983. Isolation and characterization of a noninfectious virion-like particle released from cells infected with human strains of cytomegalovirus. *Virology* **130**:118-133.
100. **Jacobson, M. A. and J. Mills.** 1988. Serious cytomegalovirus disease in the acquired immunodeficiency syndrome (AIDS): Clinical findings, diagnosis, and treatment. *Annals of Internal Medicine* **108**:585-594.
101. **Jarvis, M. A. and J. A. Nelson.** 2002. Human cytomegalovirus persistence and latency in endothelial cells and macrophages. *Current Opinion in Microbiology* **5**:403-407.
102. **Jordan, R., L. Schang, and P. A. Schaffer.** 1999. Transactivation of herpes simplex virus type 1 immediate-early gene expression by virion-associated factors is blocked by an inhibitor of cyclin-dependent protein kinases. *Journal of Virology* **73**:8843-8847.
103. **Joseph, S. and J. MacDermot.** 1991. Thrombin signalling in U937 human monocytic cells is coupled to inositol phosphate formation but not to thromboxane B2 synthesis nor to inhibition of adenylate cyclase: distinct differences in thrombin signalling between U937 cells and platelets. *European Journal of Pharmacology* **208**:149-156.
104. **Joseph, S. and J. MacDermot.** 1993. The N-terminal thrombin receptor fragment SFLLRN, but not catalytically inactive thrombin-derived agonists, activate U937 human monocytic cells: evidence for receptor hydrolysis in thrombin-dependent signalling. *Biochemical Journal* **290**:571-577.
105. **Kalafatis, M. and D. O. Beck.** 2002. Identification of a binding site for blood coagulation factor Xa on the heavy chain of factor Va. Amino acid residues 323-331 of factor V represent an interactive site for activated factor X. *Biochemistry* **41**:12715-12728.
106. **Kaner, R. J., R. V. Iozzo, and N. A. Kefalides.** 1987. *Clinical Research* **35**:289A.
107. **Kari, B. and R. Gehrz.** 1992. A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope.

Journal of Virology **66**:1761-1764.

108. **Keay, S. and B. Baldwin.** 1991. Anti-idiotypic antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. *Journal of Virology* **65**:5124-5128.
109. **Keay, S. and B. R. Baldwin.** 1996. Evidence of the role of cell protein phosphorylation in human cytomegalovirus/host cell fusion. *Journal of General Virology* **77**:2597-2604.
110. **Key, N. S., R. R. Bach, G. M. Vercellotti, and C. F. Moldow.** 1993. Herpes simplex virus type 1 does not require productive infection to induce tissue factor in human umbilical vein endothelial cells. *Laboratory Investigation* **68**:645-651.
111. **Klemola, E., R. von Essen, G. Henle, and W. Henle.** 1970. Infectious mononucleosis-like disease with negative heterophil agglutination test. Clinical features in relation Epstein-Barr virus and cytomegalovirus. *Journal of Infectious Disease* **121**:608-614.
112. **Koo, B. H., K. H. Chung, K. C. Hwang, and D. S. Kim.** 2002. Factor Xa induces mitogenesis of coronary artery smooth muscle cell via activation of PAR-2. *FEBS Letters* **523**:85-89.
113. **Kostavasili, I., A. Sahu, H. M. Friedman, R. J. Eisenberg, G. H. Cohen, and J. D. Lambris.** 1997. Mechanism of complement inactivation by glycoprotein C of herpes simplex virus. *Journal of Immunology* **158**:1763-1771.
114. **Kowalik, T. F., B. Wing, J. S. Haskill, J. C. Azizkhan, A. S. Baldwin, and E. S. Huang.** 1993. Multiple mechanisms are implicated in the regulation of NF-kappaB activity during human cytomegalovirus infection. *Proceedings of the National Academy of Science USA* **90**:1107-1111.
115. **Krishnaswamy, S. and A. Betz.** 1997. Exosites determine macromolecular substrate recognition by prothrombinase. *Biochemistry* **36**:12080-12086.
116. **Krishnaswamy, S., K. A. Field, T. S. Edgington, J. H. Morrissey, and K. G. Mann.** 1992. Role of the membrane surface in the activation of human coagulation factor X. *Journal of Biological Chemistry* **267**:26110-26120.
117. **Krishnaswamy, S., K. A. Field, J. H. Morrissey, T. S. Edgington, and K. G. Mann.** 1991. Activation of factor X by the extrinsic pathway. *Thrombosis and Haemostasis* **65**:296.
118. **Krishnaswamy, S., K. C. Jones, and K. G. Mann.** 1988. Prothrombinase complex assembly. Kinetic mechanism of enzyme assembly on phospholipid

- vesicles. *Journal of Biological Chemistry* **263**:3823-3834.
119. **Krishnaswamy, S., M. E. Nesheim, E. L. G. Prydzial, and K. G. Mann.** 1994. Assembly of the prothrombinase complex. *Methods in Enzymology* **222**:260-280.
 120. **Krishnaswamy, S., G. D. Russell, and K. G. Mann.** 1989. The reassociation of factor Va from its isolated subunits. *Journal of Biological Chemistry* **264**:3160-3168.
 121. **Krummenacher, C., I. Baribaud, J. F. Sanzo, G. H. Cohen, and R. J. Eisenberg.** 2002. Effects of herpes simplex virus on structure and function of nectin-1/HveC. *Journal of Virology* **76**:2424-2433.
 122. **Laquerre, S., R. Argnani, D. B. Anderson, S. Zucchini, R. Manservigi, and J. C. Glorioso.** 1998. Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. *Journal of Virology* **72**:6119-6130.
 123. **Le, D. T., S. I. Rapaport, and L. V. M. Rao.** 1992. Relations between factor VIIa binding and expression of factor VIIa/tissue factor catalytic activity on cell surfaces. *Journal of Biological Chemistry* **267**:15447-15454.
 124. **Leong, L., R. A. Henriksen, J. C. Kermode, S. E. Rittenhouse, and P. B. Tracy.** 1992. The thrombin high-affinity binding site on platelets is a negative regulator of thrombin-induced platelet activation. Structure-function studies using two mutant thrombins, Quick I and Quick II. *Biochemistry* **31**:2567-2576.
 125. **Lever, W. F., Orfanos, C. E. and G. Schaumberg-Lever.** 1974. Dermal and epidermal types of erythema multiforme: A histopathological study of 24 cases. *Archives of Dermatology* **109**:682-8
 126. **Ligas, M. W. and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate cells. *Journal of Virology* **62**:1486-1494.
 127. **Lim, T. K., V. A. Bloomfield, and G. L. Nelsestuen.** 1977. Structure of prothrombin- and blood clotting factor X-membrane complexes. *Biochemistry* **16**:4177-4181.
 128. **Lollar, P., G. J. Knutson, and D. N. Fass.** 1985. Activation of porcine factor VIII:C by thrombin and factor Xa. *Biochemistry* **24**:8056-8064.

129. Lubinski, J., L. Wang, D. Mastellos, A. Sahu, J. D. Lambris, and H. M. Friedman. 1999. In vivo role of complement-interacting domains of herpes simplex virus type 1 glycoprotein C. *Journal of Experimental Medicine* **190**:1637-1646.
130. Lubinski, J. M., M. Jiang, L. Hook, Y. Chang, C. Sarver, D. Mastellos, J. D. Lambris, G. H. Cohen, R. J. Eisenberg, and H. M. Friedman. 2002. Herpes simplex virus type 1 evades the effects of antibody and complement in vivo. *Journal of Virology* **76**:9232-9241.
131. Lubinski, J. M., L. Wang, A. M. Soulika, R. Burger, R. A. Wetsel, H. Colten, G. H. Cohen, R. J. Eisenberg, J. D. Lambris, and H. M. Friedman. 1998. Herpes simplex virus type 1 glycoprotein gC mediates immune evasion in vivo. *Journal of Virology* **72**:8257-8263.
132. Luciano, R. L. and A. C. Wilson. 2002. An activation domain in the C-terminal subunit of HCF-1 is important for transactivation by VP16 and LZIP. *Proceedings of the National Academy of Science USA* **99**:13403-13408.
133. Mann, K. G., S. Butenas, and K. Brummel. 2003. The dynamics of thrombin formation. *Arteriosclerosis Thrombosis and Vascular Biology* **23**:17-25.
134. Mann, K. G. and M. R. Downing. 1977. Thrombin generation. pp. 11-21. In: Lundblad, R.L., et. al., ed. *Chemistry. and biology. of thrombin*. Ann. Arbor., Ann. Arbor. Science,.
135. Mann, K. G. and M. Kalafatis. 2003. Factor V: a combination of Dr Jekyll and Mr Hyde. *Blood* **101**:20-30.
136. Mann, K. G., B. H. Odegaard, S. Krishnaswamy, P. B. Tracy, and M. E. Nesheim. 1987. *Proteases in biological control and biotechnology*. Alan R. Liss, Inc..
137. Martin, D. M., C. W. Boys, and W. Ruf. 1995. Tissue factor: Molecular recognition and cofactor function. *FASEB J.* **9**:852-859.
138. Mastromarino, P., L. Cioe, S. Rieti, and N. Orsi. 1990. Role of membrane phospholipids and glycolipids in the Vero cell surface receptor for rubella virus. *Medical Microbiology and Immunology* **179**:105-114.
139. Masuda, M., S. Nakamura, T. Murakami, Y. Komiyama, and H. Takahashi. 1996. Association of tissue factor with a gamma chain homodimer of the IgE receptor type I in cultured human monocytes. *European Journal of Immunology* **26**:2529-2532.
140. McEver, R. P. 1991. GMP-140: a receptor for neutrophils and monocytes on

- activated platelets and endothelium. *Journal of Cell Biochemistry* **45**:156-161.
141. **Mcgee, M. P. and L. C. Li.** 1991. Functional difference between intrinsic and extrinsic coagulation pathways - Kinetics of factor X activation on human monocytes and alveolar macrophages. *Journal of Biological Chemistry* **266**:8079-8085.
 142. **McMillan, T. N. and D. C. Johnson.** 2001. Cytoplasmic domain of herpes simplex virus gE causes accumulation in the trans-Golgi network, a site of virus envelopment and sorting of virions to cell junctions. *Journal of Virology* **75**:1928-1940.
 143. **McNamara, C. A., I. J. Sarembock, L. W. Gimble, J. W. Fenton, S. R. Coughlin, and G. K. Owens.** 1993. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. *Journal of Clinical Investigations* **91**:94-98.
 144. **McSorley, J., L. Shapiro, M. H. Brownstein, and K. C. Hsu.** 1974. Herpes simplex and varicella-zoster: comparative histopathology of 77 cases. *International Journal of Dermatology* **13**:69-75.
 145. **Melnick, J., E. Adam, and M. E. DeBakey.** 1990. Possible role of cytomegalovirus in atherogenesis. *JAMA* **263**:2204-2207.
 146. **Melnick, J. L., B. L. Petrie, G. R. Dreesman, J. Burek, C. H. McCollum, and M. E. DeBakey.** 1983. Cytomegalovirus antigen within human arterial smooth muscle cells. *Lancet* **2**:644-647.
 147. **Melnick, J. L. and A. Schattner.** 1992. Viruses and Atherosclerosis. *Israel Journal of Medical Science* **28**:463-465.
 148. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. *Journal of Virology* **76**:1537-1547.
 149. **Miletich, J. P., C. M. Jackson, and P. W. Majerus.** 1978. Properties of the factor Xa binding site on human platelets. *Journal of Biological Chemistry* **253**:6908-6916.
 150. **Miranda-Saksena, M., P. Armati, R. A. Boadle, D. J. Holland, and A. L. Cunningham.** 2000. Anterograde transport of herpes simplex virus type 1 in cultured, dissociated human and rat dorsal root ganglion neurons. *Journal of Virology* **74**:1827-1839.
 151. **Mocarski, E. S.** 1993. Cytomegalovirus biology and replication, p. 173-226. *In* B. Roizman, R. J. Whitley, and C. Lopez (eds.), *The human herpesviruses*.

Raven Press, New York.

152. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**:427-436.
153. **Moons, A. H. M., M. Levi, and R. J. G. Peters.** 2002. Tissue factor and coronary artery disease. *Cardiovascular Research* **53**:313-325.
154. **Morgan, D. M., J. D. Pearson, and J. L. Gordon.** 1989. Lysosomal hydrolases of human vascular cells: response to agonists of endothelial function. *Biochimica Biophysica Acta* **1010**:184-190.
155. **Morrison, E. E., Y. F. Wang, and D. M. Meredith.** 1998. Phosphorylation of structural components promotes dissociation of the herpes simplex virus type 1 tegument. *Journal of Virology* **72**:7108-7114.
156. **Mossman, K. L. and J. R. Smiley.** 1999. Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. *Journal of Virology* **73**:9726-9733.
157. **Mueller, B. M. and W. Ruf.** 1998. Requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. *Journal of Clinical Investigation* **101**:1372-1378.
158. **Muggeridge, M. I., T. T. Wu, D. C. Johnson, J. C. Glorioso, R. J. Eisenberg, and G. H. Cohen.** 1990. Antigenic and functional analysis of a neutralization site of HSV-1 glycoprotein D. *Virology* **174**:375-387.
159. **Murphy, S., R. A. Kahn, S. Holmes, G. L. Phillips, W. Sherwood, W. Davisson, and D. H. Buccholz.** 1982. Improved storage of platelets for transfusion in a new container. *Blood* **60**:194-200.
160. **Nagashunmugam, T., J. Lubinski, L. Y. Wang, L. T. Goldstein, B. S. Weeks, P. Sundaresan, E. H. Kang, G. Dubin, and H. M. Friedman.** 1998. In vivo immune evasion mediated by the herpes simplex virus type 1 immunoglobulin G Fc receptor. *Journal of Virology* **72**:5351-5359.
161. **Nelsestuen, G. L.** 1976. Role of gamma-carboxyglutamic acid. An unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid. *Journal of Biological Chemistry* **251**:5648-5656.
162. **Nemerson, Y.** 1988. Tissue factor and hemostasis. *Blood* **71**:1-8.
163. **Nesheim, M. E., C. Kettner, E. Shaw, and K. G. Mann.** 1981. Cofactor

dependence of factor Xa incorporation into the prothrombinase complex. *Journal of Biological Chemistry* **256**:6537-6540.

164. **Nesheim, M. E., J. B. Taswell, and K. G. Mann.** 1979. The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *Journal of Biological Chemistry* **254**:10952-10962.
165. **Nesheim, M. E., R. P. Tracy, P. B. Tracy, D. S. Boskovic, and K. G. Mann.** 1992. Mathematical simulation of prothrombinase. *Methods in Enzymology* **215**:316-328.
166. **Neuenschwander, P. F., M. M. Fiore, and J. H. Morrissey.** 1993. Factor VII autoactivation proceeds via interaction of distinct protease cofactor and zymogen cofactor complexes - Implications of a 2-dimensional enzyme kinetic mechanism. *Journal of Biological Chemistry* **268**:21489-21492.
167. **Neuenschwander, P. F. and J. H. Morrissey.** 1993. Analysis of tissue factor-induced alterations in factor VIIa enzymatic activity. *Thrombosis and Haemostasis* **69**:612.
168. **Nieto, F. J., E. Adam, P. Sorlie, H. Farzadegan, J. L. Melnick, G. W. Comstock, and M. Szklo.** 1996. Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical restenosis. *Circulation* **94**:922-927.
169. **Nowlin, D. M., N. R. Cooper, and T. Compton.** 1991. Expression of a human cytomegalovirus receptor correlates with infectibility of cells. *Journal of Virology* **65**:3114-3121.
170. **Ollivier, V., S. Bentolila, J. Chabbat, J. Hakim, and D. de Prost.** 1998. Tissue factor-dependent vascular endothelial growth factor production by human fibroblasts in response to activated factor VII. *Blood* **91**:2698-2703.
171. **Orning, L., B. E. Arbo, P. M. Fischer, and K. S. Sakariassen.** 1998. A peptide sequence from mouse tissue factor inhibits human tissue factor dependent factor X activation. *Thrombosis Research* **92**:135-140.
172. **Packham, M. A. and J. F. Mustard.** 1984. *Blood Platelet Function and Medical Chemistry.* Elsevier Biomedical, New York.
173. **Payne, M. A., P. F. Neuenschwander, A. E. Johnson, and J. H. Morrissey.** 1996. Effect of soluble tissue factor on the kinetic mechanism of factor VIIa: Enhancement of p-guanidinobenzoate substrate hydrolysis. *Biochemistry* **35**:7100-7106.
174. **Pearson, J. D., J. S. Carleton, and A. Hutchings.** 1983. Prostacyclin release

stimulated by thrombin or bradykinin in porcine endothelial cells cultured from aorta and umbilical vein. *Thrombosis Research* **29**:115-124.

175. **Peppelenbosch, M. P. and H. H. Versteeg.** 2001. Cell biology of tissue factor, an unusual member of the cytokine receptor family. *Trends in Cardiovascular Medicine* **11**:335-339.
176. **Perera, L., T. A. Darden, and L. G. Pedersen.** 2002. Predicted solution structure of zymogen human coagulation FVII. *Journal of Computational Chemistry* **23**:35-47.
177. **Peterson, E. A., M. R. Sutherland, M. E. Nesheim, and E. L. G. Pryzdial.** 2002. Thrombin induces cell surface exposure of the plasminogen receptor, annexin 2: A novel link between coagulation and fibrinolysis (in press). *J. Cell Science*.
178. **Petrovan, R. J. and W. Ruf.** 2000. Role of specific residue side chains in reversing the zymogen-like conformation of coagulation factor VIIa. *Blood* **96**:257A.
179. **Petrovan, R. J. and W. Ruf.** 2002. Role of zymogenicity-determining residues of coagulation factor VII/VIIa in cofactor interaction and macromolecular substrate recognition. *Biochemistry* **41**:9302-9309.
180. **Phinney, P. R., S. Fligiel, Y. J. Bryson, and D. D. Porter.** 1982. Necrotizing vasculitis in a case of disseminated neonatal herpes simplex infection. *Archives of Pathology and Laboratory Medicine* **106**:64-67.
181. **Pike, A. C. W., A. M. Brzozowski, S. M. Roberts, O. H. Olsen, and E. Persson.** 1999. Structure of human factor VIIa and its implications for the triggering of blood coagulation. *Proceedings of the National Academy of Science USA* **96**:8925-8930.
182. **Pryzdial, E. L. and K. G. Mann.** 1991. The association of coagulation factor Xa and factor Va. *Journal of Biological Chemistry* **266**:8969-8977.
183. **Pryzdial, E. L. G. and J. F. Wright.** 1994. Prothrombinase assembly on an enveloped virus: evidence that the cytomegalovirus surface contains procoagulant phospholipid. *Blood* **84**:3749-3757.
184. **Ramars, A. S. S., S. Mukhopadhyay, and D. Dash.** 2002. Regulation of postaggregation events induced by protease-activated receptor 1 ligation in human platelets: Evidence of differential signaling pathways. *Archives of Biochemistry and Biophysics* **398**:253-260.
185. **Raynor, C. M., J. F. Wright, D. M. Waisman, and E. L. G. Pryzdial.** 1999.

Annexin II enhances cytomegalovirus binding and fusion to phospholipid membranes. *Biochemistry* **38**:5095.

186. **Resnick-Roguel, N., A. Eldor, H. Burstein, E. Hy-Am, I. Vlodavsky, A. Panet, M. A. Blajchman, and M. Kotler.** 1990. The envelope glycoprotein of avian hemangioma retrovirus induces a thrombogenic surface on human and bovine endothelial cells. *Journal of Virology* **64**:4029-4032.
187. **Riewald, M., V. V. Kravchenko, R. J. Petrovan, P. J. O'Brien, L. F. Brass, R. J. Ulevitch, and W. Ruf.** 2001. Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. *Blood* **97**:3109-3116.
188. **Riewald, M. and W. Ruf.** 2001. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proceedings of the National Academy of Science USA* **98**:7742-7747.
189. **Rodger, G., J. Boname, S. Bell, and T. Minson.** 2001. Assembly and organization of glycoproteins B, C, D, and H in herpes simplex virus type 1 particles lacking individual glycoproteins: No evidence for the formation of a complex of these molecules. *Journal of Virology* **75**:710-716.
190. **Roizman, B. and A. E. Sears.** 1993. Herpes simplex viruses and their replication, p. 11-68. *In* B. Roizman, R. J. Whitley, and C. Lopez (eds.), *The human herpesviruses*. Raven Press, New York.
191. **Roop, C., L. Hutchinson, and D. C. Johnson.** 1993. A mutant herpes simplex virus type-1 unable to express glycoprotein-L cannot enter cells, and its particles lack glycoprotein H. *Journal of Virology* **67**:2285-2297.
192. **Rottingen, J. A., T. Enden, E. Camerer, J. G. Iversen, and H. Prydz.** 1995. Binding of human factor VIIa to tissue factor induces cytosolic Ca²⁺ signals in J82 cells, transfected Cos-1 cells, Madin-Darby canine kidney-cells and in human endothelial cells induced to synthesize tissue factor. *Journal of Biological Chemistry* **270**:4650-4660.
193. **Ruf, W. and T. S. Edgington.** 1991. An anti-tissue factor monoclonal antibody which inhibits the TF-VIIa complex is a potent anticoagulant in plasma. *Thrombosis and Haemostasis* **66**:529-533.
194. **Ruf, W. and T. S. Edgington.** 1994. Structural biology of tissue factor, the initiator of thrombogenesis *In Vivo*. *Faseb Journal* **8**:385-390.
195. **Ruf, W., A. Rehemtulla, and T. S. Edgington.** 1991. Phospholipid-independent and -dependent interactions required for tissue factor receptor

and cofactor function. *Journal of Biological Chemistry* **266**:2158-2166.

196. **Rux, A. H., H. Lou, J. D. Lambris, H. M. Friedman, R. J. Eisenberg, and G. H. Cohen.** 2002. Kinetic analysis of glycoprotein C of herpes simplex virus types 1 and 2 binding to heparin, heparan sulfate, and complement component C3b. *Virology* **294**:324-332.
197. **Rux, A. H., W. T. Moore, J. D. Lambris, W. R. Abrams, C. Peng, H. M. Friedman, G. H. Cohen, and R. J. Eisenberg.** 1996. Disulfide bond structure determination and biochemical analysis of glycoprotein C from herpes simplex virus. *Journal of Virology* **70**:5455-5465.
198. **Sahu, A. and J. D. Lambris.** 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. *Immunological Reviews* **180**:35-48.
199. **Saldanha, C. E., J. Lubinski, C. Martin, T. Nagashunmugam, L. Y. Wang, H. Van Der Keyl, R. Tal-Singer, and H. M. Friedman.** 2000. Herpes simplex virus type 1 glycoprotein E domains involved in virus spread and disease. *Journal of Virology* **74**:6712-6719.
200. **Sarmiento, M., M. L. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses: role of glycoprotein VP7(B2). *Journal of Virology* **29**:1149-1158.
201. **Schechter, A. D., P. L. A. Giesen, O. Taby, C. L. Rosenfield, M. Rossikhina, B. S. Fyfe, D. S. Kohtz, J. T. Fallon, Y. Nemerson, and M. B. Taubman.** 1997. Tissue factor expression in human arterial smooth muscle cells - TF is present in three cellular pools after growth factor stimulation. *Journal of Clinical Investigation* **100**:2276-2285.
202. **Sevinsky, J. R., L. V. M. Rao, and W. Ruf.** 1996. Ligand-induced protease receptor translocation into caveolae: A mechanism for regulating cell surface proteolysis of the tissue factor-dependent coagulation pathway. *Journal of Cell Biology* **133**:293-304.
203. **Shankar, R., C. A. de la Motte, and P. E. Diczorleto.** 1992. Thrombin stimulates PDGF production and monocyte adhesion through distinct intracellular pathways in human endothelial cells. *American Journal of Physiology* **262**:C199-C206.
204. **Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. M. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear.** 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**:13-22.

205. **Shukla, D. and P. G. Spear.** 2001. Herpesviruses and heparan sulfate: an intimate relationship in aid of viral entry. *Journal of Clinical Investigation* **108**:503-510.
206. **Sichler, K., D. W. Banner, A. D'Arcy, K. P. Hopfner, R. Huber, W. Bode, G. B. Kresse, E. Kopetzki, and H. Brandstetter.** 2002. Crystal structures of uninhibited factor VIIa link its cofactor and substrate-assisted activation to specific interactions. *Journal of Molecular Biology* **322**:591-603.
207. **Siscovick, D. S., S. M. Schwartz, L. Corey, J. T. Grayston, R. Ashley, S. P. Wang, B. M. Patsy, R. P. Tracy, L. H. Kuller, and R. A. Kronmal.** 2000. Chlamydia pneumoniae, herpes simplex virus type 1, and cytomegalovirus and incident myocardial infarction and coronary heart disease death in older adults - The Cardiovascular Health Study. *Circulation* **102**:2335-2340.
208. **Sisk, W. P., J. D. Bradley, R. J. Leipold, A. M. Stoltzfus, M. P. Deleon, M. Hilf, C. Peng, G. H. Cohen, and R. J. Eisenberg.** 1994. High-Level Expression and Purification of Secreted Forms of Herpes-Simplex Virus Type-1 Glycoprotein Gd Synthesized by Baculovirus-Infected Insect Cells. *Journal of Virology* **68**:766-775.
209. **Sissons, J. G. P., M. Bain, M. R. Wills, and J. H. Sinclair.** 2002. Latency and reactivation of human cytomegalovirus. *Journal of Infection* **44**:73-77.
210. **Skepper, J. N., A. Whiteley, H. Browne, and A. Minson.** 2001. Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment -> deenvelopment -> reenvelopment pathway. *Journal of Virology* **75**:5697-5702.
211. **Slobbevandrinen, M. E. P., R. C. R. M. Vossen, F. M. D. Cuwenberg, M. M. Hulsbosch, J. W. M. Heemskerk, M. C. E. Vandammieras, and C. A. Bruggeman.** 1997. Activation of protein kinase C enhances the infection of endothelial cells by human cytomegalovirus. *Virus Res.* **48**:207-213.
212. **Smith, T. J., L. A. Morrison, and D. A. Leib.** 2002. Pathogenesis of herpes simplex virus type 2 virion host shutoff (vhs) mutants. *Journal of Virology* **76**:2054-2061.
213. **Sorensen, B. B., P. O. Freskgard, L. S. Nielsen, L. V. M. Rao, M. Ezban, and L. C. Petersen.** 1999. Factor VIIa-induced p44/42 mitogen-activated protein kinase activation requires the proteolytic activity of factor VIIa and is independent of the tissue factor cytoplasmic domain. *Journal of Biological Chemistry* **274**:21349-21354.
214. **Speir, E., R. Modali, E. S. Huang, M. B. Leon, F. Shawl, T. Finkel, and S. E. Epstein.** 1994. Potential role of human cytomegalovirus and p53 interaction

in coronary restenosis. *Science* **265**:391-394.

215. **Stenflo, J.** 1999. Contributions of Gla and EGF-like domains to the function of vitamin K dependent coagulation factors. *Critical Reviews in Eukaryotic Gene Expression* **9**:59-88.
216. **Sutherland, M. R., C. M. Raynor, H. Leenknecht, J. F. Wright, and E. L. G. Pryzdial.** 1997. Coagulation initiated on herpesviruses. *Proceedings of the National Academy of Science USA* **94**:13510-13514.
217. **Tal-Singer, R., C. Peng, M. Ponce de Leon, W. R. Abrams, B. W. Banfield, F. Tufaro, G. H. Cohen, and R. J. Eisenberg.** 1995. Interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *Journal of Virology* **69**:4471-4483.
218. **Taubman, M. B., J. T. Fallon, A. D. Schecter, P. Giesen, M. Mendlowitz, B. S. Fyfe, J. D. Marmur, and Y. Nemerson.** 1997. Tissue factor in the pathogenesis of atherosclerosis. *Thrombosis and Haemostasis* **78**:200-204.
219. **Thiec, F., G. Cherel, and O. D. Christophe.** 2003. Role of the GLa and first epidermal growth factor-like domains of factor X in the prothrombinase and tissue factor-factor VIIa complexes. *Journal of Biological Chemistry* **278**:10393-10399.
220. **Toso, R., M. Pinotti, K. A. High, E. S. Pollak, and F. Bernardi.** 2002. A frequent human coagulation Factor VII mutation (A294V, 052) in loop 140s affects the interaction with activators, tissue factor and substrates. *Biochemical Journal* **363**:411-416.
221. **Toti, F., N. Satta, E. Fressinaud, D. Meyer, and J.-M. Freyssinet.** 1996. Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder. *Blood* **87**:1409-1415.
222. **Tracy, P. B., L. L. Eide, and K. G. Mann.** 1985. Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. *Journal of Biological Chemistry* **260**:2119-2124.
223. **Tracy, P. B., M. E. Nesheim, and K. G. Mann.** 1981. Coordinate binding of factor Va and factor Xa to the unstimulated platelet. *Journal of Biological Chemistry* **256**:743-751.
224. **Tremoli, E., M. Camera, V. Toschi, and S. Colli.** 1999. Tissue factor in atherosclerosis. *Atherosclerosis* **144**:273-283.
225. **Trybala, E., T. Bergstrom, B. Svennerholm, S. Jeansson, J. C. Glorioso,**

- and S. Olofsson. 1994. Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulfate proteoglycan. *Journal of General Virology* **75**:743-752.
226. Trybala, E., J. A. Liljeqvist, B. Svennerholm, and T. Bergstrom. 2000. Herpes simplex virus types 1 and 2 differ in their interaction with heparan sulfate. *Journal of Virology* **74**:9106-9114.
227. Trybala, E., A. Roth, M. Johansson, J. A. Liljeqvist, E. Rekabdar, O. Larm, and T. Bergstrom. 2002. Glycosaminoglycan-binding ability is a feature of wild-type strains of herpes simplex virus type 1. *Virology* **302**:413-419.
228. van Dam-Mieras, M. C. E., C. A. Bruggeman, A. D. Muller, W. H. M. Debie, and R. F. A. Zwaal. 1987. Induction of endothelial cell procoagulant activity by cytomegalovirus infection. *Thrombosis Research* **47**:69-75.
229. van Dam-Mieras, M. C. E., A. D. Muller, V. W. M. van Hinsbergh, W. J. H. A. Mullers, P. H. H. Bomans, and C. A. Bruggeman. 1992. The procoagulant response of cytomegalovirus infected endothelial cells. *Thrombosis and Haemostasis* **68**:364-370.
230. van Leeuwen, H., G. Elliott, and P. O'Hare. 2002. Evidence of a role for nonmuscle myosin II in herpes simplex virus type 1 egress. *Journal of Virology* **76**:3471-3481.
231. Vangenderen, I. L., R. Brandimarti, M. R. Torrisi, G. Campadelli, and G. Vanmeer. 1994. The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. *Virology* **200**:831-836.
232. Vercellotti, G. M. 1990. Proinflammatory and procoagulant effects of herpes simplex infection on human endothelium. *Blood Cells* **16**:209-216.
233. Vercellotti, G. M., A. Kovacs, N. S. Key, L. A. Dandeleit, and H. S. Jacob. 1997. Human cytomegalovirus infection of endothelial cells induces tissue factor expression. *Thrombosis and Haemostasis Supplement*:203 (abs).
234. Versteeg, H. H., B. B. Sorensen, S. H. Slofstra, J. H. M. Van den Brande, J. C. Stam, P. M. P. V. Henegouwen, D. J. Richel, L. C. Petersen, and M. P. Peppelenbosch. 2002. VIIa/tissue factor interaction results in a tissue factor cytoplasmic domain-independent activation of protein synthesis, p70, and p90 S6 kinase phosphorylation. *Journal of Biological Chemistry* **277**:27065-27072.
235. Visser, M. R., P. B. Tracy, G. M. Vercellotti, J. L. Goodman, J. G. White, and H. S. Jacob. 1988. Enhanced thrombin generation and platelet binding on herpes simplex virus-infected endothelium. *Proceedings of the National*

Academy of Science USA **85**:8227-8230.

236. **Visser, M. R. and G. M. Vercellotti.** 1993. Herpes simplex virus and atherosclerosis. *European Heart Journal* **14** (Suppl K):39-42.
237. **Walker, R. K. and S. Krishnaswamy.** 1993. The influence of factor Va on the active site of factor Xa. *Journal of Biological Chemistry* **268**:13920-13929.
238. **Weitz, J. I. and M. Crowther.** 2002. Direct thrombin inhibitors. *Thrombosis Research* **106**:V275-V284.
239. **Whatmore, J. L. and D. Allan.** 1994. Phospholipid asymmetry in plasma membrane vesicles derived from BHK cells. *Biochimica et Biophysica Acta-Biomembranes* **1192**:88-94.
240. **Whitbeck, J. C., C. Peng, H. Lou, R. Xu, S. H. Willis, M. P. de Leon, T. Peng, A. V. Nicola, R. I. Montgomery, M. S. Warner, A. M. Soulika, L. A. Spruce, W. T. Moore, J. D. Lambris, P. G. Spear, G. H. Cohen, and R. J. Eisenberg.** 1997. Glycoprotein D of herpes simplex virus binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and mediator of HSV entry. *Journal of Virology* **71**:6083-6093.
241. **Wild, P., E. M. Schraner, J. Peter, E. Loepfe, and M. Engels.** 1998. Novel entry pathway of bovine herpesvirus 1 and 5. *Journal of Virology* **72**:9561-9566.
242. **Wilkins, M. and S. Krishnaswamy.** 2002. The contribution of factor Xa to exosite-dependent substrate recognition by prothrombinase. *Journal of Biological Chemistry* **277**:9366-9374.
243. **Willis, S. H., C. Peng, M. Ponce de Leon, A. V. Nicola, A. H. Rux, G. H. Cohen, and R. J. Eisenberg.** 1997. Expression and purification of secreted forms of HSV glycoproteins from baculovirus-infected insect cells, p. 131-155. *In* S. M. Brown and A. R. MacLean (eds.), *Methods in Molecular Medicine*. Humana Press Inc, Totowa.
244. **Willis, S. H., A. H. Rux, C. Peng, J. C. Whitbeck, A. V. Nicola, H. Lou, W. F. Hou, L. Salvador, R. J. Eisenberg, and G. H. Cohen.** 1998. Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpesvirus entry mediator, using surface plasmon resonance. *Journal of Virology* **72**:5937-5947.
245. **Worfolk, L. A., R. A. Robinson, and P. B. Tracy.** 1992. Factor Xa interacts with two sites on monocytes with different functional activities. *Blood* **80**:1989-1997.

246. **Wright, J. F., A. Kurosky, E. L. G. Pryzdial, and S. Wasi.** 1995. Host cellular protein annexin II is associated with cytomegalovirus particles isolated from cultured human fibroblasts. *Journal of Virology* **69**:4784-4791.
247. **Wright, J. F., A. Kurosky, and S. Wasi.** 1994. An endothelial cell surface form of annexin II binds human cytomegalovirus. *Biochem. Biophys. Research Communication* **198**:983-989.
248. **Wu, T. C., R. H. Hruban, R. F. Ambinder, M. Pizzorno, D. E. Cameron, W. A. Baumgartner, B. A. Reitz, G. S. Hayward, and G. M. Hutchins.** 1992. Demonstration of cytomegalovirus nucleic acids in the coronary arteries of transplanted hearts. *American Journal of Pathology* **140**:739-747.
249. **Xu, F. J., J. A. Schillinger, M. R. Sternberg, R. E. Johnson, F. K. Lee, A. J. Nahmias, and L. E. Markowitz.** 2002. Seroprevalence and coinfection with herpes simplex virus type 1 and type 2 in the United States, 1988-1994. *Journal of Infectious Diseases* **185**:1019-1024.
250. **Yurochko, A. D., L. Rasmussen, S. Keay, L. Pereira, and E. S. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate rapid activation of SP1 and NF-kappa-B during infection. *Journal of Virology* **71**:5051-5059.
251. **Zentner, J., T. Albrecht, and D. Heuser.** 1991. Propofol increases amplitudes of SEP [letter]. *Functional Neurology* **6**:411-412.
252. **Zhong, D. G., M. S. Bajaj, A. E. Schmidt, and S. P. Bajaj.** 2002. The N-terminal epidermal growth factor-like domain in factor IX and factor X represents an important recognition motif for binding to tissue factor. *Journal of Biological Chemistry* **277**:3622-3631.
253. **Zhou, Y. F., M. B. Leon, M. A. Wacławiw, J. J. Popma, Z. X. Yu, T. Finkel, and S. E. Epstein.** 1996. Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *New England Journal of Medicine* **335**:624-630.
254. **Zhou, Z. H., M. Dougherty, J. Jakana, J. He, F. J. Rixon, and W. Chiu.** 2000. Seeing the herpesvirus capsid at 8.5 angstrom. *Science* **288**:877-880.
255. **Zhou, Z. H., B. V. V. Prasad, J. Jakana, F. J. Rixon, and W. Chiu.** 1994. Protein subunit structures in the herpes simplex virus A Capsid determined from 400-Kv spot scan electron cryomicroscopy. *Journal of Molecular Biology* **242**:456-469.
256. **Zwaal, R. F. A., P. Comfurius, and E. M. Bevers.** 1993. Mechanism and function of changes in membrane-phospholipid asymmetry in platelets and

erythrocytes. Biochemical Society Translation **21:248-253.**