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ORIGINAL ARTICLE

Single-chain factor XII exhibits activity when complexed to polyphosphate

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Summary. *Background:* The mechanism underpinning factor XII autoactivation was originally characterized with non-physiological surfaces, such as dextran sulfate (DS), ellagic acid, and kaolin. Several 'natural' anionic activating surfaces, such as platelet polyphosphate (polyP), have now been identified. *Objective:* To analyze the autoactivation of FXII by polyP of a similar length to that found in platelets ($polyP_{70}$). *Methods and results:* $PolyP_{70}$ showed similar efficacy to DS in stimulating autoactivation of FXII, as detected with amidolytic substrate. Western blotting revealed different forms of FXII with the two activating surfaces: two-chain αFXIIa was formed with DS, whereas single-chain FXII (scFXII; 80 kDa) was formed with $polyP_{70}$. Dissociation of scFXII from $polyP_{70}$ abrogated amidolytic activity, suggesting reversible exposure of the active site. Activity of scFXII–$polyP_{70}$ was enhanced by Zn^{2+} and was sensitive to NaCl concentration. A bell-shaped concentration response to $polyP_{70}$ was evident, as is typical of surface-mediated reactions. Reaction of scFXII–$polyP_{70}$ with various concentrations of S2302 generated a sigmoidal curve, in contrast to a hyperbolic curve for αFXIIa, from which a Hill coefficient of 3.67 was derived, indicative of positive cooperative binding. scFXII–$polyP_{70}$ was more sensitive to inhibition by H-D-Pro-Phe-Arg-chloromethylketone and corn trypsin inhibitor than αFXIIa, but inhibition profiles for C1-inhibitor were similar. Active scFXII–$polyP_{70}$ was also able to cleave its physiological targets FXI and prekallikrein to their active forms. *Conclusions:* Autoactivation of FXII by polyP, of the size found in platelets, proceeds via an active single-chain intermediate. scFXII–$polyP_{70}$ shows activity towards physiological substrates, and may represent the primary event in initiating contact activation *in vivo*.

Keywords: blood coagulation; factor XII; hemostasis; polyphosphates; zymogens.

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Introduction

Activation of the contact pathway occurs upon reciprocal proteolytic cleavage of factor XII and prekallikrein (PK) to their active forms, FXIIa and kallikrein, in the presence of a negatively charged surface. In turn, FXIIa cleaves FXI that is tethered to an anionic or membrane surface, generating active FXIa. FXIa initiates a series of ordered cleavages that feed into the prothrombinase complex and ultimately generate thrombin. FXI and PK require a non-enzymatic cofactor, high molecular weight kininogen (HK), to facilitate binding of these proteins to the activating surface, whereas FXII directly associates with the surface via the fibronectin domains in the heavy chain [1–4]. Zinc ions induce conformational changes in FXII [5–9] and HK [10–12], enhancing the interaction of these proteins with the anionic surface.

The function of FXII as a coagulation factor was contested for many years, as a deficiency in humans is not associated with a bleeding diathesis. It has now been hypothesized, through the use of mouse models, that FXII-driven coagulation is not essential for normal hemostasis, but mediates pathophysiological thrombus formation [13,14]. More recently, monoclonal antibodies (mAbs) directed against FXII have been shown to inhibit thrombus formation in primate thrombosis models [15]. These studies have redefined the function of FXII *in vivo* and highlighted it as a target for novel anticoagulant agents that could potentially prevent nascent thrombus growth with minimal bleeding complications. In addition

to its role in coagulation, FXII is likely to function in innate immunity [16], and indeed several lines of evidence indicate that these processes are inextricably linked [17–19]. A second question concerning the function of FXII *in vivo* was the absence of a suitable charged surface to stimulate activation. Several potential 'natural' activators, including platelet polyphosphate (polyP) [20,21], microparticles derived from platelets and erythrocytes [22], RNA [23], misfolded proteins [24], collagen [25] and mast cell heparin [26], have now been identified. PolyP is an anionic polymer of phosphate residues that is secreted from the dense granules of human platelets upon activation by agonists such as thrombin, ADP, and collagen [21,27]. Platelet polyP is of a defined size of 60–100 phosphate units in length [21], unlike the extremely long polymers found in bacteria [28]. Platelet polyP has been found to stimulate FXII-driven procoagulant activity *in vitro* [20,29] and *in vivo* [21] and to upregulate bradykinin-driven inflammation [21].

Binding of FXII to an anionic surface induces autoactivation, via cleavage at Arg353–Val354. This generates the active protease αFXIIa, an 80-kDa enzyme consisting of a heavy and a light chain linked by a disulfide bond. Further proteolytic cleavage at Arg334–Asn335 generates βFXIIa, a solution-phase derivative largely composed of the protease domain. βFXIIa cleaves PK, but has negligible activity towards FXI, whether surface-bound or in solution [30]. The mechanism of FXII autoactivation was characterized prior to the identification of physiological surfaces that were able to support this reaction [5,31,32]. The aim of this study was to define the ability of polyP, of similar chain length to that found in platelets, to stimulate autoactivation of FXII under physiological conditions. We show that autoactivation of FXII on a 'natural' activating surface proceeds via an active single-chain intermediate that is capable of cleaving both synthetic and physiological targets.

Materials and methods

Materials

FXII, αFXIIa, FXI, PK, kallikrein, horseradish peroxidase (HRP)-conjugated polyclonal antibody against FXII, HRP-conjugated antibody against FXI and HRP-conjugated antibody against PK were from Enzyme Research Laboratories (Swansea, UK). PolyP of average chain length 65 ($polyP_{65}$) and C1-inhibitor (C1-Inh) were from Sigma-Aldrich (Irvine, Scotland). Dextran sulfate (DS) with an average M_r of 500 000 and EDTA were from Fisher Scientific (Loughborough, UK). L-Pyr-Pro-Arg-*p*-nitroanilide (L-2145) and H-D-Pro-Phe-Arg-chloromethylketone (PCK) were from Bachem AG (Bubendorf, Switzerland), and H-D-Pro-Phe-Arg-pNA-2HCl (S2302) was from Quadratech (Epsom, UK). Corn trypsin inhibitor (CTI) was from Haematological Technologies (Vermont, NE, USA). NuPAGE 4–12% Bis-Tris gels, NuPAGE LDS sample buffer, reducing agent and Mops running buffer were from Life Technologies (Paisley, UK). Pierce spin cups with cellulose acetate filters were from Fisher-Scientific, Loughborough, UK. Unless otherwise stated, the buffer used throughout was 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl. Polymethacrylate beads (Sepabeads EC-HA) were a kind gift from Residion SRL (Milan, Italy). Experiments were performed with either $polyP_{65}$ or $polyP_{70}$ (a kind gift from BK Giulini). Comparable results were obtained with both preparations of polyP, which, for simplicity, will be described as $polyP_{70}$ throughout the article. The concentration of $polyP_{70}$ is expressed in terms of monomer concentration throughout the article [20,33].

Chromogenic assays

FXII autoactivation FXII (50 nM) was added to 96-well microtiter plates (Greiner, Stonehouse, UK) alone or in the presence of either $polyP_{70}$ (70 μM) or DS (1.5 μg mL^{-1}) with or without 10 μM ZnCl and with or without 1 mM EDTA. This assay was performed in 50 mM Tris-HCl (pH 7.4) buffer containing either 100 mM or 140 mM NaCl. The chromogenic substrate S2302 (0.5 mM) was added, and generation of activity was monitored at 405 nm every 30 s for 2 h at 37 °C in an ELx 808 plate reader (Bio-Tek, Potton, UK). Experiments were performed over a range of $polyP_{70}$ (0–2 mM), NaCl (0–1 M) and $ZnCl_2$ (0–100 μM) concentrations. A discontinuous assay for FXII autoactivation was performed by incubating FXII (50 nM) alone, with $polyP_{70}$ (70 μM) or with DS (1.5 μg mL^{-1}) at 37 °C. At various time points (0–120 min), the reaction was stopped by the addition of 1 M NaCl, and FXII activity was quantified with S2302 (0.5 mM) as described above. Gradients from the initial linear sections of absorbance vs. time graphs were calculated and plotted against the inhibitor, $polyP_{70}$, NaCl or $ZnCl_2$ concentrations with GRAPHPAD PRISM 5.03 (La Jolla, CA, USA). Data were analyzed by linear regression, and accurate line-fitting was established by analysis of residual data.

For western blot analysis of FXII autoactivation, FXII (50 nM) was incubated alone or in the presence of 70 μM $polyP_{70}$ or 1.5 μg mL^{-1} DS with or without 10 μM $ZnCl_2$ for 60 min at 37 °C. Reducing sample buffer was added, and samples were boiled for 10 min prior to being resolved on 4–12% NuPAGE gels and transferred to a poly(vinylidene difluoride) membrane. FXII was detected with a polyclonal antibody conjugated to HRP (Enzyme Research Laboratories). A commercial preparation of αFXIIa (50 nM) was included in the western blot as a positive control.

FXII binding studies $PolyP_{70}$ was immobilized on primary amine-containing polymethacrylate beads by the use of 1-ethyl-3-(3-[dimethylamine]propyl) carbodiimide as previously described [34]. FXII (2 μg) in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% bovine

serum albumin [BSA]) was incubated with $polyP_{70}$-labeled beads or BSA-labeled (control) beads for 30 min before centrifugation in Pierce spin cups at 1677 × *g* for 30 s to collect the flow-through. The beads were washed twice in binding buffer before elution with a high-salt buffer (50 mM Tris-HCl, pH 7.4, 1 M NaCl, 0.1% BSA). Samples were then separated on 4–12% gels and subjected to western blot analysis as described above.

Single-chain FXII (scFXII)–$polyP_{70}$ and αFXIIa activity

FXII (50 nM) was incubated with $polyP_{70}$ (70 μM) in the presence of increasing concentrations of S2302 substrate (0–500 μM). The activity of αFXIIa (50 nM) was monitored under comparable conditions. From the raw data, the slope of the linear portion of the reaction was derived with GRAPHPAD PRISM 5.03 by linear regression, and plotted against the substrate concentration. The data were then fitted to an allosteric sigmoidal model of enzyme kinetics in the case of scFXII–$polyP_{70}$, or the classic Michaelis–Menten equation in the case of αFXIIa [35].

Inhibition studies

FXII (50 nM) in the presence of $polyP_{70}$ (70 μM) or αFXIIa (50 nM) was incubated with various concentrations of CTI (0–500 nM), PCK (0–5 μM), or C1-Inh (0–400 nM), and activity was monitored with S2302 (0.5 mM). Gradient values were derived and normalized to activity in the absence of inhibition. Data were then analyzed by non-linear regression (log[inhibitor] vs. normalized response variable slope), allowing the IC_{50} values to be determined.

FXI activation

FXI (35 nM) was incubated with or without FXII (50 nM) and with or without $polyP_{70}$ (70 μM), and activity was quantified with a chromogenic substrate, L-2145. Additional samples were removed at 100 min, and western-blotted with an HRP-conjugated polyclonal antibody against FXI.

PK activation

PK (50 nM) was incubated with or without FXII (3 nM) and with or without $polyP_{70}$ (70 μM), and activity was quantified with S2302. Both kallikrein and αFXIIa cleave S2302; to control for this, the activating concentration of FXII (3 nM) was included with or without $polyP_{70}$ (70 μM). Samples were removed at 60 min and western-blotted with an HRP-conjugated polyclonal antibody against PK.

Data analysis

All data were analyzed with GRAPHPAD PRISM 5.03. Experiments were performed in triplicate, and results are presented as the mean ± standard deviation of at least three separate repeats. Statistical analyses were performed with *t*-tests, with $P < 0.05$ considered to be statistically significant.

Results

scFXII shows enzymatic activity when bound to $polyP_{70}$

$PolyP_{70}$ (70 μM) was found to be a potent stimulator of FXII autoactivation, as determined in a continuous reaction with a chromogenic substrate (Fig. 1A). Autoactivation of FXII was analyzed at 60 min by SDS-PAGE under reducing conditions and western blotting with an antibody against FXII (Fig. 1B). DS generated a two-chain active enzyme, composed of a 50-kDa chain and a 30-kDa chain; the bands observed were of the same molecular masses as those obtained with a commercial preparation of αFXIIa. In contrast, although FXII incubated with $polyP_{70}$ was capable of cleaving an amidolytic substrate, it was detected as a single band at 80 kDa. We performed a second chromogenic reaction under discontinuous conditions (Fig. 1C), in which FXII was incubated with $polyP_{70}$ or DS for various times (0–100 min). The reaction was stopped with NaCl (1 M) to disrupt binding of FXII(a) to the activating surface. FXII incubation with DS resulted in substantial cleavage of S2302, but, in contrast, no FXIIa activity was detected in preparations of FXII and $polyP_{70}$. To confirm dissociation of the scFXII–$polyP_{70}$ complex by high salt concentrations, we performed binding assays in which $polyP_{70}$ was directly coupled to polymethacrylate beads (Fig. 1D). FXII was depleted in the flow-through fraction, reflective of binding to immobilized $polyP_{70}$, and was subsequently eluted with 1 M NaCl. No binding of FXII to control beads was observed. The transition metal ion Zn^{2+} is known to enhance the association of FXII with activating surfaces such as DS [5,36]. We analyzed the contribution of Zn^{2+} to the interaction of FXII and $polyP_{70}$ by performing the continuous assay in the absence and presence of 10 μM Zn^{2+} and 1 mM EDTA. Zn^{2+} dramatically accelerated the activity generated by scFXII–$polyP_{70}$, and this effect was negated by EDTA (Fig. 1E). No significant differences in activity were noted with EDTA alone or when EDTA included in the reaction containing Zn^{2+}. The accelerated reaction in the presence of Zn^{2+} did not drive formation of the of two-chain αFXIIa by $polyP_{70}$, as shown by western blotting (Fig. 1C). The activity of scFXII–$polyP_{70}$ was compromised at 140 mM NaCl, but, interestingly, the effect of NaCl was overcome by addition of Zn^{2+} (Fig. 1E). These results suggest that the two activating surfaces, $polyP_{70}$ and DS, induce FXII activity by different mechanisms. They indicate that association of $polyP_{70}$ with FXII generates an active single-chain intermediate with substantial activity towards an amidolytic substrate in the absence of proteolytic cleavage to αFXIIa.

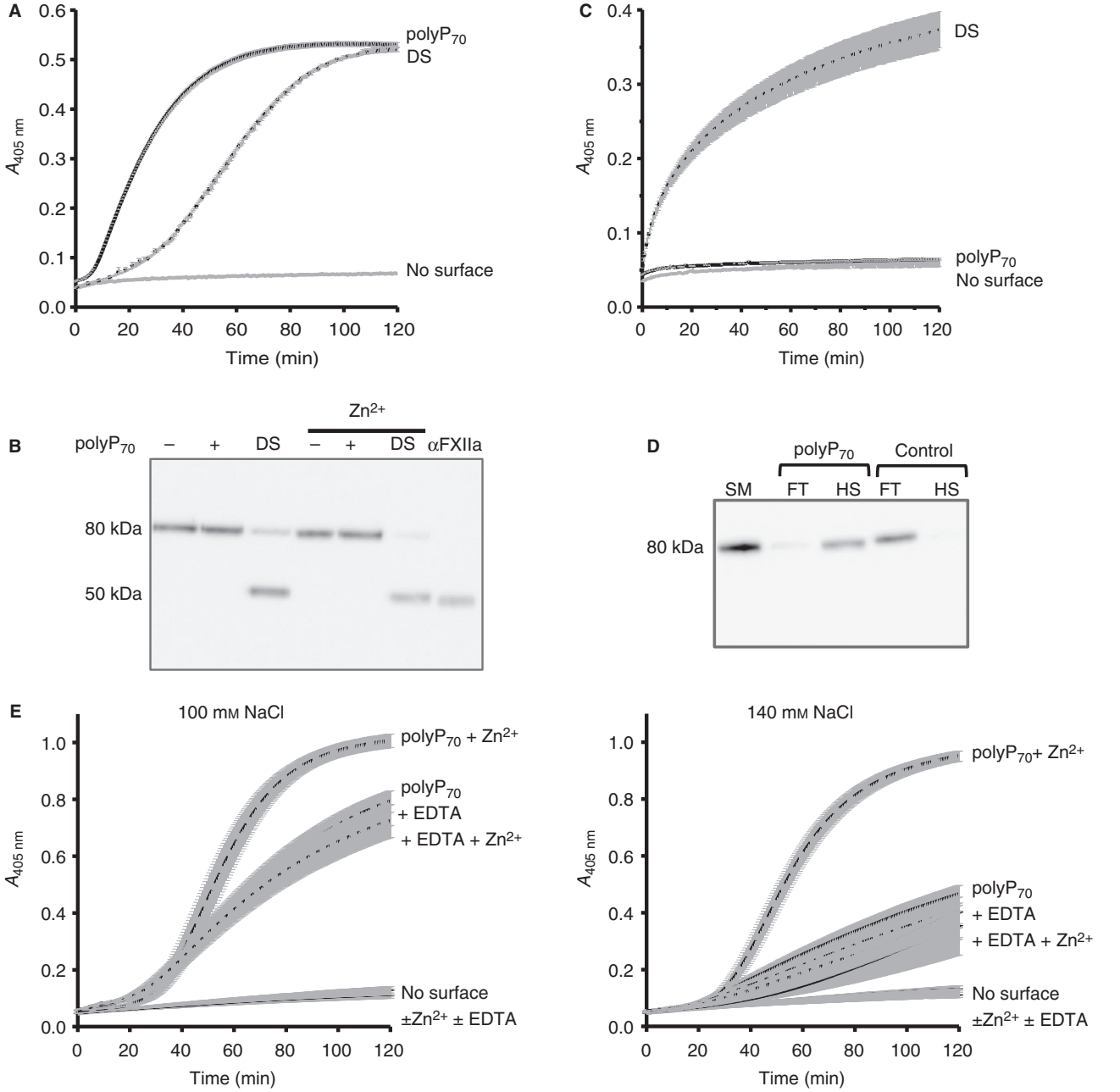

Fig. 1. Activation of FXII by polyphosphate (polyP). (A) FXII (50 nM) alone or in the presence of either $polyP_{70}$ (70 μM) or dextran sulfate (DS) (1.5 μg mL^{-1}) was added to the chromogenic substrate S2302 (0.5 mM). The reaction was monitored in a plate reader at 405 nm and 37 °C. (B) FXII (50 nM) alone or in the presence of either $polyP_{70}$ (70 μM) or DS (1.5 μg mL^{-1}) ± 10 μM ZnCl was incubated for 60 min at 37 °C. Samples were separated on 4–12% gels alongside preformed αFXIIa (50 nM) before western blotting with an antibody against FXII. (C) FXII (50 nM) was incubated at 37 °C for various times up to 120 min with $polyP_{70}$ (70 μM), with DS (1.5 μg mL^{-1}), or in the absence of a surface. After incubation, 1 M NaCl was added before quantification of activity with S2302 (0.5 mM). (D) FXII was bound to polymethacrylate beads labeled with polyP or bovine serum albumin (control). Starting material (SM), flow-through (FT) and high salt (HS) eluent were separated on 4–12% gels, and detected with an antibody against FXII. (E) FXII (50 nM) alone or in the presence of 70 μM $polyP_{70}$ ± 10 μM ZnCl ± 1 mM EDTA was added to 0.5 mM S2302, and the reaction was monitored as above in standard buffer containing either 100 mM or 140 mM NaCl. Data are expressed as mean ± standard deviation; $n = 4$.

The optimal $polyP_{70}$ polymer concentration for efficient autoactivation of FXII was 70–140 μM (Fig. 2), as determined with the continuous assay described above. As shown in Fig. 1E, the concentration of NaCl in the reaction had a dramatic impact on autoactivation of FXII by $polyP_{70}$. We investigated this more comprehensively, and found maximal autoactivation of FXII by $polyP_{70}$ at low NaCl concentrations (10–50 mM); however, considerable activity was still detectable at physiological NaCl concentrations (Fig. 2). The strong dependence on the concen-

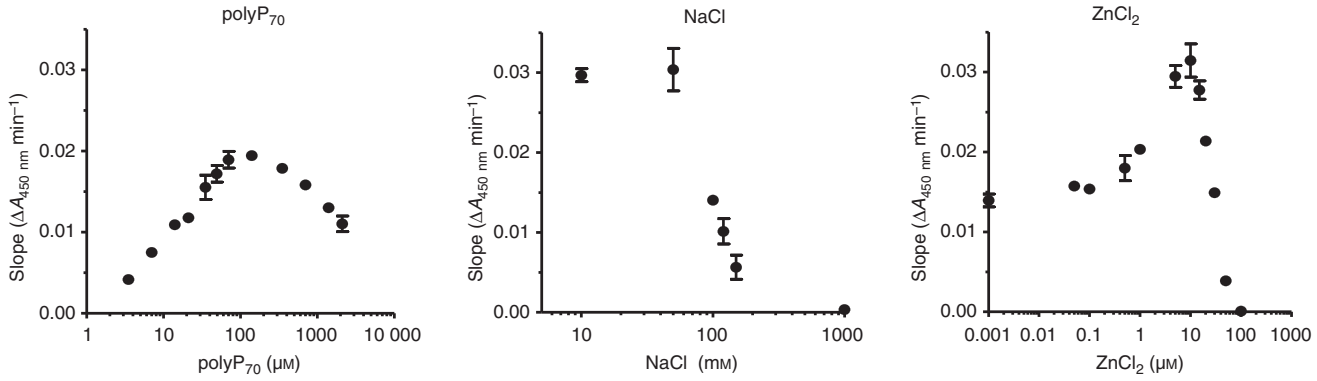

Fig. 2. Formation of single-chain FXII–polyphosphate (polyP)$_{70}$. In all reactions 50 nM FXII was added to 0.5 mM S2302 in the presence of 70 μM polyP$_{70}$, unless otherwise stated. The concentration of polyP$_{70}$ in the reaction was varied from 0 to 2 mM. The concentration of NaCl in the buffer was varied from 10 to 1000 mM, or $ZnCl_2$ was included in the reaction buffer at various concentrations (0–100 μM). Results are expressed as mean ± standard deviation; $n = 3$.

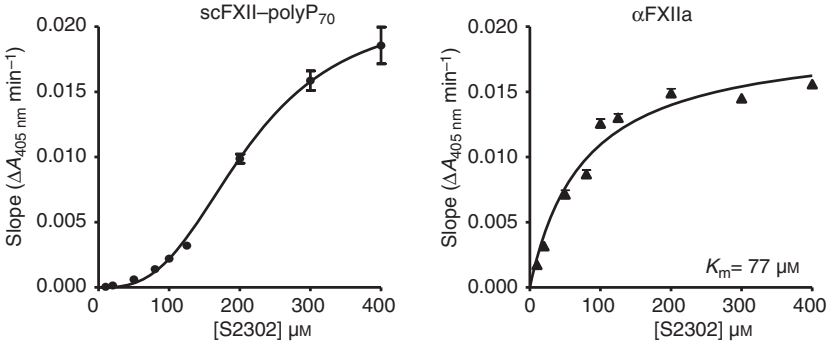

Fig. 3. Formation of single-chain FXII (scFXII)–polyphosphate (polyP)$_{70}$ follows an allosteric mode of activation. FXII (50 nM) was incubated with 70 μM polyP$_{70}$ at various S2302 substrate concentrations (10–400 μM), and generation of activity was monitored over time. Similarly, preformed αFXIIa (50 nM) was incubated with various substrate concentrations. Results are expressed as mean ± standard deviation; $n = 3$. Data were analyzed in GRAPHPAD PRISM with an allosteric sigmoidal model of enzyme kinetics for scFXII–polyP$_{70}$ or the classic Michaelis–Menten equation for αFXIIa; from these, the Hill coefficient and the K_m were derived, respectively.

tration of NaCl reflects the ionic nature of the interaction between FXII and polyP$_{70}$, as shown in Fig. 1D. We found that the optimal Zn^{2+} concentration for enhancing autoactivation of FXII by polyP$_{70}$ was 10 μM (Fig. 2). The presence of physiological concentrations of Zn^{2+} enhances the generation of scFXII–polyP$_{70}$ activity and can diminish the effect of NaCl on the reaction.

Comparison of scFXII–polyP$_{70}$ activity with αFXIIa activity

We next conducted a set of experiments to investigate the kinetics of the reaction of scFXII–polyP$_{70}$ with S2302 in comparison with a commercial preparation of αFXIIa. αFXIIa produced a classic hyperbolic curve that, when analyzed by use of the Michaelis–Menten equation, gave a K_m of 77 μM (Fig. 3). In contrast, a sigmoidal curve was obtained with scFXII–polyP$_{70}$ over a range of S2302 concentrations (10–400 μM) (Fig. 3), from which it was impossible to derive a K_m. The data were fitted to sigmoidal substrate velocity curves (GRAPHPAD PRISM), and the Hill coefficient determined to be 3.67, indicative of positive cooperative binding of polyP$_{70}$ to FXII.

Sensitivity of scFXII–polyP$_{70}$ to inhibition

We then studied inhibition of scFXII–polyP$_{70}$ by a peptidyl inhibitor (PCK), a small protein inhibitor (CTI) and a physiological inhibitor (C1-Inh) of FXIIa. FXII and polyP$_{70}$ (70 μM) or αFXIIa were incubated with increasing concentrations of inhibitors (Fig. 4). Activity was quantified with S2302 at each inhibitor concentration tested. We found that scFXII–polyP$_{70}$ was inhibited substantially faster by CTI than the two-chain form, αFXIIa (IC_{50} of 23.1 ± 0.06 nM vs. 304.4 ± 0.06 nM, respectively). Similar results were obtained with PCK, with lower IC_{50} values for scFXII–polyP$_{70}$ than for αFXIIa (IC_{50} of 533.8 ± 0.08 nM vs. 2212 ± 0.03 nM). Interestingly, no difference was noted in the inhibition of scFXII–polyP$_{70}$ and αFXIIa by C1-Inh (IC_{50} of 113 ± 0.1 nM vs. 104.4 ± 0.09 nM).

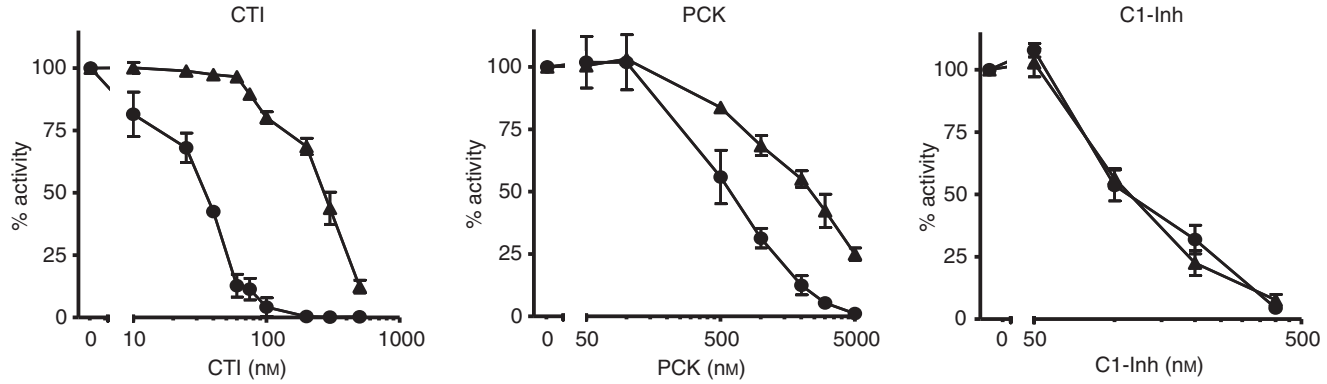

Fig. 4. Inhibition of single-chain FXII–polyphosphate (polyP)$_{70}$. FXII (50 nM) was incubated with 70 μM polyP$_{70}$ (circles) in the absence or presence of the inhibitors corn trypsin inhibitor (CTI) (0–500 nM), H-D-Pro-Phe-Arg-chloromethylketone (PCK) (0–5 μM), or C1-inhibitor (C1-Inh) (0–400 nM). Activity was monitored with the chromogenic substrate S2302, and compared with inhibition of αFXIIa (triangles). Results are expressed as mean ± standard deviation; $n = 3$. Data were analyzed as described in Materials and methods to derive the IC_{50} values.

scFXII–polyP$_{70}$ cleaves physiological substrates

We tested the ability of scFXII–polyP$_{70}$ to cleave its physiological targets, FXI and PK. FXI with or without FXII and with or without polyP$_{70}$ (70 μM) was incubated with a chromogenic substrate for FXI, and the activity was quantified over a period of 2 h. Very little FXIa activity was observed upon incubation of zymogen FXI and FXII, but inclusion of polyP$_{70}$ dramatically accelerated activation (Fig. 5A). Western blotting revealed the presence of FXIa when FXI was incubated with FXII and polyP$_{70}$ but not when it was incubated with FXII alone. In the absence of FXII, polyP$_{70}$ was unable to directly stimulate autoactivation of FXI, consistent with previous observations [21].

Similar experiments conducted with PK showed generation of kallikrein activity when PK was incubated with FXII, indicating reciprocal activation of these proteases even in the absence of an activating surface (Fig. 5B). Inclusion of polyP$_{70}$ dramatically accelerated the cleavage of PK, and bands of kallikrein at 50 kDa and 38/35 kDa were detected by western blotting. Interestingly, polyP$_{70}$ was not efficient at stimulating PK autoactivation, but these experiments were conducted in the absence of the cofactor HK, which facilitates binding of PK to an activating surface. The chromogenic substrate S2302 can be cleaved by both kallikrein and FXIIa. We therefore examined cleavage of S2302 at various FXII concentrations; minimal cleavage of S2302 was observed until the concentration of FXII exceeded 25 nM in the presence of polyP$_{70}$ (data not shown). These data clearly show that scFXII–polyP$_{70}$ has the capacity to cleave downstream physiological targets in addition to amidolytic substrates.

Discussion

FXII is known to autoactivate when bound to negatively charged surfaces. The mechanism of autoactivation has been studied with several non-physiological surfaces, such as DS [31,37], ellagic acid [38], and kaolin [39]. These studies provided valuable insights into the mechanism underpinning FXII autoactivation, but the surfaces were included at relatively high concentrations, and experiments were performed at low ionic strength. In this study, we analyzed autoactivation of FXII by the 'natural' surface polyP at physiological pH and ionic strength. It has been established that longer-chain polyP, such as those found in bacteria, are substantially more efficient at stimulating contact activation [33]. However, the aim of this study was to evaluate the ability of polyP of approximately the size found in platelets to activate FXII [21,27]. We have shown that autoactivation of FXII by polyP$_{70}$ generates an active single-chain intermediate form of FXII (scFXII), presumably by inducing a conformational change in FXII that allows the active site of the enzyme to open. If the interaction of polyP$_{70}$ and FXII is disrupted by high salt concentrations, enzymatic activity is lost, indicating that the conformational change in scFXII–polyP$_{70}$ is reversible. scFXII–polyP$_{70}$ is capable of cleaving synthetic and physiological targets, specifically FXI and PK, to their active forms, indicating that it may participate in biological reactions and could provide the initial stimulus for generating two-chain αFXIIa *in vivo*.

FXII is classified as a coagulation protein, but it closest homolog is hepatocyte growth factor [40], and it is also structurally analogous to the fibrinolytic proteins tissue-type plasminogen activator (t-PA) and single-chain urokinase plasminogen activator [41]. Interestingly, t-PA is not considered to be a 'true zymogen', as it shows catalytic activity, ~ 8% of that of the two-chain form, as a single-chain protein (single-chain t-PA [sct-PA]) [42–44]. Fibrin functions as a cofactor for sct-PA, accelerating its intrinsic enzymatic activity to such a degree that the cleaved and uncleaved forms are indistinguishable [45]. 'Zymogen activation' can therefore be

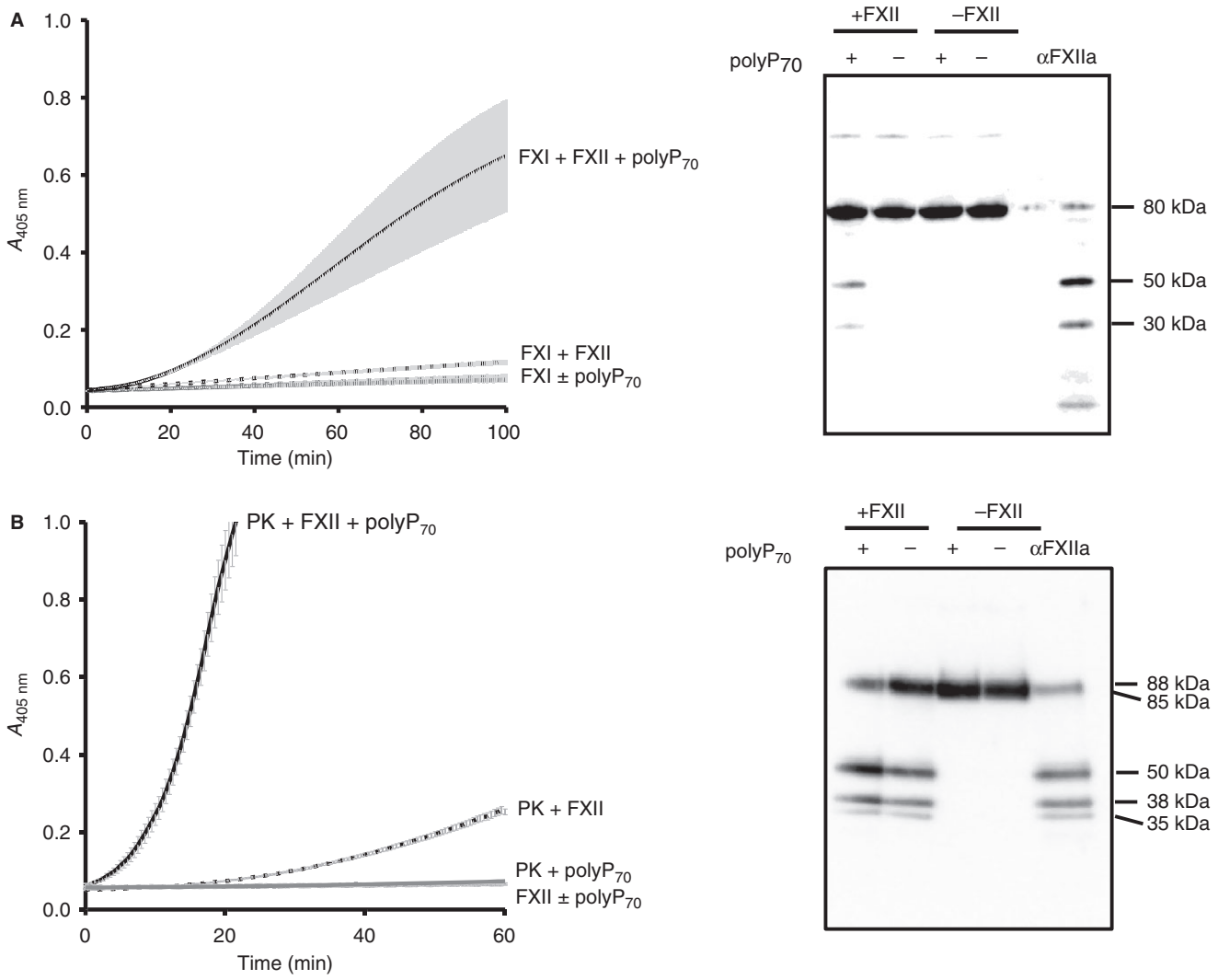

Fig. 5. Single-chain FXII–polyphosphate (polyP)$_{70}$ cleaves its physiological targets FXI and prekallikrein (PK). (A) FXI (35 nM) was incubated alone, with FXII (50 nM), with polyP$_{70}$ (70 μM) or with both in the presence of the chromogenic substrate L-2145 (0.5 mM), and the reaction was monitored at 405 nm. Data are expressed as mean ± standard deviation (SD); $n = 3$. Samples from the 120 min time point were separated under reducing conditions and western-blotted with an antibody against FXI. (B) PK (50 nM) was incubated with FXII (3 nM), with polyP$_{70}$ (70 μM) or with both in the presence of S2302 (0.5 mM). FXII (3 nM) with or without polyP$_{70}$ (70 μM) was included as a control. Data are expressed as mean ± SD; $n = 3$. Samples from the 60 min time point were western-blotted with an antibody against PK.

achieved by direct cleavage of sct-PA by plasmin or by binding of sct-PA to fibrin [45]. The results presented here indicate that a similar mechanism may exist for activation of zymogen FXII. The enzymatic activity of αFXIIa is 4200-fold higher than that of FXII [46]. However, our data reveal that, when FXII is bound to its 'cofactor', polyP$_{70}$, there is a substantial increase in enzymatic activity, roughly equivalent to that of the same concentration of cleaved αFXIIa. The hypothesis that FXII could show amidolytic activity in its single-chain form was first proposed by Ratnoff and Saito [32], after they exposed FXII to Sephadex–ellagic acid. They found that cleavage of FXII was minimal, but that coagulant and amidolytic properties could be detected. This suggests that binding of FXII to a negatively charged surface, at least to ellagic acid and polyP$_{70}$, is sufficient to induce a conformational change that exposes the active site of the protein.

The FXII heavy chain contains two surface binding regions, one in the fibronectin type I domain between Thr134 and Arg153 [2], and one at an N-terminal site located between Glu5 and Glu15 [1]. By use of a series of FXII deletion mutants, an additional discontinuous region (Pro313–Arg334, Leu334–Arg353) was identified that can participate in surface binding [4]. Autoactivation of FXII and cleavage by kallikrein is also enhanced by binding of a mAb to the kringle domain of FXII, indicating that multiple mechanisms may promote FXII activation [47]. The cooperative binding kinetics observed for FXII autoactivation by polyP$_{70}$ may be explained by the

existence of multiple binding sites for anionic surfaces, and require further studies to define the number and location of these sites.

Generation of scFXII–polyP$_{70}$ activity was dependent on the concentration of activating surface, with an optimal concentration of 70–130 μM polyP$_{70}$. Higher concentrations of polyP$_{70}$ were inhibitory, consistent with a template mechanism of activation. Substantial amidolytic activity was detected at low ionic strength (50 mM), but even at physiological salt concentrations, scFXII–polyP$_{70}$ showed significant activity. These observations are consistent with our previous reports on polyP activation of FXII in plasma [20,21]. Zinc ions bind to FXII, and are known to enhance autoactivation by several surfaces, including DS [5] and phosphatidylinositol phosphate [36]. FXII has the capacity to bind a maximum of four zinc ions, with high affinity (0.6 μM), to a single class of independent, non-interacting binding sites [36]. In plasma, the concentration of Zn^{2+} is 5–20 μM; the majority is bound to albumin, with only 0.25–1 μM being available as free ion [48]. However, the concentration of Zn^{2+} in platelets is 30–60-fold higher [49], and is sensitive to changes in the extracellular concentration [50]. It has been suggested that concentrations of ~ 10 μM free Zn^{2+} could be readily achieved in the circulation following platelet activation [51,52], and concomitant release of polyP and Zn^{2+} from activated platelets may facilitate FXII activation *in vivo*. In line with previous reports on autoactivation of FXII by DS [5], our experiments indicate that, in the presence of Zn^{2+}, the impact of NaCl on the interaction of FXII and polyP$_{70}$ activity is diminished.

It has previously been shown that autoactivation of FXII can be induced by low molecular mass polysaccharides, but the rate is dramatically accelerated with polysaccharides of 10 000 Da and above [31]. The increased level of autoactivation is explained by the existence of multiple binding sites for FXII on larger polysaccharide chains. In this study, we used polyP of an average chain length of 70. By assuming a P–O bond length of 1.5 Å, we can derive the length of polyP$_{70}$ as ~ 20 nm. The diameter of FXII is 5–6 nm (M_r = 80 000), based on the assumption that the protein molecule is approximately spherical [31]. These calculations are approximate; however, they imply that up to four FXII molecules could bind per chain of polyP$_{70}$, which may account for the levels of autoactivation and activity observed.

scFXII–polyP$_{70}$ was markedly more sensitive to inhibition by CTI and PCK than two-chain αFXIIa. Interestingly, there was no difference in the inhibition of scFXII–polyP$_{70}$ and αFXIIa by the serpin C1-Inh. It is interesting to speculate on the differences in inhibition of scFXII-polyP$_{70}$ by these inhibitors, which are markedly different in structure and mode of inhibition. It is possible that, in the case of C1-Inh, the polyanion binding site negates the effect of polyP$_{70}$, whereas with PCK a charge interaction may occur between the arginine of the peptide-based inhibitor PCK and polyP$_{70}$, drawing it into the active site and facilitating inhibition. CTI is a unique inhibitor, which is relatively specific for trypsin and FXIIa, however, like PCK the reactive site region of CTI has a net positive charge due to an abundance of arginine residues [53]. The positive charge associated with the reactive sites of CTI and PCK could help to explain the rapid inhibition of scFXII–polyP$_{70}$ by these inhibitors.

These results are the first to document the mechanism of autoactivation of FXII by a 'natural' surface under physiological conditions. Our data indicate that when FXII is in complex with its cofactor, polyP$_{70}$, it can show enzymatic activity in the absence of proteolytic cleavage. These data provide novel insights into the subtleties that regulate FXII autoactivation by naturally occurring anionic surfaces. It remains to be established whether binding of other physiological activators of FXII, such as RNA and misfolded proteins, induces similar enzymatic activity in the single-chain form of FXII. Complex formation between polyP released during platelet activation and plasma FXII may provide the initiating event to stimulate reciprocal activation of PK, subsequently leading to generation of αFXIIa by kallikrein.

Addendum

R. Engel performed research and analyzed data. C. Brain performed research and analyzed data. J. Paget performed research and analyzed data. A. S. Lionikiene performed research and analyzed data. N. J. Mutch designed research, analyzed and interpreted data, and wrote the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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