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Modifications of Extrinsic Pathway Inhibitor (EPI) and Factor Xa that Affect their Ability to Interact and to Inhibit Factor VIIa/Tissue Factor: Evidence for a Two-Step Model of Inhibition

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Key words

Extrinsic pathway inhibitor – Tissue factor – Factor VIIa – Factor Xa – γ-Carboxyglutamic acid

Summary

Inhibition of factor VIIa/tissue factor (TF) by extrinsic pathway inhibitor (EPI) requires the participation of factor Xa. Through this inhibition, factor Xa generated initially may feed back to suppress continuing generation of factor Xa via the extrinsic pathway during hemostasis. We have utilized chemical modifications of EPI and factor Xa to study the reactions responsible for inhibition. The data are consistent with a two-step model. First, EPI binds to factor Xa in a Ca^{2+} independent reaction in which the gla-domain of factor Xa does not participate. A functional active site on factor Xa and arginine residues on EPI are essential for this step. Then the factor Xa/EPI complex binds to factor VIIa/TF with resultant inhibition of its enzymatic activity. The gla-domain of factor Xa is essential for this step. Intact positively charged lysines on factor Xa may also be important.

Introduction

A factor VIIa/tissue factor (TF) enzyme-cofactor complex initiates the extrinsic pathway of blood coagulation through the activation of factor IX and factor X. In a purified system a low concentration of factor VIIa/TF will catalyze complete activation of factors IX and X. However, in a system containing plasma, activation of these substrates is turned off shortly after its initiation by a plasma inhibitor of factor VIIa/TF that requires factor Xa for its function (1–3). We termed this inhibitor extrinsic pathway inhibitor (EPI); Broze and colleagues, who purified the inhibitor from the supernatant of HEP G2 hepatoma cells (4), have recently called it lipoprotein-associated coagulation inhibitor or LACI (5).

An enzymatic role for factor Xa in the inhibitory reaction has not been demonstrated, yet active site-blocked factor Xa can not substitute for active factor Xa in the inhibitory reaction (2, 3). Broze et al. (5) have reported that EPI forms a complex with factor Xa in a reaction that does not require Ca^{2+}. They also found that bovine gamma carboxyglutamic acid (Gla) domainless factor Xa, factor Xa(-GD), formed a complex with the purified inhibitor. In a preliminary communication, we have reported similar findings of complex formation between human factor Xa (-GD) and EPI from plasma (6). We describe this obervation further herein together with evidence that arginine(s) in EPI is required for EPI to bind and neutralize factor Xa. The data provide further evidence for a two-step mechanism of EPI/factor Xa inhibition of coagulation initiated by factor VIIa/TF (5, 6).

Materials and Methods

Sodium (^{3}H) borohydride and Na^{125}I were obtained from Amersham (Arlington Heights, IL, USA). Chymotrypsin, treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone to inactivate contaminating trypsin, and citraconic anhydride were purchased from Sigma (St. Louis, MO, USA). The chromogenic substrate, S2222 (benzoyl-Ile-Glu-Arg-p-nitroanilide), was purchased from Kabi (Molndal, Sweden).

Human clotting factors VII, IX, and X, were purified, activated and radiolabeled as described earlier (1, 7–9). Human brain TF was either a crude preparation [12,000 × g, 15 min, supernate of a saline brain extract (1)] or an affinity purified reconstituted preparation (10).

EPI was partially purified from the lipoprotein fraction of plasma. Apolipoproteins were fractionated by gel filtration in urea on Sephacryl S-200 (Pharmacia, Piscataway, NJ, USA), followed by ion-exchange chromatography on Q-Sepharose (Pharmacia) and preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This procedure yields two fractions with EPI activity: fraction I, ~600–1,000 fold purified (M_r ~32,400); and fraction II, ~1,200–2,000 fold purified (M_r ~40,300). Details of the purification procedure have been described elsewhere (11). EPI activity was measured in an assay based upon the ability of a test sample to inhibit factor VIIa/TF catalyzed release of activation peptide from ^{3}H-factor IX in the presence but not in the absence of factor Xa (11). Normal pooled plasma was arbitrarily assigned a value of one EPI unit/ml and was assumed to contain ~100 ng EPI/ml as per Broze (4). In one experiment barium adsorbed plasma, prepared as described earlier (1), was used as a source of plasma EPI.

The gla-containing domain of factor Xa was removed by limited chymotryptic digestion (12) utilizing 3.3 mg of chymotrypsin coupled per ml of Affi-Gel 15 beads (Bio-Rad, Richmond, CA, USA). Factor Xa (~1.1 mg) was incubated at 37° C with 300 µl of beads in 10 mM Hepes [4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid] buffer, 0.15 M NaCl, pH 7.5 and formation of gla-domainless factor Xa, factor Xa(-GD), was monitored by the loss of clotting activity. The reaction was terminated by removal of insolubilized chymotrypsin when <0.3% clotting activity remained, at which time factor Xa(-GD) esterase activity with the chromogenic substrate S2222 was similar to that of a control preparation incubated with buffer. SDS-PAGE of the reduced protein confirmed complete removal of the gal-containing domain from the light chain of factor Xa(-GD) without evidence of degradation of the heavy chain (Fig. 1, lane 2).

Abbreviations:

Factor Xa(-GD): factor Xa treated with chymotrypsin to remove the gla-containing domain; citraconylated factor Xa: zymogen factor X treated with citraconic anhydride to modify lysine residues prior to activation with Russell's viper venom; arginine modified EPI: EPI treated with cyclohexanedione to modify arginine residues; active site-blocked factor Xa: factor Xa irreversibly inactivated with the histidine-directed serine protease inhibitor, dansyl-Glu-Gly-Arg-chloromethyl ketone (Calbiochem, La Jolla, CA, USA).

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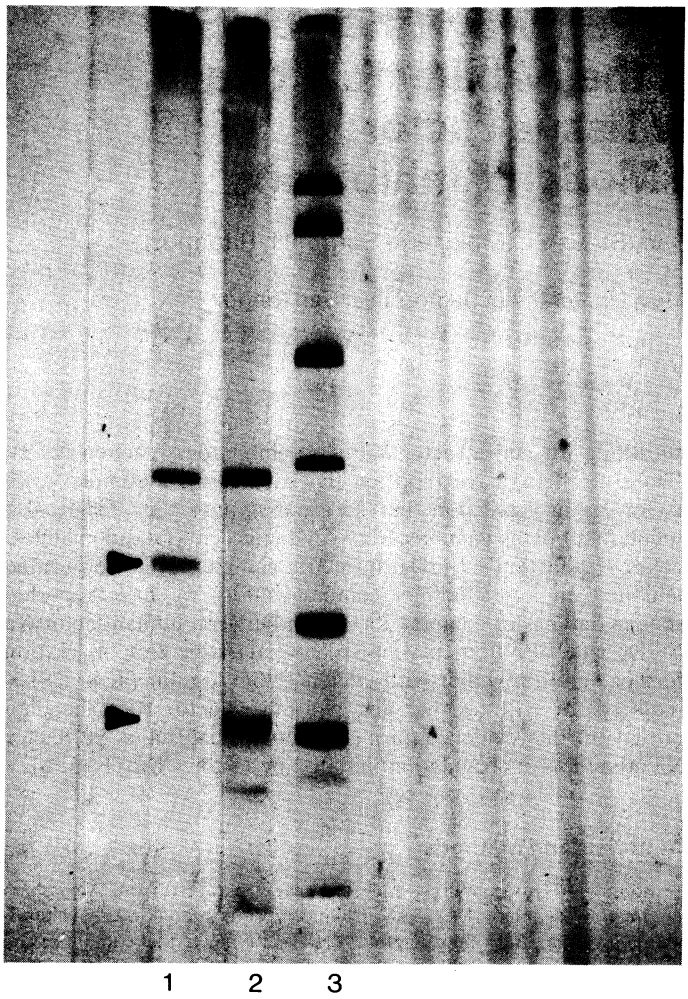

Fig. 1 SDS-PAGE of factor Xa and factor Xa(-GD). Factor Xa and Xa (-GD) were subjected to SDS-PAGE under reducing conditions. Lane 1, factor Xa; lane 2, factor Xa(-GD); lane 3, low molecular weight standards (from top to bottom; M_r = 97,400; 66,200; 42,700; 31,000; 21,500; and 14,400). Arrows indicate the position of the light chains of factor Xa and factor Xa(-GD)

The positive charge on lysine residues of factor X was reversed by limited reaction with citraconic anhydride. Conditions were: 21-fold molar excess of citraconic anhydride to lysine residues in factor X, 0.025 M citric acid buffer, pH 7.5, 37° C. The reaction was quenched with 0.1 M glycine as soon as all clotting activity was lost. The modified protein was fully activated by Russell's viper venom (RVV), yielding a citraconylated factor Xa with esterase activity similar to that of unmodified factor Xa.

The positive charge on arginine residues of EPI was selectively blocked by reaction with 1,2 cyclohexanedione (2 hr at 37° C) as described (13). Hydroxylamine was used to remove cyclohexanedione and restore the charge (13).

For binding studies EPI preparations were subjected to SDS-PAGE according to Laemmli (14) on 10% polyacrylamide slab gels and transblotted onto nitrocellulose. Nitrocellulose strips were blocked with 5% nonfat milk and then incubated overnight at 4° C with 8 ml of TBS (4 mg/ml BSA) and 0.2–0.8 μg of ^{125}I-labeled ligand (~1–6 × 10^9 CPM/mg) or 3 μg of unlabeled ligand followed by incubation with 12.5 μg/ml of rabbit anti-human factor X IgG and 0.2 μg/ml ^{125}I-protein A (~1 × 10^{10} CPM/mg). Autoradiographic exposure was carried out for 1–2 days with Kodak X-Omat film. Apparent molecular weights for EPI (fractions I and II) were determined from R_f value of the ^{125}I-factor Xa-binding protein bands on radioautographs relative to the R_f values of low molecular weight stained protein standards (Bio-Rad) subjected to SDS-PAGE on the same gel.

Results

As shown in Fig. 2, a preparation of the higher molecular weight form of plasma EPI (fraction II) neutralized the esterolytic activity of factor Xa and of factor Xa(-GD) at essentially equivalent rates. The same results were obtained with EPI preparations containing only the lower molecular weight form (fraction I) or with preparations containing both molecular weight forms (data not shown). EPI also neutralized the esterolytic activity of citraconylated factor Xa, but less completely than it neutralized unmodified factor Xa (Fig. 2).

Factor Xa(-GD) and, to a lesser extent, citraconylated factor Xa bound to both molecular weight forms of EPI immobilized on nitrocellulose (Fig. 3, lanes 3 and 4). In contrast, unactivated factor X and active site-blocked factor Xa did not bind to immobilized EPI (Fig. 3, lanes 1 and 6). Factor IXa and thrombin also failed to bind to immobilized EPI (data not shown).

Despite their ability to bind to EPI, neither factor Xa(-GD) nor citraconylated factor Xa supported EPI-mediated inhibition of factor VIIa/TF enzymatic activity (Fig. 4). Moreover, when an EPI preparation was first incubated with an ~3-fold molar excess of factor Xa(-GD), the subsequent addition of an ~5-fold molar excess of unmodified factor Xa failed to support inhibition of factor VIIa/TF (Fig. 5).

An EPI preparation was reacted with cyclohexanedione to block positively charged arginine residues. Arginine-modified EPI failed to neutralize factor Xa esterase activity and could not function as an inhibitor of factor VIIa/TF in the presence of factor Xa (Table 1). Removal of cyclohexanedione by incubation with hydroxylamine restored EPI's ability to neutralize factor Xa and to inhibit factor VIIa/TF to levels comparable to those found in a control experiment that differed only in that the cyclohexanedione was omitted from the reaction buffer (Table 1).

Table 1 Effect of arginine modification on functional activities of EPI

Reaction of EPI with		Percent neutralization[1] of	
Cyclohexanedione[2]	Hydroxylamine[2]	Factor Xa[3]	Factor VIIa/TF[4]
−	−	100	100
+	−	<6	11
+	+	42	42
−	+	26	40

[1] Relative to control.
[2] When a reactant was omitted, it was replaced with its reaction buffer.
[3] Measured as esterolytic activity with S2222.
[4] Measured in the ^{3}H-factor IX activation peptide release assay (see Materials and Methods).

In a further experiment, an EPI preparation containing both molecular weight forms of plasma EPI was subjected to SDS-PAGE and blotted onto nitrocellulose. The immobilized EPI was treated with cyclohexanedione to modify arginine residues and overlaid with ^{125}I-factor Xa to determine the effect of arginine modification upon factor Xa binding (Fig. 6, lane 4). Controls included both untreated EPI (Fig. 6, lane 5) and treated EPI after reversal of cyclohexanedione modification with hydroxylamine (Fig. 6, lane 3). As Fig. 6 shows, modification of the arginine residues of EPI markedly impaired its ability to bind factor Xa. This was true for both molecular weight forms of plasma EPI.

Discussion

The data reported herein confirm and extend the report of Broze et al. (5) that EPI binds to and neutralizes the catalytic

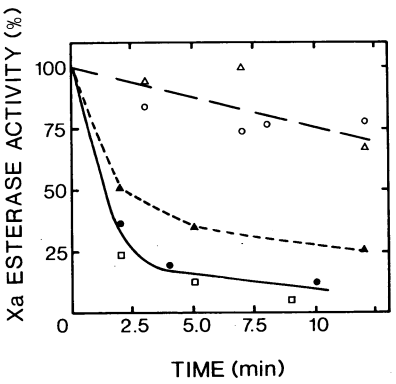

Fig. 2

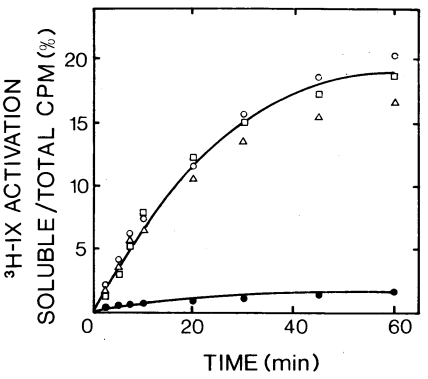

Fig. 4

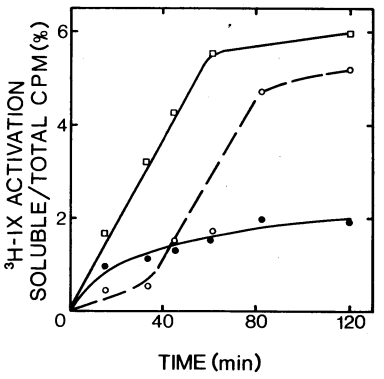

Fig. 5

Fig. 2 EPI neutralizes the esterase activities of factor Xa, factor Xa (-GD), and citraconylated factor Xa. Factor Xa, factor Xa(-GD), or citraconylated factor Xa was incubated at room temperature in buffer with ~ equimolar EPI (fraction II, M_r ~40,300) or with 0.5 mg/ml BSA (control). Aliquots were removed from these mixtures at the times indicated to measure esterolytic activity with S2222. Symbols are as follows: (●), 475 ng of factor Xa with 3.5 U of EPI (~350 ng); (□), 475 ng of factor Xa(-GD) with 3.5 U of EPI (~350 ng); (▲), 408 ng of citraconylated factor Xa with 2.9 U of EPI (~290 ng); (○), 475 ng of factor Xa with BSA; (△), 408 ng of citraconylated factor Xa with BSA. Note that a single dashed line has been drawn to fit the control incubations of both factor Xa (○) and citraconylated factor Xa (△) with BSA. Note also that a single solid line has been drawn to fit the incubations of factor Xa (●) and factor Xa(-GD) (□) with EPI

Fig. 4 Failure of factor Xa(-GD) and citraconylated factor Xa to support EPI-mediated inhibition of factor VIIa/TF. Release of activation peptide from ^{3}H-factor IX (5 μg/ml) was followed as a function of time in reaction mixtures containing: factor VII (0.1 μg/ml), TF (7.3 ng/ml purified reconstituted TF), Ca^{2+} (10 mM), and barium adsorbed plasma as a source of EPI (67%, v/v). Other additions were: (○), no factor XA; (●), factor Xa; (□), factor Xa(-GD); (△), citraconylated factor Xa. Modified factor Xa preparations were added at concentrations yielding esterase activity equivalent to factor Xa at 0.66 μg/ml. Full activation of ^{3}H-factor IX corresponds to ~35% soluble/total CPM

Fig. 5 Incubation of EPI with factor Xa(-GD) prevents EPI-mediated inhibition of factor VIIa/TF when unmodified factor Xa is subsequently added. Q-Sepharose EPI (11), 0.07 U (~7 ng), was incubated at room temperature for 30 min with: (●), 31 ng of factor Xa; (□), 31 ng of factor Xa(-GD); or (○), control buffer. Factor Xa (52 ng) was then added to the factor Xa(-GD) mixture and factor Xa (21 ng) was added to the factor Xa mixture (resulting in 52 ng factor Xa in both experimental mixtures). ^{3}H-factor IX (5 μg/ml), factor VII (0.1 μg/ml), TF (0.75% v/v, crude TF), and Ca^{2+} (5 mM) were added to all reaction mixtures and activation of ^{3}H-factor IX was monitored by release of the tritiated activation peptide. The initial lag in the progress curve of activation of factor IX in the control mixture (○) reflects the slow back activation of factor VII by factor IXa which, as described in detail elsewhere (15), is bypassed in the experimental mixtures by rapid factor Xa catalyzed activation of factor VII

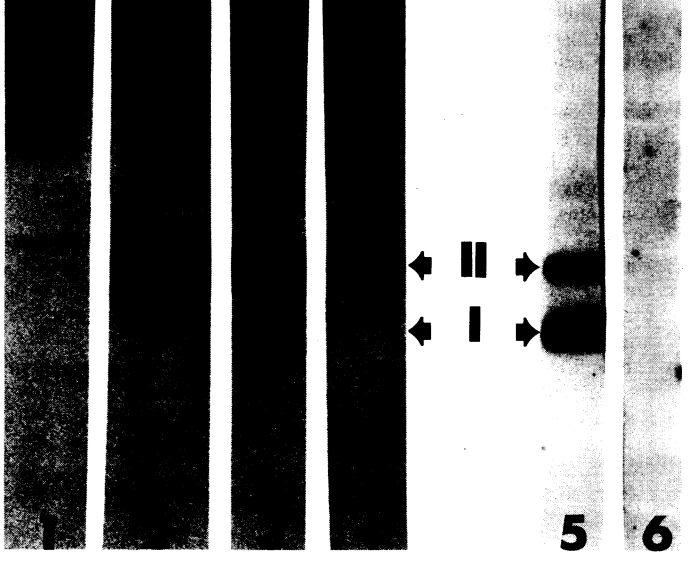

Fig. 3 Factor Xa, factor Xa(-GD) and citraconylated factor Xa bind to EPI immobilized on nitrocellulose. Q-Sepharose preparations of EPI (11) were subjected to SDS-PAGE, transblotted onto nitrocellulose, incubated with ligands overnight at 4° C as described, and subjected to radioautography (2 days at −70° C). Lanes 1–4 (~50 ng EPI containing primarily fraction II) were incubated with 3 μg ligand and subsequently developed with rabbit anti-human factor X IgG followed by ^{125}I-protein A (see Materials and Methods). Lane 1, factor X; lane 2, factor Xa; lane 3, factor Xa(-GD); lane 4, citraconylated factor Xa. Lanes 5–6 (~7 ng EPI containing both molecular weight fractions) were incubated with 0.8 μg of ^{125}I factor Xa (lane 5) or ^{125}I-active site-blocked factor Xa (lane 6). Arrows indicate positions of EPI fractions I and II

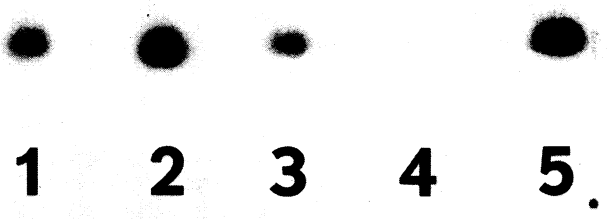

Fig. 6 Requirement for intact positively charged arginine(s) on EPI to bind ^{125}I-factor Xa. A polypreparative SDS-PAGE fraction of EPI (containing both molecular weight forms, 0.3 U, ~13 μg protein) was subjected to SDS-PAGE and blotted onto nitrocellulose. Nitrocellulose strips were treated as follows: lane 1, treated with buffer/reversed with hydroxylamine; lane 2, treated with buffer; lane 3, treated with cyclohexanedione/reversed with hydroxylamine; lane 4 treated with cyclohexanedione; lane 5, untreated. Then the nitrocellulose strips were blocked with nonfat milk and incubated with 0.24 μg/ml ^{125}I-factor Xa at 4° C overnight. Radioautographs were developed at −20° C for 16 hours

activity of factor Xa in a Ca^{2+} independent reaction that requires the presence of a functional factor Xa active site. Broze and colleagues also reported that EPI neutralized bovine factor Xa (-GD) but at a 7-fold slower rate than observed with unmodified bovine factor Xa. They suggested that the gla-domain of factor Xa, although not essential for factor Xa's interaction with EPI, facilitated optimal binding of EPI to factor Xa. The data reported herein show that EPI isolated from plasma can neutralize human factor Xa and human factor Xa(-GD) esterolytic activity at equivalent rates. We believe, therefore, that the gla-domain of human factor Xa plays no physiologically significant role in the ability of EPI to interact with human factor Xa.

Although factor Xa(-GD) bound readily to EPI, an EPI/factor Xa(-GD) complex could not inhibit factor VIIa/TF catalyzed activation of factor IX (Fig. 4). Moreover, adding factor Xa to a reaction mixture containing EPI/factor Xa(-GD) failed to restore the ability of EPI to inhibit factor VIIa/TF activity (Fig. 5). These findings are in agreement with the observations of Broze et al. (5). It would appear that the gla domain of factor Xa is required only for a subsequent binding of EPI/factor Xa to factor VIIa/TF that then inhibits the TF pathway of coagulation (6).

Earlier work had established that active site-blocked factor Xa could not support EPI-mediated inhibition of factor VIIa/TF (2, 3). However, the reason was not clear, since an enzymatic role for factor Xa in the inhibitory reaction could not be identified (2). Broze et al. (5) provided the first evidence that factor Xa's active site needed for factor Xa to bind to EPI. The present data, which demonstrate that neither unactivated factor X nor active site-blocked factor Xa could bind to immobilized EPI (Fig. 3), confirm that factor Xa's active site is essential for factor Xa to bind to EPI. Moreover, our finding that modification of arginine residues on EPI prevents the binding of factor Xa fits with this conclusion. Factor Xa is known to recognize and cleave arginyl peptide bonds in its biological substrates. In analogy to a number of other well characterized serine proteinase inhibitors (16), an arginyl peptide bond in EPI could serve as a reactive site peptide bond (scissile bond) that is cleaved when EPI neutralizes its cognate enzyme, factor Xa. The very recent report that EPI contains three tandem Kunitz-type inhibitory domains (17) supports this mechanism of interaction of EPI and factor Xa.

When the positive charge on lysine residues of factor Xa was reversed by reaction with citraconic anhydride EPI could still partially bind and neutralize factor Xa (Figs. 2 and 3). However, the resultant EPI/factor Xa complex could not inhibit factor VIIa/TF (Fig. 4). This provides further evidence that factor Xa participates at two steps in the process of EPI-mediated inhibition of factor VIIa/TF.

As discussed elsewhere (1, 2, 18), EPI-mediated inhibition of factor VIIa/TF enzymatic activity could well function as a major regulator of blood coagulation via the TF pathway during hemostasis. As factor VIIa/TF generates factor Xa, the factor Xa could bring EPI into play to suppress continuing generation of factor Xa. A two step model best fits the data on the mechanism of the inhibition. In the first step, factor Xa binds to EPI. This binding is a prerequisite for the second step, in which an EPI/factor Xa complex then binds to and inhibits factor VIIa/TF. The initial binding of factor Xa to EPI requires the active site of factor Xa and the participation of arginine residues on EPI and results in loss of the catalytic activity of the bound factor Xa. This step is independent of Ca^{2+} and can proceed in the absence of the gla-domain of factor Xa. The second step, in which a putative Ca^{2+} dependent EPI/factor Xa/factor VIIa/TF complex is formed, requires the gla-domain of factor Xa. It also appears to require unmodified lysine residues on factor Xa.

Acknowledgements

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