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Generation of Gla-domainless FVIIa by cathepsin G-mediated cleavage

Else Marie Nicolaisen, Lars Christian Petersen, Lars Thim, Jes Kristian Jacobsen, Mogens Christensen and Ulla Hedner

Novo Nordisk, Novo Alle, DK-2880 Bagsvaerd, Denmark

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Coagulation factor VII contains ten γ-carboxyglutamic acid residues in the N-terminal region (Gla-domain) which are essential for the hemostatic function of FVII. The present study shows that granulocyte cathepsin G degrades the Gla-domain of FVIIa in vitro. Characterization of the truncated FVIIa by SDS-PAGE and N-terminal amino acid sequence analysis revealed that cleavage had occurred between Tyr-44 and Ser-45 and that further cleavage was only obtained on extensive cathepsin G exposure. Cleavage of vitamin K-dependent coagulation factors by cathepsin G may play a role in vivo, and it offers a convenient way of obtaining proteins deprived of their Gla-domain for functional and structural studies.

FVIIa; Cathepsin G; Gla-domainless; Isoelectric focusing; SDS-PAGE; Amino acid sequence analysis

1. INTRODUCTION

Biosynthesis of functional coagulation factor VII requires a vitamin K-dependent post-translational γ-carboxylation of 10 glutamic acid residues in the N-terminal part of the molecule [1]. The Gla-domain is involved in a Ca^{2+}-dependent binding of FVII to negatively charged phospholipids associated with cell surface-bound tissue factor [2]. Binding strongly promotes the conversion of the FVII zymogen to FVIIa, and also the enzymatic activity of FVIIa towards its substrates, FIX and FX, is profoundly enhanced [3,4].

Recent studies have shown that other vitamin K-dependent proteins, FX and protein C, were deprived of their Gla-domain upon exposure to cathepsin G [5,6]. This proteinase is one of several present in the azurophilic granules of the polymorph nuclear granulocytes (neutrophils). When the neutrophils are activated by external stimuli, these active proteinases are secreted. Systemic activation and excessive release of cathepsin G occurs in various diseased states, such as septicemia and leukaemia. The cathepsin G-mediated cleavage of the Gla-domains of coagulation factors may contribute to the deranged hemostatic balance observed in such conditions. We have investigated the effect on coagulation factor VIIa of exposure to cathepsin G under various conditions.

Abbreviations: Gla, γ-carboxyglutamic acid; GD-L, Gla-domainless light chain; L, light chain; H, heavy chain; IEF, isoelectric focusing; r, recombinant, RP, reverse phase; Rt, retention time; TFPI, tissue factor pathway inhibitor; ATIII, antithrombin III.

Correspondence address: E.M. Nicolaisen, BPD Res. Coagulation, Novo Nordisk, Novo Alle, DK-2880 Bagsvaerd, Denmark. Fax: (45) 4449 0555.

2. EXPERIMENTAL

Recombinant FVIIa [7], 1 mg/ml was dialysed against 10 mM Tris-HCl, pH 8.6 containing 75 mM NaCl and 5 mM $CaCl_2$. Calcium ions were complexed with EDTA, added in an excess of 5 mM, before the incubation with cathepsin G at 37°C. Cathepsin G, purified from human neutrophiles according to the method of Baugh and Travis [8], was a gift from Drs. H.J. Flodgaard and O. Nordfang (Novo Nordisk, Copenhagen).

Specific clotting activity was determined in a one-stage FVII clotting assay, using a FVII immunodepleted human plasma as test base and rabbit thromboplastin.

Isoelectric focusing (IEF) was performed at 10°C, in ultrathin polyacrylamide gels (0.25 mm) (T=4%, C=5%) on a Multiphor II system (Pharmacia LKB, application note 320). The rehydrated gels contained Pharmalyte 3–10 (4% w/v). The anode and cathode solutions were aspartic acid (40 mM) and NaOH (1 M), respectively. 10 μg of protein were applied on a pre-focused gel (30 min at 500 V) and 1,000 V for 60 min followed by 1,500 V for 75 min were applied. The focused protein was precipitated for 20 min with 20% TCA and stained for 20 min in 0.05% Coomassie R-250 in 25% isopropanol, 10% acetic acid. The destaining solution was 40% methanol, 10% acetic acid.

SDS-PAGE was performed as described in [9]. 10 μg of protein were applied on a linear gradient gel, 10–15% w/v with 4.5% w/v stacking gel. A 45 mA constant current was applied for 3 h. The pI (17-0471-01) and molecular weight markers (17-0446-01) were purchased from Pharmacia-LKB.

Reverse-phase high performance liquid chromatography (RP-HPLC), amino acid analysis and amino acid sequence analysis were performed as previously described [7,10].

3. RESULTS

3.1. *SDS-PAGE and clotting activity*

Fig. 1 shows the SDS-PAGE pattern during 120 min incubation of recombinant (r) FVIIa with cathepsin G in the presence and absence of calcium ions. The controls (lanes 1–4) show the intact light (L) and heavy (H) chain. The preparation contained 5% H-chain degradation products (molecular weight 15–17 kDa), generated

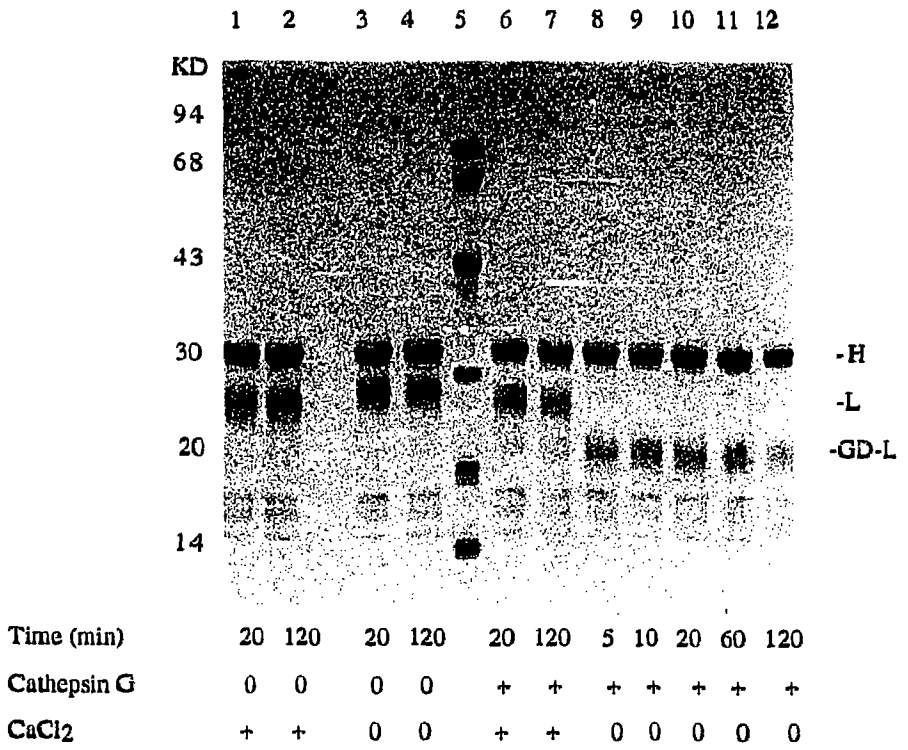

Fig. 1. SDS-PAGE of cathepsin G-degraded rFVIIa. rFVIIA (1 mg/ml) was incubated at 37°C in the presence and absence of calcium ions (5 mM $CaCl_2$), with and without cathepsin G (1/500 w/w). At the indicated intervals, aliquots were either diluted into 1% SDS, 1% DTT and boiled for SDS-PAGE or diluted in Ca^{2+} containing buffer, on ice, for clotting analysis (Table I). Molecular weight markers, lane 5.

during the activation of the rFVII and co-purifying with the intact rFVIIa [7]. After 2 h exposure to cathepsin G (1/500 w/w) in the presence of calcium ions, a slight degradation of the L-chain to a lower molecular weight form (lanes 6 and 7) was detectable. If calcium ions were omitted, the cleavage of the L-chain was completed within 5 min (lane 8). With a 10-fold increased ratio of cathepsin G to rFVIIa (1/50 w/w) an additional cleavage of the H-chain could be demonstrated after 60 min. This H-chain cleavage was completed within 120 min (data not shown). The reduction in molecular weight corresponded to removal of the Gla-domain, and the FVII clotting activity was lost accordingly (Table I).

3.2. *RP-HPCL and N-terminal amino acid sequencing*

The products of cathepsin G-mediated cleavage of rFVIIa were isolated by RP-HPCL (Fig. 2). Amino acid analysis of the minor peaks recovered prior to the cathepsin G peak (Rt 22 min), indicated that the Gla-domain had been further degraded, and SDS-PAGE showed that the peaks, located in between the cathepsin G peak and the main protein peak (Rt 27 min) corresponded to H-chain degraded forms.

N-Terminal amino acid sequencing of the protein from the main peak showed two N-terminal sequences, one from the L-chain, starting at position Ser-45, and one from the H-chain, starting at Ile-153 (Table II), confirming that the Gla-domain was lost under the applied conditions.

3.3. *Isoelectric focusing*

The effect of the Gla-domain on the IEF pattern is shown in Fig. 3. Intact rFVIIa is precipitated when applied at the anodic site (lane 2). Smearing, observed after application at the cathodic site (lane 5), indicates that steady-state conditions had probably not been obtained. In contrast the Gla-domainless rFVIIa, applied

Table I

Percentage of FVII clotting activity after exposure to cathepsin G

Cathepsin G	$CaCl_2$	Incubation time (min)				
		5	10	20	60	120
0	+	ND	ND	100%	ND	97%
0	0	ND	ND	100%	ND	95%
+	+	ND	ND	72%	ND	77%
+	0	1.5%	0.6%	0%	0%	0%

The data were obtained from the experiment described in Fig. 1. Aliquots diluted in Ca^{2+}-containing buffer were stored frozen until the time for analysis. FVII clotting activity is given as a percentage of the initial specific activity (26 U/μg rFVIIa). ND, not determined.

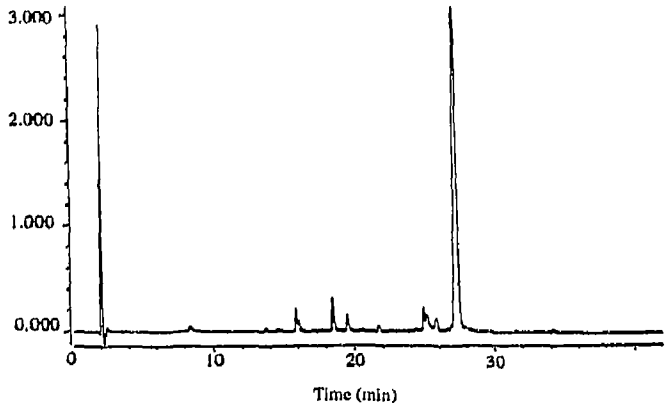

Fig. 2. RP-HPLC of cathepsin G-degraded rFVIIa 0.5 mg of rFVIIa, incubated for 20 min at 37°C with cathepsin G (1/50 w/w), were applied. Fractions absorbing at 214 nm were collected and concentrated by evaporation.

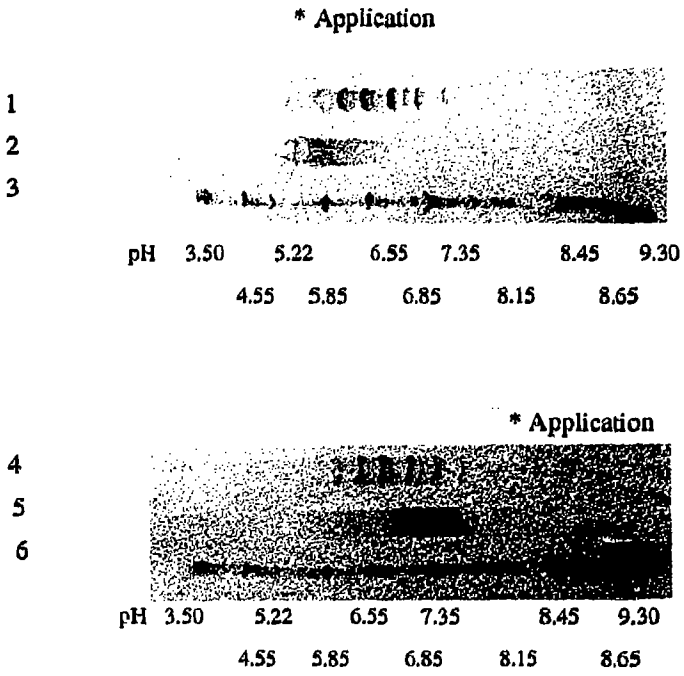

Fig. 3. IEF of rFVIIa and GD-rFVIIa. Intact rFVIIa (lanes 2 and 5) and cathepsin G-treated rFVIIa (lanes 1 and 4) were applied anodic (lanes 1 and 2) and cathodic (lanes 4 and 5). Marker proteins were applied in lanes 3 and 6.

3 cm from either electrode, resulted in an identical IEF pattern, consisting of eight sharply focused bands, with pI values ranging from 5.8 to 7.8 (lanes 1 and 4).

4. DISCUSSION

Cathepsin G is a serine protease from human granulocytes, involved in connective tissue degradation. The enzyme cleaves predominantly after Phe, Ala or Leu, with a substantial preference for Phe over the other two aromatic amino acids, Tyr and Trp [11].

Cathepsin G cleaves human FX and protein C between Phe-40 and Trp-41 [5,6]. This sequence is also present in FVII (Fig. 4). The FVIIa might be cleaved in this position too, however, under the conditions used, the main product contained only the Ser-45 N-terminal from the FVII L-chain, favouring cleavage after Tyr-44.

The homologous position in human protein C contains a His, in contrast to the other vitamin K-dependent coagulation enzymes (Fig. 4). Human FX has a lysine residue, adjacent to Tyr-44, which might lower the affinity for the cathepsin G [11].

Other methods for producing Gla-domainless forms of FVIIa have been tried. In our hands chymotrypsin cleavage seems to introduce a more extended proteolysis (results not shown), as also observed during chymotrypsin cleavage of bovine FX [12]. The incubation of rFVIIa with EDTA for 48 h results in a Gla-domainless form which lacks the first 38 amino acids (des-1-38-rFVIIa) and without clotting activity [2]. Using a low enzyme to substrate ratio (1/500 w/w), the cathepsin G cleavage, reported here, gives a des-1-44-rFVIIa within 5 to 10 min, in the absence of calcium ions, with no significant H-chain cleavage during the 120 min observation period. H-chain cleavage requires a longer incubation time or an increased amount of cathepsin G.

Cathepsin G cleavage of FVIIa for preparing a Gla-domainless form is found to be a convenient method. It might be utilized for obtaining consistent IEF patterns, as well as for other structural and functional studies of coagulation factors.

The cathepsin G cleavage of human FX and protein C [5,6] and the chymotrypsin cleavage of bovine FX [12] was found to be inhibited by calcium ions.

Residues 1–48 of FVII are homologous with the comparable N-terminal sequences of factors II, IX and X and the proteins designated C, S and Z. Only one of these so called Gla-domains, present as part of the prothrombin fragment 1 of FII, has been crystallized and analysed by X-ray diffraction [13]. In the absence

Table II

Amino acid sequence analysis of cathepsin G-degraded RFVIIa

Cycle no.	Light chain (45–152)		Heavy chain	
	PTH-aa	Yield (pmol)	PTH-aa	Yield (pmol)
1	Ser	324	Ile	835
2	Asp	383	Val	904
3	Gly*	(633)	Gly*	(633)
4	Asp	400	Gly	688
5	Gln	563	Lys	682
6	Cys**	ND	Val	818
7	Ala	525	Cys**	ND

*In cycle no. 3 the same amino acid residue appears in both light (45–152) and heavy chain. In this case the amount is equally divided.
**PTH-Cys is not determined.
ND, not determined.

Protein	Residue no.												Reference
	35		37			40	41			44	45		
FVII	γ	R	T	K	L	F	W	I	S	Y	S	D	G	[1]
Protein C	D	D	T	L	A	F	W	S	K	H	V	D	G	[14]
FX	γ	K	T	N	γ	F	W	N	K	Y	K	D	G	[15]
FIX	γ	K	T	T	γ	F	W	K	Q	Y	V	D	G	[16]
FII	T	A	T	D	V	F	W	A	K	Y	T	A	-	[17]

γ = γ-carboxylated glutamic acid residue.

Fig. 4. Homologous sequences of human vitamin K-dependent coagulation enzymes.

of calcium ions the Gla-domain of prothrombin is highly disordered. Addition of calcium ions induces a folding of the polypeptide and these cations interact with the γ-carboxy residues to provide a stable conformation of the domain [13]. This picture of the Gla-domain is easily compatible with the result obtained in this and previous studies. We suggest that the removal of the calcium ions at the same time induces an unfolding of the Gla-domain, thereby increasing the accessibility of proteolytic attack, and produces a high negative charge density, leading to an increased interaction between the Gla-domain and an area of high positive charge density known to be present near the substrate binding site of cathepsin G.

Degradation of vitamin K-dependent coagulation factors by granulocyte proteinases reported in this and in other studies [6,12] indicate an inhibitory role for neutrophils on coagulation. On the other hand, leukocyte elastase-mediated degradation of coagulation inhibitors, such as ATIII [18] and TFPI [19], as well as the cathepsin G-mediated degradation of protein C [5], are expected to have the opposite effect. Until the relative importance of the various leukocyte proteinase-mediated reactions have been quantified, it is not possible to evaluate the net result of neutrophil stimulation on blood coagulation. An important aspect in this context is the modulatory role of Ca^{2+} and glycosaminoglucans.

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