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Human inter-α-inhibitor is a substrate for factor XIIIa and tissue transglutaminase ☆

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ABSTRACT

In this study, we show that inter-α-inhibitor is a substrate for both factor XIIIa and tissue transglutaminase. These enzymes catalyze the incorporation of dansylcadaverine and biotin–pentylamine, revealing that inter-α-inhibitor contains reactive Gln residues within all three subunits. These findings suggest that transglutaminases catalyze the covalent conjugation of inter-α-inhibitor to other proteins. This was demonstrated by the cross-linking between inter-α-inhibitor and fibrinogen by either factor XIIIa or tissue transglutaminase. Finally, using quantitative mass spectrometry, we show that inter-α-inhibitor is cross-linked to the fibrin clot in a 1:20 ratio relative to the known factor XIIIa substrate α2-antiplasmin. This interaction may protect fibrin or other Lys-donating proteins from adventitious proteolysis by increasing the local concentration of bikunin. In addition, the reaction may influence the TSG-6/heavy Chain 2-mediated transfer of heavy chains observed during inflammation.

1. Introduction

Inter-α-inhibitor (IαI) is a proteoglycan composed of bikunin and two homologous heavy chains referred to as heavy chain 1 (HC1) and heavy chain 2 (HC2) [1]. The three components are covalently cross-linked by an under-sulfated chondroitin-4-sulfate, which originates from Ser10 of bikunin (Fig. 1) [2–5]. The C-terminal carboxyl groups of the HCs form ester bonds with the C6 atoms of the internal N-acetylgalactosamines and these bonds are referred to as protein–glycosaminoglycan–protein (PGP) cross-links [3].

IαI has been implicated in several biological processes, such as ovulation, cell migration and inflammation [6]. The suggested roles of IαI are based on its interactions with hyaluronan-rich extracellular matrix (ECM) or the protease inhibitory activity associated with bikunin. The bikunin subunit is composed of two Kunitz-A domains (http://merops.sanger.ac.uk) [7] and inhibits a broad range of serine proteases, including plasmin [8]. The concentration of circulating bikunin and its K_i indicates that IαI is not an effective protease inhibitor in plasma [8]. However, several studies have indicated that the inhibitory capacity of bikunin is increased by interaction with tumor necrosis factor stimulated gene-6 protein (TSG-6) [9–11].

TSG-6 also mediates the covalent interaction between the HCs and hyaluronan. Two sequential transesterifications mediated by TSG-6/HC2 transfer the HCs from the bikunin-associated chondroitin-4-sulfate to hyaluronan [12–14]. The role of the HC–HA complexes has yet to be determined, but evidence suggests a role in arthritis, as the concentration of HC–HA covalent complexes is increased in the synovial fluid [15]. In addition, these complexes appear to play a role in the attachment of leukocytes during inflammation [16].

The transglutaminases, including factor XIIIa (FXIIIa) and tissue transglutaminase (TTG), belong to a family of calcium-dependent enzymes (EC 2.3.2.13) and catalyze the formation of Nε(γ-glutamyl)lysine cross-links [17]. The generation of these isopeptide bonds increases mechanical stability and resistance to adventitious proteolysis. FXIII circulates in the plasma as a non-covalently associated tetramer composed of two a-chains and two b-chains [18]. In the final phase of blood coagulation, thrombin-activated FXIIIa cross-links the fibrin clot into an acid and urea stable polymer [19]. Following fibrin cross-linking, FXIIIa may further covalently incorporate a number of different proteins into the fibrin clot, including α2-antiplasmin (α2AP) [20], factor V [21], thrombin-activatable fibrinolysis inhibitor (TAFI) [22], von Willebrand factor [23] and plasminogen activator inhibitor type 2 (PAI-2) [24].

Abbreviations: IαI, Inter-α-inhibitor; HC, Heavy Chain; TSG-6, Tumor necrosis factor stimulated gene-6 protein; FXIIIa, Activated Factor XIII A subunit; FXIII, Factor XIII zymogen; TTG, tissue transglutaminase; ChonABC, Chondroitinase ABC; α2AP, α2-antiplasmin

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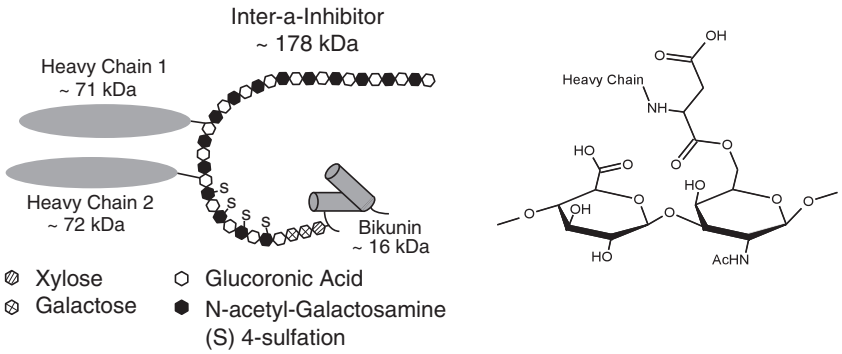


Fig. 1. Schematic representation of IαI. *Left*, IαI (~178 kDa) is a proteoglycan composed of bikunin (approximately 16 kDa) and two homologous heavy chains (HC1, approximately 71 kDa, and HC2, approximately 72 kDa). The three components are covalently cross-linked by protein–glycosaminoglycan–protein (PGP) cross-links mediated by the under-sulfated chondroitin-4-sulfate that originates from Ser10 of bikunin [3,4]. The ratio between 4-sulfated and unsulfated N-acetyl-galactosamine in the under-sulfated chondroitin-4-sulfate is 1:3, and most of the sulfated moieties are located near the reducing end [5]. *Right*, Chemical structure of the PGP cross-link. The C-terminal carboxyl groups of the HCs form ester bonds with the C6 atoms of internal N-acetyl-galactosamines [3]. The PGP-crosslink can be broken by either hydrolysis of the ester-linkage or enzymatic cleavage of the chondroitin-sulfate chain.

FXIIIa deficiency leads to severe bleeding complications, reduced wound healing and recurring miscarriages [25].

In this study, we show that IαI is a substrate for both FXIIIa and TTG. Using a combination of transglutaminase-mediated biotin–pentylamine incorporation and mass spectrometry, we have identified sites of modification in all three IαI subunits. Furthermore, we present evidence for the cross-linking of IαI and fibrinogen and show that IαI is cross-linked to the plasma clot in a 1:20 ratio relative to the known FXIIIa substrate α2-antiplasmin. These data suggest that transglutaminases cross-link IαI and other substrate proteins in general, a reaction that is likely to introduce additional cross-links and thereby increase the mechanical stability and resistance to adventitious proteolysis. Moreover, our data indicate that IαI plays a direct role in the formation and stability of the plasma clot.

2. Experimental procedures

2.1. Materials

Dansylcadaverine was purchased from Biochemika, biotin–pentylamine (EZ-link) was from Pierce, immobilized monomeric avidin was from Thermo, biotin was from Fluka and dansyl-PGGQQIV-OH was from New England Peptide.

Proteins human IαI was purified from human plasma obtained from Aarhus University Hospital, Skejby, Denmark as previously described [1]. Chondroitinase ABC (EC 4.2.2.4) was from Seikagaku. Guinea pig tissue transglutaminase (EC 2.3.2.13), human fibrinogen, thrombin and trypsin were from Sigma. Antibodies were from DAKO, and recombinant human Factor XIII (A subunit) was a kind gift from Sanofi Aventis.

2.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were boiled in SDS sample buffer in the presence of 50 mM dithiothreitol (DTT). SDS-PAGE was performed on 5–15% gradient gels (10 cm × 10 cm × 0.15 cm) using the glycine/2-amino-2-methyl-1,3-propanediol/HCl system described previously [26].

2.3. Transglutaminase catalyzed incorporation of dansylcadaverine or dansyl-PGGQQIV-OH

IαI (3 μg) was titrated with increasing amounts of either TTG of FXIIIa (0, 0.15, 0.3, 0.6 or 1.2 μg) for 2 h at 37 °C. The reaction was carried out in 20 mM Tris–HCl (pH 7.4) containing 137 mM NaCl, 10 mM $CaCl_2$ and 0.6 mM dansylcadaverine or dansyl-PGGQQIV-OH. After the addition of EDTA (10 mM final concentration), the samples were analyzed by SDS-PAGE, and the reaction products were visualized under UV light using the FluorChem Q imaging system (Cell Biosciences). All SDS-PAGE-based experiments were repeated at least five times.

2.4. Activation of FXIII

FXIII (8 mg/ml) was activated with thrombin (1 mUnit thrombin/1 μg FXIII) in 20 mM Tris–HCl (pH 7.4), 137 mM NaCl and 0.1 mM DTT. The sample was incubated at 37 °C for 15 min prior to use.

2.5. Dissociation of IαI by NaOH or chondroitinase ABC (ChonABC)

IαI was chemically dissociated at alkaline pH by incubating the protein in 100 mM NaOH at 0 °C. The sample was neutralized after 15 min by addition of Tris–HCl (pH 7.4) to a final concentration of 400 mM.

IαI was enzymatically dissociated by the addition of ChonABC. IαI was incubated with 3.3 mUnits ChonABC/μg IαI at 37 °C for 16 h. The dissociated IαI was labeled with dansylcadaverine as described above using 3 μg IαI and 0.6 μg transglutaminase.

2.6. Identification of reactive Gln residues by incorporation of biotin–pentylamine

IαI and either TTG or FXIIIa were incubated at a 5:1 ratio (w/w) in 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, and 10 mM $CaCl_2$ containing 0.1 mM DTT and 5 mM biotin–pentylamine. After 6 h at 37 °C, the reaction was stopped by addition of EDTA to a final concentration of 50 mM. The sample was reduced in 20 mM Tris–HCl and 6 M guanidine–HCl (pH 8) containing 5 mM DTT and carboxyamidomethylated by the addition of 15 mM iodoacetamide.

The reduced and carboxyamidomethylated sample was dialyzed against 20 mM ammonium bicarbonate and digested with trypsin (1:40 w/w) for 16 h. The tryptic peptides were lyophilized and dissolved in 20 mM phosphate (pH 7.4), 100 mM NaCl and 1 mM phenylmethanesulfonyl fluoride (PMSF). The sample was applied to a monomeric avidin affinity column equilibrated in 20 mM phosphate (pH 7.4) and 100 mM NaCl. After extensive washing, the biotin–pentylamine-labeled peptides were eluted with 2 mM biotin and further separated by reverse phase HPLC using a Vydac C18/5 μm, 300 Å (250 × 2.1 mm) column connected to an Äkta Explorer system (GE HealthCare). The peptides were eluted using a 0.1% trifluoroacetic acid/acetonitrile buffer system with a linear gradient. The eluate was monitored at 220 nm, and peptides were collected manually.

2.7. Matrix-assisted laser desorption/ionization mass spectrometry

Aliquots of the fractions from the Vydac C18 column were lyophilized, dissolved in matrix solution containing 70% acetonitrile, 0.03% trifluoroacetic acid, and 0.4% cyano-4-hydroxycinnamic acid and spotted onto a MALDI target.

MALDI-MS Analyses MS or MS/MS spectra were collected using a Q-TOF Ultima Global mass spectrometer (Micromass/Waters Corp.) calibrated over the *m/z* range of 50–3000 using a polyethylene glycol mixture. External calibration of each MS spectrum was performed using Glu-fibrinopeptide B (*m/z* 1570.6774). The MS or MS/MS data obtained were processed using the Masslynx 4.0 software (Micromass). Data were submitted to a local Mascot server (UniProt database) and manually interpreted. The GPMAW software (http://www.gpmaw.com) was used to generate theoretical biotin–pentylamine-labeled peptides to assist in the manual selection of peptides for MS/MS analysis. Biotin–pentylamine labeling, peptide separation and MS analysis were repeated 3 times for both TTG and FXIII.

2.8. Multiple protein sequence alignment

Sequence analyses were performed on the IαI subunits from the following species: *Meriones unguiculatus* (Mongolian jird); *Mesocricetus auratus* (Golden hamster); *Pleuronectes platessa* (Plaice); *Xenopus tropicalis* (Western clawed frog); *Salmo salar* (Atlantic salmon); *Oncorhynchus mykiss* (Rainbow trout); *Esox lucius* (Northern pike); *X. tropicalis* (Western clawed frog); and *Danio rerio* (Zebrafish). The multiple protein sequence alignment was made using the CLC main Workbench (CLCBIO).

2.9. Transglutaminase-mediated cross-linking of IαI to fibrinogen

A microtiter plate (Nunc Maxisorp™) was coated with 25 μg/ml fibrinogen in 50 mM Tris–HCl and 100 mM NaCl (pH 7.4) (buffer A) for 16 h. The wells were subsequently blocked by adding 300 μl 5% skim milk in buffer A. After 1 h, the plates were washed 3 times in buffer A, and a fixed amount of IαI (1 or 4 μg) was added to each well. The cross-linking reaction was then initiated by the addition of a fixed (1 μg) or variable (0.04–20 μg) amount of FXIII and 2 mUnits of thrombin/μg FXIII to the wells. All cross-linking reactions were performed in buffer A containing 10 mM $CaCl_2$. The samples were incubated at 37°C for 4 h, and the reaction was stopped by the addition of EDTA. To remove non-covalently bound IαI, the plate was sequentially washed 3 times with buffer A containing 6 M guanidine–HCl and 3 times with buffer A. The plate was incubated for 16 h at 4 °C in buffer A containing 1% skim milk, 0.1% Tween and a rabbit anti-IαI antibody (1:2000) (DAKO).

The plate was washed 3 times with buffer A before the addition of a goat anti-rabbit HRP-conjugated antibody (1:5000) (DAKO) in buffer A containing 1% skim milk and 0.1% Tween. The plate was incubated for 2 h at 25 °C, washed with buffer A and developed using 0.4 mg/ml *o*-Phenylenediamine dihydrochloride in 24.3 mM citric acid, 51.4 mM Na_2HPO_4 and 0.4 μl/ml H_2O_2. The plate was analyzed at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech). All measurements were performed in triplicate.

2.10. Quantifying the relative amount of IαI in a plasma clot

Freshly drawn blood from a healthy male was fractionated by centrifugation at 900 *g* for 2 min. The plasma fraction was removed and allowed to clot for 2 h at 37 °C. After clot formation, the clot was removed and extensively washed 3 times for 20 min in each of the following buffers: 1) 50 mM 50 Tris–HCl and 100 mM NaCl (pH 7.4); 2) 10% acetic acid; 3) 6 M guanidine–HCl, 2 M NaCl, and 50 mM Tris–HCl (pH 7.4) and 4) H_2O. The remaining clot was boiled for 10 min in 1% SDS with 5 mM DTT and alkylated by addition of acrylamide at a final concentration of 15 mM. The sample was loaded onto an SDS-PAGE gel. After running the gel, the material in the top of the stacking gel was prepared for in-gel digestion using a microspin column [27,28]. The washed sample was digested with trypsin (1:20 w/w) for 16 h, and the tryptic peptides were purified using GELoader tips (Eppendorf) packed with Poros R2 [29]. In short, the sample was acidified using 0.1% formic acid and loaded onto the tip. The tip was washed using 0.1% formic acid, and the sample was eluted using 80% acetonitrile with 0.1% formic acid. Acetonitrile and formic acid were removed by lyophilization.

The lyophilized peptides were suspended in 0.1% formic acid. Approximately 2 μg of peptide was used for each SRM run. The peptides were loaded onto an EASY-nano LC system (Proxeon, Denmark). They were trapped on a 2 cm, 100 μm inner diameter, and 360 μm outer diameter ReproSil-Pur C18 AQ 5 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) reversed phase capillary column and separated using a 15 cm, 100 μm inner diameter, and 360 μm outer diameter ReproSil-Pur C18 AQ 3 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid and 90% acetonitrile) over 40 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in nano-electrospray mode. For ionization, a 2300 V spray voltage and a capillary temperature of 200 °C were used. The selectivity for Q1 was set at 0.7 and Q3 at 0.1 Da (FWHM). The collision gas pressure of Q2 was set at 1.5 mTorr argon. The collision energy was calculated by Pinpoint 1.1 (Thermo Scientific). Data acquisition was performed in iSRM mode [30] using an overall cycle time of 1 s. A total of 87 primary transitions and 164 secondary transitions were used to target 33 peptides. All raw files were processed by Pinpoint 1.1. The targeted peptides were verified by comparing the transition intensities to a MS/MS spectra library [31]. For each targeted peptide that fulfilled the verification criteria, the software computed the integrated peak areas of all primary ions for quantification and calculated coefficient of variation for three technical replicates.

3. Results

3.1. IαI is a substrate for transglutaminases

The ability of IαI to act as a substrate for FXIIIa or TTG was tested by co-incubation of dansylcadaverine, IαI and increasing amounts of FXIIIa or TTG. The samples were incubated for 2 h at 37 °C, analyzed by SDS-PAGE and visualized under UV light (Fig. 2A and B). It is evident that both FXIIIa and TTG labeled IαI with dansylcadaverine in a concentration-dependent manner (Fig. 2A and B, lanes 1–5), indicating the existence of reactive Gln residues in IαI. To test whether IαI was able to act as an amine donor (i.e., whether it contains reactive Lys residues), we co-incubated the transglutaminases with IαI in the presence of the known transglutaminase substrate dansyl-PGGQQIV-OH (Fig. 2C and D). The transglutaminase-dependent incorporation of dansyl-PGGQQIV-OH demonstrates that IαI contains reactive Lys residues, but the level of labeling of IαI relative to dansylcadaverine was significantly lower.

3.2. The three IαI subunits are all substrates for TTG and FXIIIa

To determine whether all three IαI subunits (HC1, HC2 and Bikunin) contained reactive Gln residues, IαI was dissociated by NaOH or ChonABC treatment. The reaction products were then incubated with either TTG or FXIIIa in the presence of dansylcadaverine and analyzed by SDS-PAGE (Fig. 3). NaOH dissociation (lane 2) and ChonABC treatment (lane 4) of IαI revealed two fluorescently labeled bands following TTG incubation. These data indicate that both HC1 and HC2 contain reactive Gln residues. TTG is autocatalytic (lane 5) and co-

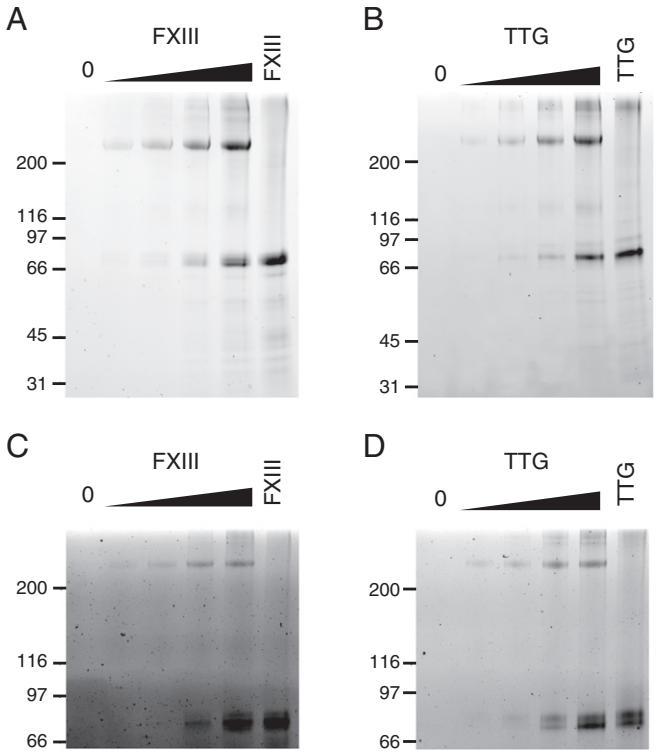


Fig. 2. IαI is a substrate for tissue transglutaminase and FXIIIa. To test for reactive Gln residues, a fixed amount of IαI was incubated with increasing amounts of either FXIIIa (A) or TTG (B) in the presence of the amine donor dansylcadaverine. To test for the presence of reactive Lys residues, IαI was incubated with increasing amounts of either FXIIIa (C) or TTG (D) in the presence of the amine acceptor dansyl-PGGQQIV-OH. The samples were incubated for 3 h at 37 °C, and the reaction was stopped by addition of EDTA. The products were separated by reducing SDS-PAGE and visualized under UV light. The samples designated "0" lack transglutaminase. FXIIIa and TTG are samples without IαI and present the autocatalytic activities of FXIIIa and TTG.

migrates with HC1; therefore, we can only conclude that HC2 is a substrate of TTG. However, the intensities of the HC1 band (lanes 2 and 4) and the TTG band (lane 5) indicate that HC1 is a substrate for TTG. Similarly to TTG, FXIII generated two fluorescently labeled bands in IαI treated with either NaOH or ChonABC (lanes 7 and 9). FXIIIa is also autocatalytic and co-migrated with HC1 (lane 10). Based on the band intensities, we were not able to conclude that

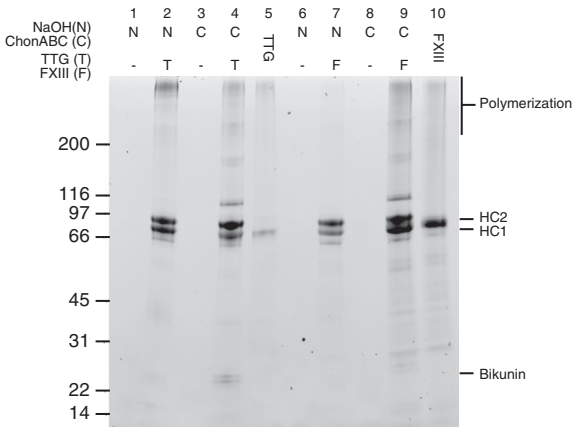


Fig. 3. All three subunits of IαI are substrates for tissue transglutaminase and FXIIIa. IαI was dissociated by either NaOH (N) or ChonABC (C) treatment. Dissociated IαI was incubated without transglutaminase (−) or with either TTG (T) or FXIIIa (F) in the presence of dansylcadaverine. The products were analyzed by reducing SDS-PAGE and visualized under UV light. TTG and FXIII indicate samples without IαI and show the autocatalytic activities of each enzyme.

HC1 is a substrate of FXIIIa; however, if HC1 is a substrate, FXIIIa does not label it to the same extent as TTG.

Bikunin liberated by NaOH contains the GAG chain and migrates as a fuzzy band during SDS-PAGE, which makes it difficult to assess the level of dansylcadaverine incorporation. However, if IαI was enzymatically dissociated using ChonABC and incubated with TTG, fluorescently labeled bands corresponding to bikunin were apparent, suggesting that bikunin is a substrate for TTG (Fig. 3, lane 4). During the experiment using dissociated IαI, polymerization of IαI subunits was observed (Fig. 3, lanes 2, 4 and 9). While the polymerization is most likely due to direct cross-linking of the reactive Lys and Gln residues in the IαI subunits, the existence of transglutaminase/IαI complexes cannot be ruled out.

3.3. Identification of the reactive Gln residues in IαI

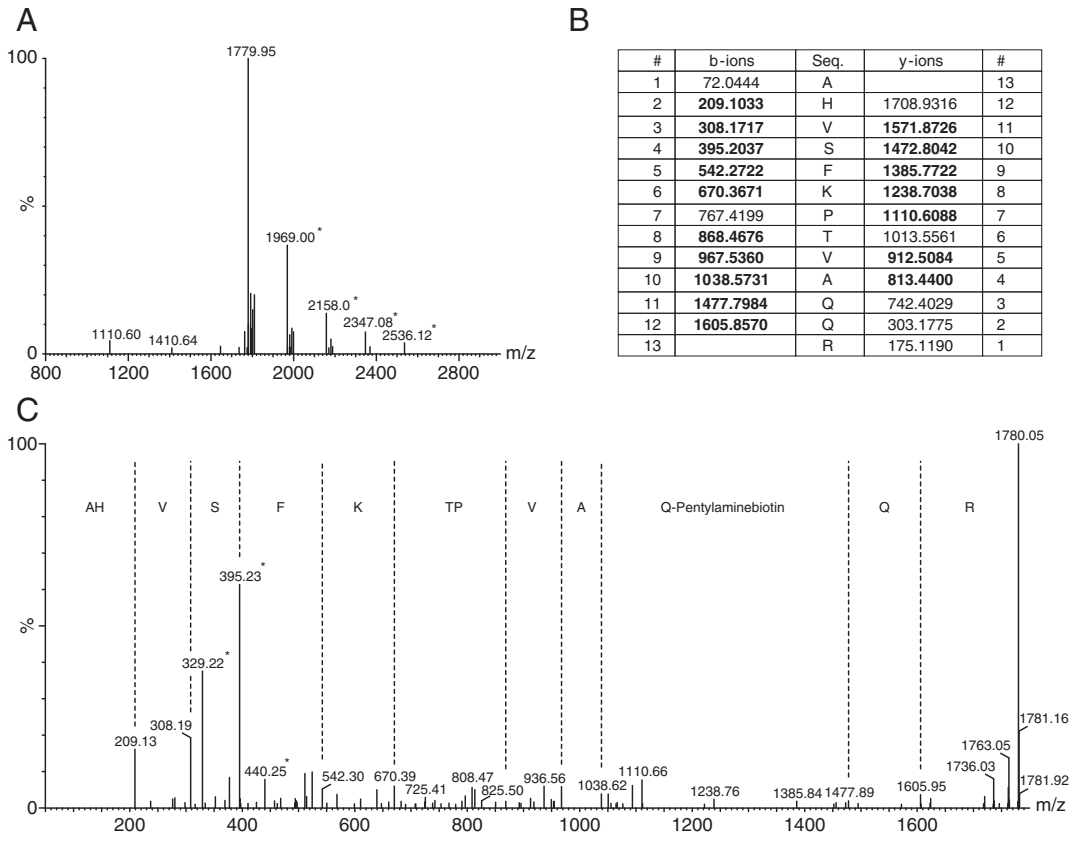
IαI was incubated with either FXIIIa or TTG in the presence of biotin–pentylamine. After incubation, the sample was digested with trypsin, and the biotin–pentylamine-containing peptides were extracted using a monomeric avidin column. The biotin-containing peptides were further fractionated using reverse phase HPLC (data not shown), and the fractions were analyzed by MALDI-MS (Fig. 4A). The *m/z* values of all of the tryptic peptides containing the biotin–pentylamine modification at all Gln residues were calculated in silico (GPMAW, Lighthouse data, http://www.gpmaw.com). The theoretical and measured *m/z* values were compared, and the fractions containing peptides with a measured *m/z* value within 50 ppm of the theoretical value were characterized by MALDI-MS/MS (Fig. 4B). The collected mass spectra were manually inspected, and a peaklist was used to query the UniProt database using a local Mascot server (Matrix Science). The MS/MS spectra of all of the biotin–pentylamine-labeled peptides contained fragment ions at *m/z* 329, 395 and 440, which correspond to fragments from the biotin–pentylamine-modified Gln residue. These three fragments were used as a signature to identify the labeled peptides. The combined MS and MS/MS analyses identified 10 FXIIIa- and 12 TTG-reactive Gln residues (Fig. 5 and Table 1), 9 of which were shared by both transglutaminases. Bikunin contained a single Gln residue utilized by both transglutaminases. HC1 contained 3 TTG-reactive Gln residues, and the most N-terminal residue also served as a substrate for FXIIIa. HC2 contained 7 residues modified by both transglutaminases and 2 individual Gln residues that were substrates for either TTG or FXIIIa. Four of the substrate sites in HC2 were located in a cluster across 18 residues within the primary sequence (Fig. 5). This hot spot in HC2 is C-terminal to the von Willebrand factor type A domain.

A multiple protein sequence alignment of the available species found in the UniProt database (UniProt release 15.10) revealed that 5 out of the 13 residues were fully conserved (Bikunin Gln74; HC1 Gln468; HC2 Gln204, Gln420, and Gln431). Furthermore, Gln residues 90, 203, 315 and 438 in HC2 were conserved in all species, except for *Xenopus laevis* (African clawed frog) and *Xenopus tropicalis* (Western clawed frog). The sequence identity of HC2 homologues compared with humans varied between 54 and 89%. All sites in HC2, except for Gln299, were conserved between *D. rerio* (Zebrafish) and humans, even though the HC2s in these species only have 54% sequence identity.

In addition to the modification sites identified in IαI, autocatalytic labeling gave rise to the identification of modification sites in both TTG and FXIIIa. Four reactive Gln residues were identified in FXIIIa, and 2 sites were found in TTG (Table 1).

3.4. Transglutaminase-mediated cross-linking of IαI and Fibrinogen

The biological relevance of the described observations was investigated by testing the ability of FXIIIa to cross-link IαI to fibrinogen. A microtiter plate was coated with fibrinogen, and IαI was added with or without FXIIIa. The plate was washed using denaturing



#	b-ions	Seq.	y-ions	#
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2	**209.1033**	H	1708.9316	12
3	**308.1717**	V	**1571.8726**	11
4	**395.2037**	S	**1472.8042**	10
5	**542.2722**	F	**1385.7722**	9
6	**670.3671**	K	**1238.7038**	8
7	767.4199	P	**1110.6088**	7
8	**868.4676**	T	1013.5561	6
9	**967.5360**	V	**912.5084**	5
10	**1038.5731**	A	**813.4400**	4
11	**1477.7984**	Q	742.4029	3
12	**1605.8570**	Q	303.1775	2
13		R	175.1190	1

Fig. 4. Identification of transglutaminase modification sites by mass spectrometry. IαI was incubated with TTG or FXIIIa in the presence of biotin–pentylamine. The reaction was quenched by addition of EDTA and subsequently reduced, alkylated and digested with trypsin. The tryptic peptides were separated with monomeric avidin resin and reverse phase HPLC. The purified labeled peptides were analyzed by MALDI-MS. The figure shows a representative example of a tryptic peptide labeled with biotin–pentylamine. A) MALDI-MS of a tryptic peptide with an m/z corresponding to AHVSFKPTVAQQR + biotin–pentylamine. B) Table showing (in bold) the confirmed y- and b-ions from MALDI-MS/MS of *m/z* 1779.96, which identified the labeled peptide as AHVSFKPTVAQ$Q_{Biotin\text{-}pentylamine}$R. C) MALDI-MS/MS of the *m/z* 1779.96 signal with the b-ion series indicated. The intense *m/z* 329, 396 and 440 signals originate from the biotin–pentylamine–modified glutamine residue. *m/z* 396 overlaps with a b-ion. **m/z* corresponding to matrix adduct.

conditions to remove non-covalently bound IαI. The presence of FXIIIa significantly increased the amount of IαI present in the well following extensive washing (Fig. 6A). Similarly, a titration of FXIIIa was performed using a fixed concentration of IαI, and a dose-dependent relationship was observed (Fig. 6B).

The amount of cross-linked IαI leveled out at FXIIIa concentrations of greater than 25 μg/ml. The relatively low concentration of fibrinogen in the wells probably limited the number of available amine donor sites. The plasma concentration of IαI has been estimated as 600–1100 μg/ml [32], and the plasma concentration of FXIIIa is approximately 15 μg/ml [33]. Therefore, the concentration of FXIII in this experiment is similar to the plasma concentration, but the IαI concentration used is at least 30 times lower than the normal plasma concentration. Together, these results support the hypothesis that FXIIIa is able to cross-link IαI to fibrinogen at physiological concentrations.

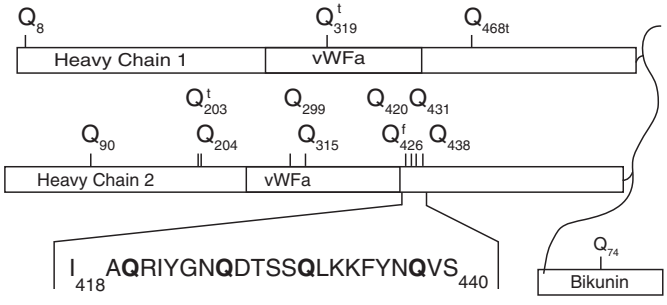

Fig. 5. Schematic representation of IαI with the reactive glutamine residues. Both TTG and FXIIIa utilize the majority of the identified sites. Three sites were only utilized by TTG (t), and one site was specific for FXIIIa (f). Reactive glutamine residues are found in all three subunits, indicating that all three can be cross-linked to other proteins. The inserted sequence is the hot spot found in HC2, which contained four reactive glutamine residues. vWFa is the von Willebrand factor type A domain.

3.5. Quantifying the relative amount of IαI in a plasma clot

To support the presence of FXIIIa-mediated cross-linking of IαI in plasma clots, we used a triple quadrupole instrument to quantify the amount of IαI relative to the known FXIIIa substrate α2-antiplasmin [20]. Extensive washing, reduction and denaturing gel electrophoresis were used to isolate the cross-linked proteins in a plasma clot. Four peptides for each IαI subunit and α2AP were selected for quantification based on SRM analysis. The relative amounts of each IαI subunit relative to α2AP were calculated (Fig. 7) from the obtained SRM data. Relative to α2AP (100%), there was 3.3% HC1, 5.2% HC2 and 6.1% Bikunin in the clot. Because the IαI subunits are cross-linked, cross-linking of a single subunit would result in cross-linking of all three subunits. The higher amount of HC2 and Bikunin compared with HC1 may result from hydrolysis of the PGP cross-link during sample preparation and/or the presence of the HC2/bikunin complex in the clot [1].

4. Discussion and conclusions

In this study, we show that IαI is a substrate for both TTG and FXIIIa. Incorporation of dansylcadaverine or biotin–pentylamine demonstrated the presence of reactive Gln residues (amine acceptors). We have determined the location of these residues and established that all three

Table 1
Identification of modification sites in IαI. The table summarizes the MALDI-MS and MS/MS data for the identified substrate sites (see Fig. 3). 'Reactive Q' corresponds to the position in the mature polypeptide. (') indicates the modified glutamine residue. The expected masses include the biotin–pentylamine modification and were calculated using the GPMAW software.

Polypeptide	Reactive Q	Peptide	FXIII	TTG	Measured	Expected	ppm
HC2	90	ALAQ'AR	+	+	1103.60	1103.60	0
	203	AHVSFKPTVAQ'Q'R	−	+	2091.11	2091.14	14
	204	AHVSFKPTVAQQ'R	+	+	1779.96	1779.97	6
	299	AEDHFSVIDFNQ'NIR	+	+	2116.05	2116.03	−9
	315	TQ'VADAKR	+	+	1199.65	1199.66	8
	420	LSNENHGIAQ'R	+	+	1549.77	1549.79	13
	426/431	IYGNQ'DTSSQ'LK	+	−	1976.01	1976.00	−5
	431	IYGNQDTSSQ'LK	+	+	1664.82	1664.83	6
	438	FYNQ'CSTPLLR	+	+	1648.91	1648.89	−12
HC1	8	Q'AVDTAVDGVFIR	+	+	1701.88	1701.90	12
	319	GSLVQASEANLQ'AAQDFVR	−	+	2315.17	2315.18	4
	468	Q'YYEGSEIVVAGR	−	+	1781.88	1781.89	6
Bikunin	74	ECLQ'TCR	+	+	1277.57	1277.58	8
FXIII	32	AVPPNNSNAAEDDLPTVELQ'GVVPR	+	−	2913.56	2913.55	−3
	246	AQ'MDLSGR	+	−	1188.59	1188.59	0
	484	FQ'EGQEEER	+	−	1462.66	1462.66	0
	724	HVYGELDVQ'IQR	+	−	1767.94	1767.92	−11
TTG	237	VVSAMVNCNDDQGVLQ'GR	−	+	2273.08	2273.07	−4
	470	EEAQ'EETGVAMR	−	+	1660.75	1660.77	12

subunits of IαI are substrates for transglutaminases. The majority of the sites have been highly conserved throughout evolution. In addition, we presented evidence for the in vitro cross-linking of IαI and fibrinogen.

Finally, we presented evidence for the cross-linking of IαI to the fibrin clot. It has previously been shown that approximately 30% of the α2AP present in plasma (1 μM) is cross-linked to the fibrin clot [34,35]. The normal human plasma concentration of fibrinogen is 9 μM, which gives a molar ratio of α2AP to fibrin of 3:100. Based on our quantification, the ratio of IαI to fibrin is approximately 1.5:1000. However, because IαI is known to stabilize the ECM and act as a protease inhibitor, a relatively small amount of IαI could affect clot formation and stability. Further studies are needed to characterize the cross-linking between IαI and fibrin and to identify the cross-linking sites to examine the effect of IαI on the covalent network in the clot.

These findings indicate that transglutaminases are able to catalyze the cross-linking of IαI and other proteins implicated in the organization of connective tissue, wound healing and fibrinolysis.

Studies of the biological function of the HCs have been focused on the interaction of HCs with hyaluronan and the importance of the HC–HA complex during development of the cumulus matrix [36–38]. HC2/TSG-6 transfers the HCs from bikunin chondroitin-4-sulfate to HA through transesterification reactions involving Ser28 of TSG-6 [13,39,40]. However, the molecular mechanisms by which the transferred HCs stabilize the ECM are not understood. It seems that both HC–HA complexes [16,36] and IαI are important for the TSG-6-independent formation of HA-rich ECM and cell adhesion [41,42]. The self-associated IαI/HA ECM is likely to become more stable because of the introduction of transglutaminase-generated cross-links between HCs and/or other HA binding proteins.

Wound healing involves inflammation, proliferation and remodeling [43], which all involve the activity of transglutaminases [44,45]. Cross-linking of IαI to the tissue during wound healing is likely to influence the wound healing process. During inflammation and proliferation, cross-linking of the HCs to the ECM might provide a

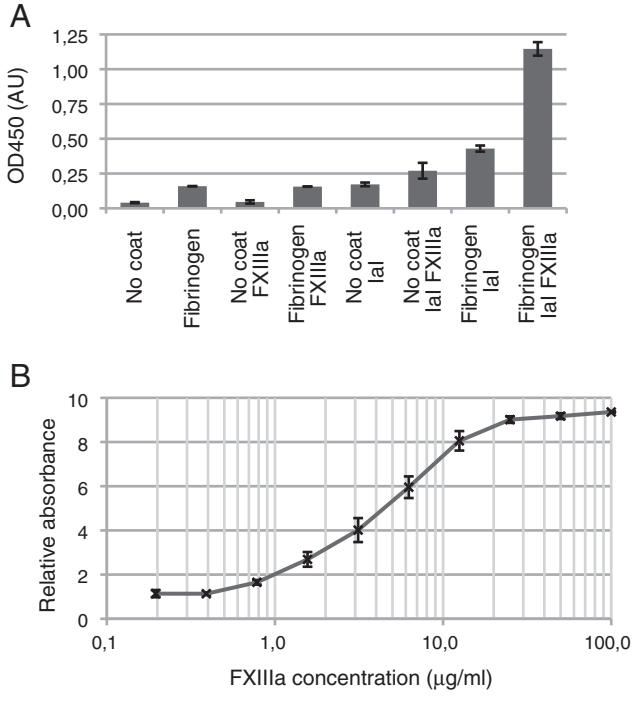

Fig. 6. IαI is cross-linked to fibrinogen by FXIIIa. A) A microtiter plate was coated with or without (No coat) fibrinogen. Fixed amounts of IαI and FXIIIa were added as indicated. B) A fixed amount of IαI (20 μg/ml) was cross-linked to fibrinogen using increasing concentrations of FXIIIa (0.2–100 μg/ml). The graph shows the absorbance relative to a sample without FXIIIa. Cross-linked IαI was detected using a rabbit anti-IαI antibody. The indicated values are the averages of three measurements, and the standard deviation is indicated.

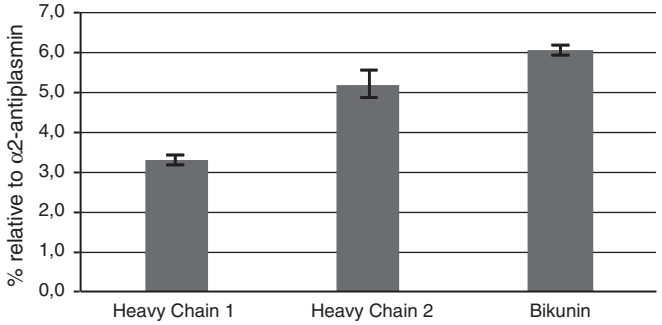

Fig. 7. Relative quantification of IαI in a plasma clot. The covalently cross-linked proteins in a plasma clot were isolated, and the three IαI subunits were quantified relative to α2AP using a triple quadrupole mass spectrometer. The measurements are based on the total intensity of four peptides from each subunit/protein with three technical replicates. The standard deviation is indicated.

scaffold for migrating cells, a characteristic previously described for the HC–HA complex [16,41]. Perforation of the skin allows bacteria to gain access to a wound. These bacteria are normally removed by neutrophils during inflammation [43], but cross-linking of IαI and particularly bikunin during wound healing might provide an initial level of protection against adventitious proteolysis from bacterial or endogenous proteases.

The inhibitory capacity of bikunin has been shown to be low relative to its concentration; thus, the physiological relevance of bikunin-mediated inhibition of several proteases, including plasmin, has been questioned [8]. Therefore, it is not likely that circulating IαI plays a role as a fibrinolysis inhibitor. The action of FXIIIa could potentiate the inhibitory activity of bikunin in two ways; i) bikunin directly cross-linked to fibrin could generate a higher local concentration of bikunin or increase plasmin inhibition as seen in its interaction with TSG-6 [9–11] and ii) because plasmin(ogen) contains at least one transglutaminase reactive Lys and Gln residue [46], it is possible that cross-linking of bikunin to plasmin(ogen) results in enhanced inhibition.

The observations made in this study suggest that transglutaminases may generate a covalent bond between IαI and other proteins, in addition to the covalent bond to glycosaminoglycans that is mediated by TSG-6. Cross-linking between IαI and other ECM proteins may increase the mechanical and proteolytic stability of the ECM.

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