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Polyphosphate accelerates factor V activation by factor XIa

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Summary

Factor Va enhances the rate of prothrombin activation by factor Xa by four to five orders of magnitude. Production of initiating levels of factor Va from its precursor, factor V, is a critical event early in haemostasis, as factor V exhibits negligible cofactor activity. While thrombin is the most potent physiological back-activator of factor V, the first prothrombinase complexes require a source of factor Va prior to thrombin generation. A recent study by Whelihan et al. (*J Thromb Haemost* 2010; 8:1532–1539) identified factor XIa as a candidate for the initial thrombin-independent activation of factor V, although this reaction was slow and required relatively high concentrations of factors V and XIa. Activated platelets secrete polyphosphate, which we previously showed to be potently procoagulant. We now report that polyphosphate greatly accelerates factor V activation by factor XIa, and that this is supported by polyphosphate polymers of the size secreted by activated human platelets. This finding provides additional evidence that factor XIa-mediated generation of factor Va may contribute to the initiation of haemostasis.

Keywords

Polyphosphate; factor V; factor XI; prothrombinase

Introduction

Coagulation factor V (FV) circulates in plasma as the single-chain procofactor with a molecular weight of 330 kDa (1, 2), comprising three A domains, a very large B domain, and two C domains, organised in an A1-A2-B-A3-C1-C2 structure (3). FV is converted by FXa or thrombin into the active cofactor via limited proteolysis at positions Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ (4, 5). This removes the B domain to generate mature factor Va (FVa), consisting of an N-terminal heavy chain (A1–A2, 105 kDa) associated via Ca²⁺ ions to the C-terminal light chain (A3-C1-C2, 74 kDa) (1–3, 6). FVa is the essential protein cofactor for factor Xa (FXa) in the prothrombinase complex that rapidly converts prothrombin to thrombin (7–10). In the absence of FVa, the rate of thrombin generation by FXa is reduced by four to five orders of magnitude (8, 9).

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Conflicts of Interest

The authors are a co-inventors on patents and pending patent applications on medical uses of polyP.

FV is unable to function as a cofactor for FXa, so generation of initiating levels of FVa is a critical early event in haemostasis (9). Since cleavage of FV by FXa is relatively slow (4), and since the generation of thrombin is dependent on the participation of FVa, it is not entirely clear which enzyme generates the first few FVa molecules that allow sufficient thrombin for back-activation of the remaining FV. A few proteases with potential physiological significance have been reported to cleave FV including calpain (11), neutrophil elastase (12, 13), the factor VIIa/tissue factor complex (14), and platelet-localised proteases (15). However, not all the cleaved forms of FV derived from these proteases are functional in FVa cofactor assays.

Recently, Whelihan et al. described cleavage of FV by factor XIa (FXIa) that yielded a FVa species with FXa-cofactor activity in human plasma (16). Our laboratory has shown that polyphosphate (polyP) accelerates the activation of FV by thrombin and FXa (17). More recently, we reported that polyP released from activated platelets greatly accelerates FXI activation by thrombin, and that polyP may therefore be the “missing” cofactor that allows this reaction to occur at physiologically relevant rates (18). We now report that platelet-size polyP also potentially accelerates FV activation by FXIa.

Materials and methods

Materials

Size-fractionated polyP preparations of very narrow size distributions were prepared as previously described (19), and are referred to by their polymer length followed by “mer” (for example, 167mer). All polyP concentrations in this study are given in terms of phosphate monomer (monomer formula: NaPO_3). Platelets were single-donor apheresis platelet concentrates (leukoreduced and irradiated) from Community Blood Services of Illinois. Platelet releasates were prepared from platelets activated with 5 μM thrombin receptor agonist peptide as described (18). Purified human prothrombin, FXIa, FV, FVa, corn trypsin inhibitor (CTI), dansylarginine *N*-(3-ethyl-1,5-pentanedyl)amide (DAPA) and anti-human FV heavy chain AHV-5146 monoclonal antibody were from Haematologic Technologies (Burlington, VT, USA). Some additional purified human prothrombin was a kind gift from Dr. Craig Jackson. Human FXa and a-thrombin were from Enzyme Research Laboratories (South Bend, IN, USA). Rivaroxaban was from Selleckchem (Houston, TX, USA). Peroxidase-conjugated goat anti-mouse immunoglobulin (IgG) and enhanced chemiluminescence (ECL)-plus blotting substrate were from Pierce (Rockford, IL, USA). Liposomes consisting of 20 % phosphatidylserine/80 % phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) were made by sonication or extrusion. Spermine, hirudin and aprotinin were from Sigma-Aldrich (St. Louis, MO, USA). The isolated polyP-binding domain of recombinant *Escherichia coli* exopolyphosphatase fused to maltose-binding protein and His₆ tag (PPXbd) was expressed and purified as described (20).

Surface plasmon resonance

Surface plasmon resonance (SPR) binding studies were performed at 25 °C using a Biacore 3000 instrument (Biacore, Uppsala, Sweden). Streptavidin was bound to CM5 sensorchips by standard amine coupling; after blocking and washing, biotinylated polyP was captured on

the surface. Varying concentrations of FV in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.005 % surfactant P20 were flowed over the surface at 50 μ l/minute (min) using a 3-min association phase and 5-min dissociation phase, with background subtraction using a streptavidin-coated reference cell without polyP. The sensorchip was regenerated by washing with 1 M NaCl between injections. Binding kinetics were analysed according to the 1:1 Langmuir binding model. K_D values were calculated from the quotient of the derived dissociation (k_{diss}) and association (k_{ass}) rate constants.

SDS-PAGE and Western blot analysis of FV activation

Varying concentrations of FXIa, FV and polyP were incubated at 37 °C. In some experiments, control reactions included 500 μ g/ml PPXbd to inhibit polyP. At various time points, aliquots were removed into SDS sample buffer and resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA) and either stained via silver stain (21) or transferred to polyvinylidene difluoride (PVDF) membranes and probed with 100 ng/ml anti-FVa heavy chain antibodies followed by 420 ng/ml peroxidase-conjugated anti-mouse IgG. Antibody binding was visualised using ECL-plus.

For experiments utilising purified human FV, reactions typically contained 20 nM FV and varying concentrations of FXIa with polyP or platelet releasate (as a source of polyP) at 37 °C in 30 mM Hepes pH 7.4, 50 mM NaCl, 1.2 mM $CaCl_2$, 2 % bovine serum albumin. Some platelet releasate samples were boiled for 20 min to denature proteins, then cooled to room temperature before use.

For experiments utilising human plasma as the source of FV, blood was collected via atraumatic venipuncture from normal volunteers into hirudin (15 μ g/ml final) to inhibit thrombin (and anticoagulate the blood). Blood was centrifuged at $3,000 \times g$ for 10 min to collect platelet-poor plasma, to which a final concentration of 100 μ g/ml CTI was then added to inhibit factor XIIa, and 50 μ g/ml rivaroxaban to inhibit FXa. Informed consent was obtained from all human volunteers in a protocol approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign.

Analysis of FV activation via cofactor activity in prothrombinase

FXIa was incubated with FV at 37 °C with or without polyP (0–80 μ M) of various polymer lengths in 30 mM Hepes pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, 1 % bovine serum albumin. At various time points, aliquots were removed and quenched by addition of an ice-cold solution containing 1.5 mg/ml spermine to neutralise polyP and 10 μ M aprotinin to inhibit FXIa. The cofactor activity of the FXIa-derived FV(a) species was measured via its ability to stimulate FXa-catalyzed thrombin generation. FXIa-treated FV was added to reactions containing 100 μ M liposomes, 2 μ M prothrombin, and 30 μ M DAPA in 30 mM Hepes pH 7.4, 100 mM NaCl, 2 mM $CaCl_2$, 1 % bovine serum albumin. The reaction was initiated with 2.5 nM human FXa and thrombin generation was measured by DAPA fluorescence (280 nm excitation, 545 nm emission, 515 nm cutoff filter) using a SpectraMax microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA). Rates of increase in fluorescence were converted to FVa concentrations using a standard curve. Control experiments indicated

that at the concentrations used, neither spermine nor aprotinin affected the prothrombinase assays.

Results

PolyP binds with high affinity to FV

We previously reported that polyP binds FXI and FXIa with high affinity (K_D values of 6.4 and 1.5 nM, respectively) (18). Using SPR, we now report that FV bound tightly to immobilised polyP (Figure 1), yielding association and dissociation rate constants of $2.88 \times 10^6 \text{ M}^{-1}\text{second}^{-1}$ (k_{ass}) and $3.07 \times 10^{-3} \text{ second}^{-1}$ (k_{diss}), respectively, with a calculated K_D of 1.06 nM.

PolyP enhances FV cleavage by FXIa

Previous studies have shown that FXIa activates FV, but the reaction was slow and required relatively high concentrations of FV or FXIa to achieve appreciable rates of activation (16). We hypothesised that polyP may serve as a physiologic cofactor for this reaction. Accordingly, we measured FV cleavage at physiologically relevant concentrations of FV (20 nM) and FXIa ($< 1 \text{ nM}$) in the presence or absence of polyP. On SDS-PAGE, the cleaved fragments of FXIa/polyP-treated FV migrated in an identical fashion with the heavy and light chains of thrombin-derived FVa (Figure 2 A). The FV fragment profile of these reactions was also visualised by using Western blotting with an antibody directed against the FVa heavy chain. Figure 2 B shows that the cleavage of FV and appearance of the newly-generated FVa heavy chain (105 kDa) was dependent on the presence of both FXIa and polyP 211mer. Control experiments in which FXIa was omitted from the reaction showed no cleavage of FV, indicating that the observed activity was not due to protease contamination of the FV preparation.

We next wanted to investigate polyP-accelerated cleavage of FV by FXIa using endogenous FV in human plasma. To eliminate interference from feedback activation of FV by other proteases, plasmas contained hirudin, rivaroxaban and CTI to inhibit thrombin, FXa and FXIIa, respectively. Consistent with the results with purified proteins, addition of FXIa and polyP (211mer) to plasma strongly enhanced the appearance of FVa heavy chain at pM FXIa concentrations (Figure 3). These results show that polyP accelerates the cleavage of FV by FXIa in plasma in a manner that is dependent on FXIa even when the activities of thrombin, FXa and FXIIa are blocked by inhibitors.

FV cleavage products exert prothrombinase cofactor activity

The above results suggest that polyP supports the cleavage of FV by FXIa at physiologically relevant enzyme concentrations. The cleavage products observed on the Western blots were consistent with those produced by other physiologically relevant enzymes (FXa and thrombin), but we wanted to determine whether the cleavage products were functionally active FVa. We reacted FV with FXIa and polyP, then evaluated whether the generated material was capable of supporting prothrombinase activity. In the absence of polyP, FVa cofactor activity was negligible over the time period in which FXIa was incubated with FV at the concentrations used (Figure 4 A–C). However, progress curves of the rate of

appearance of FVa cofactor activity were markedly enhanced by inclusion of polyP of three different polymer lengths (Figure 4A, 85mer; Figure 4 B, 305mer; and Figure 4 C, 673mer). The polyP enhancement of this reaction is clearly seen when initial rates of FV activation are plotted versus polyP concentration (Figure 4D). Because FV activation was so slow in the absence of polyP, for comparison we also quantified FV activation in the absence of polyP but at a much higher FXIa concentration (80 nM FXIa and 20 nM FV), and normalised to FXIa concentration. This revealed that 80 μ M polyP 637mer enhanced the rate of FV activation by FXIa by 62-fold.

Figure 4E shows that, while all tested polyP preparations strongly enhanced the rate of FVa generation, longer polyP polymers (305mer to 673mer) supported FVa generation at a rate that was about 2.6-fold higher than that observed with shorter polyP polymers (31mer to 130mer).

Platelet releasates enhance FV cleavage by FXIa

Activated human platelets release polyP with polymer lengths ~ 60 to 100 phosphates (22, 23). We found that polyP polymers in this size range greatly enhanced the rate of FV activation by FXIa (Figure 4 E). We therefore hypothesised that polyP secreted from activated platelets could serve as a cofactor for FV activation by FXIa. We found that releasates from activated platelets appreciably accelerated cleavage of FV by FXIa (Figure 5). Although platelets secrete their own stores of FV/FVa, control experiments (not shown) demonstrated that, at the dilutions used, any platelet-derived FVa was undetectable and therefore did not contribute to the FVa signal in these blots. In addition to polyP, activated platelets release a host of granule contents including platelet-derived proteases (11). Boiled platelet releasate still supported FXIa-mediated cleavage of FV, arguing that a protein in the releasate was unlikely to be responsible for the observed enhancement of FV cleavage by FXIa. Additionally, treatment of platelet releasate with the polyP inhibitor PPXbd diminished the enhancement of FV cleavage by FXIa (Figure 5). These findings are consistent with the idea that polyP secreted from activated platelets greatly accelerates FV activation by FXIa.

Discussion

The conversion of FV from an inactive procofactor to an active cofactor (FVa) is critical for normal haemostatic responses. The availability of FVa controls the rate of thrombin generation, and consequently the ability to produce fibrin and activate platelets (24). The importance of FV is underscored by the bleeding diathesis in humans with FV deficiency (25), and the fact that complete FV deficiency in mice is associated with massive haemorrhage and death during embryogenesis (26). Generation of initiating levels of FVa from FV is important in the initiation phase of coagulation, as intact FV containing the full B domain (with both the acidic and basic regions) is unable to function as a cofactor for FXa catalysis of the conversion of prothrombin to thrombin (9). The B-domain of procofactor FV is thought to prevent premature procoagulant activity prior to proteolytic processing by blocking binding sites on intact FV (27, 28). Removal of the B domain via thrombin-mediated cleavage of FV at Arg⁷⁰⁹, Arg¹⁰¹⁸, and Arg¹⁵⁴⁵ produces a fully functional

cofactor FVa with a heavy and light chain of 105 KDa and 74 KDa respectively. Cleavage by FXIa in the absence of polyP appears to result in the same cleavage products (16).

Thrombin is likely the major back-activator of FV during the propagation phase of coagulation, but the enzymatic source of the initial FVa molecules that allow for the initial generation of small amounts of thrombin for feedback reactions has remained uncertain. FXa cleaves FV at Arg⁷⁰⁹ and Arg¹⁰¹⁸, resulting in a molecule without the basic region of the B domain, but still containing the acidic region (4). A similar version of partially cleaved FV is released from platelet granules (29). Recent work using a tick salivary protein that specifically inhibits FXa-mediated cleavage of FV suggests that the contribution of FXa to production of FVa is physiologically relevant, particularly in the initiation phase of coagulation (30). Further, tissue factor pathway inhibitor-alpha (TFPI), while unable to inhibit prothrombinase containing FVa generated by thrombin, is able to effectively inhibit the prothrombinase complex early in the initiation phase, when it contains versions of FVa retaining the acidic region of the B domain (29).

A recent report suggested that a component of the intrinsic or contact pathway may moderate the tendency for haemorrhage in severe FV deficiency (31). Computational modelling and empirical data evaluating *in vitro* coagulation during the activated partial thromboplastin time assay suggests that limited activation of FV is required in the pre-incubation portion of the assay (16). This finding suggests that FVa molecules are generated during pre-incubation by an enzyme whose production is not calcium-dependent. Previous work by Whelihan et al. (16) proposed that FXIa may fill this role. Results from our study confirm and extend previous observations regarding FXIa-mediated activation of FV, and suggest that polyP secreted by activated platelets may be important in this process.

We showed that polyP augments FXIa-mediated FV activation in both a purified system and in plasma, and furthermore that polyP allows this reaction to proceed at the plasma concentration of FV and low pM concentrations of FXIa, compared to supraphysiologic amounts of FV and/or FXIa required in the absence of polyP (16). The size-dependence of the ability of polyP to support FXIa-mediated activation of FV indicates that polyP polymers in the size range secreted by platelets (~60 to 100mers) support robust FV activation by FXIa, although longer polymers (> 200mer) supported even faster rates of FXIa-mediated FV activation. Thus, we propose that polyP released from platelets greatly enhances FXIa-mediated generation of FVa, while longer-chain polyP released from damaged tissues or bacteria might provide even more effective FVa generation under pathologic conditions.

We previously demonstrated that polyP accelerates FV activation by thrombin and FXa (17). PolyP also potently abrogates the inhibitory function of TFPI (17, 19), possibly via blockage of charge-dependent interactions between TFPI and the acidic region of the B domain of FV (29). Taken together with our current findings that polyP augments FV activation by FXIa, we speculate that platelet polyP may be involved in this release-from-inhibition mechanism by interacting with or facilitating the removal of the highly-basic inhibitory sequences within the B-domain of FV. PolyP, platelet FV, and TFPI are all released from platelet granules upon activation, but are stored in different locations within the platelet. Dynamic regulation of thrombin generation to address varying haemostatic conditions may be a

function of the contribution of various enzymes capable of activating FV, with contributions to that regulation by differential release of platelet granule contents (32, 33).

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What is known about this topic?

- Factor XIa has been shown to activate factor V, but with slow kinetics that require supraphysiologic concentrations of factors V or XIa.
- Polyphosphate is released from activated platelets.
- Polyphosphate modulates certain blood clotting reactions.

What does this paper add?

- Polyphosphate accelerates the rate of factor V activation by factor XIa by more than 60-fold.
- Platelet releasates also accelerate factor V activation by factor XIa, and the activity appears to be attributable to polyphosphate released from the platelets.
- This adds to our understanding of how polyphosphate enhances blood clotting.

Figure 1. Binding of FV to polyP

Binding of FV was quantified using SPR, with biotinylated polyP bound to streptavidin sensorchips, over which varying FV concentrations were flowed. Curves are representative sensorgrams for 1 to 10 nM FV (as indicated). The calculated K_D was 1.06 nM.

Figure 2. Cleavage of purified FV by FXIa in the presence of polyP
A) Purified human FV (30 nM) was incubated with FXIa (80 pM) and for 10 min at 37 °C in the absence or presence polyP (0–80 μ M, 65 or 96 mer, as indicated). Reaction samples were resolved on SDS-PAGE and visualised via silver stain. For comparison, purified human FVa produced via thrombin cleavage of FV is shown to indicate the migration of the heavy chain (HC) and the light chain (LC). B) Purified human FV (20 nM) was incubated with FXIa (10–160 pM, as indicated) for 10 min at 37 °C in the absence or presence of 211mer polyP. Reaction samples were resolved on SDS-PAGE and analysed by Western blotting using a monoclonal antibody to the FVa heavy chain. Mobility of protein molecular weight standards is indicated on the far left.

Figure 4. Activation of purified FV by FXIa in the presence of polyP of varying polymer lengths FV (10 nM) was incubated with 5 nM FXIa for varying times at 37 °C in the absence or presence of polyP. Aliquots were removed and quenched in ice cold buffer containing spermine and aprotinin, after which cofactor activity of the quenched samples was measured and converted to FVa concentrations. Data are mean $\pm$ standard error (n=4). A–C) Progress curves of FVa generation in the presence of polyP of three different lengths; polymer sizes were: (A) 85mer, (B) 305mer, or (C) 673mer, at the following concentrations: 80 μ M (●), 40 μ M (□), 20 μ M (■), 10 μ M (◆), 5 μ M (▲) or no (○) polyP. D) Initial rates of FVa generation (from the progress curves in panels A–C) in the presence of 85 mer (□), 305mer (▼), or 673mer (○) polyP at the indicated concentrations. E) Initial rates of FVa generation in the presence of 80 μ M polyP of the indicated polyP polymer lengths. The rate in the absence of polyP is indicated by the point at “0”.

Figure 5. Cleavage of purified FV by FXIa in the presence of platelet releasate

Platelets were activated at a platelet concentration of $3 \times 10^6/\mu\text{l}$ (about 10-fold higher than the platelet concentration in normal blood), then removed via centrifugation to produce platelet releasate. Purified human FV (20 nM) was incubated with 4.8 nM FXIa and diluted platelet releasate (fivefold to 160-fold, as indicated) for 10 min at 37 °C. As a control, some platelet releasate was either boiled to denature proteins and then cooled, or preincubated with a polyP inhibitor (PPXbd) before use. Reaction samples were resolved on SDS-PAGE and analysed by Western blotting using a monoclonal antibody to the FVa heavy chain. The leftmost lane is FV incubated with FXIa but without platelet releasate. Mobility of protein molecular weight standards is indicated on the far left. Control experiments (not shown) demonstrated that, at the dilutions of platelet releasates used, any platelet-derived FV/FVa was undetectable.