Polyphosphate co-localizes with factor XII on platelet-bound fibrin and augments its plasminogen activator activity

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Key points

- Polyphosphate significantly augments the plasminogen activator capacity of factor XIIa.
- Platelet-bound fibrin acts as a reservoir for plasminogen, factor XII(a) and polyphosphate.

Abstract

Activated factor XII (FXIIa) has plasminogen activator capacity but its relative contribution to fibrinolysis is considered marginal compared to urokinase and tissue plasminogen activator. Polyphosphate (polyP) is released from activated platelets and mediates FXII activation. Here we investigate the contribution of polyP to the plasminogen activator function of αFXIIa. We show that both polyP$_{70}$, of the chain length found in platelets (60-100-mer) and platelet-derived polyP, significantly augment the plasminogen activation capacity of αFXIIa. PolyP$_{70}$ stimulated auto-activation of FXII and subsequent plasminogen activation, indicating that once activated, αFXIIa remains bound to polyP$_{70}$. Indeed, complex formation between polyP$_{70}$ and αFXIIa provides protection against autodegradation. Plasminogen activation by βFXIIa was minimal and was not enhanced by polyP$_{70}$; highlighting the importance of the anion binding site. PolyP$_{70}$ did not modulate plasmin activity but stimulated activation of Glu- and Lys- forms of plasminogen by αFXIIa. Accordingly, polyP$_{70}$ was found to bind to FXII, αFXIIa and plasminogen, but not βFXIIa. Fibrin and polyP$_{70}$ acted synergistically to enhance αFXIIa-mediated plasminogen activation. The plasminogen activator activity of the αFXIIa-polyP$_{70}$ complex was modulated by C1 inhibitor and histidine rich glycoprotein, but not plasminogen activator inhibitors (PAI-1 and PAI-2). Platelet polyP and FXII were found to co-localize on the activated platelet membrane in a fibrin-dependent manner and decorated fibrin strands extending from platelet aggregates. We show that in the presence of platelet polyP and the downstream substrate fibrin, αFXIIa is a highly efficient and favorable plasminogen activator. Our data is the first to document a profibrinolytic function of platelet polyP.

Introduction

The contact pathway is comprised of factor XII (FXII), prekallikrein (PK), factor XI (FXI) and a non-enzymatic cofactor, high molecular weight kininogen (HK). Reciprocal proteolytic activation of FXII and PK to their active forms, FXIIa and kallikrein respectively, occurs via interaction with a negatively charged surface and is enhanced by Zn^{2+}.[1-5] FXI and PK circulate in complex with HK,[6,7] which assembles these proteases on the activating surface. FXIIa can cleave FXI stimulating the intrinsic pathway and downstream generation of thrombin, leading to its classification as a coagulation factor. However, FXIIa is reported to participate in multiple pathways including inflammation, complement and fibrinolysis.[8]

The profibrinolytic effects of the contact pathway are multifaceted involving direct and indirect interactions. Kallikrein liberates the vasoactive peptide bradykinin from HK which induces a host of vascular responses, including release of tissue plasminogen activator (tPA) from endothelial cells.[9] Kallikrein also directly cleaves single-chain urokinase (scuPA)[10,11] to active uPA. FXII shows distinct homology to tPA and uPA[12-14] and in accordance with this FXIIa exhibits plasminogen activator activity. The kinetics of the reaction are considered unfavourable[13,15] but it is present in plasma at four orders of magnitude higher concentrations compared to tPA and uPA.[16,17] During evolution, redundancy has developed in the fibrinolytic system, underscored by the relatively mild abnormalities associated with deficiency in tPA or uPA, while the double knockout exhibits a more acute phenotype.[18] Under certain circumstances or within specific milieus it is plausible that FXIIa contributes to plasmin generation to complement or compensate for tPA and uPA activity and may be potentially relevant *in vivo*.[19]

Several natural surfaces facilitate FXII activation including polyphosphate (polyP),[20,21] RNA,[22] misfolded proteins[23] and collagen.[24] PolyP is an ancient biomolecule that is ubiquitous in nature[25] it is highly anionic and consequently acts as a propitious surface for activation of the contact pathway.[20,21] PolyP is localized in platelet dense granules[26] and is secreted upon activation[21] alongside ADP, serotonin, and metal ions, including Zn^{2+}. Platelet polyP is approximately 60-100 mers[21] which is significantly shorter than the long-chain polymers (1000-2000 mers) in bacteria.[27] Polymer length is crucial to polyP's biological activity with shorter chains showing reduced capacity to activate the contact pathway.[28] PolyP also

interacts with fibrin(ogen) and accumulates in clots altering their structural properties and susceptibility to tPA-mediated fibrinolysis.[29,30] The half-life of polyP in plasma is relatively short (1.5-2 h)[20] but could be preserved within the microenvironment of the thrombus.

The plasminogen activator function of FXIIa is augmented by artificial surfaces and Zn^{2+}.[31] Here, for the first time, we show that a natural surface, polyP, amplifies the plasminogen activator function of FXIIa. Indeed polyP, of approximate chain length of that found in platelets, binds both FXII(a) and plasminogen, indicative of a template mechanism of activation. Importantly, we also demonstrate that platelet-associated fibrin acts as a reservoir for FXII(a), plasminogen and platelet-released polyP.

Methods

Collection of blood and preparation of plasma & platelets

For platelet experiments, peripheral blood was collected into acid citrate dextrose (ACD) solution A vacuettes (Greiner Bio-one LTD); the first 3 ml was discarded. Platelets were washed and counted as described.[32] Pooled normal plasma (PNP) that is essentially free of platelets was prepared from whole blood of 20 normal donors collected into 3.2% trisodium citrate.[33]

PolyP preparation

PolyP was extracted from platelets as described.[28] Experiments were performed with platelet-derived polyP, synthetic $polyP_{65}$ (Sigma-Aldrich) or $polyP_{70}$ (a kind gift from BK Giulini, GmbH). Similar results were obtained with both synthetic preparations and for simplicity are described as $polyP_{70}$ throughout and the concentration quoted as phosphate monomer (monomer formula, $NaPO_3$).

Clot lysis

Plasminogen-depleted fibrinogen (3.8 μM), Glu- or Lys- plasminogen (0.24 μM) and αFXIIa (200 nM) were added ± $polyP_{70}$ (70 μM) in 10 mM Tris-HCl [pH 7.4], 140 mM NaCl and 0.01% Tween[20] to 96-well plates (Greiner). Clotting was initiated with 0.25 U/ml human thrombin and 10 mM $CaCl_2$. In an analogous set of experiments 200 nM FXII was pre-incubated with $polyP_{70}$ (140 μM) at ambient temperature for 30 min. In some cases tPA (1 pM) was also included. Some experiments incorporated the inhibitors PAI-1 (0.1-1 nM), PAI-2 (0.1-1 nM), C1 inhibitor (125-500 nM) or histidine-rich glycoprotein (HRG; 0.25-1 μM). $PolyP_{70}$ was replaced by HeLa RNA (10 μg/ml) or equine type 1 (Horm) collagen (5 μg/ml) in some assays. Absorbance at 405 nm was recorded every minute using a BioTek PowerWave plate reader and BioTek Gen5 software.

Plasminogen activation assay

Plasminogen activation was analysed as described[30] with the following modifications. αFXIIa (200 nM), Glu- or Lys- plasminogen (0-400 nM) and S2251 (0.5 mM) were added to microtiter plates in 10 mM Tris-HCl [pH 7.4], 140 mM NaCl and 0.01% Tween[20] ± polyP$_{70}$ (0-70 μM). Alternatively, 200 nM FXII was added ± polyP$_{70}$ (140 μM). Plasmin activity was detected by measuring the absorbance at 405 nm every 1 min. In a parallel set of assays the following inhibitors; PAI-1 (0.1-1 nM), PAI-2 (0.1-1 nM), C1 inhibitor (125-500 nM) and HRG (0.25-1 μM) were incorporated. In some experiments soluble fibrin (3.8 μM), prepared as described[34] was coated onto plates overnight at 4°C before performing activity assays. In some cases RNA (10 μg/ml) or collagen (5 μg/ml) were included in place of polyP$_{70}$.

FXIIa activity assay

FXIIa activity was analysed as described,[35] briefly αFXIIa (50 nM) and S2302 (0.25 mM) ± polyP$_{70}$ (70 μM) were added to microtiter plates and absorbance read at 405 nm for 4 h, with addition of 5 μl (5 mM) S2302, or 5 μl (50 nM) protein at the 2 h mid-point.

Gels and binding assays

In autodegradation experiments, αFXIIa (50 nM) was incubated ± polyP$_{70}$ (1 μM) for up to 360 min before resolving on 4-12 % Bis-Tris gels (NuPAGE, Life Technologies) under non-reducing conditions and protein stained using Instant*Blue*™.

PolyP$_{70}$ was bound to Sepabeads as described[36] and incubated with 5 μg of FXII, αFXIIa, βFXIIa or plasminogen. Flow through material and subsequent low salt (50 mM NaCl) and high salt (1 M NaCl) washes were collected and fractions analysed by Western blotting as described[37] using HRP-conjugated goat anti-human FXII (ERL) or HRP-conjugated goat anti-human plasminogen (ERL).

Visualization of FXII and polyP on single platelets

Slides were coated with collagen (20 µg/ml) and thrombin (100 nM) and subsequently blocked with 5% bovine serum albumin. Platelets (5 x 10^7/ml) were added to coated slides for 45 min with DAPI (25 µg/ml; Ex. 358, Em. 525), DyLight 488 (Pierce, Thermo Scientific)-labelled human FXII (DL488-FXII; 365 nM; Ex. 493, Em. 518) and Alexafluor 647® conjugated annexin V (AF647-annexin V; 1/20, Life Technologies; Ex. 594, Em. 633) in the presence of $CaCl_2$ (2 mM). In control experiments Benzonase nuclease (Sigma) was included during stimulation to degrade contaminating DNA and RNA. Platelets were visualized by fluorescent confocal microscopy on a Zeiss LSM710 confocal microscope with 63x 1.40 oil immersion objective. Images were recorded on bright field and on separate channels for each wavelength and analyzed using Zen 2012 software.

FXII and platelet polyP distribution in plasma clots

Washed platelets (6.35 x10^8/ml; final concentration in clot 1.5 x10^8/ml) were incubated with DAPI (20 µg/ml) and AF647-annexin V (1/20) before activating with collagen (100 µg/ml), TRAP-6 (100 µM) and $CaCl_2$ (4 mM). Activated platelets were added to PNP (50% final concentration) in the presence of DL488-FXII (365 nM) and DyLight 550 (DL550; Pierce, Thermo Scientific)-labelled human fibrinogen (120 nM; Ex. 562, Em. 576). Thrombin (24 nM) and $CaCl_2$ (10 mM) were added and clots were allowed to form in Ibidi µ-slide $VI^{0.4}$ ibiTreat chambers for 2 h. Images were recorded as detailed above for single platelets.

Flow Cytometry

DL488-FXII (365 nM), and DAPI (10 µg/ml) were added to washed platelets (2 x 10^7/ml) in Hepes resuspension buffer (pH 7.45) containing $CaCl_2$ (2 mM) and stimulated with convulxin (CVX; 100 ng/ml) and TRAP-6 (20 µM) or CVX and thrombin (100 nM) for 45 min at ambient temperature. AF647-Annexin-V (1/20) was added 5 min before the end of stimulation. In some cases 5 mM Gly-Pro-Arg-Pro (GPRP; Sigma-Aldrich,) was included to inhibit fibrin polymerisation. Analysis was performed on a Beckton Dickinson LSR II flow cytometer with FACSDiva 6.1.3

software (Beckton Dickinson) with appropriate compensation applied and 10000 events collected per sample. Data were analysed using FlowJo V.X.0.6 software. Results are expressed as mean percent of positive platelets and median fluorescent intensity (MFI) ± SEM.

Data analysis

Data analysis was performed in GraphPad Prism® 5.04. Clot lysis results are expressed as time to 50% lysis (CLT), derived from the time taken from the maximal amplitude of the clot to reach the midpoint to baseline. Alternatively, graphs were normalised and data plotted as % turbidity. Plasmin generation was calculated as described[38], briefly, absorbance at 405 nm was plotted against time-squared and the slope from the initial linear portion estimated. These values were used to calculate plasminogen activation rates using the specific activity of plasmin against S2251 that was experimentally determined to be 1.055 A_{405}/min/µM (not shown). Fold-changes in lysis were calculated from mean CLT data. Statistical analysis was performed on CLT, plasmin activity assays and flow cytometry data using student's t-test or when multiple parameters were tested one-way analysis of variance with Dunnett's Multiple comparison post-hoc test. *P* values of < 0.05 were considered significant.

Results

αFXIIa plasminogen activator activity is enhanced by polyP$_{70}$

Artificial surfaces can enhance the plasminogen activator capacity of αFXIIa.[31] This prompted us to examine the effect of the 'natural' activator polyP, of approximate size (60-100-mers) of that found in platelets, in αFXIIa-mediated plasminogen activation. In line with previous observations we show that αFXIIa is a relatively weak plasminogen activator.[13,15] However, inclusion of polyP$_{70}$ significantly augments the ability of αFXIIa to drive fibrinolysis, decreasing the CLT by 2.3-fold (105 ± 6 min vs. 238 ± 14 min; *P*<0.0001; Figure 1A). Similarly, polyP$_{70}$ accelerated αFXIIa-mediated plasmin generation (Figure 1B; *P*<0.0001) in a dose-dependent manner, with concentrations as low as 4.4 μM demonstrating a significant enhancement over αFXIIa alone (Figure 1C; *P*<0.0001). PolyP had no direct effect on the activity of preformed plasmin (Figure 1D; *P*=0.93). This indicates that polyP$_{70}$ accelerates αFXIIa-mediated conversion of plasminogen to plasmin. The derivative, βFXIIa, lacks the surface binding domain and is less efficient at stimulating plasminogen activation.[16] The low level of plasmin generation observed with βFXIIa was not augmented by polyP$_{70}$, reflecting the requirement of the anion binding domain of αFXIIa for interaction with polyP$_{70}$ (Figure 1E; *P*=0.71). Inclusion of Zn^{2+} in the reaction buffer did not further enhance the cofactor function of polyP$_{70}$ in this reaction (not shown). We found that platelet-derived polyP extracted from human platelets similarly enhanced the plasminogen activator capacity of αFXIIa (Figure 1 F).

As polyP is a known activator of FXII[20,21,35] we examined its capacity to both stimulate FXII activation and subsequently stimulate its plasminogen activator function. CLT were significantly longer with FXII (>300 min) compared to αFXIIa (238 ± 58 min) and similarly, rates of plasmin generation were significantly lower with FXII compared to αFXIIa, presumably reflecting the time for transition of zymogen to protease. Nevertheless, polyP$_{70}$ exhibits significant cofactor activity in clot lysis and activity assays when initiated with FXII-polyP rather than FXII alone (Figure 2A).

We next investigated binding of polyP$_{70}$ to FXII derivatives and found it complexed with FXII and αFXIIa, but not βFXIIa; confirming the critical role of the anion binding

site for this interaction (Figure 2B). When in complex with polyP$_{70}$ autodegradation of αFXIIa was delayed (Figure 2C). The protective effect of polyP$_{70}$ on αFXIIa was analysed using S2302 substrate (Figure 2D). Addition of excess substrate to the αFXIIa reaction at 2 h generated a further increase in absorbance in the presence of polyP$_{70}$ while no change was observed with αFXIIa alone. No further increase in activity was observed when additional αFXIIa was added to reactions with polyP$_{70}$ at 2 h, confirming that protein is not limiting (not shown). We further analysed changes in αFXIIa activity in terms of cleavage of FXI, but were unable to detect any changes in FXI activation after 360 min incubation in the absence or presence of polyP$_{70}$ (not shown). These data indicate that while polyP$_{70}$ confers protection against autodegradation the differences may be too minor to alter functional activity toward physiological targets.

αFXIIa enhances activation of Glu- and Lys- forms of plasminogen

We found that plasminogen bound immobilized polyP$_{70}$, and could be released by washing with high salt buffer (Figure 3A). This is indicative of an electrostatic interaction with the polymer, as previously shown for other proteins.[30,34,36] Plasminogen circulates in two forms; the predominant Glu-plasminogen is described as the 'closed' conformation, while the truncated Lys-plasminogen, cleaved at the C-terminus by plasmin, is in an 'open' conformation and exhibits a shorter half-life. Enhanced binding of Lys-plasminogen to fibrin[39] and activators tPA and uPA results in more rapid plasmin generation[40,41]. We observed faster plasmin generation and clot lysis with Lys-plasminogen (catalytic efficiency (CE) = 257.5 ± 21.0 $M^{-1}s^{-1}$) than Glu-plasminogen (CE = 18.5 ± 4.2 $M^{-1}s^{-1}$) when activated with αFXIIa (note the different scales in Figure 3B-C). PolyP$_{70}$ significantly augmented αFXIIa-mediated activation of Glu-plasminogen (CE = 41.1 ± 6.0 $M^{-1}s^{-1}$) and Lys-plasminogen (CE = 629.5 ± 75.6 $M^{-1}s^{-1}$) (Figure 3B) and accelerated clot lysis with both forms by ~2.6-fold (Figure 3C). These data suggest that polyP does not impact on the transition of the closed (Glu) to open (Lys) conformation of plasminogen, but directly facilitates cleavage to plasmin.

PolyP$_{70}$ and fibrin augment αFXIIa-mediated plasminogen activation

We have previously shown that polyP binds to fibrin(ogen) and alters the structure of the fibrin network[34] and therefore assessed the impact on αFXIIa-mediated-plasminogen activation. Fibrin significantly enhanced the plasminogen activator function of αFXIIa (Figure 4A) and when combined, fibrin and polyP$_{70}$ acted in concert to further amplify αFXIIa-mediated plasminogen activation (Figure 4A; *P*<0.001).

We analysed whether other natural surfaces that stimulate FXII activation, such as RNA[22], and collagen[24] modulated the plasminogen activator function of αFXIIa. RNA slightly enhanced plasmin generation by αFXIIa (Figure 4B; *P*<0.001), but not to the same magnitude as polyP$_{70}$, while collagen was unable to stimulate activation. However, neither collagen nor RNA was effective in shortening the CLT like polyP$_{70}$ (Figure 4C; *P*<0.001).

We next addressed whether αFXIIa acted in concert with tPA to mediate fibrinolysis. Inclusion of αFXIIa with a concentration of tPA (15 pM) sufficient to induce clot lysis further shortened the CLT from 69 ± 8.6 min to 47 ± 2.7 min (*P*<0.01; CLT of αFXIIa alone >300 min). We then performed assays at a suboptimal dose of tPA (1 pM) which alone were not sufficient to induce lysis (CLT>300 min). Addition of αFXIIa to clots containing 1 pM tPA significantly shortened the CLT (180 ± 9.4 min), but not to the extent of polyP$_{70}$ (106 ± 6.5 min *P*<0.0001; Figure 4D). A marginal decrease in CLT was observed with both αFXIIa and t-PA in the presence of polyP$_{70}$ (97 ± 1.4 min; *P*<0.01). Similarly, visualisation of lysis in real-time (Video 1) demonstrated significantly faster lysis in the presence of both αFXIIa and tPA compared to tPA alone. Interestingly, the pattern of lysis in the presence of αFXIIa was different, with clots lysing from the edge inwards, rather than proceeding with a defined lysis front as observed with tPA alone. These data illustrate that fibrin degradation is enhanced in the presence of both tPA and αFXIIa, compared to either activator alone.

C1 inhibitor and HRG regulate the plasminogen activator activity of αFXIIa

Inclusion of the plasminogen activator inhibitors, PAI-1 and PAI-2, at plasma concentrations did not impact αFXIIa-mediated lysis (Figure 5A) or plasmin generation (Figure 5B) in the absence (not shown) or presence of $polyP_{70}$ (Figure 5A). In contrast, C1 inhibitor significantly attenuated αFXIIa-mediated clot lysis (Figure 5A; *P*<0.0001) and plasmin generation (Figure 5B; *P*<0.001) ± $polyP_{70}$. HRG was also effective in down-regulating CLT (Figure 5A; *P*<0.0001) and plasminogen activation (Figure 5B; *P*<0.001) by αFXIIa ± $polyP_{70}$. The inhibition of αFXIIa-mediated plasminogen activation by α_2AP could not be examined due to its dominant inhibition of plasmin, but we found no discernible effects on direct inhibition of αFXIIa ± $polyP_{70}$ using S2302 (not shown).

FXII and polyP bind to the surface of stimulated platelets

We examined the interaction of DL488-FXII with platelets by flow cytometry and found that binding to CVX/thrombin stimulated platelets was significantly augmented compared to unstimulated (62.6 ± 6.9% versus 22.4 ± 13.5%; *P*<0.0001; Figure 6A). Platelets stimulated with CVX/TRAP-6 displayed a reduced capacity to bind DL488-FXII (44.7 ± 8.9%; *P*<0.01). Inclusion of GPRP, to impede fibrin polymerisation, markedly decreased the percentage of CVX/thrombin stimulated platelets that bound DL488-FXII (33.4 ± 6.9%; *P*<0.0001). Together these data suggest platelet-bound fibrin plays a crucial role in the association of FXII with the activated platelet surface.

We found a significantly higher degree of positivity for platelet-derived polyP on the surface of CVX/thrombin compared to unstimulated (52 ± 2% versus 8.1 ± 4.0%; *P*<0.0001). Surface-bound polyP was slightly decreased when platelets were stimulated with CVX/TRAP-6 (37.1 ± 7.2%; *P*<0.01) and upon inclusion of GPRP (31.5 ± 12.1%; *P*<0.01) suggesting a role for platelet-bound fibrin in its retention on the activated platelet membrane (Figure 6B).

Using fluorescence confocal microscopy we examined the localization of FXII on the activated platelet surface stimulated with CVX/thrombin. Phosphatidylserine (PS)-positive platelets bound DL488-FXII in a single protruding ‘cap’ on the platelet surface that is also rich in PS (Figure 6C and Video 2). DL488-FXII also bound to PS-negative, spread platelets in a central diffuse pattern over the area of the

granulomere (Figure 6D). Staining for platelet-derived polyP was dispersed over the activated membrane of PS-positive platelets (Figure 6E). Control experiments performed with nuclease, to degrade contaminating DNA and RNA, did not alter DAPI staining on activated platelets (Supplemental Figure S1).

Platelet-derived polyP associates with the platelet surface in clots and co-localises with FXII on adjacent fibrin fibres

The location of platelet-derived polyP and FXII was studied in plasma clots formed in the presence of activated platelets. Platelet-derived polyP associates with the surface of activated platelets, particularly procoagulant PS-positive platelets and on platelet-bound fibrin (Figure 6F.) No DAPI staining was observed in clots formed in the absence of platelets (data not shown). DL488-FXII also decorated fibrin fibres extending from platelet aggregates in clear co-localization with platelet-derived polyP.

Discussion

In the present study we reveal a cofactor function for platelet-derived polyP in modulating the plasminogen activator activity of αFXIIa. Platelet-derived polyP co-localised with αFXIIa on the fibrin matrix extending from platelet aggregates. Blocking fibrin polymerisation with GPRP reduced binding of FXII, highlighting its importance in accrual of FXII in platelet-rich areas. Our findings demonstrate that in the presence of platelet polyP, αFXIIa is an efficient plasminogen activator. In addition we show that fibrin augments the plasminogen activator capacity of αFXIIa in a synergistic manner to polyP. There have been several reports on modulation of fibrinolysis by polyP[20,29,30] but to our knowledge this is the first study to document a profibrinolytic function of platelet-derived polyP.

The plasminogen activator function of αFXIIa has been described previously[15,16,31], but has been largely ignored due to unfavourable kinetics. Despite displaying a 20-fold lower catalytic efficacy for plasminogen than uPA the relative abundance of FXII (375 nM) in plasma implies it may be relevant as a plasminogen activator.[17] Nevertheless, studies on the role of αFXIIa as a direct plasminogen activator are limited.[19,42,43] Here we demonstrate that polyP, of approximately the size found in platelets, and platelet-derived polyP, significantly augment αFXIIa-mediated plasmin generation. PolyP accelerates αFXIIa-mediated activation of Glu- and Lys-plasminogen to a similar degree indicating that it does not facilitate transition of the closed to open conformation and nor does it directly impact on plasmin activity. We observed binding of polyP to FXII(a) and plasminogen indicating that the cofactor function of this polymer is potentially mediated via a template mechanism on direct conversion of plasminogen to plasmin (Figure 7). PolyP also stimulates autoactivation of FXII to αFXIIa[35] and indeed affords some protection against autodegradation. This could be relevant in terms of time frame, as FXII may become activated during clot formation, however by interacting with platelet-derived polyP its activity may be protected allowing it to subsequently participate in clot degradation. Indeed a recent publication highlighted the dual role of FXII in supporting fibrin formation and degradation of the clot.[44] It is interesting to speculate on the complex role of this enzyme in modulating both fibrin formation, via generation of thrombin, and fibrin degradation via plasmin. Further work is necessary to define the

procoagulant and profibrinolytic properties of αFXIIa and the role of effector molecules, such as polyP, in these processes. Indeed opposing functions of a haemostatic enzyme is not an uncommon phenomenon. The central enzyme thrombin is a prime example as its activity can be directed from procoagulant to anticoagulant processes depending on the effector molecule bound to exosite I or exosite II of the protease.[45]

Fibrin also amplifies αFXIIa-mediated plasminogen activation, with maximal stimulation observed when both fibrin and polyP are present; suggesting that these molecules act synergistically to drive this process. PolyP has the capacity to bind to fibrin[29,30] and FXII(a) but these observations indicate that their respective binding sites on FXII(a) must be distinct. Fibrin is a well-established cofactor for tPA-mediated plasminogen activation. αFXIIa was able to act in conjunction with tPA to significantly accelerate fibrinolysis, suggesting that incorporation of these activators into the forming fibrin network may facilitate clot degradation, as depicted in the model shown in Figure 7. Interestingly, other surfaces that promote FXII activation and procoagulant function, specifically collagen[24] and RNA[22], were not effective in promoting αFXIIa-mediated fibrinolysis. This suggests that cofactors molecules may drive αFXIIa activity toward distinct downstream target substrates.

Antifibrinolytic functions of polyP have been described in the past.[20,29,30] The first of these relates to enhanced activation of thrombin activatable fibrinolysis inhibitor (TAFI), due to acceleration of thrombin generation in the presence of polyP.[20] Activated TAFI down-regulates fibrinolysis by removing C-terminal lysine residues on fibrin that are important for binding plasminogen and tPA.[46] PolyP also exerts antifibrinolytic function by altering the structure of the forming fibrin network.[29,30] Our recent work has shown that this arises from impaired fibrin polymerisation.[47] The modifications to the fibrin architecture by polyP alter its capacity to bind tPA and plasminogen, particularly that of partially degraded fibrin, thereby reducing tPA mediated plasmin generation.[30] The profibrinolytic capacity of polyP described here, in terms of augmenting αFXIIa-mediated plasminogen activation, is intriguing particularly in light of the fact that fibrin also plays a role in this process. It seems that the functions of polyP are more diverse than first thought and that this polymer

may exert different levels of control over the haemostatic system depending on timing and perhaps local concentrations of available reactants.

Interestingly, the plasminogen activator inhibitors, PAI-1 and PAI-2 were ineffective in modulating polyP-αFXIIa-mediated plasminogen activation and fibrinolysis while C1-inhibitor and HRG both effectively neutralised activity. C1-inhibitor is the predominant inhibitor of the contact pathway and regulates tPA-mediated plasminogen activation in situations when tPA is in excess over PAI-1.[48-50] Interestingly C1-inhibitor has also been shown to bind to polyP.[51] Recent data highlighted the important role of HRG in modulating FXIIa activity and function.[52-54] HRG binds to FXIIa with incredibly high affinity in the presence of Zn^{2+} ions[52] and both are released from platelet α-granules upon activation.[55,56] HRG also associates with DNA and RNA and attenuates nucleic acid-driven activation of FXII.[53] Here we show for the first time that HRG dampens αFXIIa activity directed toward the fibrinolytic pathway, adding to the complexity of this unusual adapter protein in regulation of haemostatic pathways.

To date the binding sites for αFXIIa on platelets have not been elucidated but unlike other coagulation factors it does not bind directly to PS-positive platelets via Gla domains.[57] We have previously shown that FXII(a) binds to fibrin and is actively incorporated into clots[58], and elegant flow studies confirmed that FXII(a) interacts with platelet-associated fibrin.[57] We observed significantly more FXII on the surface of platelets activated with thrombin/CVX than TRAP-6/CVX. Blocking fibrin polymerisation with GPRP during thrombin/CVX stimulation, but not TRAP-6/CVX stimulation, significantly reduced the amount of FXII associated with platelets. Together these data indicate that fibrin anchors FXII to the activated platelet membrane. PS-positive platelets display a cap of FXII on the activated surface that we have recently shown to be rich in fibrin(ogen) and plasminogen.[59] We also show that platelet-derived polyP is retained on the membrane of PS-positive platelets, but unlike FXII, fibrinogen and plasminogen, it is homogenously distributed. Inhibition of fibrin polymerisation attenuates association of polyP with the platelet surface, consistent with its known affinity for fibrin[30] but does not completely abrogate binding. This suggests the presence of a second, as yet unidentified mechanism, that mediates retention of polyP on activated platelets. Nevertheless, we also show

that polyP can translocate from the ‘hot-spots’ of PS-positive platelets into the surrounding fibrin network. FXII also decorates fibrin strands accentuating the importance of platelet-bound fibrin in localising FXII and plasminogen[59] in the vicinity of activated platelets. Release of polyP from stimulated platelets could stimulate activation of FXII on fibrin and enhance the plasminogen activator capacity of αFXIIa; in this sense fibrin will be acting as a surface for its own destruction, as it does in t-PA mediated plasminogen activation.

Thrombi are composed of different regions, the inner ‘core’ and the outer ‘shell’ which differ in their levels of platelet activation, aggregation and packing.[60] The ’core’ is comprised of tightly packed, degranulated platelets encased in fibrin.[60] ADP released from dense granules regulates α-granule secretion[61-63] forming dense regions that stabilise the platelet aggregate.[64] Concomitant release of polyP with ADP from dense granules suggests it may be retained in these low solute transport areas. α-granule release occurs at lower agonist concentrations than dense granule release[65,66] suggesting platelets in the outer core and shell may release fibrinogen and bind fibrin prior to polyP secretion thereby providing an anchor to retain polyP in the locale of the activated platelet aggregates.

Multiple questions remain over how the pleotropic effects of FXII(a) are mediated in biological systems. Further investigations on the role of αFXIIa as a plasminogen activator *in vivo* are warranted, particularly in light of the current interest in FXII as an antithrombotic target. It is plausible that different surfaces act as cofactors that direct the function of FXIIa to procoagulant and profibrinolytic pathways, analogous to mechanisms seen with other proteins in the haemostatic cascade. Platelet-bound fibrin acts as reservoir for polyP and αFXII(a) and within the thrombus microenvironment may preserve their functional activity. Clearly there is still much to be learnt about the mysterious FXII(a) and its contribution to various physiological processes.

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Authorship Contributions

J.L.M. performed the research, analyzed the data and wrote the manuscript; A.S.L, A.K., G.G. & CB performed the research and analyzed data; P.Y.K analyzed data and N.J.M. provided the inital concept of the study, supervised the research, analyzed the data and wrote the manuscript.

Conflict of Interest Disclosures

The authors have no conflicts to declare.

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Legends

Figure 1: αFXIIa plasminogen activator activity is enhanced by polyP$_{70}$. (A) Clots were formed with 3.8 μM fibrinogen, 0.24 μM Glu-plasminogen, 200 nM αFXIIa in the absence (grey line) and presence (black line) of 70 μM polyP$_{70}$. Clotting was initiated with 0.25 U/ml thrombin and 10 mM $CaCl_2$ and subsequent lysis monitored at 405 nm. Mean data ± SEM is expressed as % turbidity of *n=5*, *P*<0.0001. (B) Plasminogen activation was analysed by incubating 200 nM αFXIIa and 200 nM plasminogen in the absence (light grey line) or presence of 70 μM polyP$_{70}$ (black line). Plasmin activity was detected using the chromogenic substrate S2251 at 405 nm. Data represent mean and SEM of *n=3 P*<0.0001. (C) αFXIIa-mediated plasminogen activation was analysed in the presence of various concentrations of polyP$_{70}$ by incubating 200 nM αFXIIa and 200 nM plasminogen in the absence (dashed line) or presence of 70 μM polyP$_{70}$; 35 μM polyP$_{70}$; 17.5 μM polyP$_{70}$; 4.4 μM polyP$_{70}$. Plasmin activity was detected using the chromogenic substrate S2251 at 405 nm. Data represent mean ± SEM of *n=3 P*<0.0001. (D) Similarly, direct effects of polyP on pre-formed plasmin (6.25 nM) were analysed in the absence (grey line) and presence (black line) of 70 μM polyP$_{70}$ with S2251. Data represent mean ± SEM of *n=3*, *P=0.93*. (E) Activation of plasminogen (200 nM) by βFXIIa (200 nM) was monitored in the absence (grey line) and presence (black line) of 70 μM polyP$_{70}$ and detected using S2251. Data represent mean ± SEM of *n=4 P=0.71*. (F) Plasminogen activation was analysed by incubating 200 nM αFXIIa, 200 nM plasminogen in the absence (light grey line) or presence of 70 μM platelet-derived polyP (dark grey line). Plasmin activity was detected using the chromogenic substrate S2251 at 405 nm. Data represent mean and SEM of *n=3 P<0.0001*.

Figure 2: PolyP$_{70}$ stimulates FXII activation and modulates its plasminogen activator function (A) *PolyP induces autoactivation of FXII* - Left panel - Clots were formed with 3.8 μM fibrinogen, 0.24 μM Glu-plasminogen and 200 nM FXII in the absence (grey line) or presence (black line) of 140 μM polyP$_{70}$. Clotting was initiated with 0.25 U/ml thrombin and 10 mM $CaCl_2$ and subsequent lysis monitored at 405 nm. Mean data ± SEM are expressed as % turbidity *n=3, P<0.0001*. Right panel – FXII (200 nM) and Glu-plasminogen (200 nM) were incubated in the absence (grey line) or presence (black line) of 140 μM polyP$_{70}$ and plasmin activity detected using

S2251. Data represent mean ± SEM of *n=3 P<0.0001*. (B) *PolyP binds to FXII and αFXII* - FXII, αFXIIa or βFXIIa (5 μg) were run through columns containing Sepabeads coated with polyP$_{70}$ before collecting the flow through fractions (FT), low (50 mM NaCl; LS) and high (1 M NaCl; HS1) salt washes and comparing with starting material (SM). Protein was detected by Western blotting with an antibody to FXII. Image is representative of 3 separate experiments. (C) *PolyP protects αFXIIa from autodegradation* - αFXIIa (5 μg) was incubated ± polyP$_{70}$ (100 μg) before resolving on 4-12 % Bis-Tris gels under non-reducing conditions. Data shown are representative of 3 separate experiments. (D) *PolyP preserves αFXIIa activity* αFXIIa (50 nM) activity was analyzed using S2302 in the absence (grey line) or presence (black line) of 70 μM polyP$_{70}$. After 2 h additional S2302 substrate was added to the reaction (arrow) and readings continued for a further 2 h. Data represent mean ± SEM of *n=3*.

Figure 3: αFXIIa enhances activation of Glu- and Lys- forms of plasminogen. (A) Binding of polyP$_{70}$ plasminogen was analysed by running Glu-plasminogen through columns containing Sepabeads coated with polyP$_{70}$ before collecting the flow through fractions (FT), low (50 mM NaCl; LS) and high (1 M NaCl; HS1) salt washes and comparing with starting material (SM). Protein was detected by Western blotting with an antibody to plasminogen. Image is representative of 3 separate experiments. (B) The rate of plasmin generation by αFXIIa (200 nM) in the presence (black line) or absence (grey line) of polyP$_{70}$ (70 μM) was quantified for Glu- (left; *P<0.05*) and Lys- (right; *P<0.01*) plasminogen. Data are expressed as mean ± SD, *n=3.* (C) Fibrin clots were formed with fibrinogen (3.8 μM), αFXIIa (200 nM) and Glu- (left) or Lys- (right) plasminogen, in the absence (grey line) or presence (black line) of polyP$_{70}$ (70 μM). Clotting was initiated with thrombin (0.25 U/ml) and $CaCl_2$ (10 mM) and lysis monitored at 405 nm. Mean data ± SEM are expressed as % turbidity *n=3, P<0.0001*. Note the different scales on the Lys-plasminogen compared to Glu-plasminogen due to the different rates of activation of the isoforms of plasminogen.

Figure 4: PolyP and fibrin augment αFXIIa-mediated plasminogen activation. (A)- Plasminogen activation was analysed by incubating 200 nM αFXIIa, and 200 nM Glu-plasminogen (dotted line) in the presence of either 3.8 μM soluble fibrin (light grey line), 70 μM $polyP_{70}$ (dark grey line) or both soluble fibrin and $polyP_{70}$ (black line). Plasmin activity was detected using the chromogenic substrate S2251 at 405 nm. Data represent mean ± SEM *n=3*, *P<0.001* (B & C) The plasminogen activator function of αFXIIa (200 nM) was analysed in the presence of different surfaces including $polyP_{70}$ (70 μM), RNA (10 μg/ml) or collagen (5 μg/ml). (B) Plasmin activity, generated from 200 nM plasminogen, was detected using S2251. (C) Fibrinolysis was analysed by forming clots from fibrinogen (3.8 μM), αFXIIa (200 nM), Glu-plasminogen (200 nM), thrombin (0.25 U/ml) and $CaCl_2$ (10 mM). Lysis was monitored at 405 nm and mean ± SEM are shown as the time from maximal absorbance of the clot to 50% lysis, *n=3* *** *P<0.001*. (D) Fibrin clots were formed and monitored as described in (C) with 70 μM $polyP_{70}$ (black line), 1 pM tPA (grey line) or both $polyP_{70}$ and tPA (dark grey line). The clots formed with tPA (dashed line) and tPA + polyP (light grey line) were formed in the absence of αFXIIa. Mean data ± SEM are expressed as % turbidity *n=4*, *P<0.0001*.

Figure 5: C1 inhibitor and HRG regulate the plasminogen activator function of αFXIIa. The impact of inhibitors on αFXIIa-$polyP_{70}$ plasminogen activator function was monitored in the absence (black line) or the presence (grey line) of PAI-1 (1 nM), PAI-2 (1 nM), C1 inhibitor (500 nM) or HRG (1 μM) by absorbance based clot lysis (A) or plasminogen activation assay, where plasmin was detected by cleavage of S2251 (B). For simplicity the controls in the absence of $polyP_{70}$ are omitted, as no lysis was observed either in the absence or presence of the inhibitor. Data represent mean and SEM of *n=3*.

Figure 6: FXII and PolyP bind to the surface of stimulated platelets. (A-B) Washed human platelets were incubated with DL488-labelled FXII and DAPI to detect platelet-derived polyP and were left unstimulated or stimulated with CVX (100 ng/ml) and thrombin (100 nM) or TRAP-6 (20 μM) ± GPRP (5 mM) for 45 min at

ambient temperature before analysing DL488-FXII (top panel) and DAPI (bottom panel) positive cells by flow cytometry. ** *P<0.01*, **** *P<0.0001* vs. unstimulated platelets. † *P< .05*, †† *P<0.01*, †††† *P<0.0001* CVX/thrombin stimulated platelets vs CVX/thrombin + GPRP. (C-E) Washed platelets (5 x 10^7/ml) were activated with 20 μg/ml collagen and 100 nM thrombin in the presence of DL488-FXII (green) and DAPI (blue - E) and stained using Alexa-fluor®647 Annexin-V to detect phosphatidylserine (red). Images represent 3D render of z-stacks. Scale bars represent 5 μm. Representative images of 3 separate experiments. (F) Platelets (1.5 x10^8 platelets/ml final concentration) were activated with 100 μg/ml collagen and 100 μM TRAP-6 in the presence of $CaCl_2$ before adding to plasma clots (50%) in the presence of DL550-Fgn (120 nM; orange), DL488-FXII (365 nM; green), DAPI (20 μg/ml; light grey) and AF647-annexin V (1/20; red). Thrombin (24 nM) and $CaCl_2$ (10 mM) were added and clots were allowed to form for 2 h. The image is representative of 3 separate experiments and displays a 3D render of a z-stack. Scale bar represents 10 μm. Images in C-F were obtained using a Zeiss LSM710 confocal microscope with a 63 X 1.40 oil immersion objective and were analysed using Zen 2012 software.

Figure 7: Interaction of αFXII(a), plasminogen, polyP and fibrin

This cartoon depicts the potential template interactions between αFXII(a), plasminogen and polyP on polymerized fibrin. Fibrin forms the initial network and acts as a template for both tPA and FXIIa-mediated fibrinolysis due to its capacity to bind FXII(a), polyP, plasminogen and tPA. PolyP binds to fibrin, αFXII(a) and plasminogen and potentially acts as an anchor to reinforce the association between these proteins. When αFXII(a) is bound to fibrin and polyP its activation and plasminogen-activator activity is enhanced, facilitating plasmin generation on fibrin and subsequent degradation of the network. The cofactor capacity of fibrin in the stimulation of tPA-mediated plasminogen activation is well documented. Binding of the polyP-FXII(a) complex may to further facilitate plasminogen activation on the fibrin surface to accelerate fibrinolysis.