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Research Article

A candidate activation pathway for coagulation factor VII

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The mechanism of generation of factor VIIa, considered the initiating protease in the tissue factor-initiated extrinsic limb of blood coagulation, is obscure. Decreased levels of plasma VIIa in individuals with congenital factor IX deficiency suggest that generation of VIIa is dependent on an activation product of factor IX. Factor VIIa activates IX to IXa by a two-step removal of the activation peptide with cleavages occurring after R191 and R226. Factor IXaα, however, is IX cleaved only after R226, and not after R191. We tested the hypothesis that IXaα activates VII with mutant IX that could be cleaved only at R226 and thus generate only IXaα upon activation. Factor IXaα demonstrated 1.6% the coagulant activity of IXa in a contact activation-based assay of the intrinsic activation limb and was less efficient than IXa at activating factor X in the presence of factor VIIIa. However, IXaα and IXa had indistinguishable amidolytic activity, and, strikingly, both catalyzed the cleavage required to convert VII to VIIa with indistinguishable kinetic parameters that were augmented by phospholipids, but not by factor VIIIa or tissue factor. We propose that IXa and IXaα participate in a pathway of reciprocal activation of VII and IX that does not require a protein cofactor. Since both VIIa and activated IX are equally plausible as the initiating protease for the extrinsic limb of blood coagulation, it might be appropriate to illustrate this key step of hemostasis as currently being unknown.

Introduction

Factor VIIa is considered the initiating protease of the coagulation process [1,2]. Under physiologic conditions, a small but measurable fraction (<1%) of the plasma *F7* gene product (the combination of factor VII and factor VIIa) circulates in the activated form, factor VIIa [3]. The identity of the protease that cleaves factor VII to yield its circulating activated form remains uncertain. Moreover, if another protease is required to generate plasma factor VIIa, it suggests that VIIa is not the initial protease in coagulation and that our current model for initiating coagulation may be inaccurate.

Measurements of factor VIIa plasma concentrations in normal human subjects and individuals with specific factor deficiencies show that individuals with factor IX deficiency have the most profound decrease in any group in their circulating levels of factor VIIa [3–7], suggesting that factor IX(a) has a physiologic role in generating plasma factor VIIa. This would be an additional physiologic function for factor IX(a), which is currently recognized only for activating factor X with a strict requirement for the cofactor, factor VIIIa [8,9]. Individuals with factor VIII deficiency do not have such a profound decrease in plasma factor VIIa levels [3], suggesting that if factor IXa is a physiologic activator of factor VII, it does so in a factor VIII-independent manner. The differing requirements for factor VIII suggest that factor IX-dependent generation of factor VIIa in plasma involves a different mechanism than does factor IX-dependent activation of factor X.

Experiments using purified plasma proteins demonstrate that fully activated factor IX, factor IXa (common name, but more precisely called factor IXaβ which is how it will be identified from now on), can activate factor VII [6,10], consistent with factor IX having a role in generating plasma factor VIIa. However, to cleave factor VII, factor IXaβ must be generated from its zymogen, factor IX.

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Measurements of factor IX activation peptide in plasma, an indicator of the amount of factor IXaβ that has been formed, indicate that factor VII is required for the generation of factor IXaβ *in vivo* [11]. This implies a reciprocal activation circuit between factors VII and IX, as has been described for the initiating steps of other protease cascades [12].

The activation of the zymogen, factor VII, to proteolytically active factor VIIa requires cleavage of the peptide bond at R152 [13]. The generation of fully activated factor IX, factor IXaβ, is more complex and requires two proteolytic cleavage events, after R191 and R226 of zymogen factor IX (Supplementary Figure S1) [14,15]. Experiments using proteins purified from plasma show that factor IX activation intermediates have different properties. Cleavage of factor IX at only R191 generates factor IXα, which lacks enzymatic activity, yet reportedly binds the light chain of factor VIIIa [14,16]. Such binding to the required cofactor, factor VIIIa, is mandatory for the known biological role of factor IXa, activation of factor X. Cleavage at only R226 generates factor IXaα, which is an active protease, yet binds poorly to factor VIIIa [14,16,17]. These data yield the concept that one of the activating cleavages yields factor VIIIa binding ability, the other cleavage yields proteolytic activity, and the combination yields fully functional factor IXaβ.

In reciprocal activation loops, determining specific molecular interactions requires experimental conditions that eliminate feedback activation that can cloud interpretation of results. To help illuminate the initial proteolytic events in the coagulation cascade, we expressed and characterized specific mutants of factor IX and factor VII to generate well-defined populations of variably activated factor IX, and factor VIIa that could not provide feedback amplification. Use of well-defined, verified recombinant molecules obviated concerns that the functional effects of individual proteolytic cleavage events might be confounded by trace amounts of fully activated proteases present in plasma-derived protein preparations [15,17]. These molecules were used to test the hypothesis, generated from observations of factor levels in human plasma [3], that it is biochemically feasible that one or more of the factor IX activation intermediates could generate plasma factor VIIa in a factor VIIIa-independent manner, and that fully activated factor IXaβ may not be strictly required.

Our data show that factor IXaβ and factor IXaα are equally capable of activating factor VII to VIIa without a protein cofactor and, therefore, are plausible candidates as an initiating protease in coagulation. In the course of these experiments, we also obtained data that contrast with previous findings (from experiments with plasma-derived proteins) characterizing the factor IX activation intermediate, factor IXα, and its interaction with factor VIIIa [16]. Our data, along with those of others, suggest that, given the apparent role of factor IX (a) in generating factor VIIa, it may not be accurate to definitively represent factor VIIa as the initiating protease in the coagulation cascade. We, therefore, pose the question of whether the initial proteolytic event in coagulation might more accurately be represented as a still unresolved question.

Experimental

Materials

Human plasma-derived factor IIa, VIIa, IXa, X, XIa, factor IXa-fluorescein EGR (FEGR-FIXa), factor IXa-EGR (EGR-FIXa), fluorescein EGR-chloromethylketone (FEGRck), and affinity-purified Sheep anti-human factor VII were from Haematologic Technologies (Essex Junction, VT). Recombinant human factor VIII (FA8, UniProtKB P00451), Helixate FS (CSL Behring LLC Kankakee, IL) and Advate (Baxter) were used as the source of factor VIII. Sheep anti-human factor XI polyclonal was from Enzyme Research Laboratories (South Bend, IN). 10H10 anti-human tissue factor monoclonal antibody [18], 1150 anti-human factor VII calcium-dependent monoclonal antibody [19], and 4G3 anti-human factor X calcium-dependent monoclonal antibody [20] were purified from hybridoma cells generously provided by James Morrissey (University of Michigan). The HPC4 calcium-dependent monoclonal antibody was purified from hybridoma cells generously provided by Charles Esmon (Oklahoma Medical Research Foundation) [21]. Peroxidase conjugated AffiniPure rabbit anti-sheep IgG was from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Chromogenic substrates were purchased as follows: SN-17c (D-Phe-Pro-Arg-ANSNH-C4H9) from Haematologic Technologies (Essex Junction, VT), Spectrozyme FXa (CH_3O-CO-D-CHG-Gly-Arg-pNA.AcOH) from Sekisui Diagnostics (Stamford, CT), and Pefachrome FIXa (CH_3SO_2-D-CHG-Gly-Arg-pNA.AcOH) from DSM Pentapharm (Switzerland). Phosphatidylserine (PS) and phosphatidylcholine (PC) were from Avanti Lipids (Alabaster, AL). PC:PS phospholipid vesicles (80:20) were prepared using Bio-Beads SM-2 (Bio-Rad Laboratories, Hercules, CA) [22]. The molar concentration of phospholipid was determined with an elemental phosphorus assay [23]. Pooled normal plasma and plasmas from individuals genetically deficient in factor IX were from George King

Bio-Medical, Inc (Overland Park, KS). Factor IX immunodepleted plasma and factor VII immunodepleted plasma were from Haematologic Technologies (Essex Junction, VT).

Mutagenesis and expression of recombinant human factor IX, VII, and X

The HEK293 VKOR cell line is HEK 293 cells (ATCC) transfected with the plasmid pIRESpuro3-VKOR, which encodes vitamin K 2, 3-epoxide reductase C1 (VKOR) [24]. This cell line was a gift of James Morrissey (University of Michigan). The plasmid was maintained by exposure to 1.75 μg/ml puromycin from EMD Millipore (Billerica, MA).

F9:pcDNA3.1 (a plasmid for expression of the *F9* gene from a CMV promoter) was a gift of John Sheehan (University of Wisconsin). This plasmid was constructed from pcDNA3.1(+) (previously sold by Invitrogen (Carlsbad, CA)) with the insertion of ORF and the first 1089 nt of the 3′UTR flanked by *EcoRI* sites into the *EcoRI* site of pcDNA3.1. The 201 nt 3′ to the EcoRI site of the *F9* cDNA are not present. Sequencing of the full insertion reveals four silent mutations in the coding sequence. Mutations were introduced into the wild-type (wt) factor IX sequence via template-directed mutagenesis according to manufacturer's instructions with a QuikChange kit from Stratagene (La Jolla, CA). Mutations of F9:pcDNA3.1 were made to produce protein with the following mutations: R191H; R191Q; R191Q and S411A (R191Q SA); R226Q; and R191 and R226Q (RQRQ) (amino acid residue numbering is based on translated protein rather than the secreted protein; to convert into secreted protein numbering, subtract 46). All *F9*-related sequences were verified by Sanger sequencing.

F7 cDNA (gift of James Morrissey, University of Michigan) was amplified by PCR with the addition of flanking *EcoRI* and *NotI* restriction sites that were subcloned into pcDNA3.1(+). Mutation to encode a S404A (amino acid residue numbering is based on the translated protein rather than the secreted protein; to convert into secreted protein numbering, subtract 60) was performed by template-directed mutagenesis. All *F7*-related sequences were verified by Sanger sequencing.

pCMV4-SS-proII-FX plasmid [25] was a gift of Rodney Camire, University of Pennsylvania. Mutation to encode a S419A was performed by template-directed mutagenesis. All transgenic sequences were verified by Sanger sequencing.

HEK293 VKOR cells were transformed with the relevant plasmid using FuGENE6 from Promega (Madison, WI). Stable transformants were grown in DMEM/F12 50/50 from Mediatech (Manassas, VA) supplemented with 10% FBS, 5 μg/ml vitamin K_1 from Sigma–Aldrich (St. Louis, MO), 1.75 μg/ml puromycin, and 400 μg/ml G418 from RPI (Mount Prospect, IL). After growth to 80% confluence, cells were washed with HBSS from Mediatech and media was replaced with DMEM/F12, 50/50 with L-glutamine from Mediatech supplemented with 1× ITS from Mediatech and 5 μg/ml vitamin K_1 until cell confluence was <10%. Harvested serum-free media was supplemented with PEG-8000 to 0.1%, supplemented with benzamidine (Acros, New Jersey) to 10 mM, and frozen on the day of collection. Batches were thawed just prior to purification.

Purification of recombinant factor VII, factor X, and factor IX

Recombinant factor VII (FA7, UniProtKB P08709) and the mutant S404A factor VII were purified from conditioned cell culture media by serial chromatography [26] with the calcium-dependent anti-factor VII antibody 1150 [19], then with the anion exchange resin monoQ (GE Healthcare Life Sciences, Pittsburgh, PA). An amount of 10 mM benzamidine was maintained in all buffers to mitigate against activation to factor VIIa. Protein was quantitated by absorbance at 280 nm using extinction coefficient 1.39 ($\varepsilon_{0.1\%}$).

Recombinant mutant S419A factor X (FA10, UniProtKB P00742) was purified from conditioned cell culture media with modifications of a previously described serial chromatography method [27]. Anion exchange chromatography using Q Sepharose was done first, followed by immunoaffinity chromatography using the calcium-dependent anti-factor X antibody 4G3 [20], followed by anion exchange on monoQ (GE Healthcare Life Sciences, Pittsburgh, PA). An amount of 10 mM benzamidine was maintained in all buffers. Protein was quantitated by absorbance at 280 nm using extinction coefficient 1.16 ($\varepsilon_{0.1\%}$).

Recombinant factor IX (FA9, UniProtKB P00740) was purified from conditioned media by either conventional anion exchange chromatography and calcium-dependent elution from Q Sepharose as described previously [28], or via affinity purification using HiTrap factor IXselect (GE Healthcare BioSciences, Uppsala, Sweden). The IXselect method was essentially as described by the manufacturer [29]. Prior to loading onto a 1 ml IXselect HiTrap column, 1–2 L conditioned media was filtered and concentrated using a 30 kDa MWCO Vivaflow 200 module (Sartorious), and the column was equilibrated in binding buffer (20 mM Tris, 150 mM

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NaCl, pH 7.4). Concentrated media was loaded onto the column at 0.5–1 ml/min. Next, the column was sequentially washed at 1 ml/min with 10 CV (column volumes) binding buffer, 10 CV wash buffer (20 mM Tris, 0.5 M NaCl, 0.01% Tween 80, pH 7.4) and 3 CV binding buffer. Factor IX was then eluted at 0.5 ml/min with 5–10 CV elution buffer (20 mM Tris, 2.0 M $MgCl_2$, pH 7.4). Elution peak fractions were pooled and dialyzed extensively in 20 mM HEPES, 100 mM NaCl, 0.1% PEG-8000, pH 7.4. Dialyzed protein was concentrated using 10 kDa MWCO Amicon centrifugal filter units, and quantitated using absorbance at 280 and an extinction coefficient of 1.32 ($\varepsilon_{0.1\%}$). The column was regenerated with 5 CV 100 mM glycine, 100 mM NaCl, pH 2.0, brought back to neutral pH with 5–10 CV of equilibration buffer, washed with water, and stored in 20% ethanol. The regenerated column was used several times without problems. To prevent cross contamination of the various recombinants, separate columns were used for each mutant.

Expression and purification of soluble tissue factor (sTF)

Recombinant soluble tissue factor (TF, UniProtKB P13726) was expressed in *E coli* and purified with a novel, N-terminal affinity tag, which was subsequently removed, leaving soluble tissue factor with only its native sequence. Synthetic, overlapping oligonucleotides that encode an artificial peptide composed of hexahistidine (HHHHHH) followed by the enterokinase substrate recognition site (DDDDK), the HPC4 epitope (EDQVDPRLIDKG), and most of the TEV protease recognition site (ENLYFQ) were designed. These were synthesized by Integrated DNA Technologies (Coralville, IA). These were allowed to anneal with each other and with synthetic primers (IDT) that generated flanking *PstI* sites. These molecules were elongated and ligated by PFU Turbo Polymerase (Promega, Madison, WI), purified, cleaved with PstI, and ligated into the *PstI* site of a pET26(+)-based plasmid in which an affinity tagged sequence of an extracellular portion of tissue factor (sTF amino acids 3–219) had been inserted [30]. This plasmid was further modified with template-directed mutagenesis to remove the pre-existing affinity tag and residue of previous restriction enzyme recognition sites. The resulting transgene was subcloned into pET26(+) to yield transgene expressed from a T7 promoter with a *pelB* leader sequence followed immediately by the transgene encoding HHHHHH-DDDDK-EDQVDPRLIDKG-ENLYFQ-sTF followed by stop codon and T7 terminator sequence. The sequence was confirmed by Sanger sequencing.

E coli strain BL21(DE3) was transformed by this plasmid, and protein was expressed in a modification of a previously described protocol [31]. Expression was induced by addition of 20 μM IPTG in shaking culture at 25°C. Culture supernatants were collected after pelleting cells at 15 000 ***g*** for 15 min at 4°C. sTF was precipitated by addition of ammonium sulfate to 60% saturation at 4°C, pelleted, resuspended with 1/10th the culture supernatant volume of 40 mM HEPES, and dialyzed against nickel binding buffer (30 mM HEPES + 300 mM NaCl + 10 mM imidazole, pH 8). The dialyzed protein was bound to Ni-NTA resin (ThermoScientific, Rockford, IL), washed with the same buffer, and eluted with elution buffer (30 mM HEPES + 300 mM NaCl + 250 mM imidazole, pH 8). Eluted protein was dialyzed into HPC4 binding buffer (20 mM HEPES + 100 mM NaCl + 5 mM $CaCl_2$, pH 7.4), bound to HPC4 antibody-coupled Sepharose column, washed with lower calcium buffer (20 mM HEPES + 100 mM NaCl + 1 mM $CaCl_2$, pH 7.4), and eluted with elution buffer (20 mM HEPES + 100 mM NaCl + 5 mM EDTA + 5 mM EGTA, pH 7.4). This yielded a pure preparation of tagged sTF, as assessed by SDS–PAGE (sodium dodecyl sulfate-polyacrylamide electrophoresis). The tag can be removed with TEV protease, leaving no residue from the tag, given that sTF begins with the acceptable P1' residue threonine (some residues will not be accommodated) [32]. sTF eluted from the HPC4 column was concentrated using a 10 kD MWCO spin concentrator (Millipore, Ireland), and buffer exchanged ≥75% with 50 mM HEPES, pH 8. The tag was removed by addition of 0.2 mg/ml TurboTEV (Accelagen) or TEV expressed and purified in house [33], incubation at 30°C for ∼50 h, dialysis against nickel binding buffer, and purification of the his-tagged TEV and cleaved tag away from the untagged sTF by repeat nickel chromatography.

Activation of factor IX

Prior to activation of factor IX, antibody resins needed for purification steps were prepared by coupling either polyclonal anti-human factor XI or monoclonal 10H10 anti-human TF to NHS-activated Sepharose 4 fast flow media (GE Healthcare Biosciences) per manufacturer's instructions. Factor VIIa-Sepharose was also made within a week of use by incubating wild-type factor VIIa with NHS-activated Sepharose overnight at 4°C in the presence of 80 mM $CaCl_2$, which increased coupling efficiency. In all cases, after coupling protein to Sepharose, the resin was washed with 20 mM HEPES, 100 mM NaCl, pH 7.4 (HBS), unreacted groups were blocked with

1 M Tris, pH 7.5, and the resulting resin was washed thoroughly with 10–15 CV of 20 mM HEPES, pH 7.4. The resin was stored at 4°C in 50% glycerol/20 mM HEPES, pH 7.4.

Purified wt factor IX was activated to factor IXaβ with human factor XIa by incubating 15 μM factor IX with 0.1 μM factor XIa for 1.5 h at 37°C, followed by removal of factor XIa via incubation with polyclonal anti-human factor XI-Sepharose [28]. Attempts to generate factor IXaα from wt factor IX or R191Q factor IX using RVV-X as described previously [15,17,34] always resulted in IXaα preparations that contained what appeared to be factor IXaβ on SDS–PAGE. Likewise, cleavage of the mutant factor IX preps with factor XIa resulted in contamination with other proteolytically active products that were apparent with biotinylated EGRck labeling (Supplementary Figures S1, S2). Therefore, purified cleavage-resistant factor IX mutant proteins (i.e. R191H, R191Q, R191Q;S411A, R226Q, R191Q;R226Q) were activated with factor VIIa/sTF as follows: the indicated form of factor IX was incubated with freshly made factor VIIa-Sepharose in the presence of 5 mM $CaCl_2$, 0.1 μM sTF, 100 μM PCPS, in 20 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG-8000 (HBSCP) for 6 h at 37°C. Activated factor IX was recovered from the supernatant after pelleting the resin. The sTF was removed from the activated factor IX via incubation with 10H10 anti-human TF-Sepharose for 30 min at room temperature. (These conditions were shown in preliminary experiments to quantitatively remove sTF from the reactions.) Activated factor IX was recovered after spinning down the resin. Protein was quantitated by absorbance at 280 nm using extinction coefficient 1.43 ($\varepsilon_{0.1\%}$). Complete or near-complete activation of wt factor IX by factor XIa was noted using SDS–PAGE, whereas cleavage by factor VIIa/sTF yielded cleavage of mutant R191H or R191Q factor IX that was ∼30% complete, as assessed by SDS–PAGE under reducing conditions. Importantly, these populations included only unreacted factor IX and the desired cleavage product for each reaction. The resultant factor IX(a) preparations contained no detectable factor VIIa or sTF, as determined by aPTT assay. The levels of detection were 0.05 nM sTF and 0.25 nM factor VIIa, respectively. Factor IX catalytic sites for wt factor IXaβ and R191H or R191Q factor IXaα were quantitated by titration with antithrombin III as described [35]. The mutants S411A, R226Q, R191Q;S411A, and R191Q;R226Q factor IX demonstrated <2% activity after incubation with VIIa/sTF.

EGRck labeling of activated factor IX active sites

The active sites of the activated factor IX proteins were labeled by incubating 1 μM factor IX with 1.25 μM fluoresceinated EGRck in 30% ethylene glycol, HBSCP for 1.5 h at 37°C. Samples were quenched with either reducing or nonreducing SDS–PAGE sample buffer, boiled 5 min, and subjected to SDS–PAGE using a 4–20% TGX Stain-Free gel (Bio-Rad). The proteins in the gel were visualized via fluorescent detection using Bio-Rad's Stain-Free technology prior to transferring to polyvinylidene difluoride (PVDF) membrane using a Bio-Rad Transblot Turbo transfer apparatus. Fluorescein labeling of active sites was visualized on the blots using a UVP imager set for fluorescein detection. The detection limit was 0.94 pmole factor IXaβ.

Chromogenic substrate hydrolysis by, and plasma coagulation activity of factor IXa

The rate of hydrolysis of Pefachrome FIXa by recombinant activated factor IX was determined at room temperature in a reaction containing final concentrations of 40 nM activated factor IX in HBSCP + 0.1% BSA (HBSCPB), 30% ethylene glycol, and 0.25 to 1.0 mM Pefachrome FIXa as described [28,35]. The initial rate of substrate cleavage was determined, and the slope of the substrate vs velocity plot used to estimate the specificity constant (k_{cat}/K_M). Coagulant activity was determined in an activated partial thromboplastin time (aPTT) with the addition of recombinant activated factor IX to factor IX immunodepleted plasma (Haematologic Technologies) just prior to recalcification. PTT-LA (Stago Diagnostica) was used as the source of thromboplastin.

Kinetic based assay for determination of the affinity of factor IXa for factor VIIIa

The affinity of the interaction between factor IXa and factor VIIIa to form the factor X-activating complex was determined by measuring complex activity at a limiting concentration of factor VIIIa as described [36]. Thrombin-activated factor VIIIa (0.05 nM) was incubated with increasing amounts of factor IXaβ (0–30 nM) or factor IXaα (0–44 nM) in the presence of 50 μM PCPS, and 200 nM factor X in HBSCPB. Activated factor IX concentration (as determined by active site titration with antithrombin III) was plotted versus the rate of

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factor Xa generation, and the data fit by nonlinear regression to a single-site binding model to determine the apparent K_D. Competition assays were done at concentrations of 4 nM wt factor IXaβ, 0.05 nM factor VIII, 200 nM factor X, 50 μM PCPS, and either 0–80 nM EGR-factor IXaβ or 0–80 nM R226Q factor IXα under identical assay conditions. Data were fit to a four-parameter logistic equation to determine the EC_{50} [37,38].

Determination of factor IXa-factor VIIIa binding affinity on a phospholipid surface

The binding affinity of factor IXa for factor VIIIa in the presence of factor X and PCPS was examined in a direct or competition binding assay utilizing FEGR-FIXa. Fluorescence anisotropy measurements were obtained on a microplate reader (TECAN M1000) using black 96 well plates (Corning 3915), an excitation wavelength of 470 nm, and an emission wavelength of 525 nm. After a 30 s thrombin activation, factor VIIIa (10 nM) was immediately added to binding reactions containing 10 nM FEGR-FIXa, 50 μM PCPS, 125 nM factor X and increasing amounts of factor IXa competitor (EGR-FIXa or R226Q factor IXα) in HBSC, as described previously [35]. The fractional change in maximum anisotropy (in the absence of competitor) was plotted versus the competitor factor IXa concentration.

Kinetics of factor X activation by the factor IXa-factor VIIIa complex

Factor X activation by the factor IXa-factor VIIIa complex was determined in a reaction containing 0.1 nM factor IXaβ or factor IXaα, 1.0 or 10 nM thrombin-activated factor VIIIa, and 50 μM PCPS in HBSCPB. The reaction was initiated by addition of the indicated amounts of factor X immediately after factor VIIIa, and incubated for 30 or 60 s at room temperature, and terminated by the addition of 0.25 M EDTA/1.1 mg/ml Polybrene. The amount of factor Xa was quantitated by comparing the rate of Spectrozyme FXa hydrolysis to a standard curve as described [36]. Kinetic constants were obtained by plotting the rate of factor Xa generation versus substrate concentration, and fitting the data by nonlinear regression to the Michaelis–Menten equation.

S404A factor VII cleavage by activated factor IX

Cleavage of factor VII by various forms of activated factor IX was examined by incubating 100 nM S404A factor VII (active site serine mutated to alanine to obviate feedback activation of partially activated factor IX) with 10 nM of the designated form of activated recombinant factor IX in HBSCP ± 0–50 μM PCPS, or ±200 nM sTF, or ±1 nM thrombin-activated factor VIII for the time indicated at 37°C. Similar reactions using 1 nM factor Xa instead of factor IXa were done ±200 nM sTF as controls. Samples were quenched with 5× SDS–PAGE reducing sample buffer, boiled for 5 min, and subjected to SDS–PAGE using a 4–20% TGX Stain-Free gel (Bio-Rad). The proteins were transferred from the gel to PVDF membrane using a Bio-Rad Transblot Turbo transfer apparatus. The blot was blocked with 0.25% gelatin in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4), probed with polyclonal sheep anti-human factor VII antibody (Haematologic Technologies, 1/10 000 dilution) overnight, and incubated with peroxidase-conjugated rabbit anti-sheep IgG (1/10 000 dilution). Bio-Rad Clarity substrate was used for detection. The detection limit for the assay was 4 nM factor VIIa.

S419A factor X cleavage by activated factor IX

Cleavage of factor X by activated factor IX was examined by incubating 100 nM S404A factor X (active site serine mutated to alanine) with 10 nM of the designated form of activated recombinant factor IX in HBSCP ± 50 μM PCPS, ±1 nM thrombin-activated factor VIII for the time indicated at 37°C. Samples were quenched with 5× SDS–PAGE reducing sample buffer, boiled for 5 min, and subjected to SDS–PAGE using a 4–20% TGX Stain-Free gel (Bio-Rad). The proteins were transferred from the gel to PVDF membrane using a Bio-Rad Transblot Turbo transfer apparatus. The blot was blocked with 0.25% gelatin in TBST, probed with polyclonal sheep anti-human factor X antibody (Haematologic Technologies, 1/10 000 dilution) overnight, and incubated with peroxidase-conjugated rabbit anti-sheep IgG (1/10 000 dilution). Bio-Rad Clarity substrate was used for detection.

Kinetics of factor VII activation by the factor IXa-phospholipid complex

The rate of factor VII activation by the factor IXa-phospholipid complex was determined for wt factor IXaβ and R191H factor IXaα as follows: 20 nM activated factor IX (as determined by active site titration) was

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incubated with 0–1500 nM recombinant wt factor VII, 200 μM PCPS in HBSCPB for 1 min at room temperature. Resulting factor VIIa activity was determined in the presence of 200 nM sTF using 100 μM SN-17c substrate. Initial rates of substrate cleavage were measured by monitoring emission at 470 nm after excitation at 352 nm every 15 s for 3 min. The amount of factor VIIa was determined by comparison to a standard curve. Background factor VIIa activity (in the absence of factor IX) was also measured and subtracted from the factor IX results. Kinetic constants were obtained by plotting the rate of factor VIIa generation versus substrate concentration and fitting the data by nonlinear regression to the Michaelis–Menten equation.

Factor VIIa dependent clotting assay

A factor VIIa dependent clotting assay [39] was used to measure the activation of factor VII by factor IX or activated factor IX in plasma. Factor IX was added to genetically factor IX-deficient plasma (George King Bio-Medical, Inc) and incubated for 10 min at room temperature. The sample plasma was diluted 1/5 with factor VII immunodepleted plasma (Haematologic Technologies) and assayed immediately using a STArt4 coagulometer to measure clotting times. Initially, 50 μl sTF solution (1 μM sTF, 200 μM PCPS, HBSCP prewarmed to 37°C) was added to the STArt4 cuvette and kept at 37°C. Then, 50 μl of the diluted plasma was added. After 30 s of mixing in the cuvette, 50 μl 25 mM $CaCl_2$ (prewarmed to 37°C) and time to clot formation was determined in a coagulometer. Clotting time was converted to factor VIIa levels by comparison to a factor VIIa standard curve constructed using the same batch of factor VII immunodepleted plasma.

Results

Expression and activation of recombinant factor IX

The following variants of factor IX, and the corresponding form of IX after activation, were expressed and purified to homogeneity as detailed in Methods — wt factor IX, which would yield factor IXaβ; R191H and R191Q factor IX, each of which would yield factor IXaα; R226Q factor IX, which would yield factor IXα; R191Q; R226Q factor IX, which would remain in the unactivated form; and designated forms of factor IX with a modified active site serine, S411A. Recombinant wt factor IX was efficiently activated to factor IXaβ by incubation with factor XIa. SDS–PAGE analysis after incorporation of fluoresceinated EGR-ck revealed a homogeneous pattern consistent with factor IXaβ (Figure 1). In contrast, treatment of the R191H and R191Q factor IX proteins with factor XIa resulted in factor IXaα preparations that appeared to contain factor IXaβ by SDS–PAGE of fluorescein-EGR-ck treated proteins (Supplementary Figure S2), and activity assays. Therefore, factor VIIa/sTF was used to generate factor IXaβ-free forms of factor IXaα and factor IXα from the specifically designed forms of factor IX. Under conditions described in Methods, ∼30% of the R191H or R191Q factor IX zymogen was activated to factor IXaα, as determined by SDS–PAGE and densitometry of stained gels. No factor IXaβ was detected by SDS–PAGE or by active site labeling with fluoresceinated EGR-ck (Figure 1). The control protein R191Q;S411A factor IX was also cleaved with factor VIIa/sTF and ran as expected on SDS–PAGE under both reducing and nonreducing conditions, but did not incorporate fluoresceinated EGR-ck, consistent with the lack of a competent active site. No cleavage products were observed upon treatment of R191Q;R226Q with factor VIIa/sTF, while R226Q factor IX was cleaved to form products consistent with factor IXα. Neither of these proteins incorporated fluoresceinated EGR-ck. The purified activated factor IX preparations were analyzed by active site titration with antithrombin in the presence of heparin. Experiments were designed according to the number of active sites present within each form of active factor IXa, or the concentration of cleaved product based on SDS–PAGE in the case of the inactive proteins.

Activity of recombinant activated factor IX forms

Factor IXaα, whether derived from R191Q or R191H factor IX, demonstrated k_{cat}/K_M values identical with those for factor IXaβ against the small molecule chromogenic substrate Pefachrome IXa (Table 1) [40]. The activated control protein, R191Q;S411A factor IXaα, however, had no detectable activity, consistent with mutation of the active site serine. Likewise, R226Q factor IXα and R191Q;R226Q factor IX that had been treated with factor VIIa/sTF exhibited no detectable activity. The lack of amidolytic activity in recombinant factor IXα agrees with the absence of EGR-ck binding to the protein, and with earlier reports on plasma-derived bovine and human factor IX activation [14,41].

Consistent with previous work, no activation of factor X by any form of factor IXa was detected in the absence of factor VIIIa in the assays described. The ability of the various forms of factor IXa to interact with

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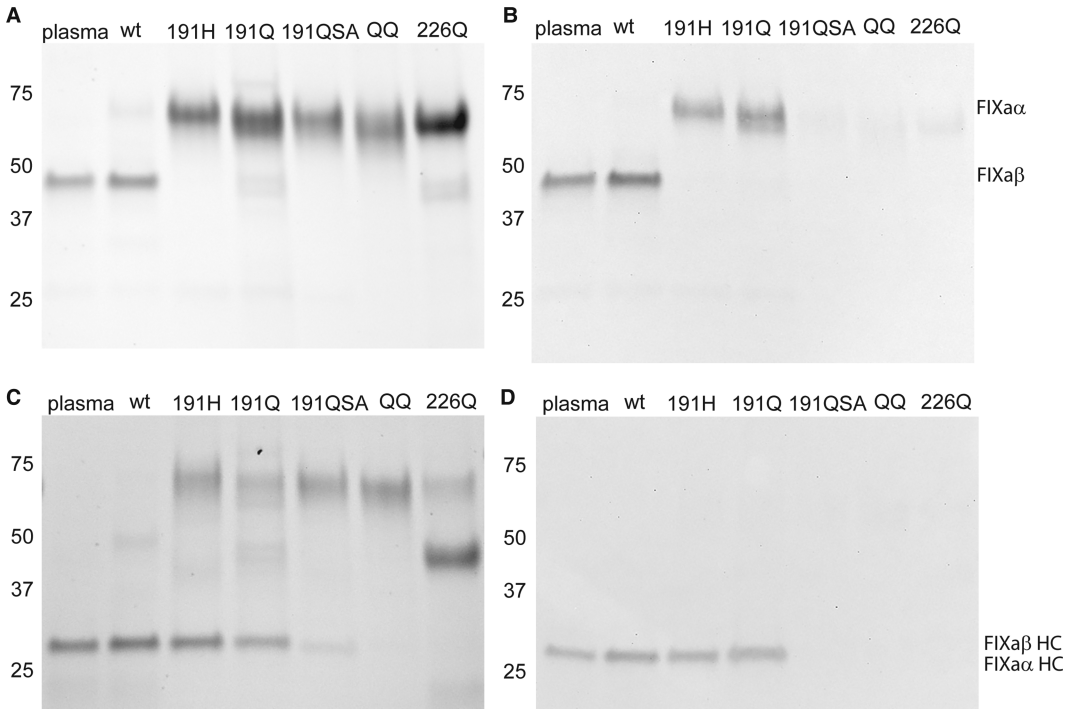

Figure 1. EGR-ck labeling of active sites.

Activated factor IX proteins (1 μM) were incubated with fluoresceinated EGR-ck (1.25 μM) in 30% ethylene glycol, HBSCP for 1.5 h at 37°C. Samples were quenched with either reducing or nonreducing sample buffer, boiled and subjected to SDS–PAGE using a 4–20% TGX Stain-Free gel (Bio-Rad). Protein was visualized via fluorescent detection using Bio-Rad's Stain-Free technology prior to transfer to nitrocellulose for fluorescein visualization. (**A**) nonreducing conditions, stained for protein, (**B**) fluorescein signal on a blot of gel run under nonreducing conditions, (**C**) reducing conditions, stained for protein (**D**) fluorescein signal on blot of gel run under reducing conditions. Activated recombinant factor IX samples were compared with plasma factor IXaβ (plasma). Recombinants were activated and purified as described in Methods — wild-type factor IXaβ (wt), R191H factor IXaα (191H), R191Q factor IXaα (191Q), R191Q;S411A factor IXaα (191QSA), R191Q;R226Q factor IX (QQ), and R226Q factor IXα (226Q).

the cofactor, factor VIIIa, was determined using the rate of factor X activation as a reporter of factor IXa-VIIIa complex formation [42]. The apparent K_D (K_D app) of factor IXaα was 8× that of factor IXaβ: 1.2 ± 0.1 nM for factor IXaβ vs 9.4 ± 0.6 nM for factor IXaα (±SEM, $n = 4$) (Figure 2A). This agrees with previous reports of factor IXaα having reduced affinity for the immobilized factor VIIIa light chain [16]. The maximal amount of

Table 1. Peptide substrate and coagulant activity for activated recombinant factor IX

'Activated' factor IX	Pefachrome IXa k_{cat}/K_M (mM^{-1} s^{-1})	Coagulant activity (%)
FIXaβ (wt)	1.5 ± 0.2	100
FIXaα (R191H)	1.5 ± 0.1	1.6 ± 0.2
FIXaα (R191Q)	1.7 ± 0.1	5.9 ± 1.4
FIXα (R226Q)	<0.1	n.d.
FIX (R191Q;R226Q)	<0.1	n.d.
FIXaα (R191Q;S411A)	<0.1	n.d.

The initial rate of Pefachrome IXa cleavage by 40 nM factor IXa was determined in HBSCPB containing 30% ethylene glycol. Coagulant activity was determined by aPTT in factor IX immunodepleted plasma. Data are expressed as the mean ± SEM of three experiments. (n.d. indicates not detected).

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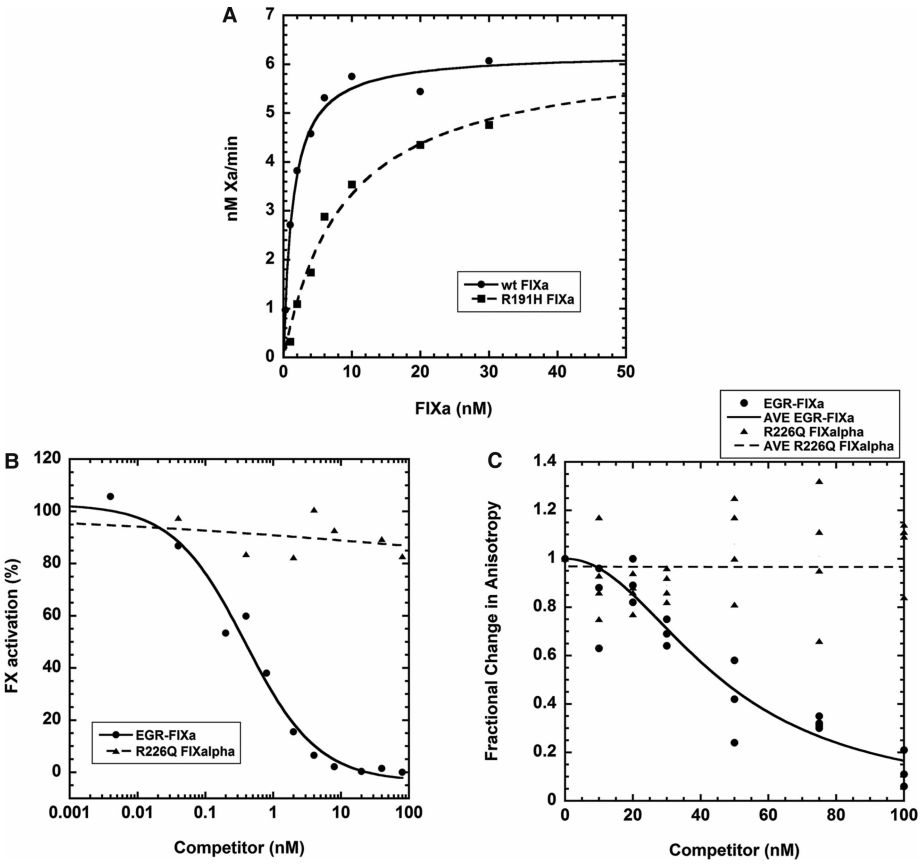

Figure 2. Apparent affinity of factor IXaβ, factor IXaα, and factor IXα for factor VIIIa on phospholipid vesicles.

(**A**) The apparent affinity (K_D (app)) for the interaction of each form of factor IXa with factor VIIIa was determined for wt factor IXaβ and R191H factor IXaα using the activation of factor X as a measure of complex formation. The rate of factor X activation by the factor IXa-factor VIIIa complex was determined by titrating the indicated form of factor IXa into a reaction containing 0.05 nM factor VIIIa, 50 μM PCPS, and 200 nM factor X in HBSCPB, as described in Methods. Representative curves for the activation of factor X by wt factor IXaβ (•) and R191H factor IXaα (■) are presented. The K_D (app) was 1.2 ± 0.1 nM for factor IXaβ vs 9.4 ± 0.6 nM for factor IXaα, while the apparent Bmax was 6.1 ± 0.3 nM FXa/min for factor IXaβ vs 5.8 ± 0.5 nM FXa/min for factor IXaα (±SEM, $n = 4$). (**B**) Competition for binding to factor VIIIa was monitored by titrating the indicated form of factor IXa into a reaction containing 4 nM wt factor IXaβ, 0.05 nM factor VIIIa, 200 nM factor X, and 50 μM PCPS, and measuring impairment of factor X activation compared with a reaction without added competitor, as described in Methods. Data are presented as the percent of maximum activity observed in the absence of competitor. Representative curves for EGR-factor IXaβ (•) and R226Q factor IXα (▲) are shown. The EC_{50} for EGR-factor IXaβ was 0.72 ± 0.09 nM (±SEM, $n = 5$); the EC_{50} for factor IXα could not be computed because of a lack of inhibition. (**C**) The ability of EGR-FIXaβ and R226Q factor IXα to compete directly with factor IXaβ for binding to factor VIIIa in the presence of factor X was determined by titrating increasing amounts of competitor into a reaction containing 10 nM FEGR-factor IXa, 10 nM factor VIIIa, 125 nM factor X, and 50 μM PCPS in HBSC as described in Methods. Data points from four experiments, and the corresponding average fitted curves for the interference of FEGR-factor IXa interacting with factor VIIIa are presented for EGR-factor IXaβ (•) and R226Q factor IXα (▲). The EC_{50} for EGR-factor IXaβ was 45.6 ± 2.0 nM (±SEM, $n = 4$); the EC_{50} for factor IXα could not be computed because of a lack of inhibition.

the saturated cofactor (apparent Bmax) was the same for factor IXaβ and IXaα (6.1 ± 0.3 nM FXa/min for IXaβ vs 5.8 ± 0.5 nM FXa/min for IXaα) suggesting both proteases, upon becoming part of the factor IXa-factor VIIIa complex, have equivalent factor X activating activity.

Since R226Q factor IXα had no catalytic activity, we measured the ability of R226Q factor IXα to bind factor VIIIa using an assay that measured the ability of factor IXα to compete with factor IXaβ for binding to factor VIIIa, as measured by a decrement in factor X activation. As a control, we measured the ability of active site inhibited EGR-factor IXaβ to compete with wt factor IXaβ for binding to factor VIIIa using activation of factor X as a readout. As expected, titration with EGR-factor IXaβ resulted in a complete competition of the signal,

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with an EC_{50} value of 0.72 ± 0.09 nM (±SEM, $n = 5$) (Figure 2B). R226Q factor IXα, however, was unable to effectively compete away the activity even when R226Q factor IXα was titrated to 100 nM (~100 fold excess).

This assay, although very commonly used, indirectly measures factor IXa-VIIIa binding via the appearance of factor Xa. To confirm the findings that factor IXα did not interact with factor VIIIa, we used a solution based anisotropy method that detects the direct interaction of factors IXα and VIIIa [35]. As shown in Figure 2C, titration with EGR-factor IXaβ effectively competed with fluoresceinated factor IXaβ binding to factor VIIIa with an EC_{50} value of 45.6 ± 2 nM (±SEM, $n = 4$). In agreement with the previous assay that imputes factor IX(a)-VIIIa binding from the appearance of enzymatic activity, R226 factor IXα was not able to effectively compete for binding to factor VIIIa as measured with a direct physical method. Thus, both approaches indicate that factor IXα is not able to effectively bind factor VIIIa.

Given the importance of the factor IXa-VIIIa complex for activation of factor X, the kinetics of factor X activation by each form of factor IXa under conditions that would favor different levels of factor IXa-VIIIa complexes was determined. Titrating factor X into reaction mixtures containing 0.1 nM factor IXa, 1 nM factor VIIIa, and 50 μM phospholipids (Figure 3A) yielded equivalent apparent K_M (K_M (app)) values for factor X activation by factor IXaβ and factor IXaα in complex with factor VIIIa; 10.8 ± 0.9 nM and 9.0 ± 1.1 nM, respectively. However, the maximum rate (Vm (app)) of factor X activation was significantly higher with factor IXaβ than with factor IXaα on a per active site basis: 6.1 ± 0.6 nM Xa/min vs 3.3 ± 0.5 nM Xa/min, respectively (±SEM, $n = 4$).

We expected this reduced Vm (app) for factor IXaα was due to a low fraction of enzyme in fully formed intrinsic tenase complexes because of the reduced affinity of factor IXaα for factor VIIIa. Thus, we repeated the experiment at 10 nM factor VIII so that factor VIIIa was saturating for factor IXaα, as well as factor IXaβ. Under these conditions, the Vm (app) was the same for both enzymes; 7.6 ± 0.7 nM Xa/min for factor IXaβ and 7.0 ± 0.6 nM Xa/min for factor IXaα (Figure 3B). The K_M (app) was 11.0 ± 1.2 nM for factor IXaβ and 7.0 ± 0.8 nM for factor IXaα (± SEM, $n = 5$).

Consistent with the above data, factor IXaα demonstrated only 1.6–6% of the coagulant activity of factor IXaβ as determined by measuring the aPTT in factor IX immunodepleted plasma (Table 1). As expected, the activated control proteins, R191Q;S411A factor IXaα, R191Q;R226Q factor IX, and R226Q factor IXα had no detectable coagulant activity. This also agrees with previous reports on the activity of factor IXaα derived from plasma factor IX [16,17].

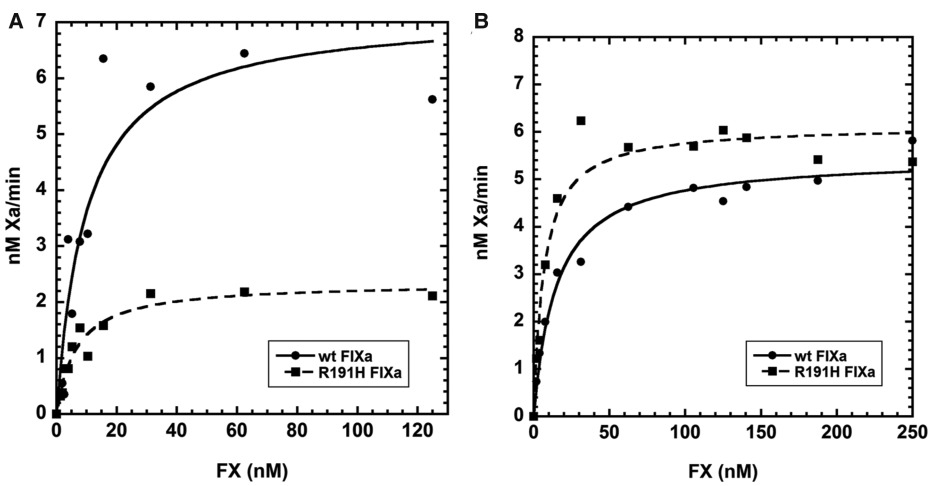

Figure 3. Kinetics of factor X activation by factor IXaβ and factor IXaα complexed with factor VIIIa.

The rates of factor X activation by wt factor IXaβ and R191H factor IXaα were determined in reactions containing 0.1 nM factor IXa, 50 μM PCPS, variable factor X in HBSCPB, and either (**A**) 1 nM factor VIIIa, or (**B**) 10 nM factor VIIIa. Representative curves for wt factor IXaβ (●) and R191H factor IXaα (■) are presented.

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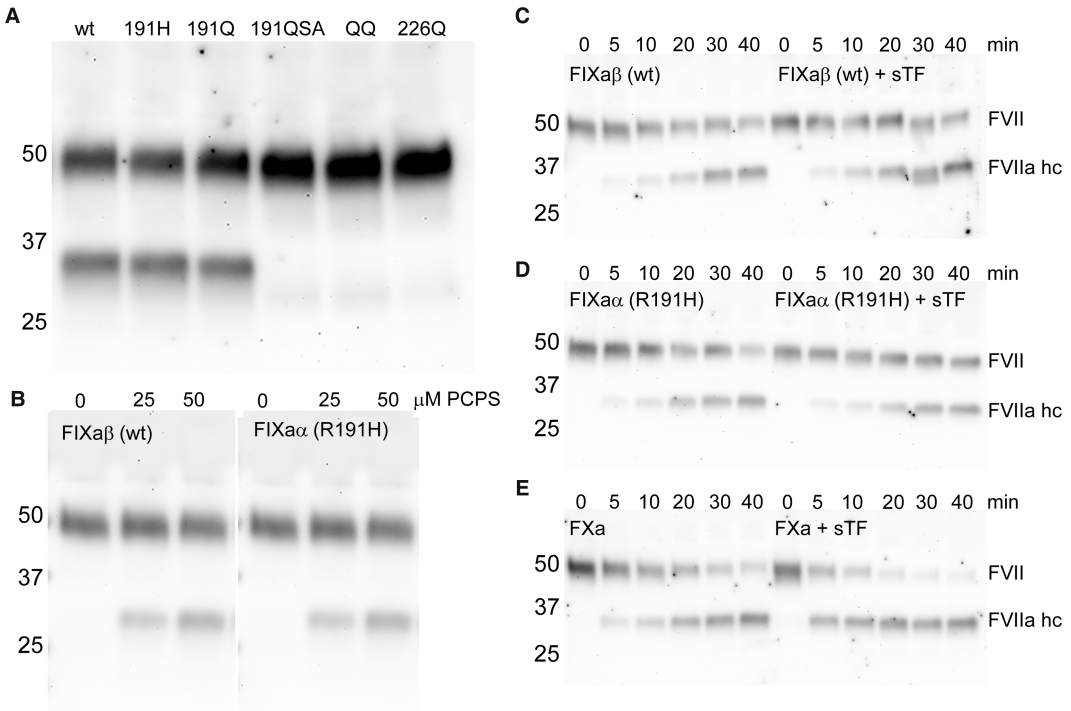

Figure 4. S404A factor VII cleavage by activated recombinant factor IX.

100 nM S404A factor VII was incubated with 10 nM of the indicated form of factor IX, in HBSCP buffer at 37°C for the indicated times under the following conditions. Reactions were quenched with reducing sample buffer, run on 4–20% SDS–PAGE, transferred to PVDF membrane, and probed with sheep anti-human factor VII polyclonal antibody as described in Methods. (**A**) activated wt, R191H, R191Q, R191Q;S411A (191QSA), R226Q, or R191Q;R226Q (QQ) factor IX were incubated with S404A factor VII in the presence of 200 μM PCPS. (**B**) wt factor IXaβ or R191H factor IXaα were incubated with S404A factor VII in the presence of the indicated concentration of PCPS. Panels **C**–**E** illustrate the effect of sTF on the cleavage of 100 nM S404A factor VII in the presence of 50 μM PCPS ± 200 nM sTF for 0–40 min at 37°C with either (**C**) 10 nM wt factor IXaβ, (**D**) 10 nM R191H factor IXaα, or (**E**) 1 nM factor Xa.

Factor VII activation by various forms of activated factor IX

The ability of each form of factor IXa to cleave factor VII was examined by incubating the indicated form of factor IXa with S404A factor VII (mutation of the active site serine to alanine to minimize possible effects of catalytically active factor VIIa) in the presence of 200 μM PCPS for 20 min at 37°C. Under these conditions, R191Q and R191H derived factor IXaα and wt factor IXaβ cleaved S404A factor VII indistinguishably (Figure 4A). The activated control proteins (R191Q;S411A factor IXaα, R226Q factor IXα, or R191Q;R226Q factor IX) yielded no detectable cleavage of S404A factor VII (detection limit for the assay was 4 nM factor VIIa). The presence of phospholipid was essential for the reaction, and the activity was similar for both factor IXaα and factor IXaβ with >25 μM PCPS (Figure 4B). Interestingly, the presence of soluble tissue factor (sTF) did not dramatically affect the cleavage rates of S404A factor VII (Figure 4C,D), unlike its effect on the activation of S404A factor VII by factor Xa (Figure 4E) [43].

The kinetics of factor VII activation by wt factor IXaβ and R191H factor IXaα were examined by titrating wt factor VII into reactions with the indicated form of factor IXa in the presence of PCPS. Activated factor VIIa was measured using the fluorogenic substrate SN-17c in the presence of sTF, as described in Methods. Similar to the kinetics of factor X activation, the apparent K_M for factor VII activation (K_M (app)) was similar for factors IXaβ and IXaα; 421 ± 37 nM vs 601 ± 63 nM, respectively (± SEM, $n = 6$). The K_M (app) for the reaction was supraphysiologic, but activation of factor VII was measurable in the physiologic range (Figure 5B).

In contrast with factor X activation by various forms of factor IXa, the maximum rate (Vm (app)) of factor VII activation was indistinguishable with each form of factor IXa; 114 ± 9 nM VIIa/min for factor IXaβ vs 103 ± 7 nM VIIa/min for factor IXaα (±SEM, $n = 6$) (Figure 5). These data indicate that factors IXaβ and IXaα

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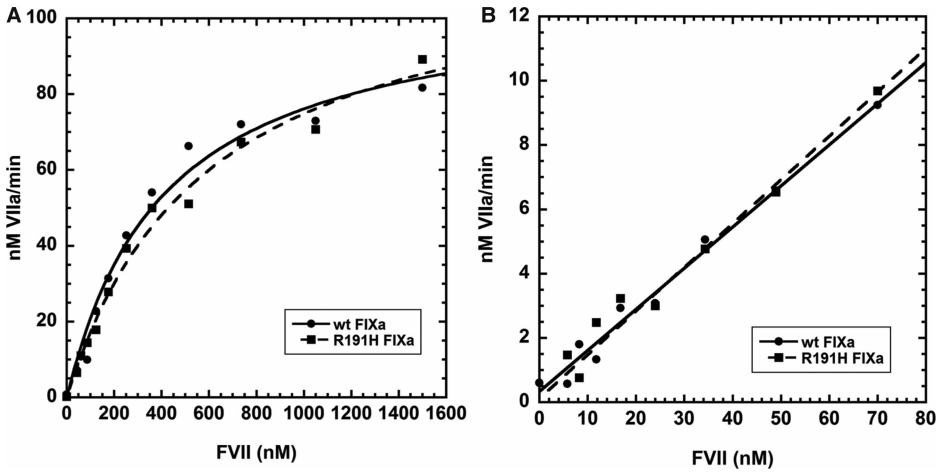

Figure 5. Kinetics of factor VII activation by factor IXaβ and factor IXaα in the presence of phospholipid.

The rates of factor VII activation by wt factor IXaβ- or R191H factor IXaα-phospholipid were determined in reactions containing 20 nM factor IXa, the indicated concentrations of factor VII, and 200 μM PCPS in HBSCPB. Activated factor VIIa was measured in the presence of 200 nM sTF by quantifying cleavage of the fluorogenic substrate SN-17c as described in Methods. (**A**) Representative curves for kinetic parameter determination (0–1500 nM factor VII) for wt factor IXaβ (•) and R191H factor IXaα (■) are presented. The apparent K_M (K_M (app)) of factor VII for cleavage by factor IXaβ was 421 ± 37 nM vs 601 ± 63 nM for factor IXaα. The maximum rate (Vm (app)) of factor VII activation was 114 ± 9 nM Xa/min for factor IXaβ vs 103 ± 7 nM Xa/min for factor IXaα (±SEM, $n = 6$). (**B**) Representative curves for the examination of low concentration substrate activity (0–70 nM factor VII) for wt factor IXaβ (•) and R191H factor IXaα (■) are presented.

interact equally well with factor VII. We then investigated whether factor VIII influenced factor IXa cleavage of factor VII and found that the presence of factor VIIIa did not significantly alter the cleavage of S404A factor VII by either form of factor IXa (Figure 6A,B). In contrast, the presence of VIIIa had a clear positive effect on the activation of S419A factor X (mutation of the active site serine to alanine) by factors IXaβ and IXaα

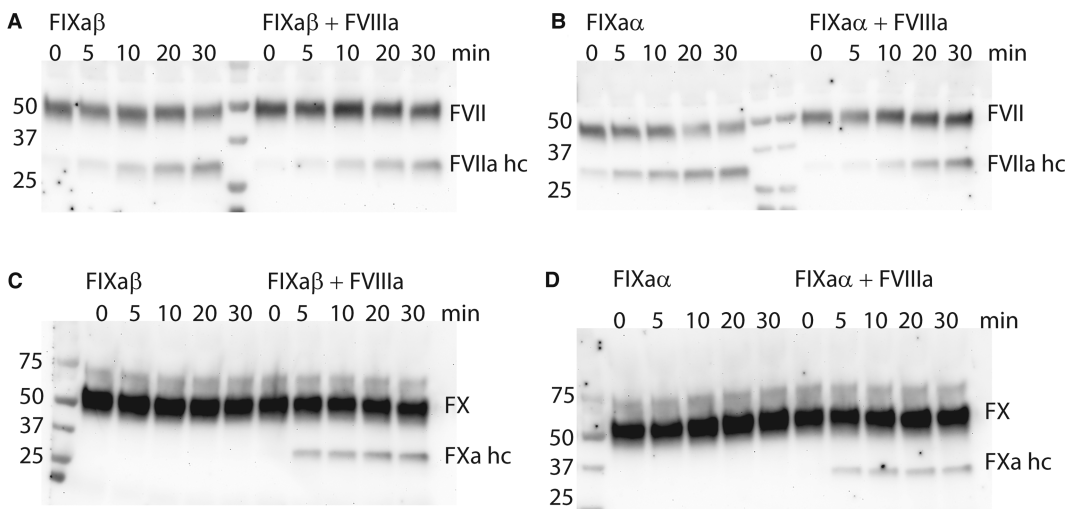

Figure 6. Effect of factor VIIIa on S404A factor VII and S419A factor X cleavage.

Thirty-minute time courses in HBSCP buffer containing 50 μM PCPS ± 1 nM thrombin-activated factor VIII were carried out at 37°C using 100 nM S404A factor VII as the substrate and (**A**) 10 nM wt factor IXaβ or (**B**) 10 nM R191H factor IXaα as the protease, or 100 nM S419A factor X as the substrate and (**C**) 10 nM wt factor IXaβ or (**D**) 10 nM R191H factor IXaα as the protease. All samples were quenched with reducing sample buffer, run on 4–20% SDS–PAGE, transferred to PVDF membrane, and probed with either sheep anti-human factor VII polyclonal antibody or sheep anti-human factor X polyclonal antibody as described in Methods.

(Figure 6C,D). These findings on factor X activation agree with kinetic assays demonstrating the importance of factor VIIIa in factor X activation by factors IXaβ and IXaα, implying that the lack of effect of factor VIIIa on factor VII activation is not a function of the assay.

To determine whether the results in reactions with purified proteins are reflected in plasma, a factor VIIa specific coagulation assay [39] was used to examine the ability of factor IX zymogen or each form of activated factor IX to generate factor VIIa in plasma. Genetically deficient factor IX plasma has been reported to be deficient in factor VIIa as well as factor IX, containing <10% of normal levels of factor VIIa [3]. Commercially obtained plasma from individuals genetically deficient in factor IX was incubated with either factor IX zymogen (90 nM) or the indicated form of activated factor IX (1 nM, to reflect levels of IXa detected in plasma [44]) and subsequently assayed for factor VIIa levels. As expected, starting levels of plasma factor VIIa were initially low (2.8 pM), but addition of either zymogen wt factor IX, R191H factor IX, and R191Q factor IX, or their corresponding activated forms: factor IXaβ, R191H factor IXaα, and R191Q factor IXaα resulted in significantly increased levels of factor VIIa in the plasmas to which they were added (Table 2). R191H and R191Q factor IXaα, however, were significantly less effective than wt factor IXaβ in generating factor VIIa in plasma. As expected, the control protein, R191Q;S411A factor IX, which does not have a competent catalytic triad, did not result in the generation of factor VIIa in plasmas to which it was added.

Discussion

Data from past studies suggest that *in vivo*, factor IX, but not factor XI nor XII, is required for the generation of basal levels of factor VIIa, and that factor VII, but not factor XI, is required for baseline levels of factor IX activation [3,7,11]. This suggests that reciprocal activation of zymogens might serve to generate the low level of active proteases that circulate in the 'idling' state of readiness that characterizes a coagulation system that is poised to respond to a hemostatic challenge [45]. A similar pattern of reciprocal zymogen activation to initiate a protease cascade is seen in other processes, including the closely related fibrinolytic cascade [46–48]. A salient question then arises regarding the proteolytic event that gets the process started. The data in this paper address the factor IX-dependent activation of factor VII and suggest that there may be functions of factor IX action that we do not fully understand, and that might shed light on the initial proteolytic events of coagulation.

Experiments in this report differ from previous reports on the interactions between factors VII(a) and IX(a) by using carefully designed and verified populations of recombinant factors VII and IX. Using these rigorously defined molecules minimized the likelihood of factor IX being activated by factor VIIa that was present in factor VII preparations, and allowed use of just the intended forms of IX(a).

Table 2. Comparison of factor VIIa levels in factor IX deficient plasma after treatment with recombinant factor IX zymogen or specific forms of activated factor IX

Factor IX zymogen	90 nM FIX addition; FVIIa (pM) detected	'Activated' Factor IX	1 nM activated FIX addition; FVIIa (pM) detected
FIX (wt)	*26.0 ± 2.0	FIXaβ (wt)	*14.2 ± 1.0
FIX (R191H)	*11.0 ± 1.5	FIXaα (R191H)	*7.3 ± 0.5
FIX (R191Q)	*7.0 ± 0.8	FIXaα (R191Q)	*9.0 ± 1.2
FIX (R226Q)	4.3 ± 0.8	FIXα (R226Q)	2.1 ± 0.2
FIX (R191Q; R226Q)	5.8 ± 2.6	FIX (R191Q; R226Q)	1.8 ± 0.2
FIX (R191Q; S411A)	3.7 ± 0.6	FIXaα (R191Q; S411A)	2.1 ± 0.4
Buffer	2.8 ± 0.5	buffer	2.4 ± 0.3

After incubation of Factor IX (90 nM, to reflect physiological levels) or 'activated' factor IX (1 nM, to reflect levels of IXa detected in plasma [44]) with factor IX genetically deficient plasma for 10 min., coagulant activity was determined using a factor VII dependent clotting assay. Clotting times were converted to factor VIIa levels using a factor VIIa standard curve. Data are expressed as the mean ± SEM of five experiments, and * indicates a significant increase over buffer baseline (Students *t*-test, 95% confidence level).

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In experiments verifying that the desired forms of factor IX(a) were generated by cleavage of mutant forms of factor IX, we found that activation with factor XIa yielded cleavage at or near the mutated activation residue, yielding populations of factor IX(a) that unexpectedly contained molecules consistent with fully activated factor IXaβ (Supplementary Figure S2). Others have demonstrated off-target cleavage with XIa [49]. Analysis of these activated recombinant factor IX species demonstrated varying effects on coagulation, intrinsic tenase activity, and factor VII activation that would have confounded interpretation of experimental results (data not shown). Hence, rather than using factor XIa to generate desired forms of factor IXa, factor VIIa/sTF was used as the activating agent. This yielded the intended products, with no evidence of off-target cleavage, or contamination by unexpected forms of factor IX(a) (Figure 1).

We cannot explain why cleavage resistant mutants of factor IX are more susceptible to cleavage by factor XIa than they are by the factor VIIa/sTF complex, as we did not explore this phenomenon further. We speculate that factor XIa, in its role catalyzing amplification of coagulation, has less constraints on substrate specificity than does factor VIIa. This would be consistent with factor XIa processivity in cleaving the two activation sites in factor IX to rapidly yield factor IXaβ. We also speculate that the factor XIa role of amplifying coagulation is poorly suited to generating the low level of basal plasma factor IX activation, which likely serves to keep hemostasis 'idling' until an initiating stimulus is introduced [50].

While studies that use mutagenesis of specific residues are certainly useful, our findings reinforce the importance of verifying the assumed characteristics of modified proteins.

Consistent with previous findings [16], cleavage of factor IX at only R226 yields factor IXaα, a fully competent enzyme, as measured by the identical rates of cleavage of a tri-peptide chromogenic substrate (Pefachrome IXa) by factor IXaβ and factor IXaα (Table 1). This suggests that cleavage at residue 226 is sufficient for the formation of the activating salt bridge between the newly formed N-terminus of the protease domain and Asp 410 (chymotrypsin numbering 194) and the subsequent stabilization of the oxyanion hole. These activation steps that yield enzymatic activity of factor IX appear to be minimally influenced by events at residue 191, the other site of factor IX activation cleavage.

In contrast with the indistinguishable abilities of factors IXaα and IXaβ to cleave a small molecule substrate that likely has no exosite interactions (Pefachrome IXa, Table 1), the activities of factor IXaβ and factor IXaα against the known physiologic macromolecular substrate, factor X, are quite different. As previously observed with plasma-derived proteins [16], factor IXaα was much less efficient than factor IXaβ at catalyzing the activation of factor X in the presence of the required cofactor, factor VIIIa (Figure 3). Given that factor IXaα and IXaβ have indistinguishable amidolytic activity, and that both forms of IX require VIIIa to activate X, these data suggest that the added cleavage at R191 imbues factor IXaβ with improved factor VIIIa binding capacity. Using a kinetic factor X activation assay, the apparent affinity for factor VIIIa was 8-fold lower for factor IXaα than for factor IXaβ (Figure 2A), indicating that cleavage at 191 is required for high-affinity factor VIIIa binding by factor IX; this is in agreement with previous studies [16]. However, contrary to this previous report, we found that cleavage at 191 is not sufficient for high-affinity factor VIIIa binding by factor IX. This is illustrated by the observations that factor IXα (factor IX cleaved only at 191) is unable to compete for factor VIIIa binding as detected in an assay with enzymatic activity as the readout (Figure 2B), or a soluble anisotropy based physical binding assay (Figure 2C). The reason(s) for this discrepancy are unknown, but may be related to differences in factor IXα homogeneity. In the earlier report [16], the factor IXα preparation, unlike our preparation and other reports [14], possessed amidolytic activity as well as factor VIII light chain binding activity, possibly indicating contamination with other activated forms of factor IX such as we observed upon activation of factor IX with factor XIa. We postulate that the previous preparation of factor IXα, was contaminated with factor IXaβ, which was then responsible for the observed factor VIII binding activity. Likewise, the previous report used the factor VIIIa light chain immobilized on a solid support as a measure of factor VIIIa binding. Using immobilized factor VIIIa light chain is more dissimilar from physiologic conditions than the current studies which used the complete factor VIIIa molecule, newly generated in phospholipid containing solution-based reactions. Moreover, the fact that two independent solution-phase assays yielded identical results strengthens the data presented in this report. Hence, in the presently described experiments the factor IXα activation intermediate exhibits no enzymatic activity, and although cleavage of factor IX at 191 may be necessary for optimum interaction with factor VIIIa, it is not sufficient for this binding function.

Interestingly, factor VIIIa has equivalent apparent Bmax values for both factor IXaα and IXaβ (Figure 2A), and upon the formation of equal numbers of factor IXa-factor VIIIa complexes on phospholipid vesicles, each form of factor IXa yielded similar factor X activation. Taken together, these findings suggest that factor IXaβ

and factor IXaα bound to factor VIIIa interact well with factor X. Since factor IXaα has a lower affinity for factor VIIIa, it forms fewer catalytically active complexes at any given concentration, and therefore, less factor X is activated to factor Xa than is the case for reactions with equivalent concentrations of factor IXaβ. This provides a mechanistic explanation for factor IXaα demonstrating <6% of the coagulant activity of factor IXaβ as determined by measuring the aPTT in factor IX immunodepleted plasma, which is a factor X dependent assay (Table 1). This pattern agrees with previous observations on the activity of factor IXaα derived from plasma factor IX [16,17].

In contrast with the differential properties of factors IXaβ and IXaα in activating factor X, the two forms of factor IXa demonstrated similar activities against another physiologically relevant macromolecular substrate, factor VII (Figure 5). This observation was unlikely due to auto activation by factor VIIa contaminating the factor VII preparation, as catalytically incompetent S404A factor VII was cleaved indistinguishably by factors IXaα and IXaβ (Figure 4). In further contrast with factor X activation, factor VII activation by either factor IXaβ or factor IXaα was unaffected by the presence of the cofactor, factor VIIIa (Figure 6). These data are in agreement with previous reports of factor IXaβ activity [10,34,51], and are novel for factor IXaα. These results also support the conclusion that cleavage of factor IX at R226 is sufficient to yield a fully competent protease, and that cleavage of factor IX at R191 does not significantly influence protease activity *per se*; rather, it allows optimal binding to factor VIIIa. Moreover, they suggest that any exosite interactions required for factor IXa activation of factor VII are equally operative for both factors IXaα and IXaβ.

Although factor VII activation by another coagulation protease, factor Xa, is enhanced by the cofactor tissue factor [43], this molecule did not influence the activation of factor VII by either form of factor IXa (Figure 4). The observation that the activation of factor VII by factor IXaβ and factor IXaα is much more efficient in the presence of phospholipids is consistent with a report that plasma factor VIIa levels increase following high-fat meals in normal individuals, factor XII deficient and factor XI deficient patients, but not in factor IX deficient patients [7]. Our experiments did not explore the question of specific phospholipid roles or requirements for factor VII activation by factor IXa [52–55]. Nonetheless, our observations are consistent with clinical observations of plasma levels of factor VIIa in individuals under various conditions.

While factors IXaβ and IXaα yielded similar cleavage of factor VII in a purified system, differences were observed in the *ex vivo* generation of factor VIIa in plasma, as measured by a factor VIIa specific clotting assay. In this plasma assay, the addition of factor IXaα resulted in 50–60% of the factor VIIa activity that was observed with the addition of factor IXaβ (Table 2). This pattern was mirrored by the relative amounts of factor VIIa detected upon addition of plasma levels (90 nM) of wt factor IX zymogen, or the R191H or R191Q factor IX mutants; more factor VIIa was detected when wt factor IX was added than with mutants that could generate only factor IXaα. We hypothesize that the higher observed factor VIIa levels after factor IXaβ exposure was most likely due to the factor VIIIa dependent superior ability of factor IXaβ to activate factor X to factor Xa, which was then also able to activate factor VII, leading to augmented factor VIIa levels when compared with the factor IXaα reactions.

In an aPTT assay using factor IX immunodepleted plasma, factor IXaα demonstrated <6% of the coagulation activity of factor IXaβ (Table 1), consistent with factor IX Chapel Hill [56]. Factor IXaα is catalytically competent, but deficient in its ability to bind factor VIIIa, thus removing most of the contribution of factor IXa to the intrinsic tenase complex, which is critical to coagulation as measured by the aPTT. Hence, the impaired ability of factor IXaα to function in the tenase has a significant impact on the aPTT.

The data presented here provide a plausible molecular mechanism for the observed dependence of plasma factor VIIa levels on factor IX. Namely, both factors IXaα and IXaβ are fully competent to activate factor VII in the absence of factor VIIIa, and thus these findings may help explain measurements in human plasma. We have no direct evidence as to which form of factor IXa is the pertinent factor VII activator *in vivo*, as to our knowledge, there has been no study differentiating between factor IXaα and IXaβ in plasma. It is tempting to speculate that factor IXaα, generated via a factor XIa independent mechanism, serves a previously unknown role of generating basal levels of factor VIIa, and factor IXaβ serves a critical role in the propagation phase of coagulation. This would also be consistent with the predominant role of factor XI serving to amplify, rather than initiate the coagulation process [57].

This raises the question of how various forms of factor IXa might be generated *in vivo*. Measurements in plasmas from factor deficient individuals indicate that factor IXa generation is dependent upon factor VII (11). However, basal levels of factor VIIa are dependent upon factor IX [3,58]. These findings are consistent with a reciprocal activation circuit between factor IX and factor VII (Figure 7). Such a circuit is a recurring pattern in

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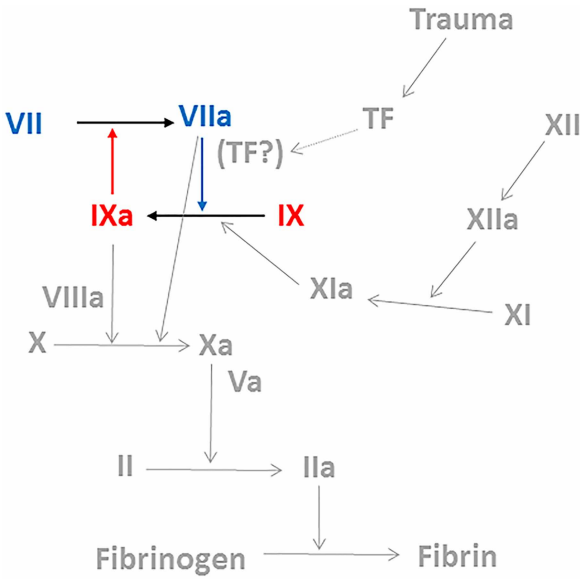

Figure 7. Schematic representation of the reciprocal activation of factors VII and IX in the coagulation cascade.

initiating serine protease cascades, such as the reciprocal activation between plasminogen and single-chain urokinase [59]. Careful experiments with well-defined molecules allowed an understanding of the initial activating events in cell surface fibrinolysis [46,48,60], but our understanding of the initiating events of the coagulation cascade is not yet at that level.

Given that it appears equally plausible that factor VIIa or factor IXa might be the initiating protease of the coagulation cascade, it might be appropriate to illustrate this key step in hemostasis as currently being unknown, until further research can establish the mechanism.

Abbreviations

CV, column volumes; EGR-FIXa, factor IX-Glu-Gly-Arg; FEGRck, fluorescein chloromethylketone; FEGR-FIXa, factor IXa-fluorescein EGR; HBS, 20 mM HEPES, 100 mM NaCl, pH 7.4; HBSCP, 20 mM Hepes, 100 mM NaCl, 5 mM CaCl2, 0.1% PEG-8000, pH 7.4; HBSCPB, HBSCP + 0.1% BSA; PC, phosphatidylcholine; Pefachrome FIXa, CH_3SO_2-D-CHG-Gly-Arg-pNA.AcOH; PS, phosphatidylserine; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SN-17c, D-Phe-Pro-Arg-ANSNH-C4H9; Spectrozyme FXa, CH_3O-CO-D-CHG-Gly-Arg-pNA.AcOH; sTF, soluble tissue factor; TBST, 10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4; VKOR, vitamin K 2, 3-epoxide reductase C1; wt FIX, wild-type factor IX.

Author Contribution

T.M.M., K.T.K., B.E.B., and E.R.N. performed experiments. T.M.M. analyzed results and made the figures. T.M.M., K.T.K., and B.S.S. designed the research and wrote the paper.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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