Chemical Footprinting Reveals Conformational Changes Following Activation of Factor XI

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Abstract

Coagulation factor XI is activated by thrombin or factor XIIa resulting in a conformational change that converts the catalytic domain into its active form and exposing exosites for factor IX on the apple domains. Although crystal structures of the zymogen factor XI and the catalytic domain of the protease are available, the structure of the apple domains and hence the interactions with the catalytic domain in factor XIa are unknown. We now used chemical footprinting to identify lysine residue containing regions that undergo a conformational change following activation of factor XI. To this end, we employed tandem mass tag in conjunction with mass spectrometry. Fifty-two unique peptides were identified, covering 37 of the 41 lysine residues present in factor XI. Two identified lysine residues that showed altered flexibility upon activation were mutated to study their contribution in factor XI stability or enzymatic activity. Lys357, part of the connecting loop between A4 and the catalytic domain, was more reactive in factor XIa but mutation of this lysine residue did not impact on factor XIa activity. Lys516 and its possible interactor Glu380 are located in the catalytic domain and are covered by the activation loop of factor XIa. Mutating Glu380 enhanced Arg369 cleavage and thrombin generation in plasma. In conclusion, we have identified novel regions that undergo a conformational change following activation. This information improves knowledge about factor XI and will contribute to development of novel inhibitors or activators for this coagulation protein.

Keywords
- factor XI
- conformational change
- chemical footprinting
- mass spectrometry

Introduction

The blood coagulation zymogen factor XI is a central player in the intrinsic coagulation pathway and contributes to haemostasis via activation of factor IX. Factor XI, a member of the serine protease family, is a 160-kDa homodimer that is cleaved at the Arg369-Ile370 bond (based on amino acid numbering of circulating factor XI; Glu1-Val607) by the physiological activators thrombin,[1–5] activated factor XIIa[6] or factor XIa (autoactivation).[3,4,7] The heavy chain and catalytic domain of factor XI(a) are linked through a disulphide bond between Cys362 and Cys482.[8] Activation results in a conformational change converting the catalytic domain into its active form and exposing exosites for its major substrate factor IX on the heavy chain.[9] Substrate recognition by the factor XIa exosites requires disulphide linking of the heavy chain and catalytic domain suggesting that the exosites for factor IX are present on both parts of factor XIa.[10]

The heavy chain of factor XI is composed of four apple domains (A1–A4) of 90 to 91 amino acids[11] with each apple

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domain containing three characteristic disulphide bonds.[8] The four apple domains form a unique disk-shaped platform around the base of the catalytic domain.[12] Although crystal structures for full-length zymogen factor XI[12] and the isolated catalytic domain from factor XIa[13–15] are available, the crystal structure of the apple domains in the active conformation is not known. Superposition of a factor XIa protease domain crystal structure on the crystal structure of the zymogen revealed that the activation loop (residues 370–376) folds into the activation pocket,[12] which is a common feature of serine proteases. Based on structural analysis of full-length factor XI(a) or its individual domains,[12–16] it is speculated that activation of the zymogen results in rearrangement of the apple domains and catalytic domain. This hypothesis is supported by biochemical data indicating the differences in binding properties between factor XI and factor XIa.[9,17,18]

Using lysine-directed chemical footprinting combined with mass spectrometry (MS), we identified novel regions in full-length factor XI that undergo conformational changes following activation. The lysine residues with the largest change in surface exposure were evaluated for their role in activation and activity of factor XI(a) in purified and plasma systems.

Methods

Materials

The cDNA for codon optimized human factor XI (FXI-WT), containing Cys11Ser to prevent possible intermolecular disulphide bridge formation, was obtained from BaseClear (Leiden, The Netherlands). QuickChange was from Agilent (Santa Clara, California, United States). HEK293 cells were from ATCC (Manassas, Virginia, United States). Cell culture media used were DMEM/F12 (Lonza, Basel, Switzerland) and Opti-MEM I reduced serum medium with Glutamax supplement (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, United States). DMRIE-C transfection reagent and Cell Factories and TripleFlasks were obtained from Thermo Fisher Scientific. Geneticin (G-418 Sulfate) was from Calbiochem (EMD Millipore Corp, Billerica, Massachusetts, United States). Peptide-IV (H-SDDDWIPDIQTDPNGLSFNPISDFPDTTSPK-OH)[19] was provided by Thermo Fisher Scientific and coupled to CNBr-Activated Sepharose 4 Fast Flow (GE Healthcare Life Sciences). A Fresenius Polysulfone low-flux dialyser F5HPS (Fresenius Medical Care, Bad Homburg von der Höhe, Germany) was used as an artificial kidney. Diisopropyl fluorophosphate (DFP) and polyethylene glycol (PEG) 6000 were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Factor XIIa was obtained from Enzyme Research Laboratories (South Bend, Indiana, United States) and thrombin was a generous gift from Dr. W. Kisiel (University of New Mexico, School of Medicine, Albuquerque, New Mexico, United States). Calcium-saturated polyphosphate with an average chain length of 100 monomers (polyP) was a generous gift of Dr. T. Renné (Karolinska Instituted, Stockholm, Sweden). All materials for MS were obtained from Thermo Fisher Scientific, unless mentioned otherwise. For SDS-PAGE NuPAGE Novex 4 to 12% Bis-Tris protein gels and Imperial protein stain were used (Thermo Fisher Scientific). Factor XI depleted plasma was purchased from Siemens Healthcare Diagnostics (Marburg, Germany) and the fluorogenic substrate Z-Gly-Gly-Arg-AMC was provided by Bachem (Bubendorf, Switzerland). Human plasma-derived C1 esterase inhibitor (C1-INH) was obtained from Sanquin (Amsterdam, The Netherlands).

Cloning, Expression and Purification of Recombinant Factor XI and Variants

The substitutions K357A, K516A, K516E, E380A, E380K or E380K/K516E were introduced into the FXI-WT construct by site-directed mutagenesis (QuickChange). Mutagenesis primers are available on request. Mutations were confirmed by sequence analysis. HEK293 cells stably expressing FXI-WT or variants were generated using DMRIE-C as a transfection reagent according to manufacturer's protocol and thereafter subjected to selective medium containing 1 mg/mL geneticin. Positive clones were propagated and tested for expression of factor XI in medium by ELISA.[20] For expression, cells were expanded and grown in a Cell Factory (6,320 cm^2) or TripleFlasks (500 cm^2) containing medium with 5% FCS. At confluence, serum-free medium (Opti-MEM) was added and collected every 48 to 72 hours.

To purify secreted recombinant factor XI, collected medium was concentrated over an artificial kidney if needed and applied to a peptide-IV affinity column.[20] Recombinant factor XI was eluted with 0.1 M citrate, pH 5.0, 1 M NaCl and 10 mM EDTA. Contamination of the eluate with factor XIa was checked with the chromogenic substrate S2366. To avoid autoactivation, the eluate was treated with the serine protease inhibitor DFP until chromogenic activity was inhibited. Purified protein was dialysed against 10 mM HEPES, pH 7.5, containing 0.5 M NaCl and stored at −80°. Concentration of recombinant protein was determined by measuring the absorbance at 280 nm using the extinction coefficient for factor XI (13.4).

Chemical Modification and MS Analysis

Recombinant factor XIa was obtained by activation of FXI-WT with factor XIIa (molar ratio 20:1), full Arg369 cleavage was confirmed by SDS-PAGE analysis and activity was checked with the chromogenic substrate S2366. FXIa-WT was purified over the peptide-IV column as described earlier. Tandem mass tag (TMT) labeling and MS analysis were performed as described previously.[21] FXI-WT and FXIa-WT were labelled for 15 minutes at 37°C with TMT-126 or TMT-127, respectively. Collision-induced dissociation (CID) spectra and higher energy collision-induced dissociation (HCD) spectra were acquired as described previously.[21,22] The identity of the peptides, including TMT labelled lysine residues, and the TMT-127/TMT-126 ratio thereof were determined using proteome discoverer 1.2 (Thermo Fisher Scientific). The protein database 25.H_sapiens.fasta was used for peptide identification. Search criteria allowed a peptide parent mass error tolerance of 30 ppm; a fragment mass error tolerance of 0.8 Da and modifications including cysteine carbamidomethylation (57.02 Da), methionine oxidation (15.99 Da) and lysine-TMT labeling (225.16 Da).

The following selection criteria were used: (1) high confidence of CID and/or HCD spectra, (2) all lysine residues are modified by a TMT label, (3) all cysteine residues are alkylated and (4) all methionine residues may be oxidized. The TMT ratio of the identified peptides was normalized against the median TMT ratio obtained within the respective experiment.

Factor XI Activation

Recombinant factor XI was incubated with thrombin (molar ratio 10:1) or factor XIIa (molar ratio 20:1) in 25 mM HEPES, pH 8.0, 127 mM NaCl, 3.5 mM KCl and 3 mM $CaCl_2$ (activation buffer) for 72 hours at 37°C. In the presence of polyP (30 µg/mL), factor XI was incubated with thrombin at a molar ratio of 1,000:1 in activation buffer supplemented with 0.1% PEG 6000 and samples were taken every 10 minutes up to 60 minutes. Factor XIa activity, in the presence of hirudin (2 µg/mL) or corn trypsin inhibitor (25 µg/mL) to inhibit thrombin or factor XIIa, respectively, was determined kinetically after addition of the chromogenic substrate S2366 for 10 minutes at 405 nm at 37°C in a SpectraMax 384 microplate reader (Molecular Devices). In addition, in some samples, the functional activity of factor XIa was determined with the ROX factor XIa assay (Rossix, Mölndal, Sweden) according to the manufacturer's instructions with the factor XIa calibrator (Rossix) as a standard. In this functional activity assay, factor XIa activates factor IX followed by activation of factor X in the presence of factor VIII, calcium ions and phospholipids. The amount of factor Xa is determined by a chromogenic substrate and is related to the factor XIa activity of the sample. Arg369-cleavage of factor XI was determined by reduced SDS-PAGE followed by a Coomassie stain (Imperial protein stain) according to manufacturer's instructions. Stained protein gels were scanned and the band intensity of factor XI (80 kDa) and heavy and light chain of factor XIa (50 + 30 kDa, respectively) was determined by ImageJ.[23] The percentage of Arg369 cleavage was calculated as follows: (band intensity of 50 + 30 kDa)/(band intensity of 80 + 50 + 30 kDa) × 100%.

FXIa-WT and FXIa-E380A were obtained by activating FXI-WT and FXI-E380A with thrombin in the presence of polyP as described earlier. Inhibition of activity by C1-INH was followed by incubating 20 nM FXIa for 0 to 120 minutes with 1.5 U/mL C1-INH after which activity was determined kinetically as described earlier. The activity of FXIa-WT or FXIa-E380A at 0 minute (in the absence of C1-INH) was set at 100% and remaining activity was calculated for additional time points. To determine the half-life, a nonlinear regression curve was fitted through the data points.

Plasma Clotting Assays

Thrombin generation in factor XI depleted plasma was determined with Calibrated Automated Thrombography (Thrombinoscope BV, Maastricht, The Netherlands) as previously described.[24] Thrombin generation was assessed in the presence of phospholipids (4 µM) with or without addition of tissue factor (TF; 0.5 pM) or factor XIIa (1.2 µg/mL). Briefly, 80 µL plasma was added to 20 µL of trigger reagent and incubated for 10 minutes at 37°C. Thrombin generation was initiated by adding 20 µL HEPES/BSA buffer containing 100 mM $CaCl_2$ and 2.5 mM fluorogenic substrate Z-Gly-Gly-Arg-AMC. Fluorescence was determined in a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific) and thrombin generation curves were calculated with Thrombinoscope software.

Clotting activity of recombinant factor XI was assessed in factor XI depleted plasma on an automated coagulation analyser (Behring Coagulation System, Siemens Healthcare Diagnostics) with APTT reagents and protocols from the manufacturer. Factor XI activity levels were corrected for concentration, assessed by A280, and FXI-WT activity levels were set at 100%.

Data Analysis

Results are expressed as mean ± standard error of the mean, unless stated otherwise. For comparison, the Kruskal–Wallis' test was used as a pretest followed by Mann–Whitney *U* test where appropriate. All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, California, United States). Values of $p < 0.05$ were considered statistically significant.

Results

Identification of Conformational Changes following Cleavage of Factor XI

TMT footprinting-based MS analysis was performed to identify lysine residues that have an altered reactivity in factor XI following Arg369 cleavage. With our TMT-MS/MS approach, we identified 51 unique peptides covering 35 out of the 41 lysine residues present in factor XI(a) (►**Table 1**). In ►**Fig. 1**, the TMT-127/TMT-126 ratio for each identified lysine residue is depicted. An enhanced TMT-127/TMT-126 indicates that the specific lysine residue is more prone to chemical modification in factor XIa, while a reduced ratio suggests that it is less reactive in the active conformation. The results reveal that most of the identified peptides in the apple domains exhibit a TMT-127/TMT-126 ratio ≥ 1. In addition, the A2 domain contains two lysine residues (Lys103 and Lys165) with a reduced TMT-127/TMT-126 ratio. Half of the identified peptides were present in the catalytic domain. It is interesting to note that the first ∼140 amino acids of the catalytic domain have a TMT-127/TMT-126 ratio around 1, after which a region of ∼50 amino acids shows a reduced TMT-127/TMT-126 ratio.

We have chosen to study the lysine residue within the peptide with the highest TMT-127/TMT-126 ratio, that is, Lys357 with a ratio of 2.5 ± 0.2, in more detail by mutating this lysine into an alanine. Lys357 is positioned at the interface between A4 and the catalytic domain in factor XI and in between the disulphide bridges Cys273/Cys356, within the A4 domain and Cys362/Cys482, connecting the heavy and light chains (►**Fig. 2A**). Arg369 cleavage of factor XI results in a conformational change which might include lifting of the protease domain from the apple domains thereby exposing residues present at the interface between A4 (starting from Cys356) and the Cys362.[12]

The peptide with the lowest TMT-127/TMT-126 ratio (0.5) contains the three lysine residues 516, 518, and 529. Based

Table 1 TMT-labelled factor XI(a) peptides identified by mass spectrometry

Identified peptide	Modified lysine residue	Identified peptide	Modified lysine residue
TPSA**k**Y	26	TLRLc**k**M	357
TPSA**k**YcQVVcTY	26	TAAHcFYGVESP**k**IL	422
SF**k**QcSHQISAcN**k**DIY	83 + 94	YGVESP**k**IL	422
VDLDM**k**GINY	103	YGVESP**k**ILRVY	422
NSSVA**k**SAQEcQERcTDDVHcHFF	113	GVESP**k**IL	422
D**k**VVSGF	165	GVESP**k**ILRVY	422
SL**k**ScAL	173	SGILNQSEI**k**EDTSF	437
SL**k**ScALSNL	173	SGILNQSEI**k**EDTSFF	437
FSQEWP**k**ESQRNL	230	GVQEIIIHDQY**k**mAESGY	455
SQEWP**k**ESQRNL	230	**k**mAESGY	455
SQEWP**k**ESQRNLcL	230	**k**MAESGYDIAL	455
P**k**ESQRNL	230	**k**LETTVNY	467
L**k**TSESGLPSTRI**kk**S**k**AL	240 + 252 + 253 + 255	TDSQRPIcLPS**k**GDRNVIY	486
kTSESGLPSTRI**kk**S**k**AL	240 + 252 + 253 + 255	TDSQRPIcLPS**k**GDRNVIYTDcW	486
kTSESGLPSTRI**kk**S**k**ALSGF	240 + 252 + 253 + 255	R**k**LRD**k**IQNTL	505 + 509
LGEELDIVAA**k**SHEAcQ**k**L	294 + 301	RD**k**IQNTL	509
GEELDIVAA**k**SHEAcQ**k**L	294 + 301	Q**k**A**k**IPLVTNEEcQ**k**RY	516 + 518 + 529
DIVAA**k**SHEAcQ**k**L	294 + 301	VTNEEcQ**k**RY	529
FTYTPAQAScNEG**k**G**k**cY	325 + 327	RGH**k**ITH**k**m	535 + 539
TYTPAQAScNEG**k**G**k**cY	325 + 327	RGH**k**ITH**k**MIcAGY	535 + 539
TYTPAQAScNEG**k**G**k**cYL	325 + 327	IcAGYREGG**k**DAc**k**GDSGGPL	550 + 554
TPAQAScNEG**k**G**k**cY	325 + 327	REGG**k**DAc**k**GDSGGPL	550 + 554
TPAQAScNEG**k**G**k**cYL	325 + 327	Sc**k**HNEVW	564
L**k**LSSNGSPT**k**IL	331 + 340	VDWILE**k**TQAV	603
SSNGSPT**k**IL	340	ILE**k**TQAV	603
SSNGSPT**k**ILHGRGGISGY	340		

Abbreviation: TMT, tandem mass tag.
Note: In the identified peptide, the modified lysine residue is shown in bold. Lowercase letters indicate that this amino acid was modified with a TMT label (k), alkylated (c) or oxidated (m).

on crystal structure analysis, we observed that Lys516 forms a salt bridge with Glu380 present in close proximity to the factor XIa activation loop, while the other two lysine residues do not.[13,25] In factor XIa, the activation loop, at least partially, covers Lys516 possibly explaining the reduced accessibility of this lysine residue in the activated form compared with the zymogen (►**Fig. 2A, B**). Lys516, and its interactor Glu380, is conserved among species, while Lys518 and Lys529 are not (►**Fig. 2C**). In addition, the amino acids Glu380 and Lys516 (chymotrypsin numbering Glu26 and Lys157) are conserved in kallikrein, but not in other serine proteases (►**Fig. 2D**) suggesting that this salt bridge is unique for factor XI(a) and (pre)kallikrein.

Importance of Lys357, Glu380 and Lys516 in Activation of Factor XI by Thrombin or Factor XIIa

First, we studied the Arg369 cleavage of factor XI-WT and variants by thrombin or factor XIIa in a purified system. Without the addition of a surface, for example, polyP, cleavage of factor XI at the Arg369-Ile370 bond is an inefficient process. After 72 hours incubation, 25 ± 3% of factor XI-WT was cleaved by thrombin, whereas Arg369 cleavage by factor XIIa was 57 ± 4% (►**Fig. 3A, B**). Arg369 cleavage was improved in FXI-K516A and -E380K by incubation with thrombin, and FXI-E380A and -E380K/K516E by both thrombin and factor XIIa. Reduced Arg369 cleavage was observed in FXI-K516E and -K357A by thrombin or factor XIIa, respectively. In contrast to enhanced Arg369 cleavage, FXI-E380A, -E380K and E380K/K516E had reduced chromogenic activity after a 72-hour incubation with either thrombin or factor XIIa. Taking a closer look at the SDS-PAGE gels shows that these three mutants are cleaved almost completely after 72 hours of incubation as indicated by disappearance of the 80 kDa band. The 30 and 50 kDa bands, however, are faint and suggest that the cleaved form of these variants is degraded.

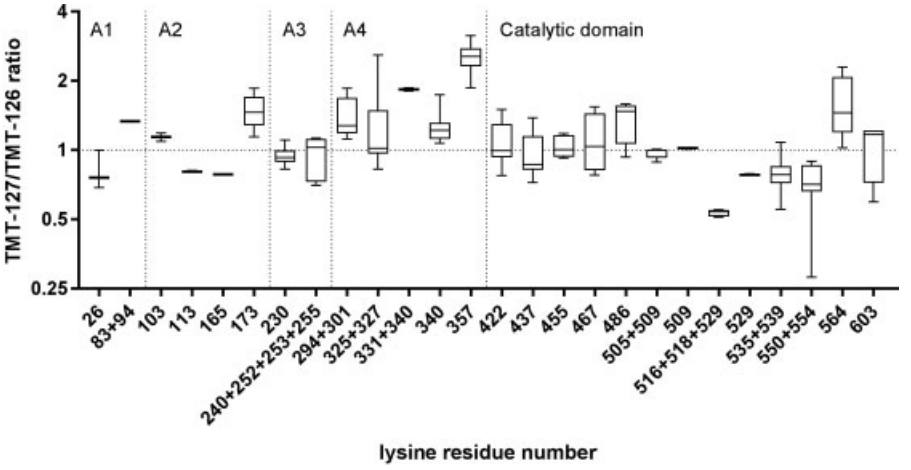


Fig. 1 TMT-127/TMT-126 ratio of the identified lysine containing peptides of factor XI(a). Factor XI was labelled with TMT-126 and factor XIa with TMT-127, proteins were mixed in a 1:1 molar ratio, digested with chymotrypsin and analysed by mass spectrometry. Lysine residue positions are indicated on the *x*-axis. TMT-127/TMT-126 >1 indicates that this lysine is more reactive in FXIa, suggesting that the involved lysine residue(s) within the peptide are more exposed to the protein surface in FXIa, while a ratio <1 indicates that this lysine is more reactive in FXI. Data are expressed as box-and-whisker plots depicting the smallest observation, lower quartile, median, upper quartile and largest observation. TMT-127/TMT-126 ratios were obtained from all the HCD spectra that belong to the indicated peptides of at least four independent experiments.

Next, we incubated FXI-WT and variants with thrombin in the presence of polyP and determined the kinetics of this activation by assessing Arg369 cleavage by SDS-PAGE and activity by chromogenic substrate S2366. As has been previously shown by Choi et al,[26] polyP enhances the activation of recombinant factor XI by thrombin. Within 60 minutes, FXI-WT and all variants except FXI-K516A were almost completely cleaved (►**Fig. 4A, B**). Arg369 cleavage of FXI-E380A was comparable with FXI-WT, while FXI-K357A, -K516E and -E380K were cleaved faster. The double mutant FXI-E380K/K516E was cleaved very efficiently; within 30 minutes, all factor XI was cleaved. To determine whether the cleaved factor XI variants were active, we assessed conversion of the chromogenic substrate S2366. Activity of all recombinant factor XI variants, except FXI-E380A and -E380K, increased in time coinciding with enhanced availability of the cleaved product (►**Fig. 4C**). Although Arg369-cleavage of FXI-K357A, -K516A and -K516E was not complete before the 50-minute time point, chromogenic activity reaches a plateau after 20 to 30 minutes of incubation. Reduction in chromogenic activity, while Arg369 cleavage is complete, is also observed for FXI-E380K/K516E. This suggests that the cleaved forms of these mutants are readily degraded. We additionally measured factor XIa activity for the biological substrate factor IX. FXI-WT and all variants were capable of converting factor IX into factor IXa (►**Fig. 4D**). The lowest activity of factor XIa was measured for FXI-E380K which also had the lowest chromogenic activity, while Arg369 cleavage was confirmed by SDS-PAGE analysis (►**Fig. 4A–C**).

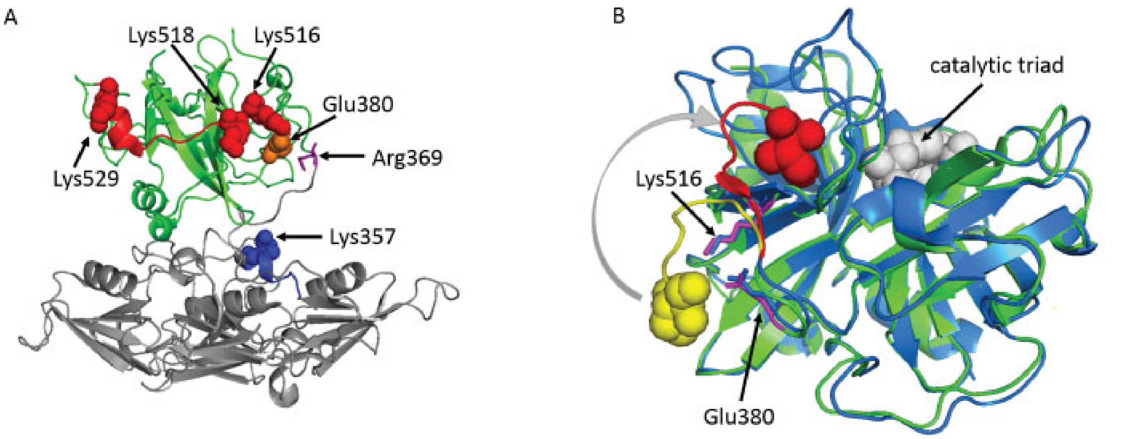
Behaviour of FXI-WT and Variants in Plasma Clotting Assays

We performed an APTT to determine the clotting activity of the recombinant factor XI variants in comparison to FXI-WT (►**Fig. 5A**). In line with previous results, FXI-E380A and -E380K had impaired clotting capability when activated via factor XIIa. This could partially be rescued by mutating Lys516 as well (i.e., FXI-E380K/K516E). In addition, a single mutation at Lys516 enhanced factor XIa activity in the APTT assay.

To understand the role of Lys357, Glu380 and Lys516 in a more physiological setting, we employed thrombin generation assays in factor XI depleted plasma supplemented with FXI-WT or variants. To study the thrombin/factor XI amplification loop, clotting was initiated by addition of TF (0.5 pM) in the presence of phospholipids (►**Fig. 5B–D**). Peak height, a thrombin generation parameter, for FXI-K516E was comparable with FXI-WT, while FXI-K357A and -E380K/K516E had lower and FXI-K516A slightly enhanced peak height (►**Fig. 5C**). In contrast to previous results from the purified system, FXI-E380A and -E380K showed highly enhanced thrombin generation, as shown in thrombin peak height (►**Fig. 5D**). Lag time was reduced in FXI-E380A, while other variants were comparable to FXI-WT (►**Fig. 5C**). Since these results showed unexpected high thrombin generation for Glu380 mutants, we hypothesized that the starting agent (TF) affects the outcome in our assays. Therefore, we employed thrombin generation assays that were started via factor XIIa in the presence of phospholipids and determined the effect of FXI-WT and the variants -E380A and -E380K (►**Fig. 5E**). Again thrombin generation was enhanced, both lag time and peak height, for the Glu380 variants. Moreover, in the absence of a triggering reagent, thrombin generation was delayed but reached higher peak thrombin height in FXI-E380A and -E380K (►**Fig. 5F**).

Impaired Inhibition of FXIa-E380A by C1-INH

Thrombin generation was enhanced in Glu380 variants while these variants have reduced activity following activation by thrombin or factor XIIa in a purified system. Plasma contains many inhibitors that rapidly inactivate factor XIa. We



C

	370									380								[illegible]
Homo sapiens	I	V	G	G	T	A	S	V	R	G	**E**	W	P	W	Q	V	T	L	H
Canis lupus familiaris	I	V	G	G	E	A	S	V	H	G	**E**	W	P	W	Q	I	T	L	H
Oryctolagus cuniculus	I	V	G	G	S	A	S	L	P	G	**E**	W	P	W	Q	V	T	L	H
Fukomys damarensis	I	V	G	G	T	V	S	V	R	G	**E**	W	P	W	Q	V	T	L	H
Bos mutus	I	V	G	G	T	R	S	V	H	G	**E**	W	P	W	Q	I	T	L	H
Bos Taurus	I	V	G	G	T	Q	S	V	H	G	**E**	W	P	W	Q	I	T	L	H
Rattus norvergicus	V	F	G	G	A	A	S	V	H	G	**E**	W	P	W	Q	V	T	L	H
Mus Musculus	V	V	G	G	A	A	S	V	H	G	**E**	W	P	W	Q	V	T	L	H
Heterocephalus glaber	I	V	G	G	T	V	S	V	R	G	**E**	W	P	W	Q	I	T	L	H

	511					516		518											[illegible]
Homo sapiens	Q	N	T	L	Q	**K**	A	K	I	P	**L**	V	T	N	E	E	C	Q	K
Canis lupus familiaris	Q	N	T	L	Q	**K**	A	N	V	P	**L**	V	T	T	E	E	C	Q	T
Oryctolagus cuniculus	Q	N	T	L	Q	**K**	A	K	I	P	**L**	L	S	N	E	E	C	Q	K
Fukomys damarensis	Q	N	T	L	Q	**K**	A	K	I	P	**L**	V	S	N	E	E	C	Q	A
Bos mutus	Q	N	T	L	Q	**K**	A	K	V	P	**L**	M	T	N	E	E	C	Q	A
Bos Taurus	Q	N	T	L	Q	**K**	A	K	V	P	**L**	M	T	N	E	E	C	Q	A
Rattus norvergicus	Q	S	T	L	Q	**K**	A	K	V	P	**L**	V	S	N	E	E	C	Q	T
Mus Musculus	Q	S	T	L	Q	**K**	A	K	V	P	**L**	V	S	N	E	E	C	Q	T
Heterocephalus glaber	K	N	T	L	Q	**K**	A	E	I	P	**L**	V	S	N	E	E	C	Q	A

D

Protein	26	157	Salt bridge possible
FXI	**E**	**K**	**Yes**
PK	**E**	**K**	**Yes**
Chymotrypsin	S	Q	
FII	M	V	
FVII	E	V	
FIX	Q	Y	
FX	E	M	
FXII	A	E	
Plasminogen	S	E	
Protein C	D	F	
tPA	S	E	
uPA	N	M	

Fig. 2 Position of the identified lysine residues in factor XI(a) crystal structure. (**A**) Structure of factor XI, in grey (the apple domains) and in green (the catalytic domain), is rendered from crystal structure 2F83. In blue, the identified peptide containing Lys357 and in red, the identified peptide containing Lys516, Lys518 and Lys529 are shown. Glu380, a possible interactor for Lys516, is shown in orange and the cleavage site (Arg369) is shown in purple. Except for the indicated residues only backbone carbons are depicted. Note that amino acid residues Tyr503-Asp508, Gly544-Cys553 and Cys581-Arg584 are lacking from the electron density map. (**B**) Overlay of factor XI catalytic domain (green, rendered from crystal structure 2F83) with factor XIa catalytic domain (blue, rendered from crystal structure 4X6P). Following Arg369 cleavage, the activation loop (yellow in factor XI and red in factor XIa) folds into the catalytic domain as indicated by the grey arrow. Ile370 (red sphere) is in close proximity to the catalytic triad (light grey spheres) in factor XIa as opposed to Ile370 (yellow sphere) in factor XI. The position of both Lys516 and Glu380 does not change after activation of factor XI (pink sticks indicate orientation in factor XI; blue sticks indicate orientation in factor XIa). (**C**) Glu380 and Lys516 in factor XI are conserved amongst species. (**D**) Chymotrypsin numbering of factor XI Glu380 (i.e., Glu26) and Lys516 (i.e., Lys157) reveals that this combination of amino acids is unique for factor XI and prekallikrein among serine proteases.

hypothesized that the main plasma inhibitor for factor XIa (i.e., C1-INH) would have impaired inhibitory capacity for FXIa-E380A and -E380K. C1-INH rapidly reduced FXIa-WT activity (►**Fig. 6**) with a half-life of 2.7 minutes. In contrast, inactivation of FXIa-E380A by C1-INH was severely impaired (half-life of inhibition of 29.5 minutes). Although FXIa-E380K was also poorly inactivated by C1-INH, the activity of this variant was extremely low and did not allow the estimation of a reliable half-life.

Discussion

Structural information about full-length factor XIa or the apple domains of factor XIa may help develop novel

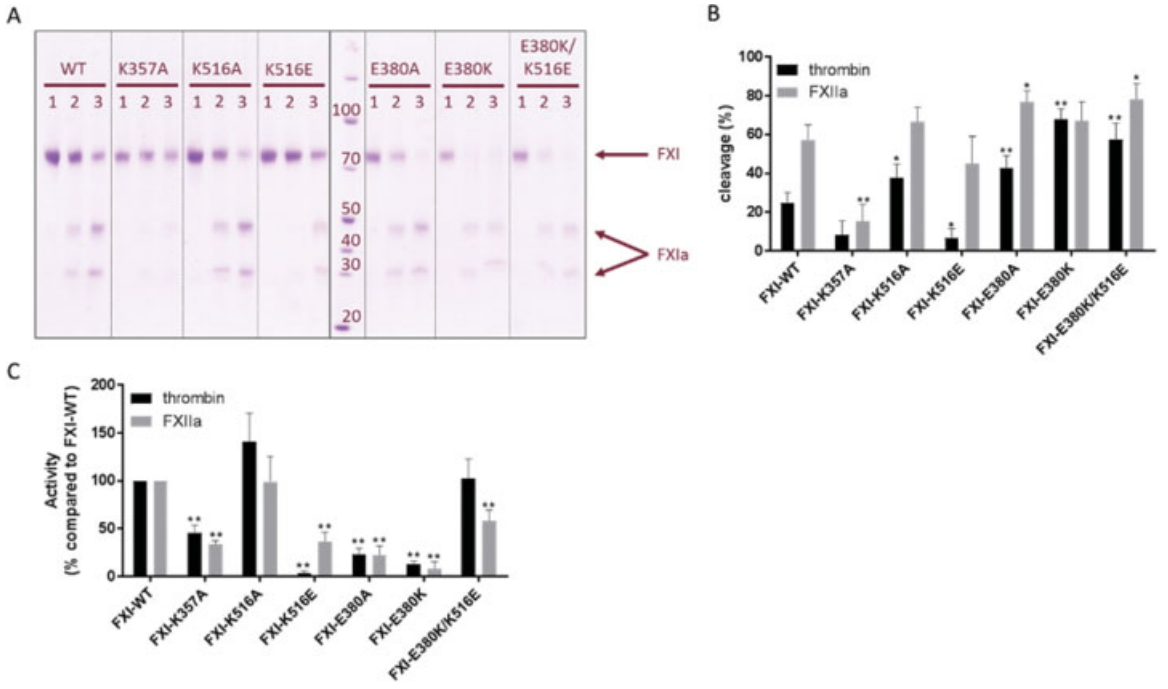


Fig. 3 Cleavage and activity of recombinant factor XI variants in a purified system. Factor XI WT or the variants K357A, K516A, K516E, E380A, E380K or E380K/K516E were incubated with thrombin (molar ratio 10:1) or factor XIIa (molar ratio 20:1) for 72 hours at 37°C. (**A**) Arg369 cleavage of factor XI was determined with SDS-PAGE under reducing conditions (1 = control; 2 = thrombin; 3 = factor XIIa incubation). (**B**) Percentage of Arg369 cleavage was determined by analysing band intensity of 80 kDa (FXI) and 50 + 30 kDa (FXIa) bands on SDS-PAGE gel. (**C**) After 72 hours, the activity of cleaved factor XI WT and variants was assessed by chromogenic activity for S2366. Results from three independent experiments were pooled, a representative SDS-PAGE gel is shown in (A). Data represent mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ versus respective FXI-WT.

procoagulant or inhibitory strategies for factor XI(a). In this study, we are the first to use chemical footprinting to determine conformational changes of factor XI following activation. We have identified several lysine residues that have an altered accessibility to the solvent in the zymogen compared with the active conformation of factor XI.

Based on the different binding properties of the zymogen and the activated form of factor XI,[9,17,18] we hypothesized that the binding sites for the proteases thrombin and factor XIIa become less accessible after activation, while the binding sites for the substrate factor IX will be more exposed. As both factors XI and XIa are bound to high molecular weight kininogen (HK) in the circulation,[27] we speculate that this particular binding site is unaltered following activation. Using mutated or chimeric recombinant factor XI, peptide mimicry or inhibitory strategies, binding sites for thrombin, factor XIIa and factor IX on factor XI(a) are proposed. Domain A1 in factor XI is the binding site for thrombin; residues Glu66, Lys83 and Gln84 form part of the binding site.[2,12,28] We did not detect a clear reduction in TMT-127/TMT-126 ratio, indicative of less reactive lysine residues, in A1. The binding site for factor XIIa is not exactly known, and the interaction may stretch among multiple domains.[29] Proposed binding sites are located on the A2[5] and A4[30] domain of factor XI. We did not see a clear reduction (TMT-127/TMT-126 < 0.5) in reactivity of lysine residues present in either of the two apple domains. Binding sites for factor IX on factor XIa were identified on the N- and C-terminus of A3[31,32] and A2 (Ala134–Leu172).[33] In addition, Lys554 is suggested to be part of the exosite for factor IX on the catalytic domain.[34] Unfortunately, except for the region in the catalytic domain, these proposed binding sites do not contain lysine residues or we were not able to detect these lysine residues. We have detected Lys554, but since it is located in a peptide containing Lys550 as well, it is difficult to determine the contribution of each of the two lysine residues to the TMT-127/TMT-126 ratio. Binding between HK and factor XI involves multiple apple domains, the strongest interaction is with A2 and a weaker interaction is detected with A1 and A4.[27,35] Recently, a peptide containing the signature DFP (Asp-Phe-Pro) that can be found in HK forms a salt bridge to Lys103 present in the A2 domain of factor XI(a).[36] Our TMT-MS/MS analysis revealed a TMT-127/TMT-126 ratio of 1.09 for Lys103, supporting unaltered conformation following activation as expected for the HK binding site. Note that we can only speculate about possible binding sites for enzymes/substrates/cofactor of factor XI(a) based on the TMT-MS/MS results comparing the zymogen factor XI with its protease. To determine binding sites using TMT-MS/MS, comparison of factor XI(a) in the presence/absence of the binding partners should be performed.

Two interesting lysine residues were determined that have not yet been described in the literature. Lys357, more prone to chemical modification in factor XIa, is adjacent the disulphide bridge connecting the N- and C-terminal part of A4 and part of the loop connecting A4 to the catalytic domain. NMR solution structure analysis has predicted that this locally strained loop connecting A4 to the catalytic

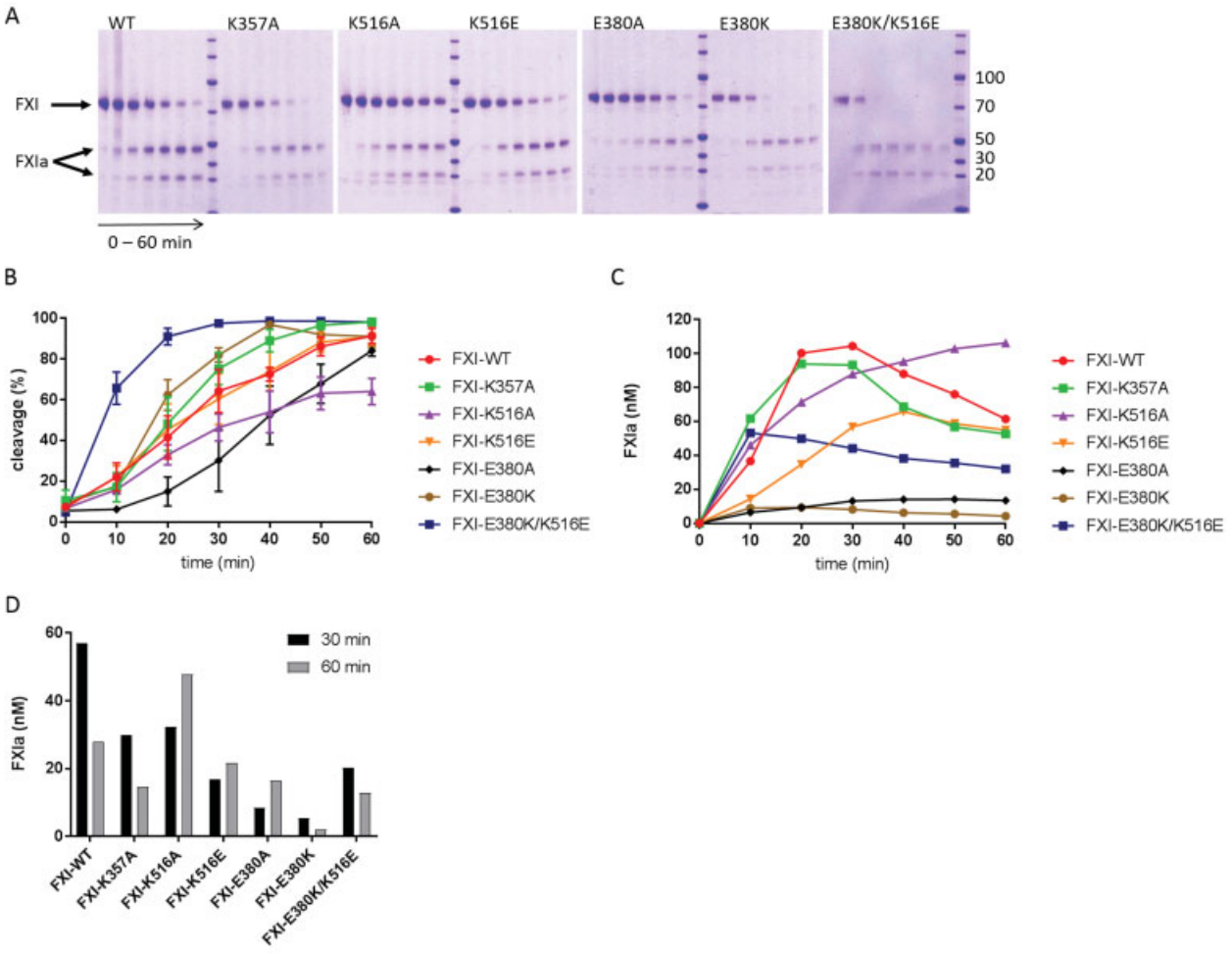


Fig. 4 Kinetics of factor XI WT and variants cleavage and activity by thrombin in the presence of polyP. Factor XI WT or the variants K357A, K516A, K516E, E380A, E380K or E380K/K516E were incubated with thrombin (molar ratio 1,000:1) in presence of polyP (30 µg/mL) for 60 minutes at 37°C and every 10 minutes a sample was drawn. Arg369 cleavage was analysed by SDS-PAGE under reducing conditions (**A**) and percentage of Arg369 cleavage was determined by analysing band intensity of 80 kDa (FXI) and 50 + 30 kDa (FXIa) bands (**B**; results from three independent experiments are combined). (**C**) Factor XIa activity was analysed by conversion of the chromogenic substrate S2366. To determine whether the cleaved recombinant proteins could also activate a biological substrate, samples were taken at 30 and 60 minutes for measuring the activity of factor XIa based on subsequent cleavage of factor IX and factor X (**D**). Data represent means (+ SEM).

domain is released following activation.[16] We have generated a K357A variant to study the role of this region in Arg369 cleavage and activity. Overall, Arg369 cleavage by thrombin or factor XIIa was impaired in the absence of polyP, but the activated form of K357A was active. In the presence of polyP, FXI-K357A Arg369 cleavage and factor XIa activity was comparable to FXI-WT. In line, plasma clotting assays revealed no obvious changes in activity. This would argue against a functional role for Lys357 in factor XI(a).

The peptide containing the lysine residues 516, 518 and 529 had the lowest TMT-127/TMT-126 ratio, indicating that these lysine residues are less prone to chemical modification in factor XIa. Although this peptide contains three lysine residues, which makes it difficult to determine the contribution of each lysine to the overall TMT ratio, together with crystal structure analysis, we hypothesized that Lys516 is covered by the factor XI activation loop. Moreover, crystal structure analysis revealed a possible salt bridge in FXI(a) between Lys516 and Glu380.[13,25] Following Arg369 cleavage of factor XI, the activation loop (Ile370-Ser376)[12] folds into the catalytic domain. Crystal structure analysis reveals that Lys516 is partly covered by this region in both factors XI and XIa; however, the orientation of this loop is shifted. Therefore, it is unlikely that reduced accessibility of Lys516 to TMT-127 is solely due to covering of this lysine by the activation loop. It might be possible that the interaction between Lys516 and Glu380 is stronger in factor XIa, and therefore (partly) protection from chemical modification by TMT-127 might contribute to reduced TMT-127/TMT-126 ratio. We hypothesized that the interaction between Lys516 and Glu380 is necessary for stability and activity of factor XIa. Overall, our mutagenesis studies showed that mutations of Lys516 or Glu380 resulted in factor XI variants with similar or improved Arg369 cleavage by thrombin (in the absence and presence of polyP), except for FXI-K516E in the absence of polyP, or factor XIIa. Strikingly, the cleaved variants FXI-K516E, -E380A, -E380K and -E380K/K516E showed reduced activity for a small substrate and for the physiological

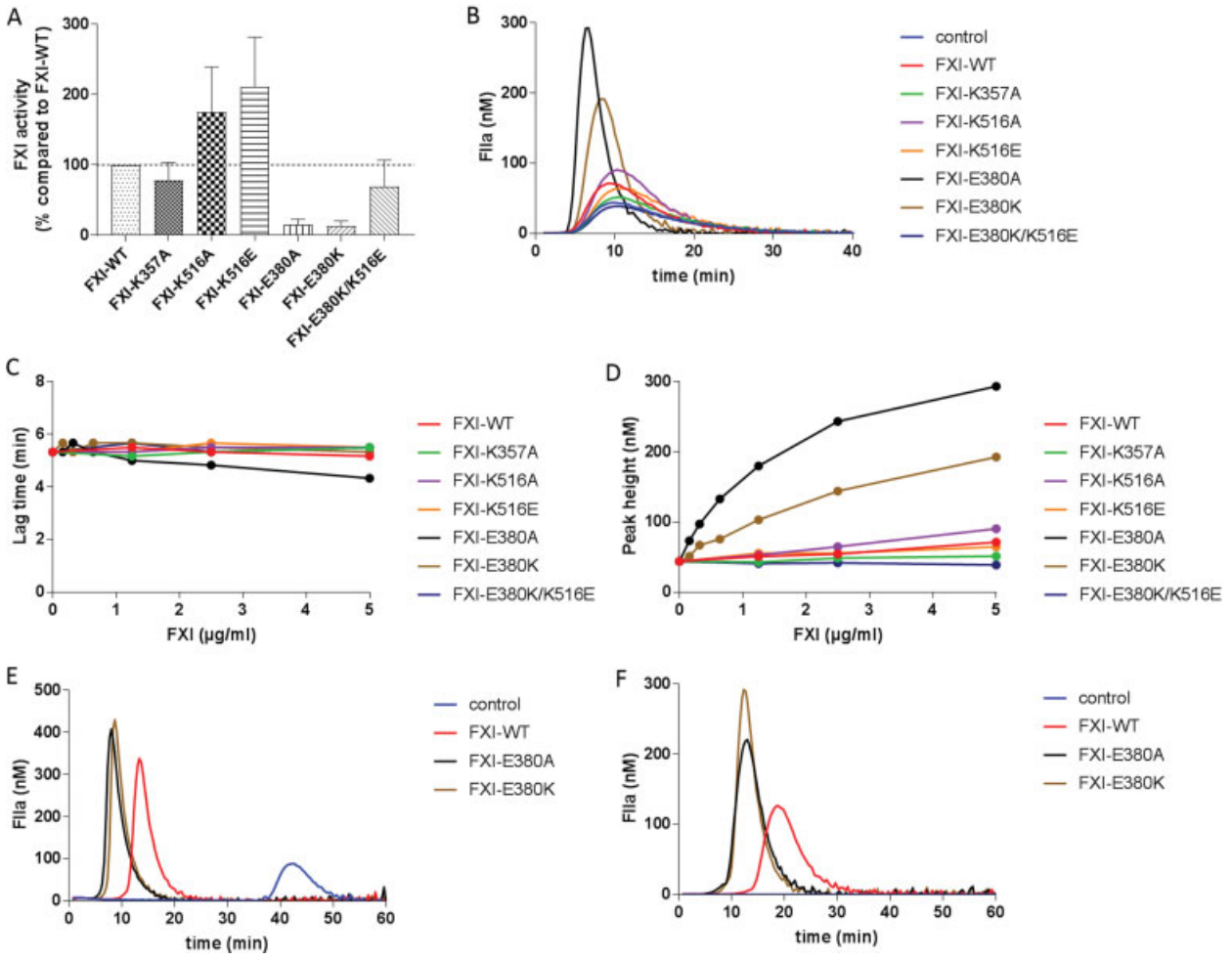


Fig. 5 Effect of factor XI WT and variants in factor XI depleted plasma on coagulation. (A) Activity of FXI-WT and variants was assessed in factor XI depleted plasma supplemented with 5 µg/mL recombinant factor XI, coagulation was initiated by the APTT reagent ActinFSL. (**B**) Thrombin generation was performed in factor XI depleted plasma supplemented with 5 µg/mL recombinant factor XI and initiated via TF (0.5 pM) in the presence of phospholipids (4 µM). Factor XI depleted plasma without addition of recombinant factor XI served as control. To assess the effect of recombinant factor XI concentrations on lag time and thrombin peak height, additional thrombin generation assays were performed with concentrations of recombinant factor XI ranging from 0 to 5 µg/mL. The correlation between factor XI concentration and (**C**) lag time and (**D**) peak height is shown. To determine whether the enhanced thrombin generation in FXI-E380A and -E380K was specific for initiation via TF, thrombin generation was initiated via factor XIIa (1.2 µg/mL) in the presence of phospholipids ((**E**) 4 µM) or (**F**) by phospholipids (4 µM) alone. Please note that the control (factor XI depleted plasma) in F is on the *x*-axis. (**A**) Data represent means + SD. Thrombin generation assays in (**B–D**) were performed twice, a representative experiment is shown.

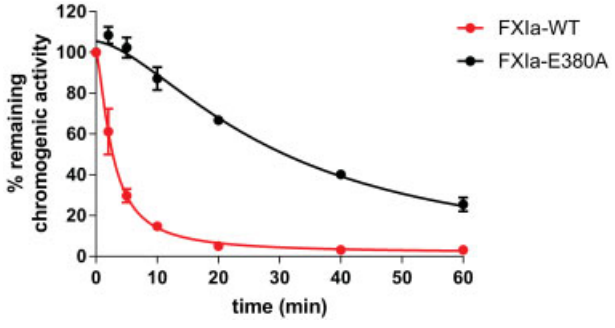


Fig. 6 Impaired inhibition of FXIa-E380A by C1-INH. Factor XI WT or the variant E380A were first activated by incubation with thrombin (molar ratio 1,000:1) in the presence of polyP (30 µg/mL) for 60 minutes at 37°C. C1-INH (1.5 U/mL) was added to FXIa-WT or FXIa-E380A (20 nM) and chromogenic activity for S2366 was determined in time. Activity at $t = 0$, in the absence of C1-INH, was set at 100%. Data represent mean ± SD, $n = 3$.

substrate factor IX. This defect in activity was most pronounced in the Glu380 variants. An APTT-based clotting assay confirmed to a great extent our results from purified protein systems. In contrast, thrombin generation was enhanced in FXI-E380A and -E380K when initiated via TF, factor XIIa or in the presence of only phospholipids (autoactivation). Plasma contains HK and various inhibitors for factor XIa, for example, C1-INH and antithrombin, which might explain differences in activity compared with purified protein systems. Factor XIa is generated fast in APTT-based assays, while it is much slower in the thrombin generation assays we have used. Variances in kinetics might explain why FXI-E380A and -E380K behave differentially in both plasma assays. Factor XIa might be inactivated quickly by inhibitors present in plasma which may lead in an APTT assay, where majority of factor XI is activated before clotting is initiated with addition of calcium ions, to reduction of factor XIa

activity. C1-INH, the major plasma inhibitor of FXIa poorly inactivated FXIa-E380A compared with FXIa-WT resulting in an 11-fold increase in half-life in the presence of C1-INH. Substitution of the basic amino acids of the autolysis loop (i.e., Arg504, Lys505, Arg507 and Lys509) for alanine enhanced inhibition of factor XIa by C1-INH.[37] We now show that substitution of an acidic residue, in close proximity to the autolysis loop, has the opposite effect. The reduced inhibition of FXIa-E380A by C1-INH explains to a large extent the high activity of the factor XI Glu380 variants in thrombin generation.

Of the 41 lysine residues present in factor XI, 6 were not identified with our MS analysis. Peptides containing these lysine residues were not identified or at low confidence. These lysine residues were located within A1 (Lys8, Lys61), A2 (Lys150, Lys162) and just before the Arg369-cleavage site (Lys365, Lys367). In addition, factor XI contains several regions that lack lysine residues. For instance, the first half of the A3 domain does not contain any lysine. This particular region, together with the C-terminus of A3, is predicted to be the binding site for factor IX on factor XIa.[9,31,32] Since factor IX binds only to the activated form of factor XI, it would be interesting to see whether the expected binding site for factor IX indeed undergoes a conformational change following activation of factor XI and is more exposed in the active form. Together, lack of information from the unidentified regions is a limiting factor in TMT-MS/MS. In conclusion, our findings provide novel insight about conformational changes in factor XI and shed new light on the role of the salt bridge between Glu380 and Lys516 in the catalytic domain.

What Is Known about This Topic

- Factor XI is activated by cleavage at the Arg369-Ile370 bond.
- Activation of factor XI leads to a conformational change exposing exosites for factor IX on the apple domains of the heavy chain. The heavy chain and protease domain are still attached via a disulphide bridge.
- Based on crystal structure analysis, conformational changes following activation of factor XI within the protease domain are described.

What Does This Paper Adds

- By using lysine-directed chemical footprinting in conjunction with MS, we have identified novel regions, both in the heavy chain and the protease domain, in factor XI that undergo a conformational change following activation.
- Lys357 is more accessible in factor XIa, mutation of this lysine did not alter factor XI activity.
- Lys516 is less accessible in factor XIa. Mutating Lys516 or its interactor Glu380 showed that although Lys516 does not contribute to factor XIa activity or stability, Glu380 plays a role in factor XIa activity.

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Conflict of Interest
None declared.

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