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(54) **FACTOR IXA: FACTOR VLLLA INTERACTION AND METHODS THEREFOR**

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(57) **ABSTRACT**

Novel agents that inhibit the interaction of factor VIIIa with factor IXa in newly discovered regions of interaction, Region 2 and Region 3, are disclosed. The novel polypeptides or derivatives of polypeptides prevent activation of factor X and have anti-coagulation activity. The agents include polypeptides or polypeptide derivatives that are homologous to factor VIIIa or factor IXa in Region 2 and/or Region 3, as well as agents that are not homologous, such as antibodies Region 2 or Region 3. Pharmaceutical compositions comprising the agents are also disclosed. Methods of treatment are also disclosed, comprising the step of determining whether the compound displaces the interaction of the above agent from factor VIII or factor IX. Methods for preventing coagulation in a blood sample are also disclosed. These methods comprise adding the above agent to the sample.

Figure 1

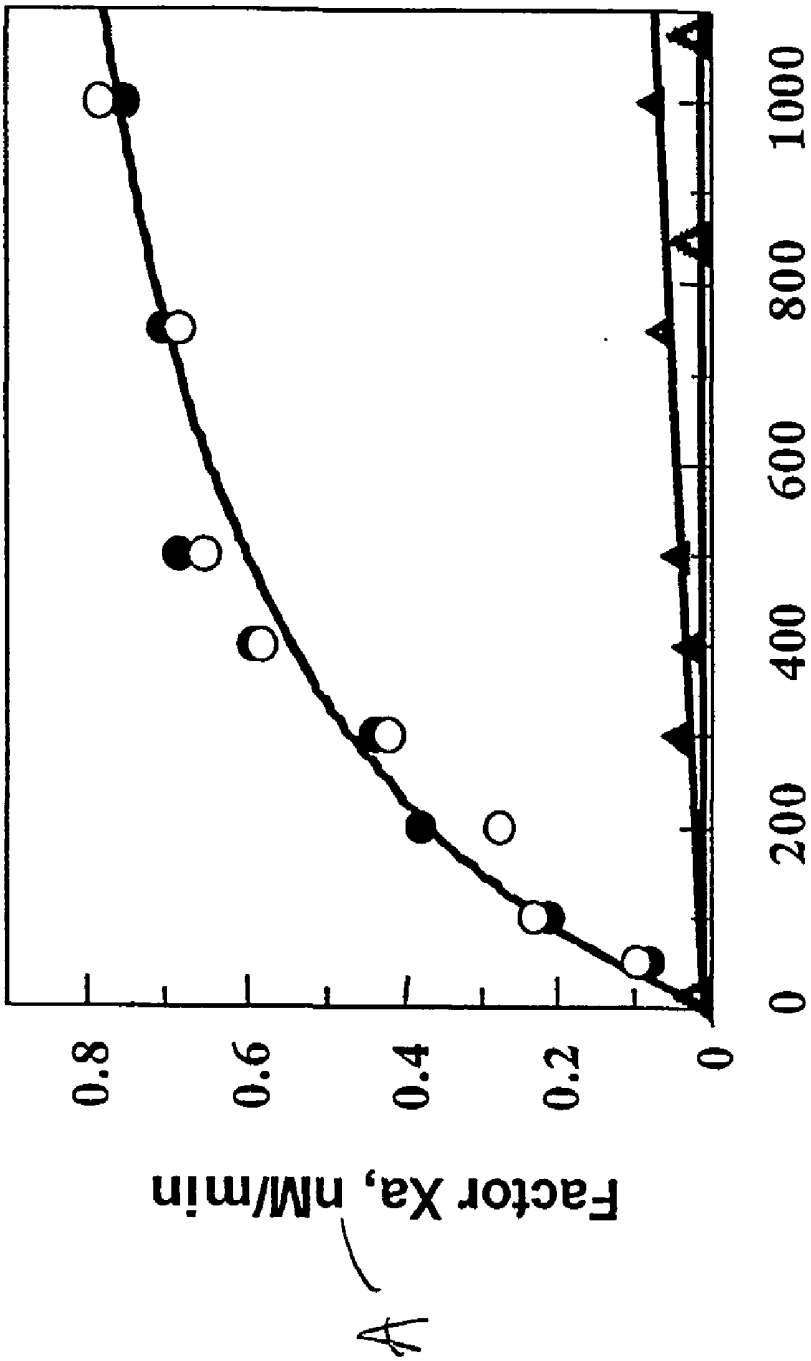
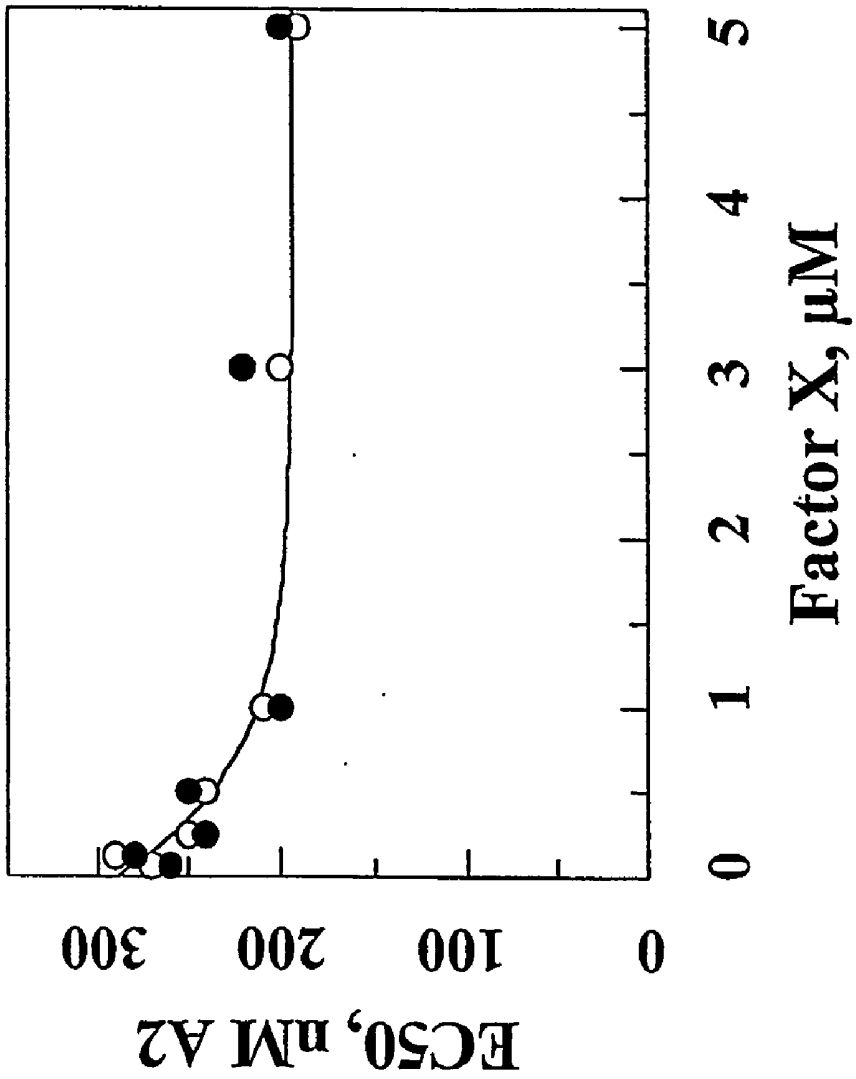

Factor Xa, nM/min
0
0.2
0.4
0.6
0.8
Factor VIIIa A2, nM
0
200
400
600
800
1000

Figure 2



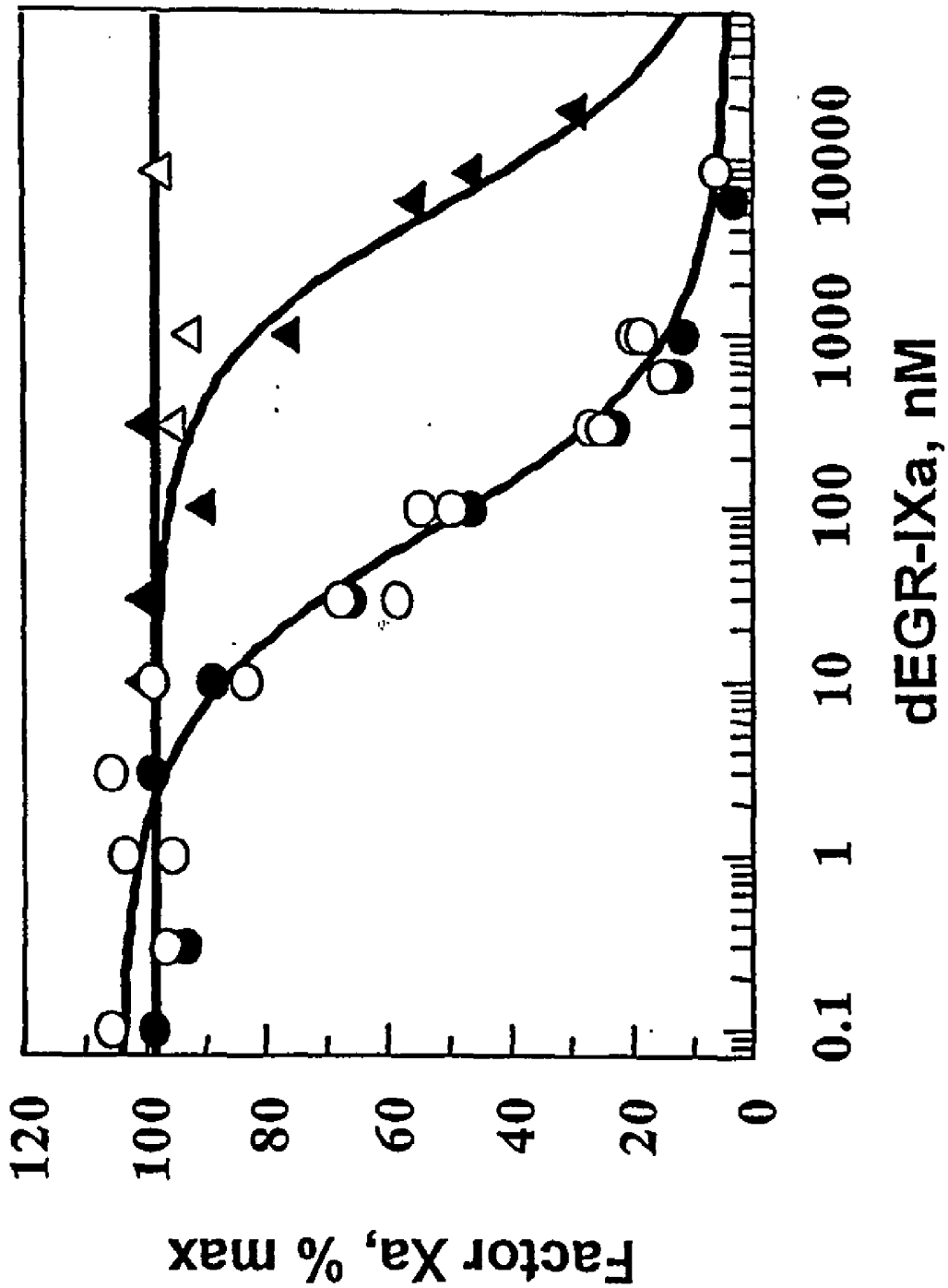

Factor Xa, % max
120
100
80
60
40
20
0
0.1
1
10
100
1000
10000
dEGR-IXa, nM

Figure 3

Figure 4

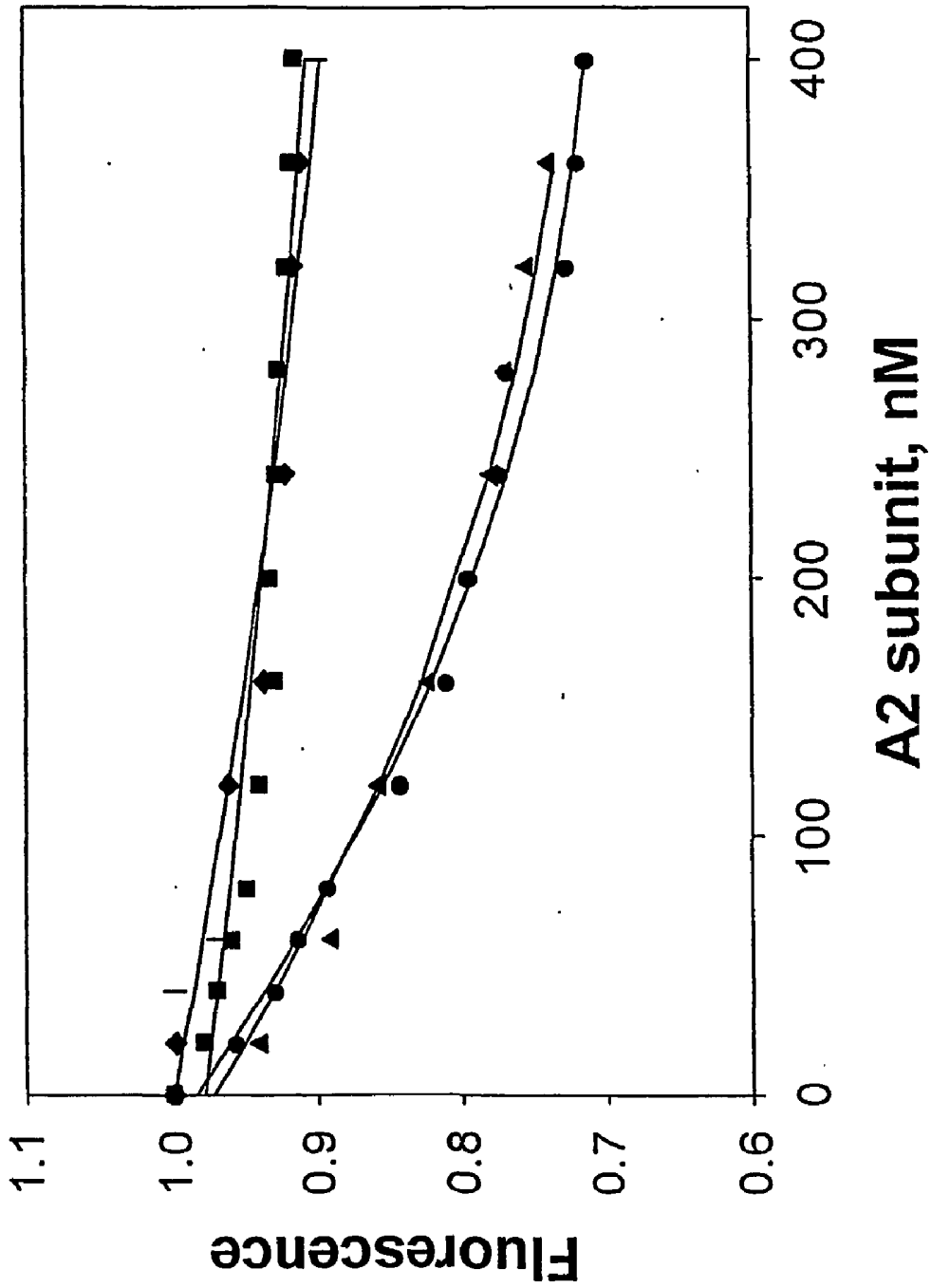


Figure 5

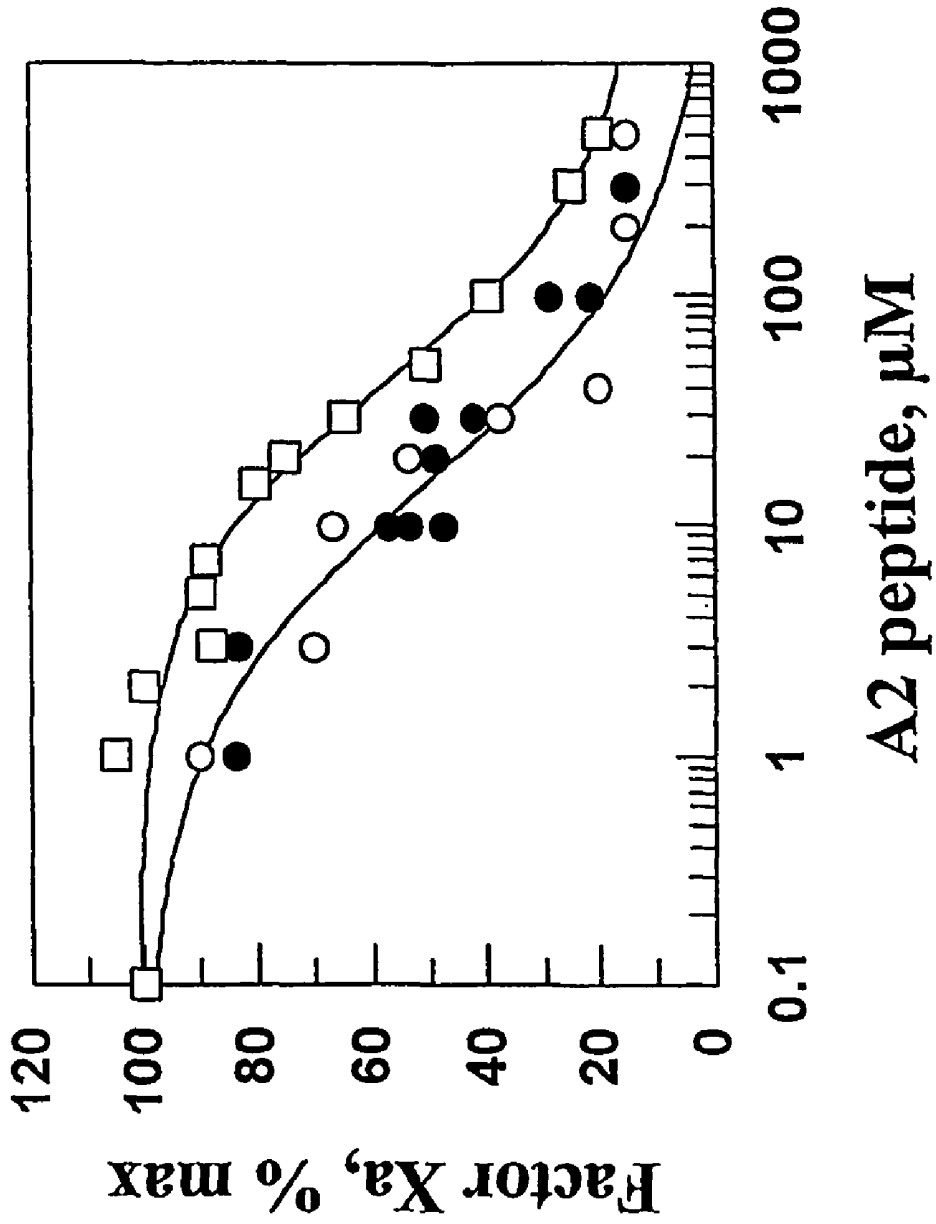
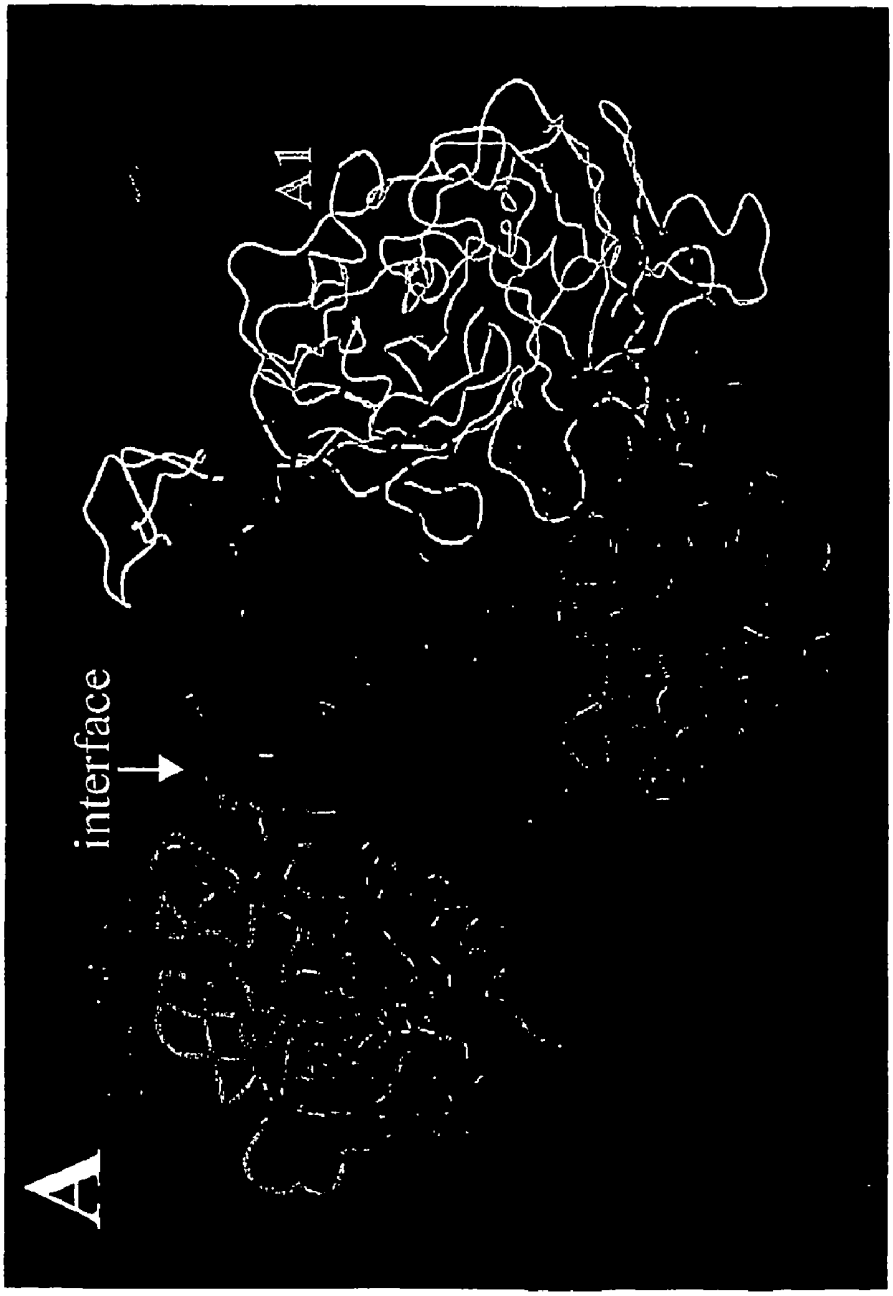


Figure 6A



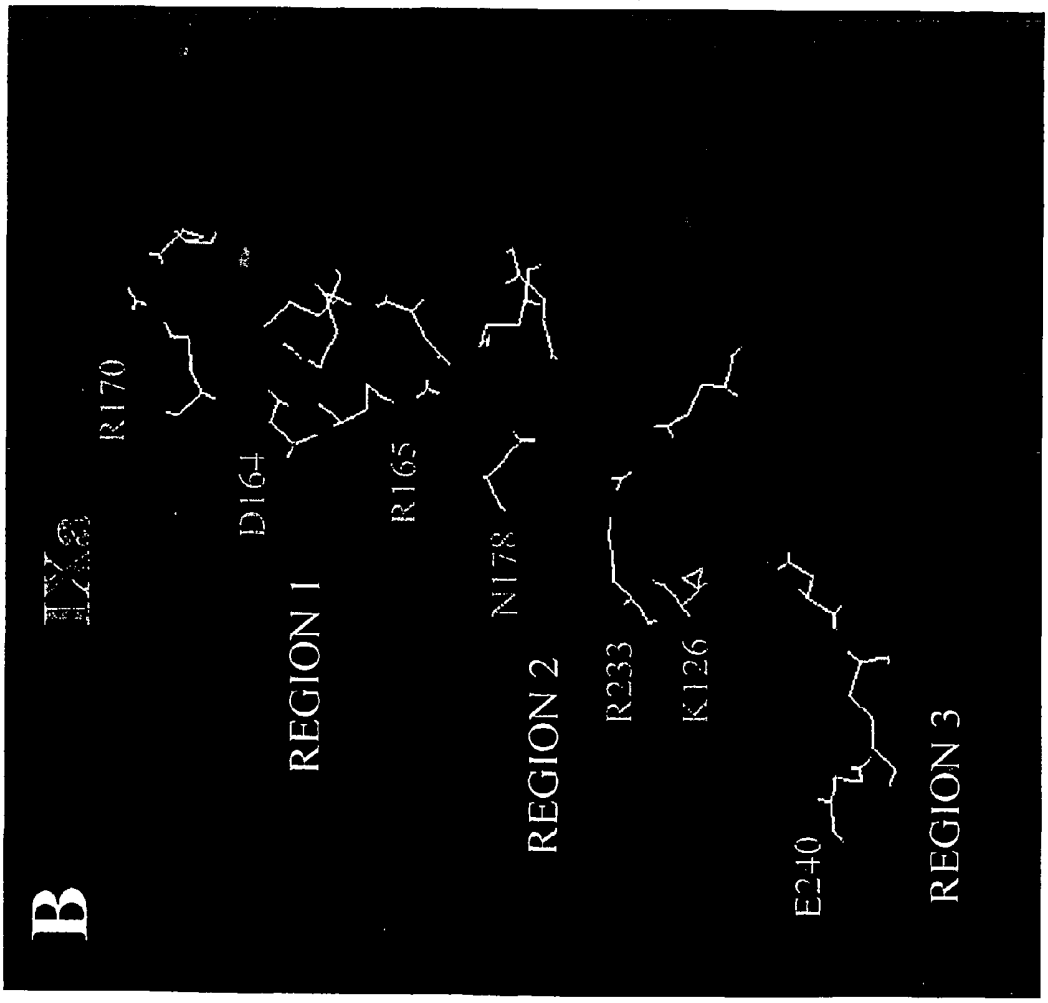

B
IX8
R170
D164
R165
N178
R233
K126
E240
REGION 1
REGION 2
REGION 3

Figure 6B

Figure 6C

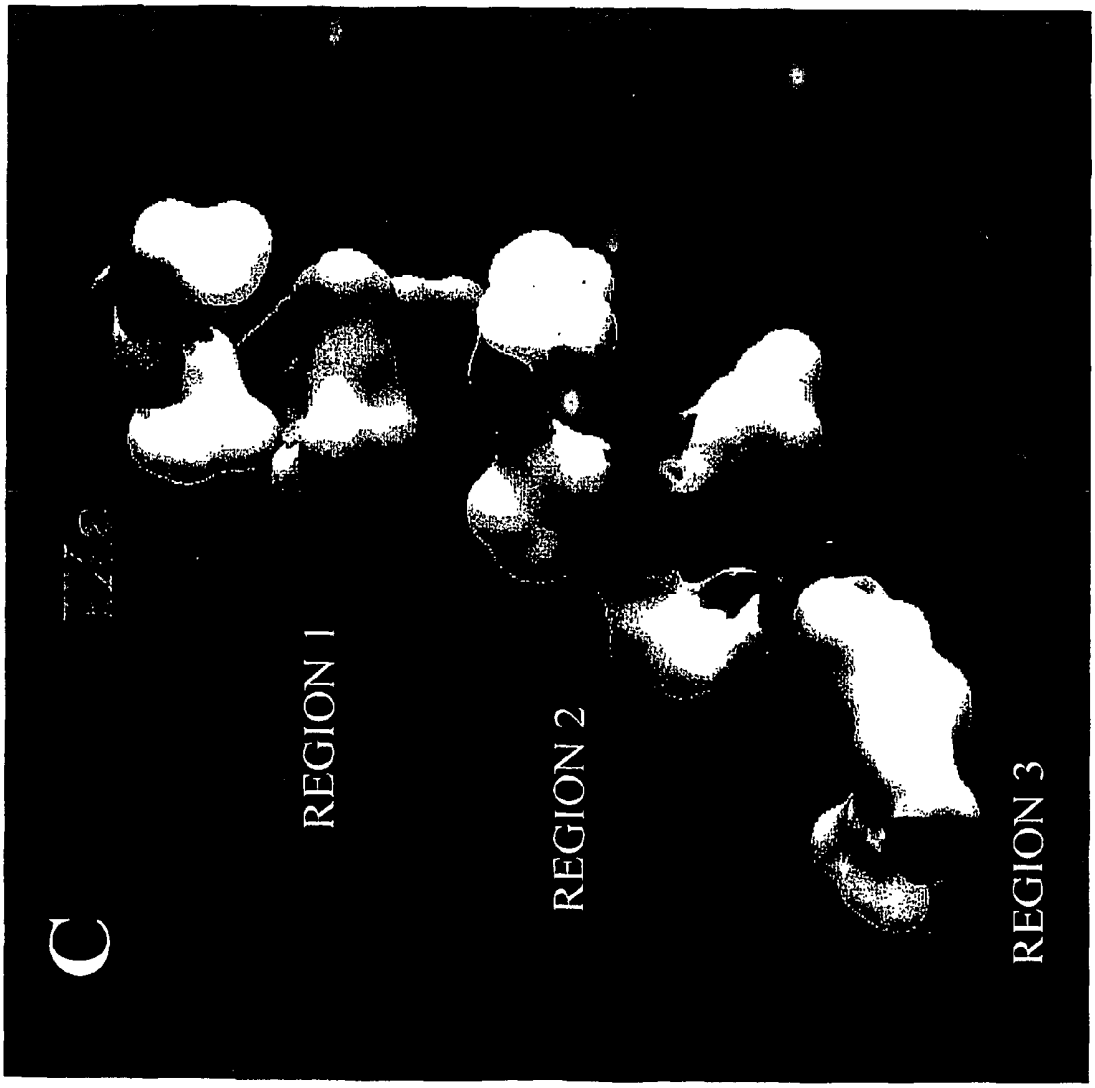


FIGURE 7A

Alignment of human (SEQ ID NO:3), mouse (SEQ ID NO:4)and pig (SEQ ID NO:5)factor VIII region 2/3

```
            1                                                          60
SEQ ID NO:3 AYTDETFKTREAIQH ESGILGPLLYGEVGD TLLIIFKNQASRPYN IYPHGITDVRPLYSR
SEQ ID NO:4 AYTDETFKTRETIQH ESGLLGPLLYGEVGD TLLIIFKNQASRPYN IYPHGITDVSPLHAR
SEQ ID NO:5 AYTDVTFKTRKAIPY ESGILGPLLYGEVGD TLLIIFKNKASRPYN IYPHGITDVSALHPG

            61                                                         120
SEQ ID NO:3 RLPKGVKHLKDFPIL PGEIFKYKWTVTVED GPTKSDPRCLTRYYS SFVNMERDLASGLIG
SEQ ID NO:4 RLPRGIKHVKDLPIH PGEIFKYKWTVTVED GPTKSDPRCLTRYYS SFINPERDLASGLIG
SEQ ID NO:5 RLLKGWKHLKDMPIL PGETFKYKWTVTVED GPTKSDPRCLTRYYS SSINLEKDLASGLIG

            121                                                        180
SEQ ID NO:3 PLLICYKESVDQRGN QIMSDKRNVILFSVF DENRSWYLTENIQRF LPNPAGVQLEDPEFQ
SEQ ID NO:4 PLLICYKESVDQRGN QMMSDKRNVILFSIF DENQSWYITENMQRF LPNAAKTQPQDPGFQ
SEQ ID NO:5 PLLICYKESVDQRGN QMMSDKRNVILFSVF DENQSWYLAENIQRF LPNPDGLQPQDPEFQ

            181                                                        240
SEQ ID NO:3 ASNIMHSINGYVFDS LQLSVCLHEVAYWYI LSIGAQTDFLSVFFS GYTFKHKMVYEDTLT
SEQ ID NO:4 ASNIMHSINGYVFDS LELTVCLHEVAYWHI LSVGAQTDFLSIFFS GYTFKHKMVYEDTLT
SEQ ID NO:5 ASNIMHSINGYVFDS LQLSVCLHEVAYWYI LSVGAQTDFLSVFFS GYTFKHKMVYEDTLT

            241                                                    294
SEQ ID NO:3 LFPFSGETVFMSMEN PGLWILGCHNSDFRN RGMTALLKVSSCDKN TGDYYEDSY
SEQ ID NO:4 LFPFSGETVFMSMEN PGLWVLGCHNSDFRK RGMTALLKVSSCDKS TSDYYEEIY
SEQ ID NO:5 LFPFSGETVFMSMEN PGLWVLGCHNSDLRN RGMTALLKVYSCDRD IGDYYDNTY
```

FIGURE 7B

Alignment of human (SEQ ID NO:6), mouse (SEQ ID NO:7), and dog (SEQ ID NO:8) factor IX region 2/3

```
            1                                                          60
SEQ ID NO:6 SYVTPICIADKEYTN IFLKFGSGYVSGWGR VFHKGRSALVLQYLR VPLVDRATCLRSTKF
SEQ ID NO:7 SYVTPICVANREYTN IFLKFGSGYVSGWGK VFNKGRHASILQYLR VPLVDRATCLRSTTF
SEQ ID NO:8 SYVTPICIADREYSN IFLKFGSGYVSGWGR VFNKGRSASILQYLK VPLVDRATCLRSTKF

            61                                                         120
SEQ ID NO:6 TIYNNMFCAGFHEGG RDSCQGDSGGPHVTE VEGTSFLTGIISWGE ECAMKGKYGIYTKVS
SEQ ID NO:7 TTYNNMFCAGYREGG KDSCEGDSGGPHVTE VEGTSFLTGIISWGE ECAMKGKYGIYTKVS
SEQ ID NO:8 TIYNNMFCAGFHEGG KDSCQGDSGGPHVTE VEGISFLTGIISWGE ECAMKGKYGIYTKVS

            121       133
SEQ ID NO:6 RYVNWIKEKTKLT
SEQ ID NO:7 RYVNWIKEKTKLT
SEQ ID NO:8 RYVNWIKEKTKLT
```

FACTOR IXA: FACTOR VLLLA INTERACTION AND METHODS THEREFOR

REFERENCE TO GOVERNMENT GRANT

[0001] This invention was made with government support under National Institutes of Health Grants HL36365, HL30616 and HL38199. The Government has certain rights in the invention.

SEQUENCE LISTING

[0002] A paper copy of the sequence listing and a computer readable form of the same sequence listing are appended below and herein incorporated by reference. The information recorded in computer readable form is identical to the written sequence listing, according to 37 C.F.R. 1.821 (f).

BACKGROUND OF THE INVENTION

[0003] (1) Field of the Invention

[0004] This invention relates generally to the prevention of coagulation. More particularly, this invention relates to compositions and methods for preventing coagulation by inhibiting binding of factor IXa to factor VIIIa, and applications utilizing these compositions and methods, including treating patients in need of anti-coagulants, and preventing coagulation in blood samples.

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[0095] (3) Description of the Related Art

[0096] Two common causes of abnormal bleeding are deficiencies of factor VII (hemophilia A) or factor IX (hemophilia B). Factor IX, a vitamin K-dependent protein, is synthesized by hepatocytes as a precursor molecule of 461 residues containing a 28 residue signal propeptide and an 18 residue leader propeptide (Yoshitake et al., 1985). During biosynthesis, the nascent protein undergoes several post-translational modifications, resulting in a single-chain protein consisting of 415 amino acids and containing 17% carbohydrate by weight (DiScipio et al., 1978). The mature protein circulates in blood as a zymogen of Mr 57,000.

[0097] Factor IX is activated during physiologic clotting to the two-chain, disulfide-linked serine protease, factor IXa, by VIIa/Ca^{2+}/tissue factor (TF) or by factor XIa/Ca^{2+} (Davie et al., 1991). The domain organization of factor IXa is similar to those of the other two enzymes (factors VIIa and Xa) involved in the TF-induced coagulation and to that of an anticoagulant enzyme termed activated protein C. The light chain of IXa consists of an amino-terminal γ-carboxyglutamic acid domain ("Gla domain", residues 1-40 out of which 12 are γ-carboxyglutamic acid residues), a short hydrophobic segment (residues 41-46), and two epidermal growth factor (EGF)-like domains (EGF1 residues 47-85, and EGF2 residues 86-127) whereas the heavy chain contains the carboxy-terminal serine protease domain with

trypsin-like specificity (Davie et al., 1991; Brandstetter et al., 1995). Activation peptide (AP) of residues 145-180, which is released upon conversion of factor IX to IXa, is rich in carbohydrate and is the least conserved region in IX from different species (Sarkar et al., 1990). Factor IXa hence formed converts factor X to Xa in the coagulation cascade; for a biologically significant rate, this reaction requires Ca^{2+}, phospholipid and factor VIIIa. The amino acid sequences of factor VIIIa and factor IXa are provided herein in the sequence listing as SEQ ID NO:1 and SEQ ID NO:2, respectively.

[**0098**] Based upon the crystal structure of the Gla domain of factor VIIa (Banner et al., 1996) and the Ca^{2+}-binding properties of factor X (Sabharwal et al., 1997), it would appear that this domain in IXa possesses several low to intermediate affinity Ca^{2+}-binding sites. In addition, the EGF1 and the protease domain each possess one high affinity Ca^{2+}-binding site (Rao et al., 1995; Bajaj et al., 1992). The Ca^{2+}-loaded conformer of the Gla domain binds to phospholipid vesicles (Freedman et al., 1996) and the EGF1 domain of IX is required for its activation by VIIa/Ca^{2+}/TF (Zhong et al., 1994). Further, Ca^{2+}-binding to the EGF1 domain has been reported to promote enzyme activity and factor VIIIa binding (Lenting et al., 1996). For proper binding of IXa to PL and VIIIa, all of the Ca^{2+}-sites in IXa must be filled (Bajaj, 1999; Mertens et al., 1999). The role of the EGF2 domain is not clear but may be involved in binding to platelets and in factor X activation (Ahmed et al., 1995). Finally, the protease domain is thought to play a primary role in binding to factor VIIIa (Astermark et al., 1994; O'Brien et al., 1995; Bajaj et al., 1993).

[**0099**] It has been demonstrated that mutations in the protease domain Ca^{2+}-binding ligands decrease the affinity of factor IXa for factor VIIIa by ~15-fold and that proteolysis at R318-S319 [residues 150-151 in the chymotrypsin numbering system] in the autolysis loop results in a further decrease in this interaction by ~8-fold (Mathur et al., 1997, *J. Biol. Chem.* 272, 23418-23426). Since residues in the protease domain Ca^{2+}-binding loop as well as those in the autolysis loop may not directly participate in binding to factor VIIIa (Hamaguchi et al., 1994), Ca^{2+} binding to the protease domain and integrity of the autolysis loop stabilize yet another region in this domain of factor IXa that directly interacts with factor VIIIa. This region has recently been identified as the 330 helix of factor IXa, comprising residues L330-R-338, corresponding to residues 162-170 using the chymotrypsin numbering system (Mathur and Bajaj, 1999; Bajaj, 1999).

[**0100**] Factor VIII is synthesized as a single chain molecule containing several domains (A1-A2-B-A3-C1-C2) (Vehar et al., 1984), with a molecular mass of approximately 300 kDa (Wood et al., 1984; Toole et al., 1984). The A domains are homologous to the ceruloplasmin domains and to the A domains of factor Va (Pemberton et al., 1997), whereas the C domains are homologous to the galactose lipid binding domain and to the regions within neuraminidase (Pratt et al., 1999). Factor VIII circulates as a divalent metal ion-dependent, noncovalent heterodimer resulting from proteolytic cleavage at the B/A3 junction that generates a heavy chain (A1-A2-B) and a light chain (A3-C1-C2). This procofactor form is cleaved by thrombin at R372-S373, R740-S741, and R1689-S1690 to yield factor VIIIa, a heterotrimer composed of A1, A2 and A3-C1-C2 subunits (Lollar and Parker, 1989; Fay et al., 1991a). The A1 and A3-C1-C2 subunits remain associated with a divalent metal ion dependent linkage whereas A2 subunit is weakly associated with the A1 and A3-C1-C2 dimer (Lollar and Parker, 1990; Fay et al., 1991b). While intact factor VIIIa is required for maximal enhancement of factor IXa activity, recent results have shown that the isolated A2 subunit stimulates factor IXa by ~100-fold (Fay and Koshibu, 1998). However, peptides of A2 residues S558-Q565, K556-N564, and Q561-D569 inhibit factor Xa generation in purified systems (Fay et al., 1994; Fay and Koshibu, 1998).

[**0101**] Ca^{2+}-dependent assembly of factor IXa and factor VIIIa on a suitable PL surface is essential for hemostasis since defects or deficiency in the proteins result in severe bleeding diatheses, namely, hemophilia A (factor VIII deficiency) or hemophilia B (factor IX deficiency) (Hemostasis Research Group, 2000; Green et al., 2000). In this assembly, Ca^{2+}-loaded form of the Gla domain of IXa binds to PL (Freedman et al., 1996) whereas $EGF1^3$/EGF2 region(s) and the protease domain are thought to interact with A3 and A2 domains of VIIIa, respectively (Fay and Koshibu, 1998; Lenting et al., 1996). VIIIa in this assembly is thought to be anchored to the PL surface via C2 domain (Pratt et al., 1999). Binding of substrate factor X to this IXa/VIIIa assembly may be partly mediated through the A1 domain of VIIIa (Lapan and Fay, 1997). Thus, although it has been shown that helix 330 of IXa and A2 domain of VIIIa interact with each other, little is known regarding the interface region(s) between these two modules, or other areas of interaction between factor VIIIa and factor IXa.

[**0102**] The identification of other sites of interaction between factor VIIIa and factor IXa would be useful for devising methods and reagents for inhibiting clotting in vitro and in vivo.

SUMMARY OF THE INVENTION

[**0103**] In accordance with the present invention, the inventors have succeeded in identifying two new areas in factor VIIIa and factor IXa that interact during factor X activation. The amino acid designations used herein are based upon a human factor VIII sequence as depicted in SEQ ID NO:1 and a human factor IX sequence as depicted in SEQ ID NO:2. These two new areas are identified herein as Region 2 and Region 3. Region 2 comprises the interaction between N346 (178 by the chymotrypsin numbering system) of factor IXa and E455 and K570 of factor VIIIa, and the interaction between R403 (233 chymotrypsin) of factor IXa and E633 of factor VIIIa. Region 3 comprises the interaction between K293 (126 chymotrypsin) of factor IXa and D712 of factor VIIIa, and the interaction between E410 (240 chymotrypsin) of factor IXa and K713 of factor VIIIa. By utilizing this knowledge, novel compositions and methods for inhibiting coagulation are disclosed.

[**0104**] Thus, in some embodiments, the present invention is directed to an agent that specifically inhibits the interaction of factor VIIIa with factor IXa in Region 2 and/or Region 3 without activating factor X. Preferably, the agent inhibits coagulation. The agent can be a polypeptide or a derivative thereof, where the polypeptide comprises an amino acid sequence of at least 3 contiguous amino acids homologous to (a) a sequence in factor VIII comprising E445, D570, E633, D712, or K713; or (b) a sequence in

factor IX comprising N346 (chymotrypsin 178), R403 (chymotrypsin 233), K293 (chymotrypsin 126), or E410 (chymotrypsin 240). Preferably, the amino acid sequence is at least 5 amino acids long, more preferably, 10 amino acids long. The agent also can comprise at least two amino acids identified in part (a) or (b) above. For example, the amino acid sequence can comprise a sequence selected from the group consisting of factor VIII sequences E445 through K570; E445 through E633, E445 through K713, K570 through E633, K570 through K713, and E633 through K713 of factor VIIIa, and factor IX sequences K293 through N346; N346 through R403; R403 through E410; K293 through R403; N346 through E410; and K293 through E410. The agent can also be a nonpeptidomimetic of these amino acid sequences.

[0105] The agent can also be a peptide comprising a sequence from any one of (a) region 2 or 3 of factor VIII (SEQ ID NO:3), (b) region 2 or 3 of factor IX (SEQ ID NO:6), (c) a sequence that is at least 88% identical to SEQ ID NO:3, and (d) a sequence that is at least 88% identical to SEQ ID NO:6, wherein the sequence is at least three amino acids long. The most preferred peptides comprise an amino acid sequence of any one of SEQ ID NOS:9-16.

[0106] The agent can also be a non-homologous binding polypeptide. Preferably, the non-homologous binding polyeptide agent has an antibody binding site that specifically binds to factor VIIIa or factor IXa in Region 2 or Region 3. The antibody binding site preferably specifically binds to the amino acid sequence of the agents above that are homologous to factor VIIIa or factor IXa in Region 2 or Region 3. In preferred embodiments, the agent is an antibody, most preferably a monoclonal antibody, particularly a humanized monoclonal antibody.

[0107] In additional embodiments, the present invention is directed to a polynucleotide encoding an amino acid sequence homologous to any one of the above-described agents, where the polynucleotide is operably linked to a control sequence that allows the polynucleotide to be translated in a mammalian cell.

[0108] In other embodiments, the present invention is directed to a composition that induces coagulation. The composition comprises the portions of the amino acid sequence of factor VIIIa that interact with factor IXa, or derivatives thereof. The composition could comprise the entire portion of the factor VIIIa amino acid sequence that encompasses the factor IXa-interacting portions (E440-K713 of SEQ ID NO:1), or it could comprise the amino acid fragments that interact with factor IXa connected by linkers designed to align the interacting portions to the proper areas of factor IXa.

[0109] The present invention is also directed to pharmaceutical compositions comprising the agents or polynucleotides encoding the agents disclosed above, in a pharmaceutically acceptable excipient. In preferred embodiments, the excipient is suitable for intravenous administration.

[0110] Additionally, the present invention is directed to a method of treatment to prevent coagulation in a patient in need thereof. The method comprises administering to the patient any of the polypeptide or polynucleotide agents disclosed above, preferably as the above-described pharmaceutical compositions. The method is particularly useful for patients suffering from a cardiovascular disorder, where the preferred disorders are thrombosis, atherosclerosis and restenosis. In most preferred embodiments, the pharmaceutical composition is administered intravenously.

[0111] The present invention is also directed to a method for identifying a compound having anti-coagulation activity. The method comprises determining whether a compound displaces the interaction of any of the above-described agents to factor VIIIa or factor IXa. Preferably, the agent is labeled with a detectable marker, most preferably a fluorescent marker, a radioactive marker, and a spin label.

[0112] In additional embodiments, the present invention is directed to a method of preventing coagulation in a blood sample. The method includes adding any of the above-described agents to the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0113] **FIG. 1** is a graph depicting the effect of the isolated A2 subunit of factor VIIIa on the rate of activation of factor X by various factor IXa proteins. The rate of formation of factor Xa by each factor IXa protein was measured as described in the Example. The reaction mixtures contained 5 nM factor IXa, 250 nM factor X and various concentrations of A2 subunit. The buffer used was TBS/BSA, pH 7.5 containing 25 μM PL and 5 mM $CaCl_2$. The proteins used are: IXa_{WT} (●), IXa_{PCEGF1} (○), IXa_{R333Q} (▲), and $IXa_{VII\text{-}helix}$ (Δ). The data were fitted to a single site binding equation (Eq 1).

[0114] **FIG. 2** is a graph depicting the effect of factor X concentration on the EC_{50} (functional Kd) of the interaction of the A2 subunit with IXa_{WT} or IXa_{PCEGF1}. The EC_{50} of the interaction of factor IXa_{WT} (●) or factor IXa_{PCEGF1} (○) with the A2 subunit was determined at various concentrations of factor X. Each point (EC_{50}) shown is the concentration of free A2 subunit (y-axis) providing 50% of the Vmax. Each EC_{50} value was obtained from a direct plot (similar to **FIG. 1**) of factor Xa generation at various concentrations of the A2 subunit and a constant concentration of factor X. The factor IXa concentration in each experiment was fixed at 5 nM. The buffer used was TBS/BSA, pH 7.5 containing 25 μM PL and 5 mM $CaCl_2$. Factor Xa concentration was measured by S-2222 hydrolysis.

[0115] **FIG. 3** is a graph depicting the abilities of various dEGR-IXa proteins to inhibit factor IXa:A2 subunit interaction as measured by a decrease in factor Xa generation in the tenase system. The reaction mixtures contained 100 nM IXa_{WT}, 30 nM A2 subunit, 250 nM factor X, 25 μM PL, and various concentrations of dEGR-IXa proteins in TBS/BSA, pH 7.5 containing 5 mM $CaCl_2$. Factor Xa generation was measured by S-2222 hydrolysis. The value of slope factor, s, was 0.9±0.1 indicating a single affinity binding site between the interacting proteins. The curves represent best fit of the data to the IC_{50} four-parameter logistic equation (Eq 2). The proteins used are: dEGR-IXa_{WT} (●), dEGR-IXa_{PCEGF1} (○), dEGR-Xa_{R333Q} (▲) and dEGR-$IXa_{VIIhelix}$ (Δ).

[0116] **FIG. 4** is a graph depicting the effect of the A2 subunit on the fluorescence emission intensity of dEGR-IXa proteins. Reactions (160 μL) were titrated with A2 subunit in buffer containing 20 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween, 200 μg/ml BSA and 100 μM PL vesicles. Fluorescence emission intensity of each dEGR-IXa

(220 nM) at a given A2 subunit concentration was determined as described in the Example. Data are presented as F/F_0, where F_0 is emission intensity in the absence of A2 and F is the intensity at a given A2 subunit concentration. Symbols are dEGR-IX_{WT} (▲), dEGR-IXa_{PCEGF1} (●), dEGR-IXa_{R333Q} (♦), and dEGR-$IX_{VIIIhelix}$ (■).

[0117] **FIG. 5** is a graph depicting the ability of the 558-565 A2 peptide to inhibit the interaction of various factor IXa proteins with the A2 subunit. The reaction mixture for factor IXa_{WT} (●) or factor IXa_{PCEGF1} (○) contained 100 nM of factor IXa protein, 30 nM A2 subunit, 250 nM factor X, 5 mM $CaCl_2$, and 25 μM PL in TBS/BSA, pH 7.5. The reaction mixture for factor IXa_{R333Q} ( ) contained 300 nM of factor IXa instead of 100 nM used for factor IXa_{WT} or factor IXa_{PCEGF1}; the concentrations of other components were unchanged. Factor Xa generation was determined by S-2222 hydrolysis, and the curves represent best fit to the IC_{50} four-parameter logistic equation (Eq. 2). The value of slope factor, s, was 0.9±0.1 indicating a single affinity binding site between the various IXa proteins and the A2 peptide.

[0118] **FIG. 6** depicts an interface model between the factor IXa protease domain and the A2 subunit of factor VIIIa. The coordinates for the human factor IXa structure are from the Brookhaven Protein Data Bank (PDB code 1RFN) and the coordinates for the A1, A2, and A3 subunits of factor VIIIa (Pemberton et al., 1997) are based upon homology models built using ceruloplasmin coordinates (PDB code 1KCW). A, Schematic representation of the interface model. The ribbon structure for each protein is depicted. The IXa protease domain is shown in light blue and the EGF2 domain is shown in red. The A1 subunit is in yellow, the A2 subunit is in magenta with residues 484-509 in white, and the A3 subunit is in cyan with the C-terminal in red. The Gla and the EGF1 domains of factor IXa and the C1 and C2 domains of factor VIIIa are not shown. The interface residues of the factor IXa protease domain and of the A2 subunit are shown as CPK space filling models. The molecules are oriented such that the Gla domain of factor IXa and the C2 domain of factor VIIIa are projecting away from the viewer. The Gla domain in factor IXa and the C2 domain of factor VIIIa bind to the PL surface. B, Detailed interface between factor IXa protease domain and the modeled A2 subunit. Only the charged residues that participate in the binding interactions are depicted. The hydrophobic residues that participate in this interaction are discussed in the text. The orientation of the molecules is the same as in A. Chymotrypsin numbering system for the factor IXa protease domain is used. Corresponding factor IX numbering system are 338 (c170), 332 (c164), 333 (c165), 346 (c 178), 403 (c233), 293 (c126) and 410 (c240). Factor IXa residues are labeled light blue and A2 subunit residues are labeled magenta. C, Electrostatic potential between the factor IXa protease domain and the A2 subunit interface as determined using the program GRASP (Nicholls et al., 1991). Blue represents positive, red represents negative and white represents neutral residues.

[0119] **FIG. 7** depicts an alignment between the sequences depicting regions 2 and 3 of various mammalian factors VIII (A) and IX (B).

DETAILED DESCRIPTION OF THE INVENTION

[0120] The amino acid numbering system that is herein used is based upon the human factor VIII sequence of SEQ ID NO:1 and the human factor IX sequence of SEQ ID NO:2, unless indicated otherwise.

[0121] The following abbreviations are used herein: TF, tissue factor; Gla, gamma-carboxyglutamic acid; EGF, epidermal growth factor; PL, phospholipid; BSA, bovine serum albumin; WT, wild type; TBS, Tris-buffered saline; dEGR-ck, dansyl-Glu-Gly-Arg-chloromethyl ketone; dEGR-IXa, IXa inactivated with dEGR-ck; S-2222, benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide; NP, normal plasma; Kd_{A2}, dissociation constant for dEGR-IXa and the A2 subunit; $Kd_{peptide}$, dissociation constant for factor IXa and the A2 558-565 peptide. The numbers in parentheses with a prefix c (e.g., c57) refers to the chymotrypsin equivalents for the protease domain of factor IXa (Bajaj and Burktoft, 1993).

[0122] In accordance with the present invention, it has been discovered that factor VIIIa and factor IXa interact at two regions, Region 2 and Region 3, which have not been previously identified as areas of interaction. Region 2 is defined herein as the interaction between N346 (178 by the chymotrypsin numbering system) of factor IXa and E445 and K570 of factor VIIIa, and the interaction between R403 (c233) of factor IXa and E633 of factor VIIIa. Region 3 is defined herein as the interaction between K293 (c126) of factor IXa and D712 of factor VIIIA, and the interaction between E410 (c240) of factor IXa and K713 of factor VIIIA. These interactions are necessary for normal conversion of factor X to factor Xa by the factor IXa protease domain, as normally occurs in clotting. The skilled artisan would thus expect anything that disrupts these interactions to inhibit clotting. It is also noted that mutations at amino acid residue 403 (c233) of factor IX has been found to cause hemophilia (Green et al. 2000). Similar findings have been shown for Region 1, defined herein as the interaction between Helix 330 (amino acid residues 330-338 [c162-c170]) of factor IXa and residues 558-565 of factor VIIIa. In that region, mutants of factor IXa inhibited the formation of factor Xa in vitro (Example; Mather and Bajaj, 1999), and short peptides with sequences identical to that region of factor VIIIa also inhibited formation of factor Xa (Fay et al., 1994) in vitro.

[0123] As used herein, "clotting" or "blood clotting" or "coagulation" means the sequential process by which the multiple coagulation factors of the blood interact in the coagulation cascade, ultimately resulting in the formation of an insoluble fibrin clot.

[0124] As used herein, "inhibiting clotting" encompasses effects where clotting is eliminated, as well as where clotting is just reduced to a significant degree. Depending on the desired goal, preferred methods and agents of the present invention will inhibit thrombosis under optimum conditions by at least 10%; more preferably, the inhibition will be at least 25%; even more preferably, at least 50%; even more preferably, at least 75%. The most preferred methods or reagents of the present invention inhibit thrombosis by 90-100% under optimized conditions; however, if desired, the conditions could be adjusted to be suboptimal if lower degrees of inhibition of thrombosis are desired.

[0125] Reduction or inhibition of clotting can be measured by any means known in the art. Nonlimiting examples of

useful methods to measure clotting include (a) methods that directly measure the interaction of factor VIIIa with factor IXa, for example by measuring changes in dansyl emission intensity (see, e.g., Example 1); (b) methods that measure the result of the factor VIIIa-factor IXa interaction, i.e., formation of factor Xa (TENase activity) (see, e.g., Example 1); and (c) methods that measure rate of thrombosis, such as the well-known thrombin time, prothrombin time, or activated partial thromboplastin time assays. See, generally, Lottenberg et al., 1981, and Ohno et al., 1980.

[0126] Thus, in some embodiments, the present invention provides an agent that specifically inhibits clotting by preventing the interaction of a factor VIIIa with a factor IXa in Region 2 or Region 3. In one group of these embodiments, the agent is a polypeptide, or a derivative thereof, that (a) is capable of interacting with factor VIIIa in Region 2 or Region 3 by virtue of its sequence homology with factor IXa, and/or (b) is capable of interacting with factor IXa in Region 2 or Region 3 by virtue of its sequence homology with factor VIIIa. Since these polypeptides or derivatives are homologous to factor VIIIa or factor IXa at regions of interaction, they would be expected to interact with factor IXa or factor VIIIa, respectively, blocking the interaction between the two factors. This would prevent formation of factor Xa and inhibiting clotting.

[0127] This group of clotting inhibitors would exclude polypeptides or derivatives that are capable of inducing coagulation to a limited degree by substituting for factor VIIIa or IXa. For example, the A2 region of factor VIII would not be an agent that inhibits clotting by preventing the interaction of factor VIIIa with factor IXa, because the A2 region is capable of inducing coagulation in a TENase reaction mixture by substituting for factor VIIIa (Fay and Koshibu, 1998; see also Example 1). The skilled artisan could easily test any polypeptide or derivative for its ability to induce coagulation by determining whether the polypeptide or derivative is capable of inducing coagulation in a TENase reaction mixture without factor VIIIa or factor IXa.

[0128] These polypeptides can be produced by any of several well-known methods, including expressing a clone of a gene that encodes the polypeptide, and chemical synthesis, for example by the classical Merrifeld method of solid phase peptide synthesis (Merrifeld, 1963) or the FMOC strategy on a Rapid Automated Multiple Peptide Synthesis system (DuPont Company, Wilmington, Del.) (Caprino and Han, 1972).

[0129] Since Region 2 and Region 3 comprise the amino acids E445, K570, E633, D712 and K713 in factor VIIIa (SEQ ID NO:1) and N346 (178 chymotrypsin), R403 (233 chymotrypsin), K293 (126 chymotrypsin) and E410 (240 chymotyypsin) in factor IXa (SEQ ID NO:2) the inhibitory peptide or derivative preferably comprises an amino acid sequence, or derivative, homologous with factor VIIIa or factor IXa that comprises at least one of those amino acid residues. As such, examples of polypeptides or derivatives that would be useful for the present invention include any amino acid sequence of at least 3 contiguous amino acids homologous to a sequence in factor VIIIa that comprises E445, K570, E633, D712 or K713, or any amino acid sequence of at least 3 contiguous amino acids homologous to a sequence in factor IXa that comprises N346, R403, K293, or E410. In preferred embodiments, the polypeptide or derivative comprises at least 5 amino acids homologous to factor VIIIa or factor IXa; more preferably, the polypeptide or derivative comprises at least 10 amino acids homologous with factor VIIIa or factor IXa.

[0130] Preferably, the peptide or derivative is homologous to an amino acid sequence from factor VIIIa or factor IXa that also encompasses other amino acid residues that are involved in the interaction of factor VIIIa with factor IXa, for example other residues from Region 2 or Region 3, residues from Region 1 (encompassing the interaction of the helix 330 [chymotrypsin 162] of factor IXa with residues 558-565 of factor VIIIa—see Example), or residues involved in calcium binding. Examples of amino acid sequences or derivatives that are particularly useful for the present invention include sequences that are derived from region 2 and 3 of factor VIII, which includes human (SEQ ID NO:3), mouse (SEQ ID NO:4), and pig sequences (SEQ ID NO:5), and sequences that are at least 88% identical to SEQ ID NOS:3-5, and sequences that are derived from region 2 and 3 of factor IX, which includes human (SEQ ID NO:6), mouse (SEQ ID NO:7), and dog sequences (SEQ ID NO:8), and sequences that are at least 88% identical to SEQ ID NOS:6-8. It is further envisioned that those sequences comprise residues E445 through K570; residues E445 through E633, residues E445 through K713, residues K570 through E633, residues K570 through K713, and residues E633 through K713 of factor VIIIa, and factor IXa sequences K293 through N346; N346 through R403; R403 through E410; K293 through R403; N346 through E410; and K293 through E410.

[0131] A preferred peptide comprises an amino acid sequence that is selected from the list consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15 and SEQ ID NO:16.

[0132] The step wise comparison and alignment of regions 2 and 3 of factors VIII and IX from various mammalian species, according to the ClustalW program or similar sequence alignment program (the parameters of which are detailed below), is depicted in **FIG. 7**. The proportion of identical amino acids between human and mouse and human and pig or dog was determined from aligned sequences and reported herein as percent identity in Table 1.

TABLE 1

Species compared	Sequence identifiers	Percent amino acid identity
Human v. mouse Factor VIII region 2/3	SEQ ID NO: 3 v. SEQ ID NO: 4	88%
Human v. pig Factor VIII region 2/3	SEQ ID NO: 3 v. SEQ ID NO: 5	88%
Human v. mouse Factor IX region 2/3	SEQ ID NO: 6 v. SEQ ID NO: 7	90%
Human v. dog Factor IX region 2/3	SEQ ID NO: 6 v. SEQ ID NO: 8	95%

[0133] Sequence identity or percent identity is intended to mean the percentage of same residues between two. sequences aligned using the Clustal method (Higgins et al, *Cabios* 8:189-191, 1992) of multiple sequence alignment in the Lasergene biocomputing software (DNASTAR, INC,

Madison, Wis.). In this method, multiple alignments are carried out in a progressive manner, in which larger and larger alignment groups are assembled using similarity scores calculated from a series of pairwise alignments. Optimal sequence alignments are obtained by finding the maximum alignment score, which is the average of all scores between the separate residues in the alignment, determined from a residue weight table representing the probability of a given amino acid change occurring in two related proteins over a given evolutionary interval. Penalties for opening and lengthening gaps in the alignment contribute to the score. The default parameters used with this program are as follows: gap penalty for multiple alignment=10; gap length penalty for multiple alignment=10; k-tuple value in pairwise alignment=1; gap penalty in pairwise alignment=3; window value in pairwise alignment=5; diagonals saved in pairwise alignment=5. The residue weight table used for the alignment program is PAM250 (Dayhoff et al., in *Atlas of Protein Sequence and Structure*, Dayhoff, Ed., NBRF, Washington, Vol. 5, suppl. 3, p. 345, 1978).

[**0134**] To determine percent sequence identity between two sequences, the number of identical amino acids in the aligned sequences is divided by the total number of amino acids that are compared. The sequence identity between human factor VIII region 2/3 and mouse or pig factor VIII region 2/3 is about 88%.

[**0135**] Based upon the comparison of human region 2/3 sequence to other mammalian homologues of region 2/3, the inventor envisions that the polypeptide or derivative that is capable of inhibiting coagulation comprises a portion of a sequence that is at least 88% identical to SEQ ID NO:3 or a portion of a sequence that is at least 88% identical to SEQ ID NO:6.

[**0136**] The polypeptide or derivative can be of any length, provided it is capable of inhibiting coagulation, and may comprise a sequence homologous to a large portion of factor VIII or factor IX, or may comprise a sequence that is homologous to factor VIII or factor IX at Region 2 or Region 3 fused to a sequence that is not homologous. For example, the polypeptide or derivative can be 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, 200, or more amino acids or amino acid derivatives long, or any length between those values.

[**0137**] As used herein, the term "derivative" includes any non-peptide compound, including peptidomimetics or non-peptidomimetics, that can substitute for a particular amino acid or polypeptide. Based on the structural features of the critical amino acid sequence of the peptides of the present invention that permit the interaction of the peptide to factor VIIIa or factor IXa, one can develop these non-peptide derivatives that are capable of binding to factor VIIIa or factor IXa and that inhibit coagulation. Thus, a non-peptide derivative includes any non-peptide chemical compound that can interact with factor VIIIa or factor IXa at Region 2 or Region 3 to inhibit clotting.

[**0138**] The techniques for development of peptidomimetics and nonpeptidomimetics are well known in the art. See for example, Navia and Peattie, 1993; Ripka et al., 1998; Kieber-Emmons et al., 1997; Freidinger, 1999; Qabar et al., 1996. Typically this involves identification and characterization of the protein target as well as the protein ligand using X-ray crystallography and nuclear magnetic resonance technology. In the case of the factor VIIIa binding domain on factor IXa, both factors have been sequenced and cloned (Wood et al., 1984; Vehar et al., 1984; Yoshitake et al., 1985). Additionally, the X-ray structure of factor IXa has been determined (Brandstetter et al., 1995) and modeling studies have elucidated characteristics of the factor VIIIa and factor IXa binding site (disclosed herein and in Mather and Bajaj, 1999). Using information learned from the structure of factor VIIIa and its polypeptide ligand, a pharmacophore hypothesis is developed arid compounds are made and tested in a routine assay system. The test compound can then be evaluated by, for example, binding to factor VIIIa or IXa, e.g., by electrophoretic mobility shift assays (Igarashi et al., 1993) or an assay system utilizing co-precipitation of the ligand and factor VIIIa or IXa. Alternatively, the compound can also be tested functionally by methods known in the art, e.g., by its ability to reduce or abolish activity of factor VIIIa or factor IXa in a coagulation based assay or in factor X activation assay. See, e.g., Examples 1 and 2 for such methods. As is well known, peptidomimetics and nonpeptidomimetics are often superior to analogous peptides in therapeutic applications because the mimetics are generally more resistant to digestion than peptides.

[**0139**] Additionally, included within the derivatives contemplated as part of the invention are the polypeptides disclosed above, wherein individual amino acids in the claimed sequence are substituted with linkers which are not amino acids but which allow other amino acids in the sequence to be spaced properly to allow binding to factor VIIIa. For example, the C of the sequence MTALLKVSS-CDKNTGDYYEDSY (SEQ ID NO:11) can be replaced with a linker to allow the other areas of the sequence to align properly with Region 3 of factor IXa. Use of such linkers is well known in the art and their design in this context would not require undue experimentation.

[**0140**] Another group of agents that can inhibit clotting according to the invention is the group of agents that comprise non-homologous binding polypeptides. These polypeptides are not homologous to factor VIII or factor IX at Region 2 or Region 3, but are able to bind to factor VIII or factor IX at those regions. This group of agents consists of (a) polypeptides that bind to Region 2 or Region 3 through an antibody binding site and (b) polypeptides that do not bind to Region 2 or Region 3 at an antibody binding site. The latter polypeptides can be identified, e.g., by random peptide libraries, such as phage display libraries (Cortese et al., 1995; Cortese et al., 1996). Peptidomimetics or nonpeptidomimetics that are derivatives of these peptides, prepared by methods known in the art, are also envisioned as being within the scope of the present invention.

[**0141**] In preferred embodiments, the non-homologous binding peptide comprises an antibody binding site that specifically binds to factor VIIIa or factor IXa in Region 2 or Region 3. Due to their ease of preparation, these agents are preferably antibodies or antibody fragments such as FAb or $F(Ab)_2$ fragments, but other types of polypeptides comprising antibody binding sites can be prepared by known methods (see, e.g., Winter and Milstein, 1991).

[**0142**] These agents comprising an antibody binding site that specifically binds to factor VIII or IX at Region 2 or Region 3 would be expected to inhibit TENase activity and

coagulation, since antibodies to other regions of factor IX have been shown to have such an effect in vitro and in vivo (Feuerstein, 1999).

[0143] The antibodies of these embodiments can be polyclonal or, preferably, monoclonal antibodies, which can be prepared by, e.g., the well-known hybridoma method (Galfre and Milstein, 1981) or by recombinant methods (Winter and Milstein, 1991). These antibodies can also be humanized by known methods to avoid immune reactivity when used in therapeutic methods (Breedveld, 2000).

[0144] The antibodies to Region 2 or Region 3 can be raised against the whole factor VIII or IX, after which antibodies can be selected by routine methods (e.g., by ELISA with monoclonal antibodies, or affinity purification with polyclonal antibodies) for binding to Region 2 or Region 3 by, e.g., determining whether the antibody binds to the polypeptide agents previously disclosed that are homologous to these regions in factor VIII or factor IX. Alternatively, the antibodies to Region 2 or Region 3 can be raised against the previously disclosed polypeptides themselves. As is well known, antibodies can be raised against a short polypeptide by conjugating the peptide to an immunogenic carrier molecule such as bovine serum albumin or keyhole limpet hemocyanin. Under these conditions, antibodies will be produced against the carrier molecule as well as the polypeptide. The antibodies to the polypeptide can then be selected by routine methods.

[0145] The utility of any particular agent in inhibiting the interaction of factor VIIIa and IXa can also be ascertained by evaluating the binding of the polypeptide or derivative to the factor VIIIa or IXa by any of a number of methods that are well known in the art. For example, the polypeptide or derivative can be labeled with a radioactive agent or a dye such as a fluorescent dye, and unbound vs. bound polypeptide or derivative can be determined by methods such as chromatography or electrophoresis, where the chromatographic or electrophoretic conditions are selected where unbound polypeptide migrates differently than polypeptide bound to factor VIIIa or factor IXa. Alternatively, bound vs. unbound polypeptide or derivative can be determined by dialysis, using a membrane which allows the passage of unbound labeled polypeptide or derivative but not polypeptide or derivative bound to factor VIIIa or factor IXa. Another alternative method for determining polypeptide or derivative bound to factor VIIIa or factor IXa is by the determination of displacement of labeled polypeptide from factor VIIIa or factor IXa that is adsorbed to a solid phase.

[0146] The inhibitory agents disclosed above can be supplied as a polynucleotide that encodes the agent, wherein the polynucleotide is operably linked to a control sequence that allow the polynucleotide to be translated in a mammalian cell. These agents are useful, e.g., in gene therapy applications. Gene therapy reagents and control sequences for cardiovascular and hematology applications are well known in the art. See, e.g., Carmeliet and Collen, 1996; Clowes, 1997; Schwartz and Moawad, 1997; and Yla-Herttuala and Martin, 2000.

[0147] As used herein, "operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0148] In other embodiments, the present invention is directed to a composition that induces coagulation. The composition comprises the portions of the amino acid sequence of factor VIIIa that interact with factor IXa, or derivatives thereof. The composition could comprise the entire portion of the factor VIIIa amino acid sequence that encompasses the factor IXa-interacting portions (for example E440-K713 of SEQ ID NO:1), or it could comprise the amino acid fragments that interact with factor IXa connected by linkers designed to align the interacting portions to the proper areas of factor IXa.

[0149] It is contemplated that the polypeptides or derivatives of the present invention are usually employed in the form of pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous.

[0150] The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmaceutically acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

[0151] It is also contemplated that certain formulations comprising the polypeptides or derivatives are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic and nucleic acid degradation and/or substances that promote absorption such as, for example, surface active agents.

[0152] In other embodiments, the present invention is directed to a method of treatment to prevent coagulation in a patient in need thereof. The method comprises adminis-

tering at least one of the agents described above in a pharmaceutically acceptable excipient. In preferred embodiments, the patient is a human, but the method could easily be adapted to any other vertebrate subject. In doing so, the non-human vertebrate Region 2 and Region 3 analogous to human Region 2 and Region 3 can be routinely identified by the modeling methods disclosed herein.

[0153] This method is useful for any disorder where inhibition of coagulation is desired. Preferred disorders are cardiovascular disorders involving inappropriate coagulation. Examples include thrombosis, atherosclerosis and restenosis. Thrombosis is defined herein as the formation, development, or presence of a blood clot in a blood vessel or the heart, including where cerebral vessels are involved, leading to stroke. Thrombosis can be usefully treated by this method to prevent further clot formation.

[0154] Atherosclerosis is useful for treatment by these methods, since clot formation, induced by tissue factor in a ruptured or fissured atherosclerotic plaque, often induces unstable angina and myocardial infarction (Toschi et al., 1997; Ardissino et al., 2000). Thus, treatment of an atherosclerosis patient with the agents disclosed above would prevent such clot formation. Similarly, restenosis, defined herein as a reformation of an occlusion in a blood vessel after an occlusion has been corrected, e.g., with angioplasty, often occurs due to tissue factor induced coagulation (Oltrona et al., 1997; Gallo et al., 1998). Thus, treatment of a patient in danger of restenosis would prevent such occlusions from occurring.

[0155] In these embodiments, the agent can be administered by any method known in the art that is capable of providing the agent to the bloodstream where clotting inhibition is desired. Intravenous administration is preferred, since that introduces the agent directly into the bloodstream. The agent can also be administered in the form of a polynucleotide encoding the agent, wherein the polynucleotide is operably linked to a control sequence that allows the polynucleotide to be translated in cells into which the polynucleotide is introduced. This gene therapy approach can also involve ex vivo introduction of the polynucleotide-control sequence combination into a cell such as a lymphocyte or macrophage, which is then transferred to the patient, where the polynucleotide is expressed and the agent is produced. For general reviews of applicable gene therapy approaches, see Carmeliet and Collen, 1996; Clowes, 1997; Schwartz and Moawad, 1997; and/or Yla-Herttuala and Martin, 2000.

[0156] The present invention is also directed to a method of identifying a compound having anti-coagulation activity. The method comprises combining the test compound with reagents that exhibit Region 2 or Region 3 interaction, then determining whether the compound displaces that interaction. For example, the compound could be combined with factor IXa and an agent that interacts with factor IXa at Region 2 or Region 3 (e.g., a peptide comprising E445 to K570 of factor VIIIa). If the compound disrupts the interaction, it would likely be a compound that would inhibit coagulation. Any of several means known in the art can be utilized to determine whether the compound displaces the agent. Preferably, that determination is made by evaluating whether the compound prevents the binding of the agent to factor VIIIa or factor IXa, wherein the agent is labeled with a detectable marker. The detectable marker can be any of a number of well-known markers, including fluorescent markers, radioactive markers, and spin labels. In preferred embodiments of this method, the agent selected should not bind with a high affinity to factor VIIIa or factor IXa at Region 2 or Region 3, since the binding of an agent with high affinity would be difficult to displace with the test compound, and test compounds that might otherwise be effective in preventing the interaction of factor VIIIa with factor IXa would not be able to displace the agent.

[0157] In additional embodiments, the present invention is directed to a method of preventing coagulation in a blood sample. The method comprises adding an agent as previously described to the sample. These agents can be homologous to factor VIII or factor IX at Region 2 or Region 3. Alternatively, the agents can be non-homologous to factor VIII or factor IX at Region 2 or Region 3. As previously discussed, the latter agent can, for example, comprise an antibody binding site or a polypeptide that does not comprise an antibody binding site.

[0158] In these methods, the polypeptide or derivative can be added to the blood sample as a liquid or dried preparation. Alternatively, the polypeptide can be present in the container that receives the blood sample (for example a vacutainer), in order for the blood sample to be exposed to the polypeptide when the sample enters the container. The quantity of the polypeptide or derivative added to the container can be determined without undue experimentation, merely by determining the quantity of the polypeptide or derivative necessary to prevent coagulation of the quantity of blood which is to be drawn in the sample.

[0159] Industrial Application

[0160] The compositions and methods of the present invention provide novel treatments to prevent coagulation in vivo, methods for preventing coagulation in blood samples, and methods for identifying agents that have anti-coagulation activity.

[0161] Preferred embodiments of the invention are described in the following example. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

[0162] The procedures disclosed herein which involve the molecular manipulation of nucleic acids are known to those skilled in the art. See generally Fredrick M. Ausubel et al. (1995), "Short Protocols in Molecular Biology", John Wiley and Sons, and Joseph Sambrook et al. (1989), "Molecular Cloning, A Laboratory Manual", second ed., Cold Spring Harbor Laboratory Press, which are both incorporated by reference.

EXAMPLE 1

[0163] This example provides evidence demonstrating the importance of the interaction of the A2 subunit of Factor VIIIa with Factor IXa at Region 1, Region 2, and Region 3.

[0164] Experimental Procedures

[0165] Reagents.

[0166] Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) was purchased from Helena Laboratories. Dansyl-Glu-Gly-Arg-chloromethyl ketone (dEGR-ck) was obtained from Calbiochem. Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), recombinant hirudin, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma. Phospholipid (PL) vesicles containing 75% PC and 25% PS were prepared by the method of Husten et al., 1987. For fluorescence experiments, phospholipid vesicles were comprised of 20% PS, 40% PC and 40% PE and were prepared using octyl glucoside as previously described (Mimms et al., 1981). Recombinant factor VIII preparations (Kogenate®) were a gift from Drs. Lisa Regan and Jim Brown of Bayer Corporation. Purified recombinant factor VIII was also a generous gift from Debra Pittman of the Genetics Institute. Normal plasma factor IX (IX_{NP}) and factor X was isolated as previously described (Bajaj and Birktoft, 1993) and factor Xa was prepared as outlined in Bajaj et al., 1981. Purified human factor XIa and thrombin were purchased from Enzyme Research Laboratories (South Bend, Ind.). A synthetic peptide corresponding to the A2 subunit residues 558-565 (Ser-Val-Asp-Gln-Arg-Gly-Asn-Gln) (SEQ ID NO:17) was obtained as described in in Fay and Koshibu, 1998, and its concentration was determined by amino acid analysis.

[0167] Proteins.

[0168] The Kogenate® concentrate was fractionated to separate factor VIII from albumin following S-Sepharose chromatography as outlined in Fay et al., 1993. Factor VIIIa was prepared from factor VIII using thrombin and subsequently purified using CM-Sepharose chromatography (Curtis et al., 1994), and the A2 subunit and A1/A3-C1-C2 dimer were separated by Mono S chromatography (Fay et al., 1991a). The A2 subunit was further purified using an anti-A2 immunoaffinity column (Fay et al., 1991a). The purified A2 subunit was essentially homogeneous (>95% pure) as judged by SDS-PAGE. For some experiments, proteins were concentrated using a MicronCon concentrator (Millipore, 10 kDa cut-off). The concentration of the A2 subunit was determined by the coomassie blue dye binding method of Bradford (1976). Wild-type factor IX (IX_{WT}), as well as mutants IX_{R333Q} (a point mutant in which Arg333 is replaced by Gln), $IX_{VIIhelix}$ (a replacement mutant in which helix 330-338 (c162-170) is replaced by that of factor VII) and IX_{PCEGF1} (a replacement mutant in which EGF1 domain is replaced by that of protein C) were constructed, expressed and purified as described earlier (Mather and Bajaj, 1999; Zhong et al., 1994; Zhong and Bajaj, 1993). Purified proteins were homogeneous on SDS-PAGE and contained normal Gla content (Mather and Bajaj, 1999; Zhong et al., 1994).

[0169] Preparation of dEGR-ck Inhibited Factor IXa Proteins.

[0170] Each factor IX protein was activated at 200 μg/ml by factor XIa (2 μg/ml) for 90 min. The buffer used was TBS, pH 7.5 (50 mM Tris, 5 mM NaCl, pH 7.5) containing 5 mM $CaCl_2$. SDS-PAGE analysis revealed full activation of each factor to factor IX to factor IXa without degradation in the autolysis loop (Mather et al., 1997). dEGR-IXa_{WT}, and various dEGR-IXa mutant proteins free of dEGR-ck were obtained as described previously (Mather et al., 1997).

[0171] Activation of Factor X by Each Factor IXa Protein in the Presence of Only Ca^{2+} and PL.

[0172] For these studies, each factor IX was activated with factor XIa/Ca^{2+} as described above. For factor X activation studies, the concentration of factor IXa was kept at 20 nM and the buffer used was TBS/BSA (TBS with 200 mg/ml BSA) containing 5 mM $CaCl_2$. The concentrations of PL used were 10, 25, 50, and 100 μM in different sets of experiments. The concentration of factor X at each PL concentration ranged from 25 nM to 3 μM. The activations were carried out for 5-15 min and the amount of factor Xa generated was measured by hydrolysis of S-2222 as described previously (Mather and Bajaj, 1999; Mather et al., 1997). The Km and kcat values were obtained using the program GraFit from Erithacus Software.

[0173] Determination of EC_{50} of Interaction of Factor IXa Proteins with A2 Subunit.

[0174] The EC_{50} (functional Kd) of binding of each factor IXa protein with A2 subunit was measured essentially as described previously for its interaction with intact factor VIIIa (Mather and Bajaj, 1999; Mather et al., 1997). For these experiments, concentrations of factor IXa and factor X were kept constant, and the rates of formation of factor Xa were determined at increasing concentrations of A2 subunit. Reaction mixtures contained 5 nM IXa protein, 250 nM factor X, 25 μM PL and various concentrations of A2 subunit in TBS/BSA, pH 7.5 containing 5 mM $CaCl_2$. Reactions were carried out at 37° C. for 5-20 minutes and stopped by adding 1 μL of 500 mM EDTA. The amount of factor Xa generated was determined by S-2222 hydrolysis as described previously (Mather and Bajaj, 1999; Mather et al., 1997). The EC_{50} was obtained by fitting the data to a single site ligand binding equation (Eq. 1) by non-linear regression analysis using the program GraFit from Erithacus Software.

$$V = \frac{V_{max} L}{EC_{50} + L} \quad \text{(Eq. 1)}$$

[0175] Where V is the rate of formation of factor Xa at a given concentration of the A2 subunit, denoted by L, and V_{max} is the rate of factor Xa formation by the factor IXa:A2 subunit complex. EC_{50} is the functional Kd defined as the concentration of free A2 subunit yielding 50% of the V_{max}. The background rate of factor Xa generation was obtained by carrying out the reaction in the absence of the A2 subunit. This represented less than 1% of the V_{max} and was subtracted before data analysis. To obtain EC_{50} values as a function of substrate concentration, a series of experiments were performed in which factor X was varied from 50 nM to 1 μM.

[0176] Determination of Kd_{A2} of Binding of dEGR-IXa Proteins to A2 Subunit.

[0177] The apparent Kd (termed Kd_{A2}) for binding of each dEGR-IXa protein to the A2 subunit was determined by its ability to inhibit factor IXa_{WT}:A2 subunit interaction in the tenase complex as described earlier for intact factor VIIIa (Mather and Bajaj, 1999; Mather et al., 1997). The reactions

were carried out as described for the EC_{50} experiments above except dEGR-IXa and IXa_{WT} were mixed prior to addition of the A2 subunit; this ensured steady state conditions. Mixtures contained 100 nM IXa_{WT}, 30 nM A2 subunit, 250 nM factor X, 25 μM PL, and various concentrations of dEGR-IXa proteins in TBS/BSA, pH 7.5 containing 5 mM $CaCl_2$. The IC_{50} (concentration of inhibitor required for 50% inhibition) was determined by fitting the data to the IC_{50} four-parameter logistic equation of Halfman (1981) given below (Eq. 2).

$$y = \frac{a}{1 + (x/IC_{50})^s} + \text{background} \quad \text{(Eq. 2)}$$

[0178] where y is the rate of Xa formation in the presence of a given concentration of dEGR-IXa protein represented by x, a is the maximum rate of factor Xa formation in the absence of dEGR-IXa, and s is the slope factor. Each point was weighted equally, and the data were fitted to Equation 2 using the nonlinear regression analysis program GraFit from Erithacus Software. The background value represented ~5% of the maximum rate of Xa formation in the absence of dEGR-IXa. To obtain Kd_{A2} values for the interaction of dEGR-IXa proteins with A2, we used the following equation (Eq. 3) as described by Cheng and Prusoff (1973) and further elaborated by Craig (1993).

$$Kd_{A2} = \frac{IC_{50}}{1 + (A/EC_{50})} \quad \text{(Eq. 3)}$$

[0179] where A is the concentration of IXa_{WT}, and EC_{50} is the concentration of factor IXa_{WT} that gives a 50% maximum response in the absence of the competitor at a specified concentration of factor X used in the experiment.

[0180] Fluorescence Quenching of the Dansyl Moiety in dEGR-IXa by the A2 Subunit.

[0181] The effect of the A2 subunit on the emission intensity of the dansyl moiety in each dEGR-IXa protein was determined using the SLM AB2 spectrofluorometer. Each reaction mixture contained 220 nM dEGR-IXa in 20 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween, 200 μg/ml BSA and 100 μM PL vesicles. The excitation wavelength was 340 nm (slit width, 8 nm) and the emission wavelength was 540 nm (slit width; 8 nm). First, blank values (in triplicate) were obtained for the buffer containing PL. dEGR-IXa was then added and the emission intensity in the absence of the A2 subunit was recorded. Each reaction mixture was subsequently titrated with the A2 subunit and the emission readings (in triplicate) were obtained at each time point. The fluorescence emission intensity at each point was corrected for increases in the reaction volume prior to analysis of the data. The volume of added A2 subunit did not exceed 10% of the total volume. Data are presented as F/F_0 where F_0 is the emission intensity in the absence of A2 subunit and F is the intensity at a given A2 subunit concentration.

[0182] Determination of the Apparent $Kd_{peptide}$ of Binding of Each Factor IXa to the A2 558-565 Peptide.

[0183] The apparent Kd (termed $Kd_{peptide}$) for binding of each factor IXa to the A2 558-565 peptide was determined by its ability to inhibit the respective IXa:A2 subunit interaction as measured by reduction in the rate of factor X activation in the tenase system. The reaction mixtures for both IXa_{WT} and IXa_{PCEGF1} contained 100 nM factor IXa, 30 nM A2 subunit, 250 nM factor X, and 25 μM PL in TBS/BSA, pH 7.5 with 5 mM $CaCl_2$. The reaction mixture for IXa_{R333Q} contained 300 nM factor IXa instead of 100 nM used for IXa_{WT} or IXa_{PCEGF1}; concentrations of other components were the same. The amount of factor Xa generated was determined by hydrolysis of S-2222. The IC_{50} values were obtained using Eq. 2. Here, y is the rate of factor Xa formation in the presence of a given concentration of the A2 558-565 peptide represented by x, and a is the maximum rate of factor Xa formation in the absence of the A2 peptide. Eq. 3 was then used to obtain the apparent $Kd_{peptide}$ values. In this context, A is the concentration of IXa_{WT}, IXa_{PCEGF1}, or IXa_{R333Q}, and EC_{50} is the apparent Kd_{A2} for the respective IXa:A2 subunit interaction.

[0184] Molecular Modeling.

[0185] The three (A1, A2, and A3) domains in factor VIIIa are homologous to the three respective domains in ceruloplasmin (Pemberton et al., 1997; Church et al., 1984). The A1, A2, and A3 domains in factor VIIIa were modeled using the coordinates of each respective domain of ceruloplasmin (Zaitseva et al., 1996). Each domain was modeled using the homology model building module from Biosym/MSI, San Diego, Calif. as well as the Swiss-Model server using the optimize mode (Peitsch, 1996; Guex et al., 1999). The two approaches used in building the homology models resulted in minor differences between the structure of each of the A subunits. However, the structure pertaining to the loop containing the 3_{10} helical turn involving residues 558-565 (Region 1) as well as the other interface regions of the A2 subunit implicated in binding to factor IXa (Regions 2 and 3) were invariant between the two models. Further, the Biosym/MSA models of all three A subunits were similar to those published earlier by Pemberton et al. (1997). Thus, we used the coordinates of Pemberton et al. (1997; also given at the Hemostasis Research Group web site, http://europium.c-sc.mrc.ac.uk/usr/WWW/WebPages/main.dir/main.htm) in building the interface between the A2 subunit and the protease domain of factor IXa. Details are provided in the Results and Discussion section.

[0186] Results and Discussion

[0187] Activation of Factor X by Various Factor IXa Proteins in the Presence of Only Ca^{2+} and PL.

[0188] The kinetic constants for the activation of factor X were obtained by various factor IXa proteins in the presence of Ca^{2+} and several concentrations of PL. This analysis was performed to establish whether or not the factor IXa proteins under investigation bind to Ca^{2+} and PL normally and possess a functional active site. The kinetic constants obtained in the absence of factor VIIIa are listed in Table 2. All mutants activated factor X normally and the specificity constant (kcat/Km) for each mutant at different PL concentrations did not differ appreciably from that observed for IXa_{WT} or IXa_{NP}. The increase in Km values at higher concentrations of PL for WT or for a given mutant may reflect binding of factor IXa and factor X to different PL vesicles (Mann et al., 1988; van Dieijen et al., 1981). Further, our Km and kcat values are in close agreement with the earlier published data (Fay and Koshibu, 1998; van Dieijen et al.

1981). Consistent with earlier observations (van Dieijen et al. 1981), we also observed a slight increase in kcat for each factor IXa protein at higher concentrations of PL. Cumulatively, our data presented in Table 2 indicate that the factor IXa mutants under investigation interact with Ca^{+2} and PL normally. Further, in the absence of factor VIIIa, activation of factor X by these IXa mutants is not impaired.

TABLE 2

Kinetic parameters of factor X activation in the absence of factor VIIIa. The concentration of each reagent in the reaction mixture was: 20 nM factor IXa, 5 mM $CaCl_2$, and varying concentrations of factor X ranging from 25 nM to 3 μM.

Protein	PL (μM)	Km (μM)	kcat (min^{-1})	kcat/Km (μM^{-1} min^{-1})
IXa_{NP}	10	0.10	0.012	0.108
	25	0.16	0.022	0.138
	50	0.21	0.032	0.156
	100	0.63	0.062	0.098
IXa_{WT}	10	0.09	0.010	0.110
	25	0.12	0.012	0.102
	50	0.24	0.024	0.100
	100	0.57	0.038	0.066
IXa_{PCEGF1}	10	0.13	0.010	0.076
	25	0.23	0.018	0.080
	50	0.26	0.028	0.110
	100	0.54	0.038	0.070
IXa_{R333Q}	10	0.11	0.012	0.108
	25	0.18	0.018	0.100
	50	0.23	0.030	0.130
	100	0.61	0.040	0.066
$IXa_{VIIhelix}$	10	0.12	0.014	0.116
	25	0.20	0.020	0.100
	50	0.25	0.028	0.112
	100	0.55	0.042	0.076

[0189] A42 Subunit Mediated Enhancement of Factor X Activation by Various IXa Mutants.

[0190] In this section, we evaluated the ability of the A2 subunit to augment factor X activation by various factor IXa mutants. These data are presented in **FIG. 1**. The presence of the A2 subunit in the reaction mixtures enhanced the factor X-activating activity of IXa_{PCEGF1} to the same extent as that of IXa_{WT}. However, the ability of the A2 subunit to potentiate the activity of IXa_{R333Q} was severely impaired and it was absent for the $IXa_{VIIhelix}$. Next, we determined the EC_{50} (functional Kd) values for interaction of each factor IXa protein with the A2 subunit using Eq. 1. Fitting the data to a single site-binding model yielded an apparent Kd of 257±31 for both IXa_{WT} and IXa_{PCEGF1}; for IXa_{R333Q} or $IXa_{VIIhelix}$, it could not be calculated. These data strongly indicate that the helix 330 (c162) of factor IXa interacts with the A2 subunit of VIIIa.

[0191] In further experiments, we measured the EC_{50} values for interaction of IXa_{WT} and of IXa_{PCEGF1} with the A2 subunit using different concentrations of factor X ranging from 50 nM to 5 μM. These data are presented in **FIG. 2**. At each concentration of factor X, the concentration of IXa was held constant at 5 nM and the rate of factor Xa generation was determined in the presence of increasing concentrations of the A2 subunit. The EC_{50} values ranged from ~300 nM at lower concentrations of factor X (<150 nM) to ~200 nM at higher concentrations of factor X (>1 μM) for both IXa_{WT} and IXa_{PCEGF1}. Our functional Kd (EC_{50}) values ranging from 200-300 nM for the interaction of IXa_{WT} (or IXa_{PCEGF1}) and the A2 subunit employing different factor X concentrations are consistent with the EC_{50} values obtained earlier using similar conditions for IXa_{NP} and the A2 subunit (Fay and Koshibu, 1998). From these observations, we conclude that factor X does not appreciably influence the functional Kd of IXa:A2 subunit interaction. This is in contrast to the results obtained using factor VIIIa where factor X reduces the functional Kd of IXa:VIIIa interaction by ~10-fold (Mather et al., 1997). More importantly, our data with the IXa_{PCEGF1} mutant indicate that the EGF1 domain of factor IXa does not interact with the A2 subunit of factor VIIIa.

[0192] Determination of Apparent Kd_{A2} Values for the Interaction of A2 Subunit with dEGR-IXa Proteins.

[0193] Here, we investigated the steady state inhibition of IXa_{WT}:A2 interaction by different dEGR-IXa proteins. These data are presented in **FIG. 3**. The IC_{50} values were obtained using Equation 2 and the respective apparent Kd_{A2} values were obtained using Equation 3. dEGR-IXa_{WT} and dEGR-IXa_{PCEGF1} interacted with the A2 subunit with a similar Kd_{A2} of ~100 nM, whereas dEGR-IXa_{R333Q} interacted with the A2 subunit with a Kd_{A2} of ~1.8 μM and dEGR-$IXa_{VIIhelix}$ failed to compete with IXa_{WT} up to 12 μM concentration. The apparent Kd_{A2} (~100 nM) obtained from the inhibition data (**FIG. 3**) and EC_{50} values (~250 nM) obtained from the potentiation of factor X activation data (**FIGS. 1 and 2**) for the factor IXa_{WT} and IXa_{PCEGF1} are in close agreement with each other. Of significance is the observation that the mutations in the helix 330 (c162) of the protease domain of factor IXa severely impairs its interaction with the A2 subunit.

[0194] Effects of A2 subunit on the Fluorescence Emission of dEGR-IXa Proteins.

[0195] Since dansyl emission is quite sensitive to its environment, we examined the changes in dansyl emission intensity (excitation wavelength, 340 nm and emission wavelength, 540 nm) of dEGR-IXa proteins in the presence of increasing concentrations of the A2 subunit. Reaction mixtures contained 220 nM of each dEGR-IXa protein, 100 μM PL and various concentrations of the isolated A2 subunit. The results are presented in **FIG. 4**. For IXa_{WT} and IXa_{PCEGF1}, a dose-dependent decrease in the fluorescence emission of the dansyl probe was observed. However, little if any change in the emission intensity was observed when the A2 subunit was titrated into the reaction mixtures containing factor IXa_{R333Q} or $IXa_{VIIhelix}$. A nonlinear least squares fitting to the data for IXa_{WT} or IXa_{PCEGF1} to a bimolecular association model yielded a plateau value of 0.59±0.05 for F/F_0 and an apparent Kd_{A2} value of ~100 nM for each protein. These results suggest that the isolated A2 subunit interacts equivalently with these two forms of factor IXa, similarly modulating the emission of the active site-labeled dansyl probe. The apparent Kd_{A2} value of ~100 nM for factor IXa_{WT} or IXa_{PCEGF1} obtained using the fluorescence quenching measurements is in agreement with the values obtained from steady state experiments. Consistent with the data presented in **FIGS. 1 and 3**, these fluorescence results suggest that the factor IXa_{R333Q} and $IXa_{VIIhelix}$ mutants are severely impaired in their interactions with the A2 subunit.

[0196] Determination of Apparent $Kd_{peptide}$ Values for Binding of Factor IXa Proteins to the A2 558-565 Peptide.

[0197] The data presented thus far strongly indicate that the A2 subunit interacts with the residues of the helix 330

(c162) of factor IXa. Previous studies also suggest that residues 558-565 of the A2 subunit are involved in binding to factor IXa (Fay and Koshibu, 1998). However, it is not known whether the 558-565 peptide region of the A2 subunit represents the site of direct interaction with the helix 330 of factor IXa. We investigated this possibility by measuring the affinity of the A2 558-565 peptide for IXa_{WT}, IXa_{PCEGF1}, and IXa_{R333Q}. These data are presented in **FIG. 5**. The A2 558-565 peptide inhibits the interaction of IXa_{WT} and IXa_{PCEGF1} with similar IC_{50} values of ~8 μM. The present IC_{50} value [~8 μM] for the A2 peptide inhibition of the IXa_{WT}:A2 subunit interaction is five-fold lower than the IC_{50} value [~40 μM] obtained from the inhibition studies of the A2 subunit enhancement of IXa_{NP} activity [Fay and Koshibu, 1998]. The difference in IC_{50} values is most likely due to the different concentrations (30 nM in the present study vs. 240 nM in the previous study) of the A2 subunits used in the two studies. However, the A2 558-565 peptide inhibited the IXa_{R333Q}:A2 subunit interaction with an IC_{50} value of ~70 μM, which is ~9-fold higher than the value obtained for IXa_{WT} or IXa_{PCEGF1} (**FIG. 5**). The Cheng and Prusoff relationship (Cheng and Prusoff, 1973; Craig, 1993) was then used to obtain apparent $Kd_{peptide}$ values for each factor IXa protein. These apparent $Kd_{peptide}$ values along with the changes in Gibbs free energy are listed in Table 3. Notably, the increase in apparent Kd_{A2} or $Kd_{peptide}$ for IXa_{R333Q} is similar (~15-fold) as compared to the apparent Kd_{A2} or $Kd_{peptide}$ obtained for IXa_{WT} (or IXa_{PCEGF1}). Further, the difference in ΔG° for the interaction of the A2 subunit with IXa_{WT} (or IXa_{PCEGF1}) and IXa_{R333Q} is 1.72 kcal mol^{-1}. This difference in ΔG° is essentially the same as that (1.62 kcal mol^{-1}) obtained for the interaction of A2 peptide with IXa_{WT} (or IXa_{PCEGF1}) and IXa_{R333Q}. If the A2 558-565 peptide bound to a different region than the helix 330 of factor IXa, then one would expect it to bind to IXa_{R333Q} with the same affinity as that for IXa_{WT}. Since this is not the case, our data support a conclusion that the helix 330 (c162) in factor IXa is most likely in direct contact with the 558-565 region of the A2 subunit.

TABLE 3

Apparent Kd and Gibbs free energy values for the interaction of various factor IXa proteins with the A2 subunit and the A2 558–565 peptide. Apparent Kd_{A2} values are from FIG. 3 and apparent $Kd_{peptide}$ values are from FIG. 5.

Protein	App Kd_{A2} – nM	ΔG°_{A2}[b] kcal mol^{-1}	App $Kd_{peptide}$ – μM	$\Delta G^\circ_{peptide}$[b] kcal mol^{-1}
IXa_{WT}	100 ± 11	9.54	4 ± 1	7.36
IXa_{PCEGF1}	114 ± 15(1)[a]	9.47(0.07)[c]	4 ± (1)[a]	7.36(0.00)[c]
IXa_{R333Q}	1850 ± 82(18)	7.82(1.72)	62 ± 9(15)	5.74(1.62)
$IXa_{VIIhelix}$	$>10^3$	ND[d]	ND	ND

[a]The fold-change in apparent Kd values (mutant/WT) is given in parentheses.
[b]Gibbs free energy values were calculated using the equation, $\Delta G^\circ = RT\ln Kd$, where R is the gas constant (1.987×10^{-3} kcal mol^{-1} deg^{-1}), T is the absolute temperature (298 Kelvin), and Kd is the dissociation constant.
[c]The change in ΔG° values between the mutant and WT is given in parentheses.
[d]ND, not determined

[**0198**] Modeling of the Interface Between the Protease Domain of Factor IXa and the A2 Subunit of Factor VIIIa.

[**0199**] Based upon the preceding information, we modeled the interface between the protease domain of factor IXa (Hopfner et al., 1999; PDB code 1RFN) and the A2 subunit (see Experimental Procedures) by bringing together the helix 330 of factor IXa and the 3_{10} helical turn in residues 558-565 of the A2 subunit and maximizing the interaction among the charged residues. Emphasis was also given for interactions involving hydrogen bonds and hydrophobic contacts. An important guiding principle in the construction of this interface model was that the Gla domain of factor IXa and the C2 domain of factor VIIIa must be oriented such that each may contact the PL surface. To achieve this, the A2 structure (along with the A1 and A3 subunits) was rotated and translated as a rigid body. The principal approach used was that described earlier by Tulinsky and coworkers in building the prothrombin model from the structures of fragment 1 and prethrombin (Arni et al, 1994). Minor adjustments in the side chains of both proteins were also made. All residues in the interface of both proteins were checked for distances to insure no improper contacts (Laskowski et al., 1993). The interface model that resulted from this approach is shown in **FIG. 6A**. In this display, the Gla domain of factor IXa and the C2 domain of factor VIIIa are projecting away from the viewer.

[**0200**] In addition to the A2 558-565 region and the factor IXa 330-338 region, two other regions that apparently also play a role in the interaction of A2 subunit and the protease domain (Region 2 and Region 3) were identified. The details of the three interface regions are shown in **FIG. 6B**. It appears that electrostatic forces might play a significant role in the interaction between the A2 subunit and the protease domain, and an electrostatic potential for the interface calculated using the program GRASP (Arni et al., 1994) is shown in **FIG. 6C**. Further, in addition to the electrostatic interactions outlined in **FIG. 6**, hydrophobic and polar uncharged interactions between T343 (c175) and Y345 (c177) of factor IXa and H444 of the A2 subunit were observed. Moreover, a hydrogen bond between N258 (c93) of factor IXa and S709 of the A2 subunit could also be formed. Importantly, a significant hydrophobic patch involving I566 and M567 in the A2 subunit and I298 (c129B), Y295 (c128), F299 (c130), F302 (c133), F378 (c208), and F98 (EGF2 domain) in factor IXa was noted. Thus, it appears that the hydrogen bonds as well as the hydrophobic and electrostatic interactions all play important roles in the interface between factor IXa and the A2 subunit. In this context, an apparent Kd_{A2} of ~100 nM observed for this interaction reflects the net change in free energy involved in making and breaking such bonds.

[**0201**] A factor IXa-interactive site comprised of residues 484-509 in the A2 subunit that was identified using a monoclonal antibody (Fay and Scandella, 1999) does not appear to contact the protease domain in the interface model. However, it should be noted that in a previous study, Lollar et al. (1994) concluded that this same monoclonal antibody does not interfere with the IXa:VIIIa interaction. The reason(s) for the differing results obtained in the two studies (Fay and Scandella, 1999 and Lollar et al., 1994) is not fully understood. Further in the interface model shown in **FIG. 6A**, the 484-509 region in the A2 subunit is not in close proximity to the 558-565 interface region. Therefore it is likely that this monoclonal antibody prevents the association of the A2 subunit with factor IXa through steric interference.

[**0202**] Analysis of Hemophilia Databases.

[**0203**] Of significance is the observation that numerous mutations in the helix 330 (c162) of factor IXa cause

hemophilia B (Green et al., 2000; Mathur and Bajaj, 1999) while several mutations in or near factor VIII residues 558-565 result in hemophilia A (Hemostasis Research Group, 2000). Arg333 (c1 65) in Region 1 of our interface model (**FIG. 6**) interacts with the Asp560 residue of the A2 subunit, and mutations in the Arg333 (c165) residue that eliminate the charge (e.g., Arg Glu or Leu) cause severe hemophilia B (Green et al., 2000). Further, Asn346 (c178) of factor IXa interacts with both Lys570 and Glu445 of the A2 subunit, and a mutation of Asn346 (c178) to Asp causes hemophilia B (Green et al., 2000). Similarly, Arg403 (c233) in our model interacts with Glu633 of the A2 subunit and mutations in Arg403 (c233) to Trp or Gln cause hemophilia B (Green et al., 2000). Moreover, Arg338 (c 170) of factor IXa interacts with Asp560 of the A2 subunit and mutations in both of these residues result in hemophilia (Hemostasis Research Group, 2000; Green et al., 2000). In addition, Arg562 contained within the A2 558-565 peptide region is cleaved by activated protein C (Fay et al., 1991b) and factor IXa selectively protects this site from cleavage (Regan et al., 1994). In support of this observation, Arg562 of the A2 subunit along with Gln561 interacts with Asp332 (c164) in our interface model and the mutation Asp332 (c164) to Tyr results in hemophilia B (Green et al., 2000).

[**0204**] Mutations in the hydrophobic patch of the interface model are also known to cause bleeding diathesis. Thus, change of Phe378 (c208) to Val or Leu in factor IXa causes hemophilia B (Green et al., 2000), and change of Ile566 to Thr in the A2 subunit causes hemophilia A (Hemostasis Research Group, 2000). Moreover, change of Phe302 (c133) to Ala has been shown to impair the interaction of factor IXa with factor VIIIa (Kolkman et al., 1999). The change of Phe302 (c133) to Ala and Phe378 (c208) to Val or Leu are expected to diminish the hydrophobic interactions involving Ile566 and Met567 of the A2 subunit. Although the change of Ile566 to Thr in the A2 subunit yields a dysfunctional factor VIII by creation of a new N-linked glycosylation site at Asn564 (Hemostasis Research Group, 2000) this mutation would also diminish hydrophobic interactions.

[**0205**] Concluding Remarks.

[**0206**] Previous studies have indicated that the helix 330 (c162) of the protease domain (Mather and Bajaj, 1999) and 558-565 region of the A2 subunit (Fay and Koshibu, 1998) represent important determinants for the interaction of IXa and VIIIa, respectively. However, it was not known whether these two regions interact with each other in the IXa/VIIIa complex. The present study provides evidence that these two regions form an interface and interact with each other through hydrophobic as well as electrostatic forces (region 1 in **FIG. 6B**). Modeling of the interface indicates that two other regions (regions 2 and 3) also participate in the interaction of IXa with VIIIa. Several mutations in the proposed interface cause hemophilia A or B and are known to impair the IXa:VIIIa interaction. Thus, our interface model is compatible with the existing biochemical as well as with the two-dimensional electron crystallography data of Stoylova et al. (1999). We would expect that the three-dimensional cocrystal structure of the factor IXa protease domain and the A2 subunit would support this view.

EXAMPLE 2

Polypeptides that are Envisioned to Function as Anti-Coagulation Agents

[**0207**] Determination of the EC_{50} of for Factor IXa-Factor VIIIa Interaction in the Tenase Complex

[**0208**] These experiments are performed with normal factor IXa in the presence of phospholipid (PL) vesicles. 50-μl reaction mixtures in TBS-BSA (50 mM Tris-HCl, pH 7.4, 1 mg/ml BSA) with 5 mM $CaCl_2$ and 10 μM PL are prepared containing a fixed concentration of factor VIIIa (0.07 nM) and various concentrations of factor IXa (0-20 nM) at a constant concentration of factor X (480 nM), which is added last to initiate the reaction. The reaction is carried out at 37° C. for 30-120 seconds at which time 1 μl of 0.5 M EDTA is added to stop further generation of factor Xa. A 40 μl aliquot is added to a 0.1-mL quartz cuvette containing a synthetic substrate S-2222 in 75 μl of TBS-BSA, pH 7.4. The final concentration of S-2222 is 100 μM. The p-nitroaniline release is measured continuously (ΔA_{405}/min) for up to 20 minutes (Mathur et al., 1997). Factor Xa generated is calculated from a standard curve constructed using factor Xa prepared by insolubilized Russell's viper venom. In control experiments, at each factor X concentration used, the rate of factor Xa generation is also measured at various concentrations of normal factor IXa in the absence of factor VIIIa; these control values are ~5% of the experimental values in the presence of factor VIIIa and are subtracted before analysis of the data. The EC_{50} (functional Kd) is defined as the free concentration of normal factor IXa which provides 50% of the V_{max}. The EC_{50} is obtained by fitting the data to a single site ligand binding equation (Eq. 1) by non-linear regression analysis using the program GraFit from Erithacus Software.

$$V = \frac{V_{max} L}{EC_{50} + L} \qquad \text{(Eq. 1)}$$

[**0209**] Where V is the rate of formation of factor Xa at a given concentration of normal factor IXa, denoted by L, and V_{max} is the rate of factor Xa formation by the factor IXa:factor VIIIa complex. The EC_{50} obtained under above conditions is ~0.4 nM. This EC_{50} value is used to calculate the IC value for each competitor (e.g., peptide) employed.

[**0210**] Determination of the Apparent $Kd_{peptide}$ of Binding of (1) Factor VIIIa to Each Factor IXa Peptides (SEQ ID NOS:13-16) and Factor IXa to Each of Factor VIIIa Peptides (SEQ ID NOS:9-12)

[**0211**] The apparent Kd (termed $Kd_{peptide}$) for binding of (1) factor VIIIa to the factor IXa peptides of SEQ ID NO:13, 14, 15 or 16; or of (2) factor IXa to the factor Via peptides of SEQ ID NO:9, 10, 11 or 12 is determined by its ability to inhibit the IXa:VIIIa interaction as measured by reduction in the rate of factor X activation in the tenase system. The reaction mixture for normal factor IXa contains 0.2 nM factor IXa, 0.07 nM factor VIIIa, 480 nM factor X, and 10 μM PL in TBS/BSA, pH 7.4 with 5 mM $CaCl_2$ and increasing amounts of the peptide. The amount of factor Xa generated is determined by hydrolysis of S-2222. The IC_{50} (concentration of inhibitor required for 50% inhibition) is

determined by fitting the data to IC_{50} four-parameter logistic equation of Halfman (1981) given below.

$$y = \frac{a}{1 + (x/IC_{50})^s} + \text{background} \quad \text{(Eq. 2)}$$

[0212] y is the rate of factor Xa formation in the presence of a given concentration of inhibitory peptide represented by x, a is the maximum rate of factor Xa formation in the absence of inhibitory peptide, and s is the slope factor. Each point is weighted equally, and the data are fitted to Eq. 2 using the nonlinear regression analysis program GraFit from Erithacus Software. The background value represents ~5% of the maximum rate of factor Xa formation in the absence of an inhibitory peptide. To obtain an apparent $Kd_{peptide}$ value for the interaction of Factor VIIIa with Factor IXa, the following equation is employed as described by Cheng and Prusoff (1973) and further elaborated by Craig (1993)

$$Kd_{peptide} = \frac{IC_{50}}{1 + (A/EC_{50})} \quad \text{(Eq. 3)}$$

[0213] A is the concentration of normal factor IXa (0.2 nM), and EC_{50} is the concentration of normal factor IXa that gives a 50% maximum response in the absence of the competitor (0.4 nM) at a specified concentration of factor X (480 nM) used in the experiment.

[0214] The inventor envisions that any one of the peptides of SEQ ID NOS:9-16 inhibits the interaction of IXa with the factor VIIIa A2 subunit at an IC_{50} value of less than 400 μM.

[0215] All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

[0216] In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained. As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

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             20                  25                  30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
         35                  40                  45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
     50                  55                  60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
 65                  70                  75                  80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                 85                  90                  95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130                 135                 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160
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His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                165                 170                 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180                 185                 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195                 200                 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210                 215                 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                245                 250                 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275                 280                 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                325                 330                 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
            340                 345                 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370                 375                 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560
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Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
               565                 570                 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
               645                 650                 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
               725                 730                 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
            740                 745                 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
               805                 810                 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
        835                 840                 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850                 855                 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865                 870                 875                 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
               885                 890                 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
        915                 920                 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930                 935                 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
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                965                 970                 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
        995                 1000                1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu Asn
    1010                1015                1020

Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu Phe Lys
1025                1030                1035                1040

Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp Lys Asn Ala
                1045                1050                1055

Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr Thr Ser Ser Lys
            1060                1065                1070

Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly Pro Ile Pro Pro Asp
        1075                1080                1085

Ala Gln Asn Pro Asp Met Ser Phe Phe Lys Met Leu Phe Leu Pro Glu
    1090                1095                1100

Ser Ala Arg Trp Ile Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser
1105                1110                1115                1120

Gly Gln Gly Pro Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys
                1125                1130                1135

Ser Val Glu Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val
            1140                1145                1150

Gly Lys Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe
        1155                1160                1165

Pro Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu
    1170                1175                1180

Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys
1185                1190                1195                1200

Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr
                1205                1210                1215

Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr
            1220                1225                1230

Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu
        1235                1240                1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys His
    1250                1255                1260

Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu Gly Leu
1265                1270                1275                1280

Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys Thr Thr Arg
                1285                1290                1295

Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr Gln Arg Ser Lys
            1300                1305                1310

Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu Glu Thr Glu Leu Glu
        1315                1320                1325

Lys Arg Ile Ile Val Asp Asp Thr Ser Thr Gln Trp Ser Lys Asn Met
    1330                1335                1340

Lys His Leu Thr Pro Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys
1345                1350                1355                1360

Glu Lys Gly Ala Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg
                1365                1370                1375
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Ser His Ser Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys
            1380                1385                1390

Val Ser Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu
        1395                1400                1405

Phe Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys
    1410                1415                1420

Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys
1425                1430                1435                1440

Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln
                1445                1450                1455

Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr
            1460                1465                1470

Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr
        1475                1480                1485

Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys Asp
    1490                1495                1500

Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu Asp Leu
1505                1510                1515                1520

Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile Lys Trp Asn
                1525                1530                1535

Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg Val Ala Thr Glu
            1540                1545                1550

Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp Pro Leu Ala Trp Asp
        1555                1560                1565

Asn His Tyr Gly Thr Gln Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu
    1570                1575                1580

Lys Ser Pro Glu Lys Thr Ala Phe Lys Lys Lys Asp Thr Ile Leu Ser
1585                1590                1595                1600

Leu Asn Ala Cys Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly
                1605                1610                1615

Gln Asn Lys Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr
            1620                1625                1630

Glu Arg Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg
        1635                1640                1645

Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
    1650                1655                1660

Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr
1665                1670                1675                1680

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
                1685                1690                1695

His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
            1700                1705                1710

Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
        1715                1720                1725

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
    1730                1735                1740

Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
1745                1750                1755                1760

Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
                1765                1770                1775
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Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr
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Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
        1795                1800                1805

Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
    1810                1815                1820

Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
1825                1830                1835                1840

Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
                1845                1850                1855

Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr
            1860                1865                1870

Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
        1875                1880                1885

Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
    1890                1895                1900

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala
1905                1910                1915                1920

Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln
                1925                1930                1935

Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn
            1940                1945                1950

Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
        1955                1960                1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu
    1970                1975                1980

Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys
1985                1990                1995                2000

Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val
                2005                2010                2015

Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile
            2020                2025                2030

Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
        2035                2040                2045

Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr
    2050                2055                2060

Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile
2065                2070                2075                2080

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu
                2085                2090                2095

Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
            2100                2105                2110

Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly
        2115                2120                2125

Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
    2130                2135                2140

Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
2145                2150                2155                2160

Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
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Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala
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Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
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Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn
    2210                2215                2220

Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val
2225                2230                2235                2240

Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
                2245                2250                2255

Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr
            2260                2265                2270

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
        2275                2280                2285

Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg
    2290                2295                2300

Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
2305                2310                2315                2320

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                2325                2330


<210> SEQ ID NO 2
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Glu Asn Thr Glu Arg Thr Thr Glu Phe Trp Lys Gln Tyr Val Asp Gly
         35                  40                  45

Asp Gln Cys Glu Ser Asn Pro Cys Leu Asn Gly Gly Ser Cys Lys Asp
     50                  55                  60

Asp Ile Asn Ser Tyr Glu Cys Trp Cys Pro Phe Gly Phe Glu Gly Lys
 65                  70                  75                  80

Asn Cys Glu Leu Asp Val Thr Cys Asn Ile Lys Asn Gly Arg Cys Glu
                 85                  90                  95

Gln Phe Cys Lys Asn Ser Ala Asp Asn Lys Val Val Cys Ser Cys Thr
            100                 105                 110

Glu Gly Tyr Arg Leu Ala Glu Asn Gln Lys Ser Cys Glu Pro Ala Val
        115                 120                 125

Pro Phe Pro Cys Gly Arg Val Ser Val Ser Gln Thr Ser Lys Leu Thr
    130                 135                 140

Arg Ala Glu Thr Val Phe Pro Asp Val Asp Tyr Val Asn Ser Thr Glu
145                 150                 155                 160

Ala Glu Thr Ile Leu Asp Asn Ile Thr Gln Ser Thr Gln Ser Phe Asn
                165                 170                 175

Asp Phe Thr Arg Val Val Gly Gly Glu Asp Ala Lys Pro Gly Gln Phe
            180                 185                 190

Pro Trp Gln Val Val Leu Asn Gly Lys Val Asp Ala Phe Cys Gly Gly
        195                 200                 205
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Ser Ile Val Asn Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Glu
    210                 215                 220

Thr Gly Val Lys Ile Thr Val Val Ala Gly Glu His Asn Ile Glu Glu
225                 230                 235                 240

Thr Glu His Thr Glu Gln Lys Arg Asn Val Ile Arg Ile Ile Pro His
                245                 250                 255

His Asn Tyr Asn Ala Ala Ile Asn Lys Tyr Asn His Asp Ile Ala Leu
            260                 265                 270

Leu Glu Leu Asp Glu Pro Leu Val Leu Asn Ser Tyr Val Thr Pro Ile
        275                 280                 285

Cys Ile Ala Asp Lys Glu Tyr Thr Asn Ile Phe Leu Lys Phe Gly Ser
    290                 295                 300

Gly Tyr Val Ser Gly Trp Gly Arg Val Phe His Lys Gly Arg Ser Ala
305                 310                 315                 320

Leu Val Leu Gln Tyr Leu Arg Val Pro Leu Val Asp Arg Ala Thr Cys
                325                 330                 335

Leu Arg Ser Thr Lys Phe Thr Ile Tyr Asn Asn Met Phe Cys Ala Gly
            340                 345                 350

Phe His Glu Gly Gly Arg Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro
        355                 360                 365

His Val Thr Glu Val Glu Gly Thr Ser Phe Leu Thr Gly Ile Ile Ser
    370                 375                 380

Trp Gly Glu Glu Cys Ala Met Lys Gly Lys Tyr Gly Ile Tyr Thr Lys
385                 390                 395                 400

Val Ser Arg Tyr Val Asn Trp Ile Lys Glu Lys Thr Lys Leu Thr
                405                 410                 415


<210> SEQ ID NO 3
<211> LENGTH: 294
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
  1               5                  10                  15

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
             20                  25                  30

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
         35                  40                  45

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
     50                  55                  60

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
 65                  70                  75                  80

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
                 85                  90                  95

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
            100                 105                 110

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
        115                 120                 125

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
    130                 135                 140

Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
145                 150                 155                 160
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Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
                165                 170                 175

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
            180                 185                 190

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
        195                 200                 205

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
    210                 215                 220

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
225                 230                 235                 240

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
                245                 250                 255

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
            260                 265                 270

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
        275                 280                 285

Tyr Tyr Glu Asp Ser Tyr
    290


<210> SEQ ID NO 4
<211> LENGTH: 294
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 4

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Thr Ile Gln His Glu
  1               5                  10                  15

Ser Gly Leu Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
             20                  25                  30

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
         35                  40                  45

His Gly Ile Thr Asp Val Ser Pro Leu His Ala Arg Arg Leu Pro Arg
     50                  55                  60

Gly Ile Lys His Val Lys Asp Leu Pro Ile His Pro Gly Glu Ile Phe
 65                  70                  75                  80

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
                 85                  90                  95

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Ile Asn Pro Glu Arg
            100                 105                 110

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
        115                 120                 125

Ser Val Asp Gln Arg Gly Asn Gln Met Met Ser Asp Lys Arg Asn Val
    130                 135                 140

Ile Leu Phe Ser Ile Phe Asp Glu Asn Gln Ser Trp Tyr Ile Thr Glu
145                 150                 155                 160

Asn Met Gln Arg Phe Leu Pro Asn Ala Ala Lys Thr Gln Pro Gln Asp
                165                 170                 175

Pro Gly Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
            180                 185                 190

Phe Asp Ser Leu Glu Leu Thr Val Cys Leu His Glu Val Ala Tyr Trp
        195                 200                 205

His Ile Leu Ser Val Gly Ala Gln Thr Asp Phe Leu Ser Ile Phe Phe
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    210                 215                 220

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
225                 230                 235                 240

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
                245                 250                 255

Gly Leu Trp Val Leu Gly Cys His Asn Ser Asp Phe Arg Lys Arg Gly
            260                 265                 270

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Ser Thr Ser Asp
        275                 280                 285

Tyr Tyr Glu Glu Ile Tyr
    290


<210> SEQ ID NO 5
<211> LENGTH: 294
<212> TYPE: PRT
<213> ORGANISM: Pig

<400> SEQUENCE: 5

Ala Tyr Thr Asp Val Thr Phe Lys Thr Arg Lys Ala Ile Pro Tyr Glu
  1               5                  10                  15

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
             20                  25                  30

Leu Ile Ile Phe Lys Asn Lys Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
         35                  40                  45

His Gly Ile Thr Asp Val Ser Ala Leu His Pro Gly Arg Leu Leu Lys
     50                  55                  60

Gly Trp Lys His Leu Lys Asp Met Pro Ile Leu Pro Gly Glu Thr Phe
 65                  70                  75                  80

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
                85                  90                  95

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Ser Ile Asn Leu Glu Lys
            100                 105                 110

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
        115                 120                 125

Ser Val Asp Gln Arg Gly Asn Gln Met Met Ser Asp Lys Arg Asn Val
    130                 135                 140

Ile Leu Phe Ser Val Phe Asp Glu Asn Gln Ser Trp Tyr Leu Ala Glu
145                 150                 155                 160

Asn Ile Gln Arg Phe Leu Pro Asn Pro Asp Gly Leu Gln Pro Gln Asp
                165                 170                 175

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
            180                 185                 190

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
        195                 200                 205

Tyr Ile Leu Ser Val Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
    210                 215                 220

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
225                 230                 235                 240

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
                245                 250                 255

Gly Leu Trp Val Leu Gly Cys His Asn Ser Asp Leu Arg Asn Arg Gly
            260                 265                 270
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Met Thr Ala Leu Leu Lys Val Tyr Ser Cys Asp Arg Asp Ile Gly Asp
        275                 280                 285

Tyr Tyr Asp Asn Thr Tyr
    290


<210> SEQ ID NO 6
<211> LENGTH: 133
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Ser Tyr Val Thr Pro Ile Cys Ile Ala Asp Lys Glu Tyr Thr Asn Ile
  1               5                  10                  15

Phe Leu Lys Phe Gly Ser Gly Tyr Val Ser Gly Trp Gly Arg Val Phe
             20                  25                  30

His Lys Gly Arg Ser Ala Leu Val Leu Gln Tyr Leu Arg Val Pro Leu
         35                  40                  45

Val Asp Arg Ala Thr Cys Leu Arg Ser Thr Lys Phe Thr Ile Tyr Asn
     50                  55                  60

Asn Met Phe Cys Ala Gly Phe His Glu Gly Gly Arg Asp Ser Cys Gln
 65                  70                  75                  80

Gly Asp Ser Gly Gly Pro His Val Thr Glu Val Glu Gly Thr Ser Phe
                 85                  90                  95

Leu Thr Gly Ile Ile Ser Trp Gly Glu Glu Cys Ala Met Lys Gly Lys
            100                 105                 110

Tyr Gly Ile Tyr Thr Lys Val Ser Arg Tyr Val Asn Trp Ile Lys Glu
        115                 120                 125

Lys Thr Lys Leu Thr
    130


<210> SEQ ID NO 7
<211> LENGTH: 133
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 7

Ser Tyr Val Thr Pro Ile Cys Val Ala Asn Arg Glu Tyr Thr Asn Ile
  1               5                  10                  15

Phe Leu Lys Phe Gly Ser Gly Tyr Val Ser Gly Trp Gly Lys Val Phe
             20                  25                  30

Asn Lys Gly Arg His Ala Ser Ile Leu Gln Tyr Leu Arg Val Pro Leu
         35                  40                  45

Val Asp Arg Ala Thr Cys Leu Arg Ser Thr Thr Phe Thr Thr Tyr Asn
     50                  55                  60

Asn Met Phe Cys Ala Gly Tyr Arg Glu Gly Gly Lys Asp Ser Cys Glu
 65                  70                  75                  80

Gly Asp Ser Gly Gly Pro His Val Thr Glu Val Glu Gly Thr Ser Phe
                 85                  90                  95

Leu Thr Gly Ile Ile Ser Trp Gly Glu Glu Cys Ala Met Lys Gly Lys
            100                 105                 110

Tyr Gly Ile Tyr Thr Lys Val Ser Arg Tyr Val Asn Trp Ile Lys Glu
        115                 120                 125

Lys Thr Lys Leu Thr
    130
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<210> SEQ ID NO 8
<211> LENGTH: 133
<212> TYPE: PRT
<213> ORGANISM: Dog

<400> SEQUENCE: 8

Ser Tyr Val Thr Pro Ile Cys Ile Ala Asp Arg Glu Tyr Ser Asn Ile
1 5 10 15

Phe Leu Lys Phe Gly Ser Gly Tyr Val Ser Gly Trp Gly Arg Val Phe
20 25 30

Asn Lys Gly Arg Ser Ala Ser Ile Leu Gln Tyr Leu Lys Val Pro Leu
35 40 45

Val Asp Arg Ala Thr Cys Leu Arg Ser Thr Lys Phe Thr Ile Tyr Asn
50 55 60

Asn Met Phe Cys Ala Gly Phe His Glu Gly Gly Lys Asp Ser Cys Gln
65 70 75 80

Gly Asp Ser Gly Gly Pro His Val Thr Glu Val Glu Gly Ile Ser Phe
85 90 95

Leu Thr Gly Ile Ile Ser Trp Gly Glu Glu Cys Ala Met Lys Gly Lys
100 105 110

Tyr Gly Ile Tyr Thr Lys Val Ser Arg Tyr Val Asn Trp Ile Lys Glu
115 120 125

Lys Thr Lys Leu Thr
130

<210> SEQ ID NO 9
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
1 5 10 15

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val
20 25

<210> SEQ ID NO 10
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu
1 5 10 15

Phe Ser Val Phe Asp
20

<210> SEQ ID NO 11
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

mtakvsscdk ntgdyydsy 19

<210> SEQ ID NO 12
<211> LENGTH: 21

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr
1 5 10 15

Ile Leu Ser Ile Gly
20

<210> SEQ ID NO 13
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Gly Lys Tyr Gly Ile Tyr Thr Lys Val Ser Arg Tyr Val Asn Trp Ile
1 5 10 15

Lys Glu Lys Thr Lys
20

<210> SEQ ID NO 14
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Cys Leu Arg Ser Thr Lys Phe Thr Ile Tyr Asn Asn Met Phe Cys Ala
1 5 10 15

Gly Phe His Glu Gly
20

<210> SEQ ID NO 15
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Lys Val Ser Arg Tyr Val Asn Trp Ile Lys Glu Lys Thr Lys Leu Thr
1 5 10 15

<210> SEQ ID NO 16
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Ser Tyr Val Thr Pro Ile Cys Ile Ala Asp Lys Glu Tyr Thr Asn Ile
1 5 10 15

Phe Leu Lys Phe Gly
20

<210> SEQ ID NO 17
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Ser Val Asp Gln Arg Gly Asn Gln
1 5

What is claimed is:

1. A polypeptide comprising at least 3 contiguous amino acids of a sequence that is at least 88% identical to SEQ ID NO:3 or SEQ ID NO:6, wherein said polypeptide (a) inhibits the interaction of blood coagulation factor VIIIa with blood coagulation factor IXa, (b) inhibits the activation of blood coagulation factor X, or (c) inhibits blood coagulation.

2. The polypeptide of claim 1, wherein the sequence is SEQ ID NO:3 or SEQ ID NO:6.

3. The polypeptide of claim 2, wherein the amino acid sequence is at least 5 amino acids long.

4. The agent of claim 3, wherein the amino acid sequence is at least 10 amino acids long.

5. The polypeptide of claim 4, wherein the amino acid sequence comprises a sequence selected from the group consisting SEQ ID NO:9, SEQ ED NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.

6. The polypeptide of claim 5, wherein the amino acid sequence consists essentially of a sequence selected from the group consisting SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ED NO:14, SEQ ID NO:15, and SEQ ID NO:16.

7. An agent comprising an antibody binding site, wherein (a) the antibody binding site specifically binds to region 2 or 3 of blood coagulation factor VIIIa or to region 2 or 3 of blood coagulation factor IXa, and (b) the agent (i) inhibits the interaction of blood coagulation factor VIIIa with blood coagulation factor IXa, (ii) inhibits the activation of blood coagulation factor x or (iii) inhibits blood coagulation.

8. The agent of claim 7, wherein the antibody binding site specifically binds to the amino acid sequence of any one of claims **1-6**.

9. The agent of claim 8, wherein the agent is an antibody.

10. The agent of claim 9, wherein the agent is a monoclonal antibody.

11. The agent of claim 10, wherein the monoclonal antibody is a humanized monoclonal antibody.

12. A polynucleotide encoding an amino acid sequence of any one of the polypeptides of claims **1-6**, wherein the polynucleotide is operably linked to a control sequence that allows the polynucleotide to be translated in a mammalian cell.

13. A pharmaceutical composition comprising (a) the polypeptide of any one of claims **1-6**, (b) the agent of any one of claims **7-11**, or (c) the polynucleotide of claim 15, in a pharmaceutically acceptable excipient.

14. The pharmaceutical composition of claim 13, wherein the excipient is suitable for intravenous administration.

15. A method of treatment to prevent coagulation in a patient in need thereof, the method comprising administering to the patient the pharmaceutical composition of claim 13 or **14**.

16. The method of claim 15, wherein the patient is suffering from a cardiovascular disorder.

17. The method of claim 16, wherein the cardiovascular disorder is selected from the group consisting of thrombosis, atherosclerosis and restenosis.

18. The method of any one of claims **15-17**, wherein the pharmaceutical composition is administered intravenously.

19. A method for identifying a compound having anti-coagulation activity, the method comprising determining whether a compound displaces the interaction of the polypeptide of any one of claims **1-7** to factor VIIIa or factor IXa.

20. The method of claim 19, wherein the polypeptide is labeled with a detectable marker.

21. The method of claim 20, wherein the detectable marker is selected from the group consisting of a fluorescent marker, a radioactive marker, and a spin label.

22. A method of preventing coagulation in a blood sample, comprising (a) adding the polypeptide of any of claims **1-6** to the sample, or (b) adding the agent of any of claims **7-11** to the sample.

* * * * *

专利名称(译)	因子ixa：因子VII1相互作用及其方法		
公开(公告)号	US20040126856A1	公开(公告)日	2004-07-01
申请号	US10/466998	申请日	2002-01-23
[标]申请(专利权)人(译)	BAJAJ PAUL小号 FAY菲利普Ĵ		
申请(专利权)人(译)	BAJAJ PAUL S. FAY菲利普Ĵ		
当前申请(专利权)人(译)	圣路易斯大学		
[标]发明人	BAJAJ PAUL S FAY PHILIP J		
发明人	BAJAJ, PAUL S. FAY, PHILIP J		
IPC分类号	C07K14/755 C12N9/64 G01N33/50 C12N9/99 G01N33/53 C07K16/36		
CPC分类号	C07K14/755 C12N9/644 C12Y304/21022 G01N2500/00 G01N33/5002		
外部链接	Espacenet USPTO		

摘要(译)

公开了在新发现的相互作用区域2区域和区域3中抑制因子VIIIa与因子IXa相互作用的新型试剂。新的多肽或多肽衍生物阻止因子X的活化并具有抗凝血活性。所述试剂包括与2区和/或3区的因子VIIIa或因子IXa同源的多肽或多肽衍生物，以及非同源的试剂，例如抗体2区或3区。包含所述试剂的药物组合物也是披露。还公开了治疗方法，包括确定化合物是否取代上述试剂与因子VIII或因子IX的相互作用的步骤。还公开了用于防止血液样品中凝结的方法。这些方法包括将上述试剂加入样品中。