Cathepsin G, a leukocyte protease, activates coagulation factor VIII

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Summary

Neutrophils and monocytes express cathepsin G and can also bind to activated platelets, thus they can be localized to the site of active coagulation. Previous studies have suggested that cathepsin G inactivated coagulation factor VIII (FVIII) and was thus anticoagulant. But other studies have indicated procoagulant functions for cathepsin G in activation of coagulation factor V or activation of platelets among other possible mechanisms. Therefore, it remains unclear if cathepsin G is anticoagulant or procoagulant. We investigated the effects of human neutrophil cathepsin G on FVIII/VIIIa. Cathepsin G activates FVIII to a partially active form while having only a minor inactivating effect on thrombin-activated FVIIIa. This inactivation is mostly due to decreased stability of FVIIIa since a disulfide bond that prevents A2 subunit dissociation from FVIIIa prevents any loss of activity due to cathepsin G proteolysis. FVIII that has been cleaved by cathepsin G can still be activated by thrombin if A2 subunit dissociation is prevented. Cathepsin G cleavages of FVIII are limited to a few specific sites that are mostly located near known activating and inactivating cleavage sites. Cathepsin G cleavage sites near to thrombin cleavage sites likely contribute to the partial activation of FVIII. Therefore, it is possible that cathepsin G from neutrophils and monocytes may provide some pro-coagulant effect by activating FVIII.

Keywords

Factor VIII, cathepsin G, thrombosis, inflammation, neutrophils

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Introduction

Inflammation and coagulation are linked in a variety of ways (1). One potential way is through the action of proteases that are expressed by cells of the immune system. Proteolysis is central to the regulation of blood coagulation as proteolytic cleavages are utilized to both activate and inactivate many factors and cofactors in the blood coagulation pathway. Circulating neutrophils and monocytes bind to activated platelets via interaction of PSGL-1 expressed constitutively on these cells and P-selectin expressed on platelets. Cathepsin G is secreted by activated neutrophils and is also expressed in a membrane-associated form by monocytes and neutrophils (2–4). Like the coagulation proteases, cathepsin G is inactivated by protease inhibitors found in the blood (alpha-1-antitrypsin and alpha-1-antichymotrypsin). However, in its neutrophil-bound form, cathepsin G is resistant to inactivation by protease inhibitors (4). Furthermore, cathepsin G activates platelets via cleavage of PAR-4 and promotes thrombin generation and fibrin formation in a platelet-dependent manner under flow conditions (5, 6). Additionally, neutrophils are found in hemostatic plugs. Cathepsin G, along with other proteases released from these neutrophils, degrades FXIII (7). Coagulation also plays a role in inflammatory responses of neutrophils and monocytes, most notably in extravascular fibrin deposition at sites of inflammation. Therefore, cathepsin G may be delivered in an active form to sites of fibrin deposition by activated neutrophils binding to platelets in the blood or migrating to sites of inflammation. Thus it is important to understand the effects of cathepsin G and other proteases generated in response to inflammation on platelet-bound or circulating coagulation factors such as factor VIII (FVIII).

FVIIIa, the activated form of FVIII, is the cofactor for factor IXa (FIXa) in the intrinsic pathway of the coagulation system. Deficiencies of FVIII or FIX result in hemophilia. The severe consequences of these deficiencies illustrate the importance of the intrinsic factor X activating complex (FXase) and the intrinsic pathway in blood coagulation.

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Thrombin and FXa both activate FVIII via specific proteolytic cleavages (8, 9). Activated protein C (APC) inactivates FVIIIa via specific proteolytic cleavages (9). Thrombin activates FVIII by cleaving it at Arg372, Arg740 and Arg1689. This results in the creation of an active heterotrimer, FVIIIa, essentially consisting of the A1 domain (residues 1–372), the A2 domain (residues 373–740) and the light chain (residues 1690–2332) (9–11). FXa also cleaves FVIII at these same sites but additionally cleaves at several others, including at the same site as APC cleavage at Arg336, which inactivates FVIIIa (9). Therefore, FXa both activates and inactivates FVIIIa. FVIIIa is quite unstable due to spontaneous dissociation of the A2 domain, such that FVIIIa has a half-life in isolation of about two minutes (12–14). Thus, it is not entirely clear how important proteolysis is for inactivation of FVIIIa *in vivo*.

Cathepsin G activates factor V (3, 15), however, increases in factor Va activity were most evident when the resulting factor Va was assayed in purified component prothrombinase assays. But the activity increase was less striking when assayed in clotting assays in plasma, which are more physiologic than purified component assays (16). Nevertheless, this suggests a procoagulant role for cathepsin G. In addition, cathepsin G activates factor X, thus providing both proteins necessary for the prothrombinase complex (17). In fact, both the intrinsic FXase complex (FVIIIa: FIXa) and the prothrombinase complex (FVa: FXa) assemble and are highly active on monocyte surfaces and the prothrombinase complex also assembles on neutrophil surfaces (18, 19).

In contrast to this, it was reported that cathepsin G inactivates FVIII, suggesting an anticoagulant role for cathepsin G (20–22). However, this work was carried out with FVIII in complex with von Willebrand factor and in some instances time courses were up to 24 hours long. Therefore, any transient activation may have been undetected, since activating cleavages of FVIII by thrombin and FXa result in unstable FVIIIa because of dissociation of the A2 domain (23). Here we have examined the effects of neutrophil cathepsin G on the functional properties of purified human FVIII and FVIIIa in time scales relative to natural coagulation processes and using a recombinant variant of FVIII, C662-C1828 FVIII, that is resistant to inactivation by A2 subunit dissociation to help elucidate mechanisms of activity. We found that cathepsin G partially activates FVIII while cleaving at a limited number of sites. And in the absence of A2 subunit dissociation of the resulting FVIIIa, cathepsin G cleavage does not inactivate cathepsin G-activated or thrombin-activated FVIIIa.

Material and methods

Materials

FVIII deficient plasma and pooled normal human plasma were purchased from George King Biomedical Inc. (Overland Park, KS, USA). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Hirudin, human neutrophil cathepsin G and cathepsin G inhibitor I were purchased from Calbiochem/EMD Biosciences (La Jolla, CA, USA). In most experiments we used recombinant human B domain-deleted (BDD) FVIII and the disulfide-crosslinked mutant, C662-C1828 FVIII, production and characterization of which is described elsewhere (24) (the plasmid was a gift from Bayer Healthcare, Berkeley, CA, USA). In some experiments we used recombinant full-length FVIII (a gift from Bayer Healthcare, Berkeley, CA, USA). Von Willebrand factor was a gift from Dr. Zaverio Ruggeri (The Scripps Research Institute).

FVIII activity assays

FVIIIa activity was measured in a standard activated partial thromboplastin time (APTT) clotting assay with FVIII deficient plasma as described (25). FVIII (0.5 to 1 U/ml) was incubated in HBS/0.5% BSA/5 mM $CaCl_2$. In some experiments von Willebrand factor was added to a concentration of 10 µg/ml. At the zero time an aliquot was removed and assayed in the APTT assay, then for thrombin activation, 5.4 nM thrombin was added. After 1 minute, 1 U/ml hirudin was added to inactivate the thrombin. Then aliquots were removed at time points and assayed in the APTT assay. For cathepsin G reaction with thrombin-activated FVIIIa, the protease was added immediately after the hirudin. For assays of cathepsin G proteolysis of FVIII, cathepsin G was added instead of thrombin and hirudin. In control experiments with cathepsin G inhibitor, cathepsin G was pre-incubated with cathepsin G inhibitor in a 15 to 30 times control stock solution for 10 minutes. Then the final concentration of cathepsin G inhibitor in the reaction was 3–12 µM.

The APTT assay was conducted as follows. Fifty µl of FVIII-deficient plasma was mixed with 50 µl Platelin LS (bioMérieux, Durham, NC, USA) and incubated at 37°C for three minutes. A 5 µl aliquot of FVIII was added, immediately followed by 50 µl of HBS/0.5% BSA/25 mM $CaCl_2$. Clotting time was measured in a Diagnostica Stago ST4 coagulometer. Thus, the FVIII/protease mix was diluted 1 to 30 into the APTT assay. A FVIII standard curve was made using recombinant FVIII. Then FVIIIa activity was converted to relative FVIIIa activity where time zero equals one for ease of comparison of different experiments and to correct for day to day variability of the baseline FVIII activity measured in the APTT assay.

FVIIIa activity was also measured in a purified component assay of the FVIIIa: FIXa complex (FXase) as follows. First, FVIII concentration was determined by FIXa titrations as described (24). FVIII was 15 nM in HBS/5 mM $CaCl_2$/ 0.1 mM $MnCl_2$/0.5 % BSA (FXase buffer) and cathepsin G was added at the appropriate concentration at time zero. Aliquots were removed and diluted 1 to 30 in 2.5 nM FIXa, 25 µM phospholipid vesicles [40 % phosphatidyl choline (PC), 20 % phosphatidyl serine (PS), 40 % phosphatidyl ethanolamine (PE)] prepared as described (26). Then 0.5 µm factor X was added. The reaction was stopped in 30 sec by addition of EDTA and then FXa production was measured by cleavage of chromogenic substrate (S2765, Diapharma, Westchester, OH, USA).

Gel analysis

SDS-PAGE of proteolyzed FVIII/FVIIIa was performed as follows. FVIII was at the same concentration and conditions as in the FXase assays but no BSA was present in the buffer. Reactions were stopped by addition of SDS sample buffer containing 10 mM EDTA and 6 mM dithiothreitol. After boiling, samples were alkylated with iodoacetamide before electrophoresis on a 4–12 % Bis/Tris Invitrogen gel with MOPS/SDS buffer. Gels

were silver-stained with SilverXpress (Invitrogen, Carlsbad, CA, USA). Molecular weights of fragments were estimated based on the molecular weight standards (Mark 12, Invitrogen). For N-terminal sequencing, digests of FVIII were performed at 3.4 μM BDD FVIII with 142 nM cathepsin G at 37ºC to assure complete digestion before SDS-PAGE. Fragments were transferred to Bio-Rad Sequi-Blot PVDF membrane, stained with Coomassie blue R 250 or ponceau S and individual fragments were cut out and sequenced by Edman degradation at the Center for Protein Sciences, The Scripps Research Institute or at the Harvard Microchemistry Facility at Harvard University (Cambridge, MA, USA).

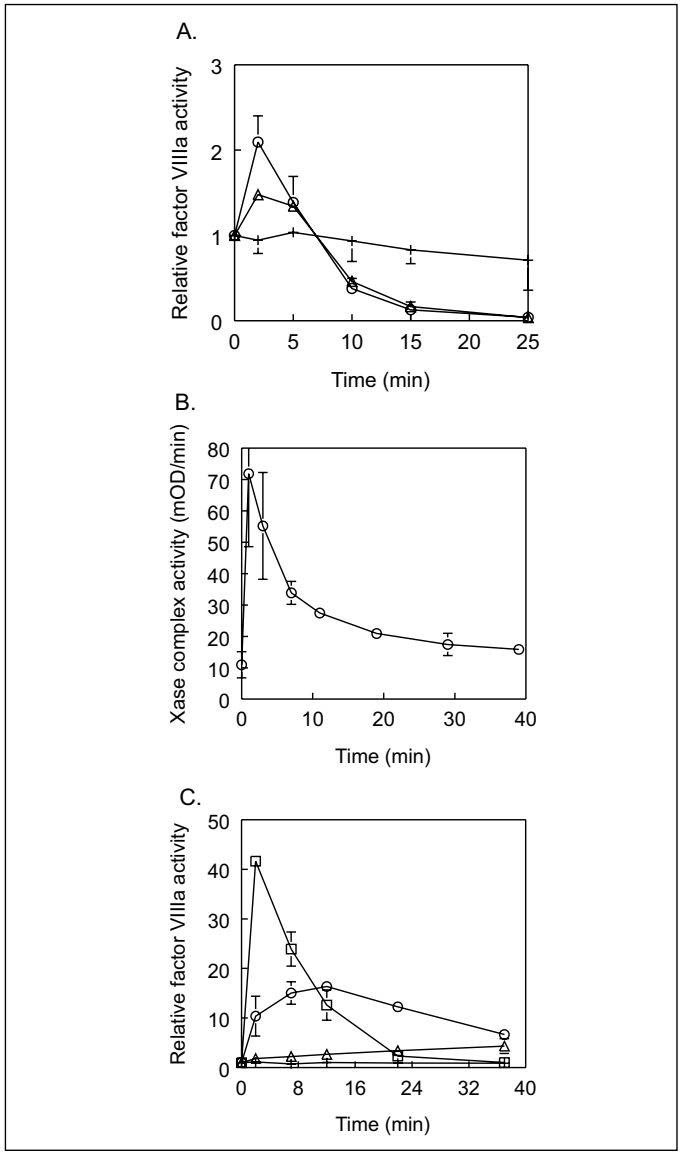

Figure 1: Cathepsin G activates FVIII. Full-length FVIII was incubated with cathepsin G and activity was monitored over time. A) FVIIIa activity measured in an APTT clotting assay, no cathepsin G (+), 3.6 nM cathepsin G (Δ), 14 nM cathepsin G (○). B) FVIIIa activity measured in a FXase assay with purified components with 14 nM cathepsin G. C) Full-length FVIII activated by thrombin, measured in an APTT assay, no thrombin (+), 0.021 nM thrombin (Δ), 0.34 nM thrombin (○), 5.4 nM thrombin (□). APTT values were converted to U/ml with a standard curve of recombinant FVIII diluted into FVIII deficient plasma and then converted to relative activity to standardize different figures. Data shown are averages of two to three experiments with standard deviations shown as error bars.

Statistics

Data were analyzed with the unpaired t-test in Excel to identify statistically significant differences relative to the reference curve or to the zero point in graphs without a reference curve.

Results

The effects of cathepsin G cleavages on human FVIII and FVIIIa function were measured using an APTT clotting assay (Fig. 1A) or a FXase assay using purified components (Fig. 1B) to monitor FVIIIa activity. Cathepsin G was incubated with full-length FVIII as described in the methods at two concentrations of cathepsin G (3.6 and 14 nM) for the APTT assay. There was an increase in FVIIIa activity at the beginning of the time course that was proportional to cathepsin G concentration. For both 3.6 nM and 14 nM cathepsin G, FVIIIa activity was statistically significantly increased relative to the no cathepsin G control at two minutes (unpaired t test). At five minutes only the 2.5 nM curve was statistically significantly increased. This increase was followed by complete loss of activity within 40 minutes. A similar profile of activation was observed with 14 nM cathepsin G as measured in the FXase assay with data points statistically significantly increased relative to the zero point from one through 19 minutes (Fig. 1B). For comparison we activated full-length FVIII with thrombin at three concentrations of thrombin (Fig. 1C). It is clear that thrombin is a more efficient activator of FVIII and thrombin-activated FVIII has higher activity.

To confirm that there was no trace thrombin in the cathepsin G that was responsible for this observed activity, in some experiments 1 U/ml hirudin was added to the FVIII before cathepsin G was added. This had no effect on the results (tested in both APTT and FXase assays). Additionally, the stock cathepsin G (2.85 μM) was incubated with pure fibrinogen for 24 hours without clotting, at which time the experiment was terminated. In identical conditions, 0.012 nM thrombin clotted fibrinogen in two hours. In an additional experiment to confirm that cathepsin G was responsible for the observed activity, the time course using both the APTT and the FXase assay was performed with cathepsin G that was pre-incubated with an excess of a specific cathepsin G inhibitor (cathepsin G inhibitor I, a highly specific competitive inhibitor of cathepsin G (EMD Biosciences)). In these experiments there was no activation or inactivation of FVIII (data not shown). The inhibitor alone had no effect on the FVIII activity assays.

In-vivo FVIII circulates in complex with von Willebrand factor (vWF). The vWF binding site in FVIII is primarily located in the acidic peptide at the beginning of the A3 domain in the light chain. When FVIII is activated it no longer binds to vWF because this peptide is released upon thrombin cleavage at Arg1689 (27). This releases FVIIIa to bind to membranes and FIXa in the FXase complex. We tested if vWF would block cathepsin G activation of FVIII in a time course in which either full-length or B domain-deleted FVIII (BDD FVIII) was incubated with 10 μg/

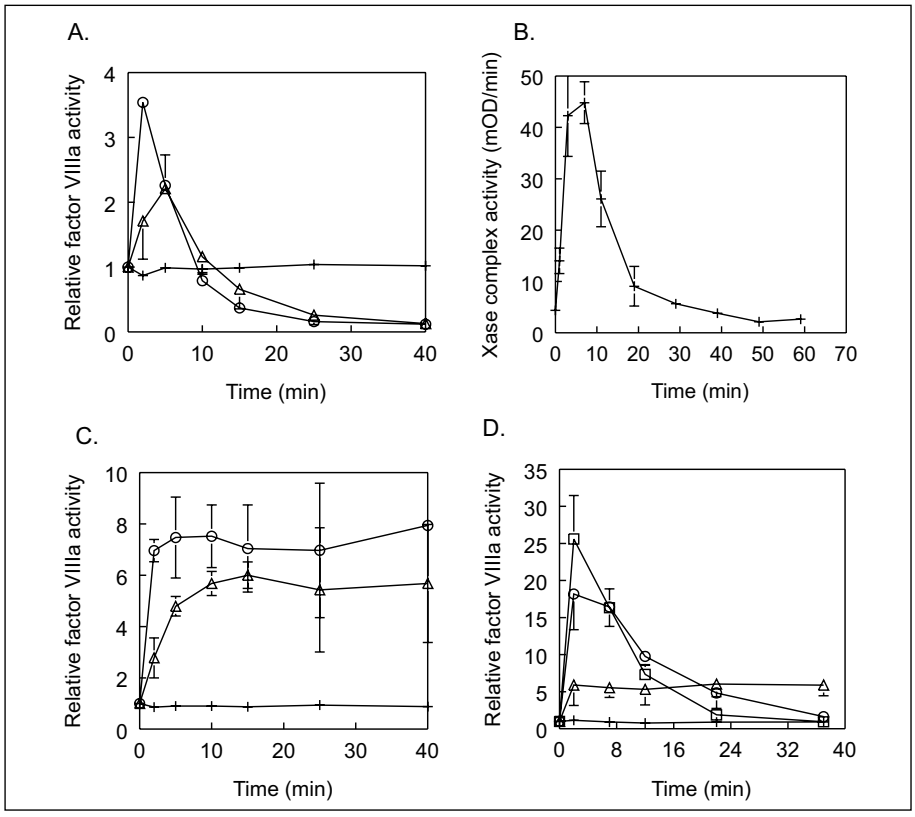

Figure 2: Cathepsin G-activated BDD FVIIIa activity is stable in the absence of A2 subunit dissociation. A) B domain-deleted (BDD) FVIII activated by cathepsin G and monitored by APTT, no cathepsin G (+), 3.6 nM cathepsin G (Δ), 14 nM cathepsin G (○). B) BDD FVIII activated by 3.6 nM cathepsin G monitored by FXase assay. C) Disulfide bond-stabilized BDD FVIII (C662-C1828 FVIII) activated by cathepsin G and monitored by APTT, no cathepsin G (+), 3.6 nM cathepsin G (Δ), 14 nM cathepsin G (○). D) BDD FVIII activated by thrombin, measured in an APTT assay, no thrombin (+), 0.021 nM thrombin (Δ), 0.34 nM thrombin (○), 5.4 nM thrombin (□). Data shown are averages of two to three experiments with standard deviations shown as error bars.

ml vWF before addition of cathepsin G. FVIIIa activity was monitored in the APTT assay. vWF did not inhibit the effect of cathepsin G on either full-length or BDD FVIII (data not shown).

We hypothesized that the loss of FVIIIa activity observed after the activation by cathepsin G was due to dissociation of the A2 subunit, as happens with thrombin-activated FVIIIa. To test this hypothesis we monitored the activation of recombinant BDD FVIII in which a disulfide bond between the A2 domain and the A3 domain was engineered (C662-C1828 FVIII). This stabilized FVIII variant does not lose activity after thrombin activation because A2 subunit dissociation is prevented by the disulfide bond (24). We first confirmed that wild type BDD FVIII was activated by cathepsin G in a similar manner as was full-length FVIII. As can be seen in Figure 2A and 2B, measured in both an APTT assay and a FXase assay, BDD FVIII is activated by cathepsin G (about 4-fold in APTT) and then loses its activity over time. In both experiments FVIIIa activity upon cathepsin G incubation was statistically significantly greater than the zero cathepsin G line (Fig. 2A) or the zero time point (Fig. 2B) in an unpaired t test. In contrast to this, C662-C1828 FVIII was activated about 7-fold by cathepsin G (Fig. 2C), but it did not lose this activity over time. This suggests that the loss of activity seen for wild type full-length or BDD FVIII after cathepsin G activation was due to A2 subunit dissociation rather than a specific inactivating cleavage. Again, for comparison we show thrombin activation of BDD FVIII measured in an APTT at three concentrations of thrombin. As was the case for full-length FVIII thrombin is a better activator of BDD FVIII than cathepsin G.

We then investigated the effects of cathepsin G on thrombin-activated FVIIIa. In Figure 3A either WT BDD FVIII or C662-C1828 FVIII was first activated by thrombin for one minute, followed by the addition of hirudin to inactivate the thrombin. Then cathepsin G was added (time 0 in the graph, Fig. 3A) and activity was monitored in an APTT assay. Cathepsin G increased the rate of inactivation of WT FVIIIa measurably (statistically significantly different from five to 40 minutes). However, cathepsin G had no effect on thrombin-activated C662-C1828 FVIIIa, in which A2 subunit dissociation was prevented. Thus, the increased inactivation rate in WT FVIIIa was likely due to faster dissociation of the A2 subunit rather than inactivating cleavages.

Furthermore, we measured the activation of C662-C1828 FVIII by thrombin after it was first cleaved by cathepsin G. In Figure 3B C662-C1828 FVIII was first incubated with 0 or 14 nM cathepsin G for 30 minutes. Cathepsin G activated the C662-C1828 FVIII to a stable plateau (open squares). After 30 minutes thrombin was added. Both the cathepsin G-activated FVIIIa and the unactivated FVIII increased in activity upon addition of thrombin but cathepsin G-activated FVIIIa was not activated by thrombin as much as FVIII, indicating that the capacity to be activated by thrombin was somewhat reduced after cleavage by cathepsin G. However, cathepsin G cleavage did not prevent thrombin activation of FVIII.

We electrophoresed complete protease digests of FVIII to visualize the fragments that resulted (Fig. 4) and performed Edman degradation sequencing of cleavage fragments to precisely identify cathepsin G cleavage sites (Table 1). Figure 4 shows digests of full-length and BDD FVIII and thrombin-activated FVIIIa by cathepsin G. Figure 5 shows the proposed cleavage sites for cathepsin G in FVIII. These digests were performed

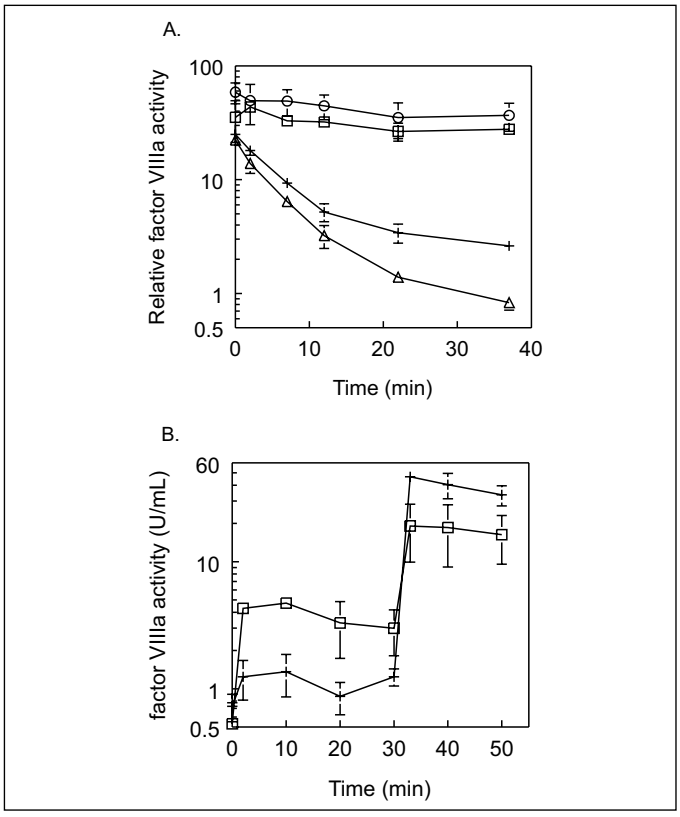

Figure 3: Cathepsin G cleavage slightly increases the inactivation rate of thrombin-activated BDD FVIIIa but does not prevent full activation of disulfide-stabilized FVIIIa by thrombin. A) Cathepsin G inactivation of thrombin-activated FVIIIa. BDD FVIIIa with no cathepsin G (+), BDD FVIIIa with 57 nM cathepsin G (Δ), C662-C1828 FVIIIa with no cathepsin G (○), C662-C1828 FVIIIa with 57 nM cathepsin G (□). Activity was monitored in an APTT clotting assay. B) Cathepsin G activation of C662-C1828 FVIIIa for 30 minutes followed by thrombin activation. No cathepsin G (+), 14 nM cathepsin G (□). Thrombin concentration was 5.4 nM. Data are averages of two to three experiments with standard deviations as error bars.

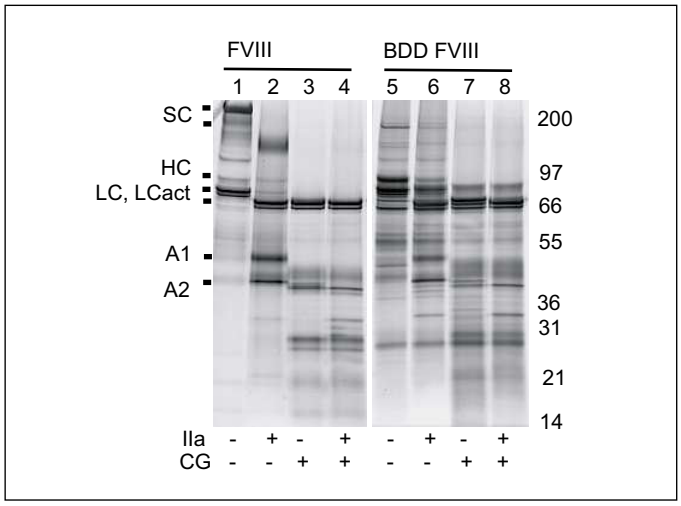

Figure 4: Silver stained SDS-PAGE of FVIII proteolysis shows limited cleavages in the heavy chain and the N terminus of the light chain by cathepsin G. Full-length FVIII (A) or BDD FVIII (B) was incubated with 5.4 nM thrombin for 40 min or with 57 nM cathepsin G for 30 min. In lanes 4 and 8 FVIII was incubated with both thrombin and cathepsin G, with the thrombin incubation first, followed by the addition of hirudin to inactivate thrombin then cathepsin G second. Reactions were incubated at room temperature. The samples were electrophoresed and stained as described in the *Methods*.

with 57 nM cathepsin G for 30 min in order to maximize digestion. Both full-length FVIII and BDD FVIII had essentially identical cleavage patterns. Unactivated FVIII (lanes 1 and 5) had a minor single chain band, which is typical in recombinant FVIII preparations, as well as various heavy chain bands in the case of full-length FVIII or a single heavy chain in BDD FVIII and the light chain of unactivated FVIII. Thrombin cleavage resulted in a light chain doublet that was shifted down to around 70 kDa due to cleavage of R1689 (lanes 2 and 6). The doublet may be due to incomplete glycosylation. Cathepsin G cleavage of FVIII without thrombin (lanes 3 and 7) resulted in light chain doublets that were similar to thrombin cleaved light chain, but there was also an additional band that was above the thrombin-cleaved light chain (lanes 3 and 7). But cathepsin G cleavage after thrombin cleavage resulted in light chain bands identical to those seen for thrombin alone (lanes 4 and 8). Therefore, cathepsin G does not appear to cleave within the thrombin-activated FVIIIa light chain. This was supported by Edman sequencing of cathepsin G cleaved FVIII (Table 1). Cathepsin G primarily cleaved at K1673, 16 amino acids before the thrombin cleavage site (R1689). Cathepsin G may also have had a secondary cleavage at R1689. Alternatively, the starting material may have been already partially cleaved at R1689.

The thrombin-cleaved A1 subunit was around 48 kDa and the A2 subunit was around 42 kDa (lanes 2 and 6). Cathepsin G cleavage resulted in a downshift of the A1 band, to around 44 kDa and the A2 band to around 41 kDa. These bands were the same regardless of the presence of thrombin. Edman sequencing of these bands confirmed their identities and showed that cathepsin G cleaved between A1 and A2 at F360 while the A1 subunit had an intact N-terminus. Cleavage at F360 would reduce the MW of cathepsin G-A1 subunit by about 1.4 kDa relative to thrombin-A1 subunit. Therefore, there was likely another cleavage site

Table 1: Edman degradation sequencing results for FVIII fragments from cathepsin G cleavages.

		Sequence	Site
Light chain	1°	KEDFD	K1673-K1674
	2°	S[F]QK[K]	R1689-S1690
A1 subunit	1°	ATRRY	NH2-A1
A2 subunit	1°	DDDNS	F360-D361
	2°	SVAKK	R372-S373
29 kDa (A1)	1°	ATRRY	NH2-A1
26 kDa (A1)	1°	SFPF	K36-S37
20 kDa (A1)	1°	QDRDA	M217-Q218

Brackets indicate probable/ reasonable residues. A dash indicates a missing residue.

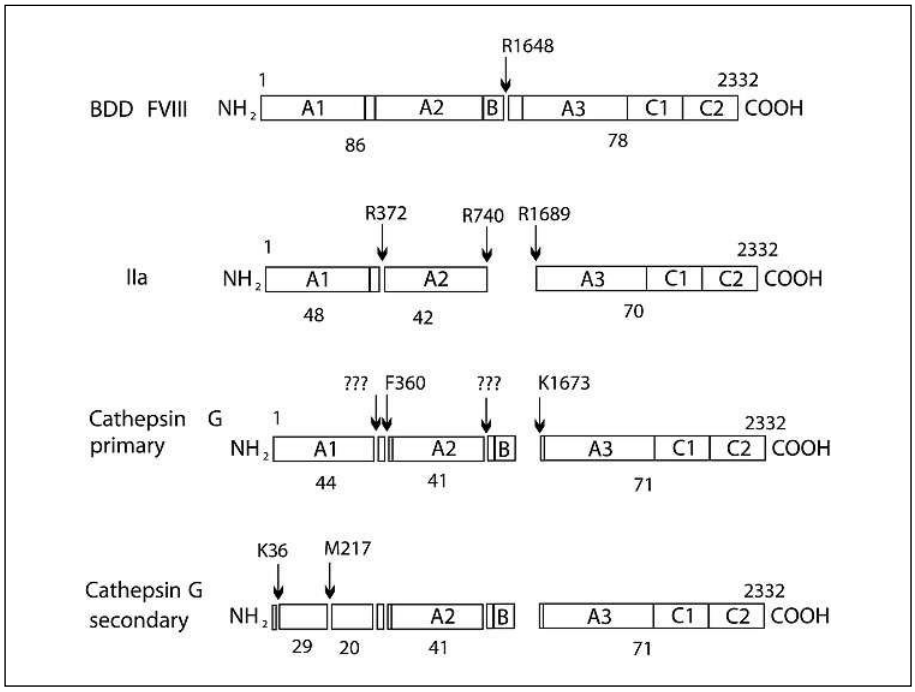

Figure 5: Cleavage sites of cathepsin G in FVIII. This schematic of the primary sequence of BDD FVIII shows the known cleavage sites in FVIII due to processing during secretion in the first diagram and thrombin activation in the second diagram. The third and fourth diagrams show cathepsin G cleavage sites identified by Edman degradation sequencing. Arrows with question marks indicate proposed cleavages based on determination of apparent molecular weights from the gels shown in Figure 4. Estimated polypeptide molecular weights in kilo Daltons are indicated below each fragment and are based on the overall analysis of N-terminal sequences and gel electrophoresis.

earlier in the C-terminal end of the A1 domain that removed approximately an additional 3 kDa. This would place that cleavage site very close to the APC cleavage site at R336.

As mentioned, the thrombin-cleaved A2 subunit had an apparent MW of 42 kDa. This was assumed to correlate with the polypeptide from 373–740. Cathepsin G-cleaved FVIII had a very similar MW in the gel (Fig. 5), but Edman sequencing analysis showed that the N-terminus of the A2 subunit was at D361 (Table 1), resulting in an additional 12 amino acids at the N-terminus of the A2 domain. Therefore, it is likely that cathepsin G also cleaved the A2 subunit about 10–15 amino acids before the normal C terminus, which is at R740. This was supported by the observation that the combined cleavages of thrombin and cathepsin G removed the upper band of the doublet, which would be expected due to cleavage at the N-terminus at R372 by thrombin and cleavage at the C-terminus 10–15 amino acids before R740 by cathepsin G. It was not possible to firmly identify this C-terminal cleavage though, because the resulting short B-domain fragment would only be a few kDa in size and was not isolated for N-terminal sequencing.

Bands that were visible at around 29 kDa, 26 kDa and 20 kDa were also sequenced. The 20 kDa band had an N-terminal sequence QDRDA (Table 1), indicating that it was the C-terminal half of the A1 subunit resulting from cleavage after M217. The 29 kDa band had the normal N terminal sequence of FVIII, consistent with the N-terminal fragment of the A1 subunit that would result from M217 cleavage. The 26 kDa band had the N-terminal sequence SFPF (Table 1), indicating cleavage after K36. This fragment is consistent with the polypeptide K37-M217 from the A1 subunit. Given that these fragments are all subsets of the A1 subunit, which is readily visible in Figure 4, these cleavages must take place more slowly than the F360 cleavage that liberates the A1 subunit from unactivated FVIII. Otherwise the 44 kDa polypeptide from the A1 subunit would not accumulate and would not be visible on the gel. Therefore, they are indicated as secondary cleavages in Figure 5. All of these cleavage sites (P1 residues = Lys, Phe, Arg or Met) are possible cathepsin G cleavage sites since cathepsin G does have clear dual trypsin- and chymotrypsin-like substrate specificity (28).

Discussion

Various studies have demonstrated procoagulant activities of monocytes and neutrophils. The mechanisms include activation of factors V and X by cathepsin G and elastase (3,19), activation of platelets via PAR-4 cleavage by cathepsin G (5), assembly of active FXase and prothrombinase on membrane monocyte or neutrophil surfaces (18,19), as well as promotion of thrombin generation and fibrin formation on platelets (6). However studies also suggested that cathepsin G inactivated FVIII, which would seem to counteract its procoagulant effects. These early studies were primarily done with FVIII: vWF complex before the nature of the components of this complex were fully understood and some were also done over multiple hour time scales. We have now determined that cathepsin G actually activates FVIII.

It is interesting to note that most of the observed cleavages by cathepsin G take place very near to known cleavage sites of thrombin, FXa and APC. It is clear that these regions of FVIII and FVIIIa are especially accessible to proteolytic cleavage. During normal activation of FVIII by thrombin, cleavage at the N-terminus of the light chain (R1689) is required to allow FVIIIa modulation of the FIXa active site (presumably affecting K_m and k_{cat} for FX) (29). Thrombin cleavages in the heavy chain at R372 and R740 are required to expose the FIXa binding site (30). While the cathepsin G cleavages do not result in optimal FVIIIa activity, apparently proteolytic separation of FVIIIa domains or sub-domains at these surface loops is sufficient to reveal significant FVIIIa cofactor activity. Optimal activation is clearly pre-

vented by the peptides that remain attached, since further cleavage by thrombin does result in more activation when A2 subunit dissociation is prevented (Fig. 3B).

Two cleavages in the A1 domain are not near to known cleavage sites of other coagulation proteases. These are the cleavage after K36 and the cleavage after M217. Since these cleavages would eliminate the cathepsin G-A1 subunit band that was observed in Figure 4 and confirmed to have the normal N-terminal sequence, we assume that they take place more slowly than the cleavage at F360 that generates the A1 subunit. However, this has not been rigorously confirmed. Apparently, these cleavages only slightly inactivate FVIIIa. In the absence of A2 subunit dissociation (using C662-C1828 FVIII), cathepsin G cleaved FVIIIa has stable activity for the length of the time course (Fig. 2C). This is further confirmed in Figure 3A where thrombin-activated FVIIIa, in which A2 subunit dissociation is prevented, is not inactivated at all by cathepsin G. However, when A2 subunit dissociation is not prevented, thrombin-activated FVIIIa does lose activity somewhat faster in the presence of cathepsin G. Since this only occurs in the absence of the stabilizing disulfide bond, we assume that cathepsin G destabilizes it somewhat. However, after 30 minutes of reaction with cathepsin G, FVIII is still capable of being activated by thrombin as long as A2 subunit dissociation is prevented by the C662-C1828 disulfide bond but it does not reach maximum activity anymore (Fig. 3B).

FXa also cleaves at K36 in the A1 domain and this cleavage appears to be responsible for a significant loss of FVIIIa activity due to a reduction in affinity for the A2 subunit resulting in increased dissociation of the A2 subunit (31–33). This is the same mechanism we propose for the increased inactivation rate of WT FVIIIa by cathepsin G since in disulfide-crosslinked FVIII this dissociation does not take place. In the porcine system FXa cleaves FVIII at R219 and R490 and one of these cleavages may be responsible for reduced activity relative to thrombin-activated FVIIIa (34). However, it has not been determined which cleavage is responsible for the loss of activity. In human FVIII residue 219 is Gln so it is not cleaved by FXa. Thus, our data suggesting that cleavages at K36 and M217 do not fully inactivate FVIII does not contradict published data.

In summary, cathepsin G does activate FVIII, though it produces an active FVIIIa that is less active than thrombin-activated FVIIIa. Cathepsin G is present in millimolar quantities in azurophil granules of neutrophils and is released from the granules upon activation of the neutrophils (35). Most of this cathepsin G is reversibly bound to the surface of the activated neutrophils via high capacity, low affinity binding sites involving chondroitin-sulfate and heparin-sulfate proteoglycans. The affinity is around 10^{-7} molar yet most of the cathepsin G remains bound, indicating that the local concentration of cathepsin G is quite high (36). In this membrane bound form, cathepsin G is resistant to plasma protease inhibitors (4). This suggests that the concentrations of active cathepsin G required in our experiments could be reached locally where neutrophils are activated *in vivo*. Cathepsin G has been considered to be procoagulant due to its interactions with platelets (6) and activation of factor V and factor X (3, 15–17). Furthermore, activated monocytes express tissue factor and both monocytes and neutrophils can provide prothrombinase binding sites (19). Therefore, these data detailing activation of FVIIIa by cathepsin G fit in with the overall procoagulant functions of neutrophil cathepsin G and activated neutrophils and monocytes. Therefore, we have elucidated one more the mechanism by which neutrophil-associated cathepsin G can promote fibrin formation, whether the neutrophils are associated with platelets in a growing thrombus or during fibrin deposition at extravascular sites of inflammation. Clearly the interactions of neutrophils and monocytes with the coagulation system are complex, but these data add one more piece to the overall picture.

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