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Human Complement C3 Is a Substrate for Transglutaminases. A Functional Link between Non-Protease-Based Members of the Coagulation and Complement Cascades

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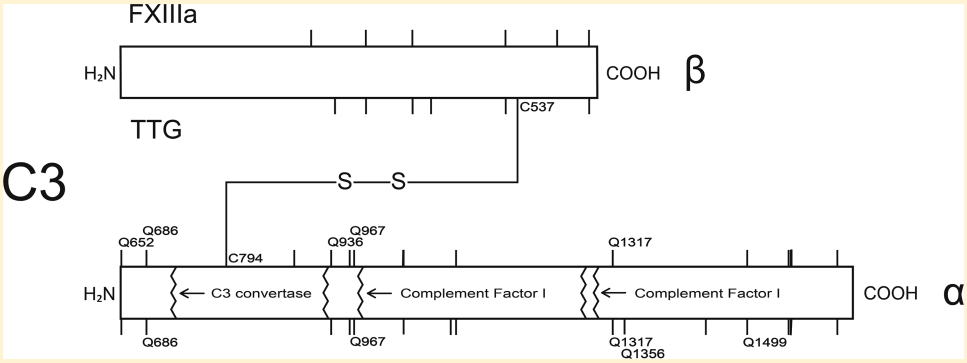

ABSTRACT: In this study, we report the finding of functional cross-talk between two non-protease components of the complement and coagulation cascades. We show that complement C3, a central component of the complement system, is associated with the fibrin clot and that C3 becomes covalently cross-linked to other proteins during coagulation. Enzymatic incorporation of dansylcadaverine and dansyl-PGGQQIV into C3 by coagulation factor XIIIa and tissue transglutaminase demonstrated that C3 is a transglutaminase substrate. This suggested that coagulation factor XIIIa covalently cross-links C3 to clot components during coagulation. Using mass spectrometry, we verified that C3 indeed is covalently associated with the fibrin clot in a ratio of 0.05:1 relative to the known coagulation factor XIIIa substrate α2-antiplasmin.

Proteolytic cascades are the foundation for several physiological processes in which rapid amplification of starting signals and tight regulation of activity are needed. Two of the most well-known proteolytic cascades are those of the coagulation and complement systems. Both are part of a primary defense that ensures homeostasis during tissue injury and wound healing. While coagulation limits blood loss and maintains tissue structure, the complement is primed for the recognition of pathogens and damaged cells and initiation of an effective immune response. When uncontrolled as during sepsis, activation of both the coagulation and complement system causes devastating damage to the host.[1]

Coagulation factor XIIIa (FXIIIa) is one of the final protease substrates of the coagulation cascade. FXIIIa is a transglutaminase (TG) and is by definition able to catalyze Ca^{2+}-dependent transamidation reactions. The most well-known of these is the formation of an isopeptide bond between a Gln residue, the amine acceptor, and a Lys residue, the amine donor, in two different proteins. This covalent cross-link generally serves to increase the mechanical stability and proteolytic resistance of protein complexes.[2] During coagulation, activated FXIIIa cross-links fibrin in the blood clot into highly insoluble polymers.[3] The severe bleeding diathesis caused by FXIIIa deficiency demonstrates the importance of this event for the generation of a stable clot.[4] FXIIIa is also able to cross-link other proteins to the fibrin clot and hereby localize their functions to the site of coagulation. These proteins include among others α_2-antiplasmin,[5] fibronectin,[6] thrombin-activatable fibrinolysis inhibitor,[7] and inter-α-inhibitor.[8] Localization of protease inhibitors such as α_2-antiplasmin is believed to protect the clot against proteolytic degradation.[5] The incorporation of fibronectin into the fibrin clot meanwhile influences cell adhesion and migration.[6]

Complement C3 is the convergence point for all complement activation pathways. During complement activation, C3 is cleaved into two functional fragments named C3a and C3b. The cleavage induces structural rearrangements in C3b that lead to exposure and activation of a reactive thioester group. The activated thioester can react with hydroxyl and amine groups in carbohydrates and proteins. This leads to covalent attachment of C3 to cell surfaces, mainly through ester bonds.[9] Surface-attached C3b and degradation products of C3b are able to promote phagocytosis, influence immune cell activity, and participate in the formation of protease complexes for amplification and completion of the cascade.[10] C3a is an

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anaphylatoxin and serves as an important inducer of inflammation.[10]

The structures of the complement and the coagulation cascades are similar, and it is believed that the cascades originate from a common ancestor.[11] On a functional level, there are further similarities and several proteases have in fact been shown to cleave and activate components of both pathways.[12,13] One important result of this functional cross-talk seems to be simultaneous activation of both cascades.[14]

In this study, we report the finding of another functional link between the coagulation and complement systems. We have found that the central complement component C3 is associated with the clot and that C3 is covalently modified during coagulation. We show that C3 is a FXIIIa substrate and that C3 can act as both an amine donor and an amine acceptor during TG-catalyzed reactions. Some of the clot-incorporated C3 survives treatment with guanidine hydrochloride and acid as well as boiling in sodium dodecyl sulfate (SDS) and dithiothreitol (DTT), suggesting that the protein is covalently linked to components of the clot. We show that covalently linked C3 is present in a ratio of 0.05:1 compared to the highly efficient FXIIIa substrate α_2-antiplasmin. This suggests that FXIIIa is able to localize C3 and C3 specific functions to the site of coagulation in a manner similar to that of α_2-antiplasmin.

EXPERIMENTAL PROCEDURES

Materials. Dansylcadaverine (DSC) was purchased from Biochemika. Dansyl-PGGQQIV (DSP) was from New England Peptide. EZ-link pentylamine-biotin and immobilized monomeric avidin were from Thermo Scientific. Polyethylene glycol 3350 was from Sigma. Polyclonal rabbit anti-human C3c antibody was from Dako. Secondary polyclonal sheep anti-rabbit Cy3-labeled antibody was from Sigma. Recombinant human FXIIIa was a kind gift from Sanofi Aventis. Plasminogen and α_2-antiplasmin were purified from human plasma as described previously.[15,16] Guinea pig tissue transglutaminase (TTG), human thrombin, and trypsin were purchased from Sigma.

Purification of Complement C3. Complement C3 was purified as previously described with minor alterations.[17] Freshly drawn plasma from a healthy donor was made 15% in polyethylene glycol 3350 and 25 nM in *p*-nitro-*p*′-guanidinobenzoate and incubated for 30 min on ice interrupted by brief vortexing. The pellet was collected by centrifugation at 33000*g* for 10 min at 5 °C and dissolved in 3 mM KH_2PO_4, 6.5 mM ethylenediaminetetraacetic acid (EDTA), 33 mM *ε*-amino-*n*-caproic acid, and 50 mM NaCl (pH 7.3) (buffer A). The sample was applied to a Mono Q 5/50 Gl anion exchange column (Amersham Biosciences) connected to an AKTA purifier system (Amersham Biosciences) equilibrated in buffer A. The column was eluted using a linear gradient from buffer A to 24% buffer B (buffer A containing 1 M NaCl) at a flow rate of 0.25 mL/min. Fractions containing C3 were pooled on the basis of Western blotting and diluted 10 times in 20 mM Tris-HCl (pH 7.4). The sample was then applied to a 5 mL Hitrap Heparin HP column (GE Healthcare) connected to an AKTA prime system (Amersham Biosciences) equilibrated in 20 mM Tris-HCl (pH 7.4). C3 was eluted using a 50 min 0 to 100% linear gradient of 20 mM Tris-HCl and 1 M NaCl (pH 7.4). Fractions containing C3 were pooled and dialyzed against 20 mM Tris-HCl and 137 mM NaCl (pH 7.4) and concentrated using Amicon Ultra Ultracel 10 K centrifugal filters (Millipore).

Identification of C3 Released from a Plasma Clot. Blood was collected into Eppendorf tubes from healthy donors by fingerstick. The blood was fractionated by centrifugation at 1900*g* for 1.5 min. The plasma fraction was decanted and allowed to clot for 3 h at 37 °C. The clot was then removed and washed for 3 × 20 min in 8 M urea, 150 mM NaCl, and 50 mM Tris-HCl (pH 7.4). The remaining clot was boiled for 10 min in sample buffer containing 2% SDS and 30 mM DTT and analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting.

Incorporation of DSC or DSP into C3 by FXIIIa or TTG. FXIIIa was activated by incubation with thrombin (1 milliunit of thrombin/μg of FXIIIa) in 20 mM Tris-HCl and 137 mM NaCl (pH 7.4) for 1 h at 37 °C. A fixed concentration of C3 (2.5 μg) was incubated with DSC or DSP in the presence of increasing amounts of either activated FXIIIa or TTG (0.07, 0.14, 0.28, or 0.11 μg). The reaction was conducted in 20 mM Tris-HCl and 137 mM NaCl (pH 7.4) containing 10 mM $CaCl_2$ and 0.5 mM DTT containing either 0.6 mM DSC or 0.6 mM DSP and allowed to continue for 1 h at 37 °C. EDTA was then added to each sample to a final concentration of 15 mM, and the samples were analyzed by SDS−PAGE using 5 to 15% acrylamide gradient gels for the DSC samples and 7.5% gels for DSP samples. The gels were scanned using UV trans-illumination and an orange filter (595 nm, 50 nm) FluorChem Q system (Cell Biosciences). α_2-Antiplasmin (2 μg) was used as an amine acceptor control, and plasminogen (2 μg) was used as the amine donor control.

SDS−PAGE and Western Blotting. Samples were boiled in SDS sample buffer containing 30 mM DTT. SDS−PAGE was performed in 5 to 15% gradient or 7.5% gels (10 cm × 10 cm × 0.15 cm) using the glycine/2-amino-2-methyl-1,3-propanediol-HCl system previously described.[18] For Western blotting, proteins were transferred to polyvinylidene fluoride membranes as previously described.[19] The membranes were blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.1% Tween (pH 7.4) (TBS-T) containing 5% dry milk overnight at 4 °C. The rabbit anti-human C3c antibody was added, and the blot was incubated for 2 h at 23 °C. The membrane was then washed for 3 × 15 min in TBS-T before the secondary anti-rabbit Cy3-labeled antibody was added in TBS-T containing 5% dry milk. The blot was incubated for 2 h, after which an image of the blot was acquired on a FluorChem Q system (Cell Biosciences).

Mass Spectrometry. NanoESI-MS/MS analyses were performed on an EASY-nLC II system (ThermoScientific) connected to a TripleTOF 5600 mass spectrometer (AB Sciex) equipped with a NanoSpray III source (AB Sciex) operated under the control of Analyst TF version 1.5.1. The trypsin-digested samples were suspended in 0.1% formic acid, injected, trapped, and desalted isocratically on a ReproSil-Pur C18-AQ column [5 μm, 2 cm × 100 μm (inside diameter) (Thermo Scientific)], after which the peptides were eluted from the trap column and separated on an analytical ReproSil-Pur C18-AQ capillary column [3 μm, 10 cm × 75 μm (inside diameter) (Thermo Scientific)] connected in-line to the mass spectrometer at a rate of 250 nL/min using a 50 min gradient from 5 to 35% phase B (0.1% formic acid and 90% acetonitrile).

Identification of Reactive Residues Using Pentylamine-Biotin. C3 was incubated with either FXIIIa or TTG at a 5:1 ratio (w/w) in 20 mM Tris-HCl and 137 mM NaCl (pH 7.4) containing 10 mM $CaCl_2$, 0.5 mM DTT, and 10 mM

pentylamine-biotin. After 6 h at 37 °C, the reaction was stopped by the addition of EDTA.

The labeled samples were denatured, reduced, and alkylated in 20 mM Tris-HCl and 6 M guanidine-HCl (pH 8) containing 5 mM DTT followed by the addition of iodoacetamide to a concentration of 10 mM. The reduced and alkylated samples were dialyzed into 20 mM NH_4HCO_3 and digested with trypsin (1:25, w/w) at 37 °C for 16 h. The remaining trypsin activity was inhibited by adding phenylmethanesulfonyl fluoride to a final concentration of 1 mM.

The samples were then dried using a speedvac (Savant), redissolved in 100 mM NaH_2PO_4 and 150 mM NaCl (pH 7), and applied to a monomeric avidin affinity column equilibrated in 100 mM NaH_2PO_4 and 150 mM NaCl (pH 7). After being extensively washed, the pentylamine-biotin-labeled peptides were eluted using 100 mM glycine (pH 2.8) and desalted on C18 stage tips (Proxion) before mass spectrometry analysis.

The collected data were processed using Analyst TF version 1.5.1 (AB Sciex) to generate the peak list used to query the Swiss-Prot database using the Mascot search engine (Matrix Science, Ltd., London, U.K.). The following search parameters were used: *Homo sapiens*, trypsin, two missed cleavages, carbamidomethyl (Cys) fixed modification, oxidation (Met) variable modification, and 2+, 3+, and 4+ charges. Peptide and MS/MS tolerances were set to 10 ppm and 0.6 Da, respectively. For reactive residue identification, pentylamine-biotin (Gln) variable modification was used. Three signature ion fragments at m/z 329, 395, and 440 arise from fragmentation at the site of biotin modification. All spectra were inspected manually. Peptides were only accepted if they contained three sequential y- or b-ions of high intensity and pentylamine-biotin signature ion fragments.

Relative Quantification of C3 Levels in a Plasma Clot. Blood was drawn by fingerstick from a healthy donor and fractionated by centrifugation at 1900*g* for 1.5 min. The plasma fraction was decanted and allowed to clot for 3 h at 37 °C. The clot was removed and washed extensively for 3 × 20 min in each of the following buffers: (i) 20 mM Tris-HCl and 137 mM NaCl (pH 7.4), (ii) 10% acetic acid, (iii) 6 M guanidine-HCl, 2 M NaCl, and 50 mM Tris-HCl (pH 7.4), and (iv) H_2O. The remaining clot was boiled for 10 min in 2% SDS containing 5 mM DTT and alkylated by adding iodoacetamide to a final concentration of 15 mM. The sample was separated by SDS–PAGE. Following electrophoresis, the protein material on the top of the stacking gel was prepared for in-gel digestion using a microspin column.[20,21] The sample was digested with trypsin (1:40, w/w) for 16 h and desalted using stagetips (Proxeon). Three technical replicates were analyzed by mass spectrometry. Quantitation was performed using a Mascot distiller version 2.4 workstation (Matrix Science) using standard settings for a Q-TOF instrument. Raw MS data were processed using Mascot Distiller for peak detection, and processed spectra were searched against the Swiss-Prot database using the Mascot search engine (Matrix Science). Search parameters were restricted to *H. sapiens*, trypsin, two missed cleavages, carbamidomethyl (Cys) fixed modification, and oxidation (Met) variable modification. Peptide and MS/MS tolerances were set to 10 ppm and 0.6 Da, respectively. Average MD was used as the quantification protocol.[22] From the search and peak picking, the three most intense peptides were used to find the average protein intensity that was then used to calculate the ratio of α_2-antiplasmin to C3.

RESULTS

C3 Is Associated with the Clot and Covalently Modified during Coagulation. A clot was generated from freshly drawn plasma. The clot was washed extensively in urea to remove loosely associated proteins, boiled in SDS sample buffer containing 30 mM DTT, and analyzed by SDS–PAGE followed by Western blotting using an anti-human C3 antibody (Figure 1, left lane). The results showed that the α- and β-

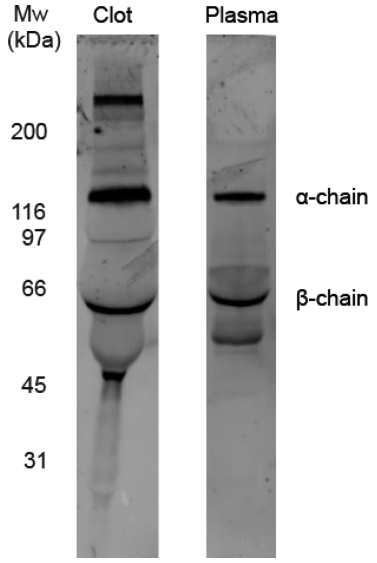

Figure 1. C3 is associated with the plasma clot. A plasma clot was washed in urea, boiled in SDS sample buffer containing DTT, and analyzed by SDS–PAGE followed by Western blotting using an anti-human C3 antibody. The resulting sample is shown in the left lane. The right lane shows a plasma control sample. Approximate molecular mass markers (Mw) are included. The left lane shows that the α- and β-chains of C3 are released from the clot and that the migration of the chains is identical to that of plasma C3. In addition to this, some C3 with an unexpectedly high molecular mass is identified in the left lane.

chains of C3 were released from the clot and migrated into the SDS–PAGE gel at the expected molecular masses compared to a plasma control sample (Figure 1, right lane). Significantly, high-molecular mass C3 was also observed. The corresponding high-molecular mass bands were excised from an identical SDS–PAGE gel and analyzed by mass spectrometry. This analysis identified both the α- and β-chains of C3 in all bands (data not shown). Because the high-molecular mass C3 resisted dissociation by urea, SDS, and DTT, it is likely that C3 had become covalently linked to other proteins during coagulation. On the basis of the obtained results, we hypothesized that C3 is a TG substrate and that C3 is cross-linked to other clot-associated proteins by FXIIIa during coagulation.

C3 Is a Transglutaminase Substrate. To investigate if C3 is indeed a TG substrate, we incubated the purified protein with DSC and increasing amounts of either of the two TGs, FXIIIa or TTG (Figure 2A,B). DSC is a widely used fluorescent amine donor in TG-catalyzed reactions and can be used to verify the presence of reactive Gln residues in a potential substrate.[23] The samples were incubated for 1 h, and the reaction was then stopped via addition of EDTA. The DSC-labeled samples were analyzed by reduced SDS–PAGE, and the incorporation of DSC was followed by UV transillumination. The data revealed that DSC is incorporated into C3 in a TG-dependent manner

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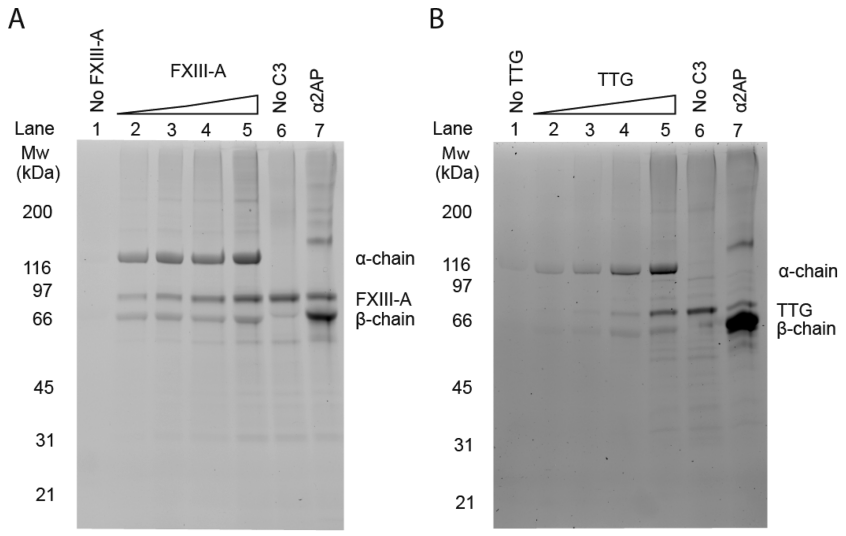

Figure 2. Dansylcadaverine is incorporated into C3 by FXIIIa and TTG. A fixed concentration of C3 was incubated with dansylcadaverine (DSC) and titrated with increasing amounts of either FXIIIa (A) or TTG (B). α_2-Antiplasmin (α2AP) was used as a positive control. The reaction was stopped after 1 h by the addition of EDTA. The samples were analyzed by reduced SDS−PAGE using UV transillumination to visualize the incorporation of DSC. Approximate molecular mass markers (Mw) are included on the left side of the gel. TG-dependent incorporation of the amine donor, DSC, into complement C3 is evident.

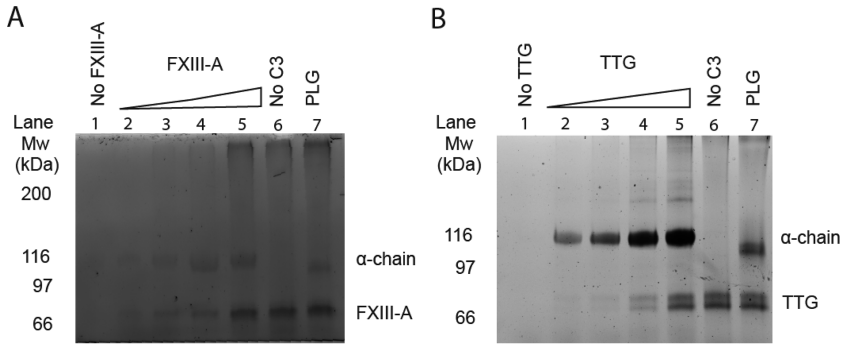

Figure 3. Dansyl-PGGQQIV is incorporated into C3 by FXIIIa and TTG. A fixed concentration of C3 was incubated with dansyl-PGGQQIV (DSP) and titrated with increasing amounts of either FXIIIa (A) or TTG (B). Plasminogen (PLG) was used as a positive control. The reaction was stopped after 1 h by the addition of EDTA. The samples were analyzed by reduced SDS−PAGE using UV transillumination to visualize the incorporation of DSC. Approximate molecular mass markers (Mw) are included on the left side of the gel. The bottom part of the gel was covered to reduce the fluorescence from surplus DSP. TG-dependent incorporation of the amine acceptor, DSP, into complement C3 is evident.

(Figure 2). Thus, C3 contains reactive Gln residues and can be subjected to TG-catalyzed modifications. Both the α- and β-chains of C3 are reactive, although the level of DSC incorporation seems to be lower for the β-chain. The level of incorporation of DSC into C3, in the case of the FXIIIa-catalyzed reaction, is comparable to that into the control substrate, α_2-antiplasmin (Figure 2, lanes 5 and 7). As the concentrations of FXIIIa and TTG increase, fluorescent high-molecular mass species appear. These must be polymerization products consisting of either C3 alone or mixed protein complexes.

An experiment similar to the one described above was performed using C3 and the fluorescent amine acceptor DSP (Figure 3A,B). DSP consists of a fluorescent group coupled to a TG-reactive peptide with the sequence PGGQQIV.[24] C3 was incubated with DSP to investigate if it also contains reactive Lys residues. The results showed TG-dependent incorporation of DSP into C3 (Figure 3). In this case, only the α-chain of C3 seems to be reactive. The level of DSP incorporation into C3 by FXIIIa is low, and C3 does not seem to be an efficient amine donor (Figure 3A, lanes 2−5). The incorporation of DSP into C3 by TTG is more efficient (Figure 3B, lanes 2−5). Polymerization and autocatalysis are evident with both FXIIIa and TTG (Figure 3A,B, lanes 5 and 6, respectively).

Identification of Reactive Residues in C3. C3 was labeled with pentylamine-biotin using either FXIIIa or TTG. Pentylamine-biotin contains a primary amine and was used to identify reactive Gln residues. The biotin-labeled C3 was digested with trypsin, and the labeled peptides were purified using a monomeric avidin resin. The purified peptides were then analyzed using nLC−MS/MS, and the resulting mass spectra were searched against the Swiss-Prot database using a local Mascot search engine (Tables 1 and 2). All spectra were inspected manually. The identified reactive residues are underlined and are numbered according to the position in the mature protein. C3 contains 21 FXIIIa-reactive Gln residues (Table 1) and 23 TTG-reactive Gln residues (Table 2). Of these reactive residues, 18 are shared by FXIIIa and TTG. Known reactive residues in FXIIIa were also identified (data not shown).[8] The spectral count includes all peptides identified containing the particular reactive residues and has also been listed (Table 1). The spectral count depends on both the

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Table 1. Identification of FXIIIa Substrate Sites in C3[a]

reactive residue	polypeptide chain	score	spectral count	sequence
Q258	β	33	3	KVEGTAFVIFGIQDGE**Q**R
Q332	β	80	3	SGIPIVTSPY**Q**IHFTK
Q395	β	93	3	LSINTHPS**Q**KPLSITVR
Q521	β	35	1	LVAYYTLIGASG**Q**R
Q591	β	22	4	LT**Q**SK
Q634	β	166	2	ADIGCTPGSGKDYAGVFSDAGLTFTSSSGQQTA**Q**R
Q652	α	34	10	SV**Q**LTEK
Q686	α	53	22	FSC**Q**R
Q886	α	58	3	SSLSVPYVIVPLKTGL**Q**EVEVK
Q936	α	133	21	EGV**Q**K
Q961, Q967	α	35	2	ILL**Q**GTPVA**Q**MTEDAVDAER
Q967	α	140	19	ILLQGTPVA**Q**MTEDAVDAER
Q1033	α	75	1	KGYT**Q**QLAFR
Q1034	α	22	2	KGYTQ**Q**LAFR
Q1105	α	95	2	QKPDGVFQEDAPVIH**Q**EMIGGLR
Q1317	α	128	10	G**Q**GTLSVVTMYHAK
Q1499	α	72	4	CAEENCFI**Q**K
Q1555, Q1558/1559	α	52	3	SGSDEV**Q**VG*QQ*R
Q1555, Q1558, Q1559	α	38	1	SGSDEV**Q**VG**QQ**R
Q1558, Q1559	α	69	3	SGSDEVQVG**QQ**R
Q1558/1559	α	89	2	SGSDEVQVG*QQ*R
Q1559	α	83	4	SGSDEVQVG**Q**QR
Q1621	α	121	2	DTWVEHWPEEDECQDEEN**Q**K

[a]The labeled residues have been underlined and are shown in bold if the residue was unambiguously assigned. If the biotin label could not be assigned to a specific residue, the possible labeling sites are shown underlined and in italics. The score recorded is for the highest-scoring peptide containing the residue. The spectral count includes all identified peptides containing the specific reactive residue.

Table 2. Identification of TTG Substrate Sites in C3[a]

reactive residue	polypeptide chain	score	spectral count	sequence
Q290	β	39	5	VLLDGV**Q**NPR
Q332	β	58	3	SGIPIVTSPY**Q**IHFT
Q395	β	93	4	LSINTHPS**Q**KPLSITVR
Q420	β	110	1	TM**Q**ALPYSTVGNSNNYLHLSVLR
Q521	β	57	2	LVAYYTLIGASG**Q**R
Q634	β	186	6	ADIGCTPGSGKDYAGVFSDAGLTFTSSSGQQTA**Q**R
Q652	α	34	9	SV**Q**LTEK
Q686	α	17	23	FSC**Q**R
Q936	α	110	8	EGV**Q**K
Q961, Q967	α	97	2	ILL**Q**GTPVA**Q**MTEDAVDAER
Q967	α	142	49	ILLQGTPVA**Q**MTEDAVDAER
Q1033	α	30	1	GYT**Q**QLAFR
Q1034	α	36	1	GYTQ**Q**LAFR
Q1097	α	89	1	QKPDGVF**Q**EDAPVIHQEMIGGLR
Q1105	α	114	4	QKPDGVFQEDAPVIH**Q**EMIGGLR
Q1317	α	89	13	G**Q**GTLSVVTMYHAK
Q1356	α	79	14	RP**Q**DAK
Q1443	α	54	5	VH**Q**YFNVELIQPGAVK
Q1499	α	71	14	CAEENCFI**Q**K
Q1555, Q1558/Q1559	α	69	1	SGSDEV**Q**VG**QQ**R
Q1555, Q1558, Q1559	α	24	1	SGSDEV**Q**VG**QQ**R
Q1558	α	83	5	SGSDEVQVG**Q**QR
Q1558/1559	α	82	8	SGSDEVQVG*QQ*R
Q1558, Q1559	α	74	6	SGSDEVQVG**QQ**R
Q1621	α	138	2	DTWVEHWPEEDECQDEEN**Q**K

[a]The labeled residues have been underlined and are shown in bold if the residue was unambiguously assigned. If the biotin label could not be assigned to a specific residue, the possible labeling sites are shown underlined and in italics. The score recorded is for the highest-scoring peptide containing the residue. The spectral count includes all identified peptides containing the specific reactive residue.

ionization efficiency and the concentration of the analyzed peptides and is therefore not a truly quantitative measure of peptide abundance. The spectral count can provide an only rough indication of the peptide abundance and consequently

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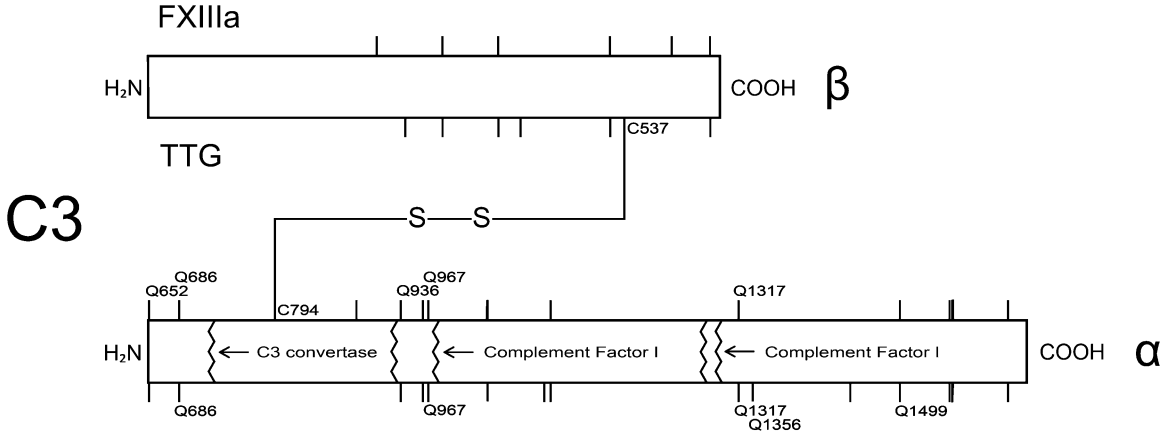

Figure 4. Schematic representation of C3 with reactive residues. The figure shows a simplified schematic representation of the chain structure of C3. C3 consists of two polypeptide chains, α and β, coupled by a single interchain disulfide bridge. Important cleavage sites in the α-chain are marked, and the responsible proteases are listed in the figure. FXIIIa and TTG-reactive residues are marked with lines above (FXIIIa) and below (TTG) the chains. The major reactive Gln residues and the Cys residues connecting the chains are numbered according to the numbering of the mature protein.

the residue reactivity. Reactive residues with spectral counts above 10 are considered possible major reactive residues. The major FXIIIa-reactive residues are Q652, Q686, Q936, Q967, and Q1317, while residues Q686, Q967, Q1317, Q1356, and Q1499 are the major TTG-reactive residues.

A simplified schematic representation of the polypeptide chain structure of C3, including the identified reactive residues, has been generated (Figure 4). C3 consists of two polypeptide chains, and one of them, the α-chain, contains several protease cleavage sites. These cleavage sites are important for the activation and inactivation of the protein and are marked (Figure 4). The reactive Gln residues are shown with FXIIIa-reactive residues above the chains and TTG-reactive residues below the chains. Reactive Gln residues were identified within both chains. This is consistent with the result obtained in the previous section (Figure 2A,B). Fewer reactive residues were identified in the β-chain. This could explain the observed lower reactivity of the β-chain. The reactive Q652 is situated in activation peptide C3a. Reactive residues are also present in fragments C3d and C3g that are cleaved off the main C3 molecule by complement factor I when C3b is converted to C3c.[9]

Identification and Relative Quantification of C3 in the Clot. Considering the association of C3 with the clot and the possible covalent modification of C3 by FXIIIa during coagulation, we wished to investigate if C3 is covalently incorporated into the clot. We therefore generated a plasma clot from freshly drawn plasma and washed it extensively to remove noncovalently attached proteins. Clot complexes that could not be dissolved by SDS and DTT were isolated using denaturing gel electrophoresis. The isolated material was digested with trypsin and analyzed by mass spectrometry to simultaneously identify C3 and quantify the amount of C3 in the clot relative to α_2-antiplasmin. The resulting data were searched and quantified with Mascot Distiller using the average method.[22] The intensities of the three most intense peptide ions from C3 and α_2-antiplasmin were calculated from integration of extracted ion chromatograms and used to calculate the average intensity for each protein. The average intensities were compared to provide an estimate of the relative amounts of the two proteins. C3 was identified in an amount corresponding to 5% ($\pm$0.8) of the amount of α_2-antiplasmin. Thus, C3 is also covalently associated with the clot.

DISCUSSION

In this study, we present evidence that C3 can serve as a FXIIIa and a TTG substrate. TG-dependent incorporation of DSC and DSP demonstrated that C3 contains both reactive Gln and Lys residues. The localizations of the reactive Gln residues were determined. Both the α- and β-chains of C3 contain reactive residues, and 18 of these are shared by FXIIIa and TTG. Not all of these residues can be expected to be equally reactive in vivo because of substrate competition. Judging by the number of times the single reactive residues have been identified, there seems to be a preference of the TGs for a few of these residues. The major FXIIIa-reactive residues are Q652, Q686, Q936, Q967, and Q1317.

We furthermore show that C3 is associated with the plasma clot. C3 and some high-molecular mass C3 species were released from the clot and identified using Western blotting. This C3 seems to be less tightly associated with the clot and could include incompletely cross-linked protein complexes generated during coagulation. C3 was also identified in the highly insoluble clot material in a ratio of 0.05:1 relative to α_2-antiplasmin. This suggests that some C3 is covalently cross-linked to the clot. Cross-linking of a small percentage of C3, whatever the effect may be, can have a large impact on complement activity at the site of coagulation because of the amplification of the cascade.

There is evidence that C3 can be activated by proteases belonging to the coagulation cascade in vitro.[25] This implies that C3 could become covalently attached to clot components through the activated thioester. To rule out the use of an ester bond, we therefore washed the clot extensively with hydroxyl amine, which efficiently breaks this bond.[26] C3 was still identified in the cross-linked material, suggesting that it remained associated with clot components through a covalent non-ester linkage (data not shown).

General cross-talk between proteases of complement and coagulation has been described previously.[14] Here we show the existence of cross-talk between two non-protease components. C3 specifically has previously been associated with coagulation events. One study showed that C3 is present in acute coronary thrombi obtained from patients with myocardial infarction.[27] This study also showed that complement activation products, C3a among others, are generated at the site of thrombus

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formation. Another study showed that C3 binds to plasma clots and clots generated from purified fibrinogen. C3 was shown to affect fibrin network structure and seems to have a negative effect on the proteolysis of the clot in vitro.[28] Both studies indicate the proximity of C3, clot components, and activated FXIIIa, but neither of the studies addressed the possibility of covalent cross-links being generated between C3 and clot components. Here we show that C3 is indeed a FXIIIa substrate and that C3 is covalently incorporated into the clot during blood coagulation.

Wound healing involves inflammation, tissue formation, and tissue remodeling, and both coagulation and complement are part of the first of these events.[29] TGs, particularly TTG and FXIIIa, are active during all events of wound healing.[30,31] In addition to cross-linking fibrin during coagulation, FXIIIa also cross-links other proteins of importance to the generated clot. FXIIIa is hereby able to alter the characteristics of the provisional matrix during wound healing. A recent study provided evidence of FXIIIa-mediated protection against infection in a mouse skin infection model.[32] This might be a result of the cross-linking and consequential entrapment of bacteria, but it also seems to involve the immune system and possibly the complement system. Activated C3 and related C3 fragments have functions in complement amplification and signaling for inflammatory responses, phagocytosis, and lysis of pathogens and damaged cells and B-cell stimulation.[10] All of these functions may be important during wound healing, and it is likely that the localization of C3 to the clot by FXIIIa is able to prime the wound environment for induction of an immune response and for removal of bacteria and damaged cells.

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ABBREVIATIONS

FXIIIa, coagulation factor XIIIa; TG, transglutaminase; TTG, tissue transglutaminase; DSC, dansylcadaverine; DSP, dansyl-PGGQQIV; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

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