The Catalytically Active Serine Protease Domain of Human Complement Factor I†

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ABSTRACT: Factor I (fI) is a major regulator of complement. As a protease it has very restricted specificity, cleaving only C3b or C4b in the presence of a cofactor such as factor H (fH). Cleavage of C3b by fI yields iC3b, a major opsonin. The cleavage occurs through the formation of a ternary complex between the enzyme, the substrate, and the cofactor. The catalytic subunit of fI, the SP domain, accommodates substrate recognition and cleavage. The role of the fI heavy chain within the catalysis complex is unknown. Using partial proteolysis and affinity chromatography an intact form of the SP domain was generated and isolated from fI in high yield. fI and the SP domain were found to have similar amidolytic activities but strikingly different proteolytic activities on C3(NH_3). fI did not cleave C3(NH_3) in the absence of fH, while in its presence it cleaved C3(NH_3) rapidly at two sites. The SP domain, however, slowly cleaved C3(NH_3) in the absence of fH, at more than two sites. Cleavage by the SP domain was inhibited, not stimulated, by fH. Pefabloc SC and antipain inhibited the proteolytic activity of both fI and the SP domain, but suramin inhibited only fI and not the SP domain. The contrast in the proteolytic activities suggests that the heavy chain domains and the cofactor must have roles in orienting the natural substrates and restricting cleavage to the two sites which yield iC3b through a highly specific catalysis.

The complement system is the chief recognition and effector component of innate immunity that participates in inflammation and acts to enhance the adaptive immune response (*1*). The role of complement is to recognize, opsonize, and promote clearance of nonhost or altered-host materials, such as microorganisms and damaged cells. Seven serine proteases, factor D (fD),[1] MASP-2, C1s, C1r, factor B (fB), C2, and factor I (fI), play key roles in the generation of complement activities in the amplification and regulation of cascade reactions (*2*−*5*). Two additional homologues of MASP-2, namely, MASP-1 and MASP-3, have been identified, but their roles in complement activation have not yet been determined. All of the proteases contain serine protease (SP) domains homologous to the trypsin family and, except fD, have additional protein modules that influence the orientation and localization of protein substrates and mediate complex formation through protein−protein interactions.

Complement fI regulates the turnover of C3, the most abundant complement protein (*4*, *5*). It converts C3b into iC3b, a major opsonin (*6*, *7*). The human fI gene (accession numbers: cDNA Y00318, genomic X78594) is localized on chromosome 4q25.

fI has restricted specificity limited to cleavage of arginyl bonds in its natural protein substrates C3b and C4b. Additional components such as factor H (fH), CR1, MCP, or C4bp are required as cofactors. During natural substrate cleavage, fI forms a ternary complex with the substrate and the cofactor (*8*, *9*). However, fI has been shown to be active in the cleavage of synthetic amide substrates in the absence of any cofactor(s) (*10*). This suggests that the catalytic subunit of the enzyme in its resting state has a conformation that accommodates substrate recognition and cleavage. Certain aspects of the fI−cofactor−substrate complex formation remain unclear, such as whether binding of the cofactor to both fI and the substrate is required for substrate orientation or is necessary for inducing appropriate conformations in either the substrate or enzyme (*2*).

fI is synthesized as a single polypeptide chain, which is glycosylated and processed prior to secretion (*11*). No

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[1] Abbreviations: BSA, bovine serum albumin; C4bp, C4-binding protein; CCP, complement control protein; fB, factor B; fD, factor D; fH, factor H; fI, factor I; dpm, disintegrations per minute; CR, complement receptor; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; ϵACA, ϵ-aminocaproic acid; EDTA, ethylenediaminetetraacetic acid; FIMAC, factor I membrane attack complex; HC, fI heavy chain; IAM, iodoacetamide; Iodogen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril; LC, fI light chain; LDLRa, low-density lipoprotein receptor type A; MASP, mannan-binding lectin-associated serine protease; MCP, membrane cofactor protein; MES buffer (for SDS−PAGE), 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 50 mM Tris, 3.4 mM SDS, and 1 mM EDTA, pH 7.3; OD, optical density; PBS, phosphate-buffered saline (Dulbecco A formula: 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 139 mM NaCl, and 3 mM KCl, pH 7.4); Pefabloc-SC, 4-(2-aminoethyl)-benzenesulfonyl fluoride; PEG, poly(ethylene glycol); PVDF, poly(vinylidene fluoride); SBTI, soybean trypsin inhibitor (Kunitz inhibitor); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SP, serine protease; SRCR, scavenger receptor cysteine rich; Tris, tris(hydroxymethyl)aminoethane; Tween-20, poly(oxyethylene) sorbitan monolaurate.

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circulating zymogen form has been identified. The mature protein consists of an N-terminal heavy chain with 317 amino acid residues and a C-terminal light chain with 244 residues that are covalently linked via a disulfide bond (*12*, *13*). Each chain contains three occupied N-linked glycosylation sites contributing 20−25% (w/w) of the apparent protein molecular weight (*14*, *15*). Analysis of the primary structure of fI reveals an unique linear arrangement of domains: an N-terminal FIMAC (factor I membrane attack complex) domain, an SRCR (scavenger receptor cysteine-rich) domain, and two LDLRa (class A low-density lipoprotein receptor) domains in the noncatalytic heavy chain (Figure 1) (*12*, *13*, *16*).

The FIMAC domain contains a Kazal-type protease inhibitor module, the role of which has not been elucidated. Kazal-type inhibitors are a diverse family of multidomain proteins with high inhibitory potency which inhibit thrombin/trypsin-like proteases (*17*). It has been suggested that the serine protease domain of fI might be inhibited in the isolated enzyme because of its proximity to the Kazal domain (*18*). If this is so, cofactor−substrate−fI complex formation might cause separation of the SP and Kazal domains, allowing fI to become fully active. However, fI has been shown to be active against synthetic substrates in the absence of cofactors (*10*).

The C-terminus of fI consists entirely of a trypsin-like serine protease light chain containing (reasoning by homology) the residues that form the His-Asp-Ser catalytic triad. In addition, residues are present that define the specificity pocket D^{501} and the extended substrate binding site S^{527}, W^{528}, and G^{529} (numbering as in Figure 1). Among complement proteases the SP domain of human fI is most similar to fD (28% amino acid sequence identity), but among all human serine proteases, it is most similar to human tissue plasminogen activator (41% sequence identity) and human plasma kallikrein (37% sequence identity).

Although the catalytic properties of fI have recently been characterized more extensively (*10*), more information is required for understanding protein−protein interactions between enzyme−cofactor−substrate. Little is known about the role of the fI heavy chain domains in complex formation, while the mechanisms of the cofactor activity have not been fully explored. It is unknown whether the catalytic subunit of fI can cleave the natural substrate in the absence of the heavy chain and in the presence or absence of cofactors.

The central objective of this work has been the structural and functional characterization of the fI serine protease domain. The first steps of this strategy included the isolation and characterization of the light chain SP domain from the native enzyme. It was found that the isolated SP domain has both proteolytic and amidolytic activity. However, its interaction with natural protein substrates is altered relative to native fI.

MATERIALS AND METHODS

The following reagents were purchased from Sigma (St. Louis, MO): bovine serum albumin (BSA), 1,4-dithiothreitol (DTT), 2-[4-(2-(hydroxyethyl)-1-piperazine)]ethanesulfonic acid (HEPES), 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen), and poly(ethylene glycol) (PEG 3350 molecular weight).

Human plasmin was bought from Kabi Diagnostica (Stockholm, Sweden). Sequencing grade endoproteinase Glu-C (V8 protease) was obtained from Promega (Madison, WI).

Ammonium hydrogen carbonate, ethylenediaminetetraacetic acid (EDTA), sodium phosphate, and tris(hydroxymethyl)aminoethane (Tris) were obtained from BDH Laboratory Supplies (Poole, U.K.). PBS tablets were purchased from Oxoid Limited (Basingstoke, Hampshire, U.K.). Calcium chloride, glycine, magnesium chloride, and sodium chloride were bought from Riedel-de Haën (Seelze, Germany) and acrylamide and sodium dodecyl sulfate (SDS) from National Diagnostics (Atlanta, GA). Spectro/Por 6 dialysis tubing was from Medical Industries Inc. (Los Angeles, CA). Microfluor white plates were from Thermo Electron Informatics (Franklin, MA). Pooled human plasma was bought from HD Supplies (Aylesbury, U.K.). Sodium [^{125}I]iodide and all of the chromatographic materials were purchased from Amersham Biosciences U.K. Ltd. (Chalfont St. Giles, Bucks, U.K.).

Protease inhibitors ϵ-aminocaproic acid (ϵACA), antipain, aprotinin, benzamidine, leupeptin, 1,10-phenanthroline, and soybean trypsin inhibitor (SBTI) were bought from Sigma, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (Pefabloc-SC) was from Pentapharm Ltd. (Basle, Switzerland), lima bean trypsin inhibitor type IIL (LBTI) was from Fluka (Buchs, Switzerland), and suramin was from Bayer AG (Leverkusen, Germany). Hirudin was obtained from Accurate Chemical and Scientific Corp. (Westbury, NY).

FGR-AMC substrate was purchased from American Diagnostica Inc. (Greenwich, CT). Precast 10% or 4−12% NuPAGE Bis-Tris gels and PVDF membrane were bought from Invitrogen (Carlsbad, CA). StrataClean resin was obtained from Stratagene (La Jolla, CA).

Purification of Proteins and Radioiodination. fI, fH, and C3 were purified, and C3(NH_3) and ^{125}I-C3(NH_3) were prepared as described in ref *10*.

SDS−PAGE Analysis. The Laemmli system (*19*) was used for SDS−PAGE analysis, but the sample preparation, SDS−PAGE sample buffer composition, and Coomassie Blue staining are as described by Fairbanks et al. (*20*). The composition of the sample buffer was 0.2 M Tris, 8 M urea, 2% SDS, and 0.002 M EDTA, pH 8.0, with 0.001% Bromophenol blue. For analysis under reduced conditions the sample buffer was made 40 mM with DTT. For some applications, such as the more detailed fragment characterization studies, samples were analyzed using the NuPAGE Bis-Tris precast gel system (Invitrogen) with MES buffer according to the manufacturer's instructions. This gel system provides separation in a wider molecular weight range.

Proteolytic Fragmentation of Human fI by V8 Protease or Plasmin. fI was digested with V8 protease as described in Figure 2 and with plasmin as described in Figure 3.

Characterization of fI Plasmin Digest Fragments by N-Terminal Sequencing. Digest samples for analysis were run on a 10% Novex Bis-Tris NuPAGE precast gel using MES buffer in a Novex X Cell II Mini-cell gel apparatus. The gel was electroblotted onto a Novex 0.2 μm PVDF membrane (Invitrogen) on a Novex blot module. The membrane was stained with Coomassie Brilliant Blue. Target protein bands were excised from the membrane and washed with 10% methanol prior to N-terminal sequencing (*21*).

They were then sequenced on an Applied Biosystems 494A Procise protein sequencer (Applied Biosystems, Warrington, U.K.) for 10 cycles each using standard sequencing cycles.

Purification of the fI SP Domain by Antibody Affinity Chromatography. Digests of fI by plasmin were prepared as described in the legend to Figure 5. A digest was passed down a 2 mL MRC-OX21 antibody−Sepharose column (*22*) preequilibrated in 25 mM Tris, 140 mM NaCl, and 0.5 mM EDTA, pH 7.4. The MRC-OX21 mAb recognizes an epitope on the heavy chain of fI. Antibody affinity chromatography was employed to ensure complete removal of uncleaved fI from the digest mixture. Fractions (1 mL) were collected (numbered 1−6), and the column was washed extensively with running buffer until the OD_{280nm} of the eluate was <0.04. Bound protein was then eluted with 3 M $MgCl_2$, pH 6.9. The fractions containing 3 M $MgCl_2$ were pooled and dialyzed against 10 mM NaCl, pH 7.2, and concentrated by freeze-drying. The unbound and bound fractions were analyzed by SDS−10% PAGE.

Characterization of the Functional Activity of fI and the Purified fI SP Domain. (A) The Proteolytic Assay: Cleavage of ^{125}I-C3(NH_3) by fI or the SP Domain. This assay was done as in ref *23* with minor modifications. Approximately 35000 dpm of ^{125}I-C3(NH_3) (15 ng) was mixed with 1.25 μg of fH and 0.47 μg of the SP domain or 1.08 μg of fI in a final volume of 100 μL of 10 mM potassium phosphate, 0.5 mM EDTA, 0.1% Tween-20, and 1.25 μM SBTI, pH 7.2. fI and the SP domain were at a final concentration of 0.12 μM. Molarity was calculated on the assumption that all of the material in the preparations is functional and on the observed MW of 88 kDa for fI and 38 kDa for the SP domain on the SDS−PAGE. All mixtures were incubated at 37 °C for variable times up to 480 min. Prior to analysis, reactions were stopped by the addition of 25 μL of SDS−PAGE sample buffer with DTT (for reduced samples only). Analysis was carried out by SDS−8.5% PAGE and autoradiography. An example can be found in ref *22* or *23*. ^{125}I-C3(NH_3) analyzed under reduced conditions runs as a two-band pattern, the highly labeled 116 kDa α chain and the β chain of 68 kDa. On incubation with fI and fH, the α chain is cleaved into two fragments, one running with the β chain and the other ∼43 kDa. The rate of cleavage of the α chain is proportional to the proteolytic activity of fI.

In some experiments unlabeled C3(NH_3) was used instead, and adjustments were made to compensate for the observation that the SP domain preparation contained some partially cleaved material. Here 10 μg of C3(NH_3) (31 μL) was mixed with 0.36 μg of fI (3.15 μL) or 0.47 μg of the SP domain (10 μL), to compare on an equimolar basis the intact molecules, in the presence or absence of 1.246 μg of fH (10 μL) in 80 μL of final reaction volume made 1.56 μM with SBTI. It was assumed for these analyses that the SP fraction contained about one-third SP domain with an intact N-terminus. For pattern comparison of the C3(NH_3) cleavage by the isolated SP domain or fI, the reactions were analyzed by SDS−PAGE reduced and nonreduced.

Various compounds were tested as inhibitors in the proteolytic assay. As described above, fI or the SP domain was preincubated with the test compound at 37 °C for 60 min prior to the addition of fH. Incubation was continued for 60 min at 37 °C, then ^{125}I-C3(NH_3) (17500 dpm) was added, and incubation was continued for a further 480 min. The final reaction volume was 100 μL. Samples were analyzed by SDS−PAGE and autoradiography as described above.

(B) The Amidolytic Assay: Cleavage of the Synthetic Substrate FGR-AMC by fI or the SP Domain. The amidolytic activities of fI and the SP domain were compared with FGR-AMC. This substrate is cleaved by fI in the absence of cofactors (*10*). Measurements were carried out in pairs of equimolar concentrations for comparison. For each reaction the substrate, 100 μM in 100 μL of 20 mM HEPES, pH 8.5, was added to 100 μL of fI or SP of variable concentration in the same buffer in white Microfluor plate wells. The amidolytic activity was measured using a microtiter plate reader (Fluoroskan; Thermo Life Sciences Ltd., Basingstoke, U.K.) by excitation at 355 nm and continuous monitoring of emission at 460 nm for 2 h at 37 °C.

RESULTS

Proteolytic Fragmentation of fI with V8 Protease. The sequence and domain organization of human fI are shown in Figure 1. Several proteases (trypsin, chymotrypsin, thrombin, and pepsin) were used in preliminary tests on fI cleavage, but these proteases did not produce suitable fragment sizes. V8 protease and plasmin produced more favorable results. fI was digested for a fixed time with increasing quantities of V8 protease. As shown in Figure 2B, the fI SP domain (LC) is quite resistant to cleavage by V8, while the heavy chain is cleaved to fragments of about 40 and 20 kDa. Further degradation of the 40 kDa fragment is seen at high V8 concentration. With nonreduced samples (Figure 2A), it can be deduced from the disulfide bridging pattern of fI (Figure 1) that the major cleavage occurs in the region of the LDLRa2 domain between C^{237} and C^{309} (Figure 1). This creates an N-terminal fragment of ∼40 kDa, leaving the SP domain attached to an ∼15 kDa C-terminal fragment of the heavy chain. The digestion therefore left a large heavy chain fragment attached to the SP domain, which was unsuitable for further processing. Plasmin digestion was therefore explored.

Proteolytic Fragmentation of fI with Plasmin. Digests of fI with plasmin were done to observe the rate and patterns of cleavage. A typical time course is shown in Figure 3A. Intact fI is cleaved (Figure 3A) so that after 160 min less than 10% remains. An intermediate product of MW ∼40 kDa (nonreduced) is formed (labeled "c"), reaches a maximum at ∼30 min, and then breaks down further. Concurrently with the appearance of product c, a band of MW ∼38 kDa appears ("d"). It was shown by N-terminal sequence (Table 1) analysis that product d contains as a major component a species with the intact N-terminus of the SP domain.

As illustrated in Figure 3B, more than 90% of the heavy chain ("a") has been cleaved at 160 min, and a large number of small fragments from ∼31 to 4 kDa are formed. The light chain ("b") appears more resistant to digestion, although after ∼40 min it clearly diminishes. Therefore, timing is critical for the optimal generation of an intact SP domain product.

Further experiments were carried out to optimize the time−course and concentration of plasmin, so as to obtain the maximum yield of band d (Figure 3A), avoiding further degradation of the light chain (as seen in Figure 3B at 80 and 160 min).

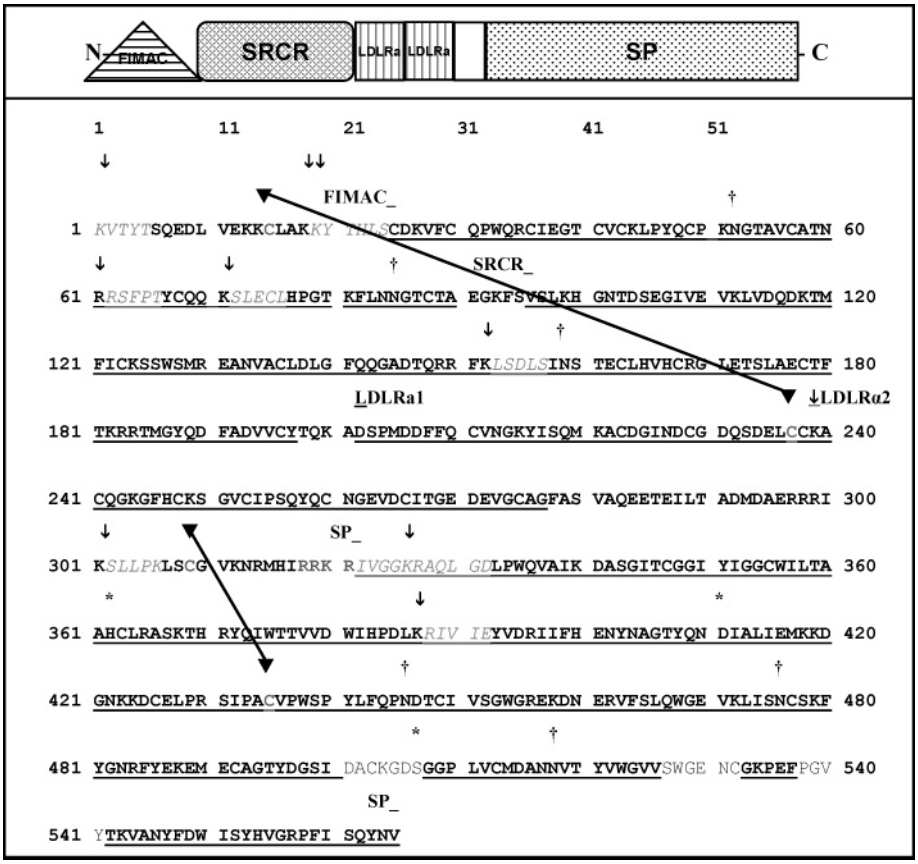

FIGURE 1: Schematic representation of fI and the primary sequence of human complement factor I. fI exhibits a unique linear arrangement of domains: an N-terminal FIMAC (factor I membrane attack complex) domain, a SRCR (scavenger receptor cysteine-rich) domain, and two LDLRa (class A low-density lipoprotein receptor) domains in the noncatalytic heavy chain. In the primary sequence, all domains are illustrated underlined, and the start of each domain is indicated by an underscore beside the domain name (e.g., SRCR_). Nonunderlined text in the heavy chain shows nondomain regions. The signal sequence (18 amino acids) is omitted, and residue numbering is from the N-terminus of the mature protein. The R^{321}-I is cleaved posttranslationally, giving rise to the fI heterodimer. Disulfide bridges between domains are shown by (▼). The full list of disulfide bridges (mainly deduced by homology) is as follows: heavy chain, $\mathbf{C^{15}-C^{237}}$, $C^{25}-C^{36}$, $C^{30}-C^{75}$, $C^{41}-C^{57}$, $C^{43}-C^{88}$, $C^{49}-C^{68}$, $C^{123}-C^{163}$, $C^{136}-C^{196}$, $C^{168}-C^{175}$, $C^{211}-C^{229}$, $C^{223}-C^{238}$, $C^{241}-C^{253}$, $C^{248}-C^{266}$, $C^{260}-C^{275}$; heavy−light chain, $\mathbf{C^{309}-C^{435}}$ (experimentally determined, see text); and light chain, $C^{347}-C^{363}$, $C^{355}-C^{513}$, $C^{426}-C^{449}$, $C^{477}-C^{492}$, $C^{503}-C^{532}$. The * shows the residues which (by homology) form the catalytic triad, and the † shows the six sites of attachment of N-linked oligosaccharides (52, 85, 159, 446, 476, and 528). The nonbold, nonunderlined sequences in the serine protease (SP) domain form the walls of the specificity pocket. The bonds that have been identified by N-terminal sequence analysis as cleavage sites for plasmin (see text) are indicated in italics, and the ↓ indicates each bond that is cleaved.

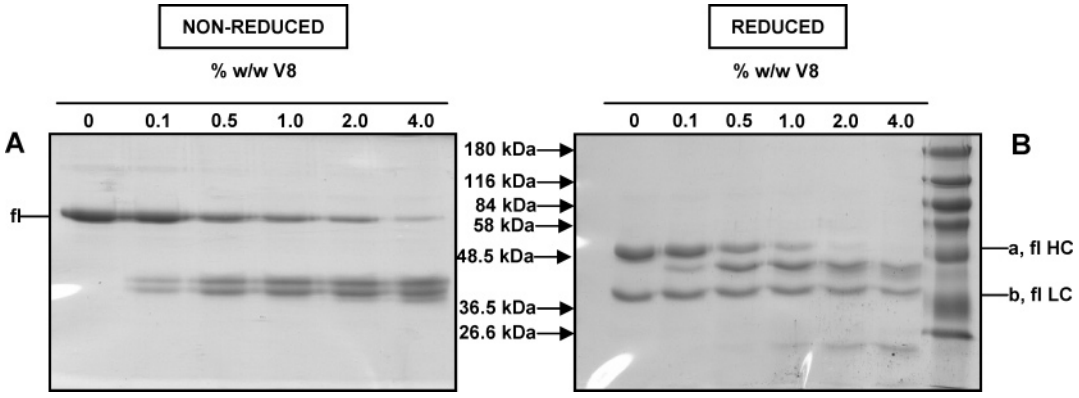

FIGURE 2: Proteolytic fragmentation of human fI by V8. Aliquots of 20 μL containing 9 μg of fI (0.45 mg/mL) in PBS and 0.5 mM EDTA, pH 7.4, were incubated with variable amounts of V8 protease at final concentrations from 0.1% to 4% (w/w). The final reaction volume was 40 μL, and reaction mixtures were incubated for 1 h at 37 °C. The reactions were stopped by the addition of 15 μL of SDS−PAGE sample buffer and analyzed by SDS−9.0% PAGE under nonreduced (A) and reduced (B) conditions. Bands a and b represent fI LC and fI HC, respectively.

The band labeled d (Figure 3A) consists of a small fragment of the heavy chain disulfide linked to the light chain (see below). fI contains no unpaired cysteines (*18*). All of the cysteines in fI can be assigned by homology to intra-

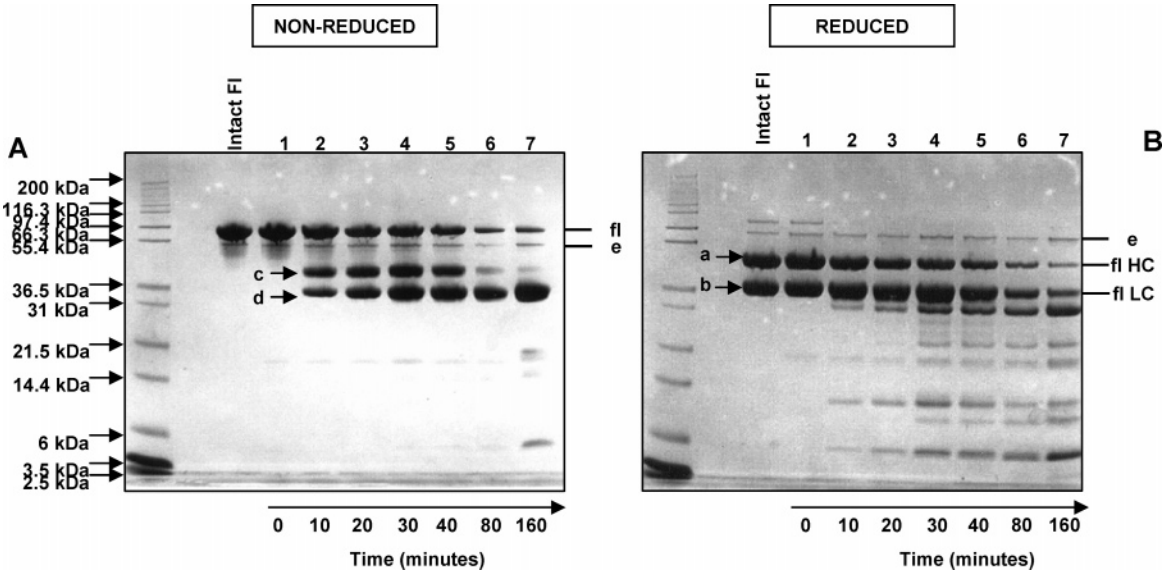

FIGURE 3: Proteolytic fragmentation of human fI by plasmin. Seven identical samples each containing 10 μg of fI (1.185 μg/μL) in PBS and 0.5 mM EDTA, pH 7.4, were incubated with 0.6 μg of plasmin [6% (w/w) plasmin/fI] (1.2 μL of 0.5 μg/μL) at 37 °C for a variable time. The reaction was blocked by the addition of a 2-fold molar excess of SBTI over plasmin and further incubation for 15 min under the same conditions. The samples were analyzed by SDS−10% PAGE (precast 10% NuPAGE Bis-Tris gel system) under nonreduced (A) and reduced (B) conditions. Tracks 1−7 represent successive time points in the digestion. The track labeled "intact fI" shows fI without plasmin, unincubated. fI HC is labeled as "a" and the LC as "b". An intermediate product, "c", of MW ~40 kDa in (A) is formed in the initial phase of incubation, reaches a maximum at ~30 min, and then breaks down further. N-Terminal sequencing results showed that product "d" in (A) contains the SP domain disulfide linked to a C-terminal fragment of the heavy chain through the disulfide bridge formed between C^{309} and C^{435}. "e" is a minor contaminant, the concentration of which, compared to fI, can be considered negligible.

Table 1: Summary of Results from N-Terminal Sequence Analysis of Species b and d in Figure 3

band species	N-terminal sequence (sequencing yield in pmol)
nonreduced[a]	IVGGKRAQLG (24.0) (light chain)
band d (Figure 3A)	RAQLGDLPWQ (21.9) (light chain)
track 3	RIVIEYVDRI (17.7) (light chain)
	SLLPKLSCGV (38.4) (heavy chain)
reduced[b]	IVGGKRAQLG (16.4) (light chain)
band b (Figure 3B)	RAQLGDLPWQ (15.3) (light chain)
track 3	

[a] Plasmin-digested fI was subjected to N-terminal sequencing under reduced and nonreduced conditions. The sequencing revealed the presence of four different species, two of which carry N-terminal sequences (RAQLGDLPWQ and RIVIEYVDRI) that represent cleaved forms of the light chain. The sequence IVGGKRAQLG represents the N-terminus of the fI light chain. The sequence SLLPKLSCGV corresponds to a fragment that consists of a heavy chain piece disulfide bridged to the light chain. Of the total light chain material detected, only 35−40% is intact (24 pmol of the IVGGKRAQLG sequence compared with 24.0 + 21.9 + 17.7 = 63.6 pmol) of the total light chain sequence. [b] Extended incubation of fI with plasmin can result in the production of a partially cleaved light chain fragment that has its N-terminal IVGGK sequence removed. Removal occurs through the cleavage of 5 or 66 residues from the N-terminus. The sequencing of the reduced preparation provided a total yield of 31.7 pmol and confirmed the light chain partial cleavage. For the reduced products the ratio of intact light chain to light chain lacking the first 5 residues was (16.4:15.3) 1.07, compared with (24.0:21.9) 1.09 calculated from the nonreduced material.

domain disulfide bridges, except cysteines 15, 237, and 309 (numbering as in Figure 1). C^{435} in the SP domain by homology should be involved in linkage to the heavy chain.

Sequence analysis of band d (Figure 3A) (Table 1) showed that it consists of a single heavy chain fragment (N-terminus beginning with S^{302}LLPK) that is linked to three SP domain-derived sequences (discussed below). This pattern is consistent with the interchain disulfide being between C^{309} and C^{435}. Therefore, the two other unassigned cysteines, C^{15} and C^{237}, form a disulfide bridge linking a region close to the N-terminus of the heavy chain to the C-terminus of the LDLRa1 (Figure 1).

Band d (Figure 3A) therefore contains the desired product, the SP domain disulfide linked to a small C-terminal heavy chain fragment. The heavy chain fragment may contain up to 20 residues (residues 302−321), depending on whether there is cleavage at the lysines in this region during the circulation of fI in vivo. This material therefore contains a much smaller heavy chain fragment than the product generated by V8 proteolysis.

Characterization of the fI Digest Fragments by N-Terminal Sequencing. fI was digested with plasmin for two different time periods (40 min to generate intermediate and end-point products and 16 h to generate end-point fragments). The digests are shown in Figure 4 as a stained blot after SDS−PAGE analysis.

The fragments formed during the 40 min digest were characterized by N-terminal sequencing (bands 1−8; no data were obtained for bands X and X′). N-Terminal sequences of bands 1−8 are shown in Table 2, and the deduced cleavage sites are shown in Figure 1. Eight cleavage sites were found in the heavy chain and two in the light chain (Figure 1). All were located at the carboxy side of lysines, except for the R^{61}R site in the heavy chain (numbering as in Figure 1). The intact light chain is in band 1 but is contaminated with a form of the light chain that has been cleaved five residues from the N-terminus (R^{327}AQLG). Considering the identified cleavage sites (Figure 1), the largest end-point fragments, observed in the 16 h digest, are the 33 kDa C-terminus of the light chain (starting at R^{338}IVIE) and the 38 kDa light chain (starting at I^{322}VGGK

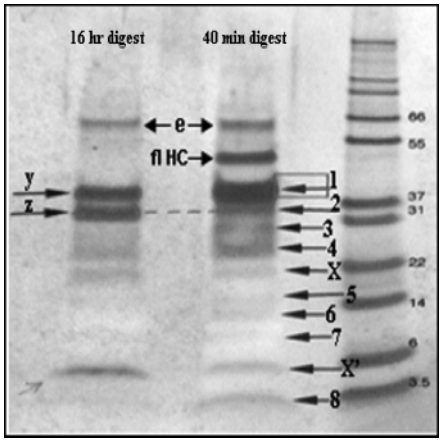

FIGURE 4: PVDF blot generated for N-terminal sequence analysis of reduced fI fragments produced by proteolysis of fI with 6% (w/w) plasmin/fI. Two digest mixtures of fI were subjected to N-terminal sequence analysis. Both mixtures were prepared with the same composition (50 μg of fI and 3 μg of plasmin in a final volume of 365 μL of PBS and 0.5 mM EDTA). One mixture was incubated for 40 min and one for 16 h at 37 °C. Each reaction was stopped by the addition of 1.5 μg of SBTI in 150 μL of the same buffer (final volume 515 μL). Seventy microliters from each preparation was concentrated with 7 μL of Strataclean resin each, and both were run reduced on a 4−12% NuPAGE gel system with MES buffer. The gel was transferred to a Novex 0.2 μM PVDF transfer membrane via Western blotting, and sequence analysis of selected bands (indicated by arrows and numbers 1−8) was done as described in the Materials and Methods section. The results of the analysis are summarized in Table 2. The presence of more than eight visible bands running below the fI light chain (1) indicates a wide diversity of breakdown products. Some are intermediate products absent from the 16 h digest and are therefore further cleaved on extended incubation. The intermediate products give rise to smaller fragments difficult to detect due to small MW. There are clear differences in product yield between the two preparations. No sequence information was obtained for bands labeled X and X′, but their sequence can be deduced (see Table 2).

Table 2: Characterization of fI Digest Fragments by N-Terminal Sequencing[a]

band in Figure 4	obsd MW (kDa)	N-terminal sequence (sequencing yield in pmol)
1	38	IVGGK (32.5), RAQLG (7.9), SLECL (7.9)
2	33	RIVIE (5.4), SLECL (2.1)
3	30	SLECL (2.5)
4	25	SLECL (7.4)
5	15	KVTYT (1.91), SLECL (1.43)
6	12	KVTYT (6.12), SLECL (4.07)
7	10	KYTHL (10.0), YTHLS (9.29), LSDLS (2.69)
8	3	SLLPK (9.31), RSFPT (3.63), IVGGK (3.24)

[a] The protein fragments numbered 1−8 in Figure 4 were sequenced. The position of these cleavage sites within the complete fI sequence is shown in Figure 1. It can be deduced from the cleavage site pattern on Figure 1 that band X (20 kDa) on Figure 4 is likely to correspond to the glycopeptide sequence between cleavage sites K^{152}−K^{301}. Band X′ might be the 62 or 63 aa segments starting at A^{240}CQKG or R^{327}AQLG.

or R^{327}AQLG). The long end-point digest cannot, however, be used to generate the intact SP domain because of the partial cleavage at position 5 of the light chain.

The results show that overall the SP domain exhibits higher resistance to plasmin than the heavy chain, but cleavages at two positions in the light chain (K^{326} and K^{387}) do occur. These cleavages contribute to three forms of the light chain: R^{327}AQLG and R^{388}IVIE and the intact light chain I^{322}VGGK. The additional sequence S^{302}LLPK (Table 1) that was detected attached to the light chain in nonreduced samples corresponds to a fragment from the C-terminal end of the heavy chain. The detection of this sequence shows that the disulfide interchain bridge forms between positions 309−435 (Figure 1). In total, 35−40% of the whole light chain material (band d, Figure 3A) had an intact N-terminus (Table 1).

Purification of the fI Light Chain by Affinity Chromatography. The plasmin digest of fI, done under conditions designed to optimize the yield of the intact SP domain (see Figure 5), was further processed through an MRC-OX21−Sepharose affinity column.

The MRC-OX21 mAb removes intact fI and some heavy chain fragments. As shown in Figure 5, the mAb column bound and retained all of the intact fI, while the SP domain-containing fragment (d on Figure 5A) was not bound on the column. A residual heavy chain fragment of ~40 kDa (c_1 on Figure 5A) also did not bind to the column. Upon reduction, c_1 was seen to break down to smaller fragments (Figure 5B).

The MRC-OX21 Epitope. From the data available (Table 2) and the high reproducibility of the gel system, it was considered useful to identify the region where the MRC-OX21 mAb binds. Band 6 (Table 2 and Figure 5) contained two heavy chain fragments with N-terminal sequences of K^1VTYT and S^{72}LECL of MW ~11.6 kDa. The SDS−PAGE analysis on Figure 5B shows that, from band 6, species 6_1 did not bind to the column and 6_2 did. Using the primary sequence data (Figure 1) and the established plasmin cleavage sites, the information collected about the preferred cleavage sites, we investigated which part of the heavy chain was likely to contain the epitope. A nonglycosylated fragment of ~11.6 kDa beginning from K^1VTYT would contain ~105 amino acids (numbering as in Figure 1). However, such a product would not form as there is a cleavage at S^{72}LECL and a glycosylation at N^{52}. The MW of the fragment K^1-VTYT to S^{72}LECL (using 110 and 3500 Da as the average MW for each amino acid and N-linked carbohydrate attachment) is 11.6 kDa, which is the MW of band 6. The other band 6 fragment begins at S^{72}LECL and with high probability ends within K^{152}LSDL (Table 2), giving a fragment of 81 amino acids with one glycosylation site (approximately 12 kDa). The MRC-OX21 mAb epitope must be present in one of these two fragments. Band 2 (Table 2) also contains two species, of which one binds to MRC-OX21 and one does not. Since the epitope is not in the light chain, the fragment from band 2 that must bind MRC-OX21 is the one with the N-terminal S^{72}LECL. Band 3 consists of only a single fragment with the same starting sequence, S^{72}LECL, and binds to MRC-OX21 (Figure 5B). These findings indicate that the epitope is located within S^{72}LECL-K^{152}, the smallest fragment (band 6_2) which binds MRC-OX21. This represents the last few residues of the FIMAC and the first half of the SRCR domain.

In conclusion, proteolytic fragmentation of fI by plasmin generated a range of fragments. Affinity chromatography was used to purify species d (Figures 3 and 5), which contains the SP domain disulfide linked to a small heavy chain peptide. Product d, however, is heterogeneous since it contains partially cleaved SP domain forms.

The presence of Ile at the N-terminus of SP domains is generally considered essential for the formation of a salt

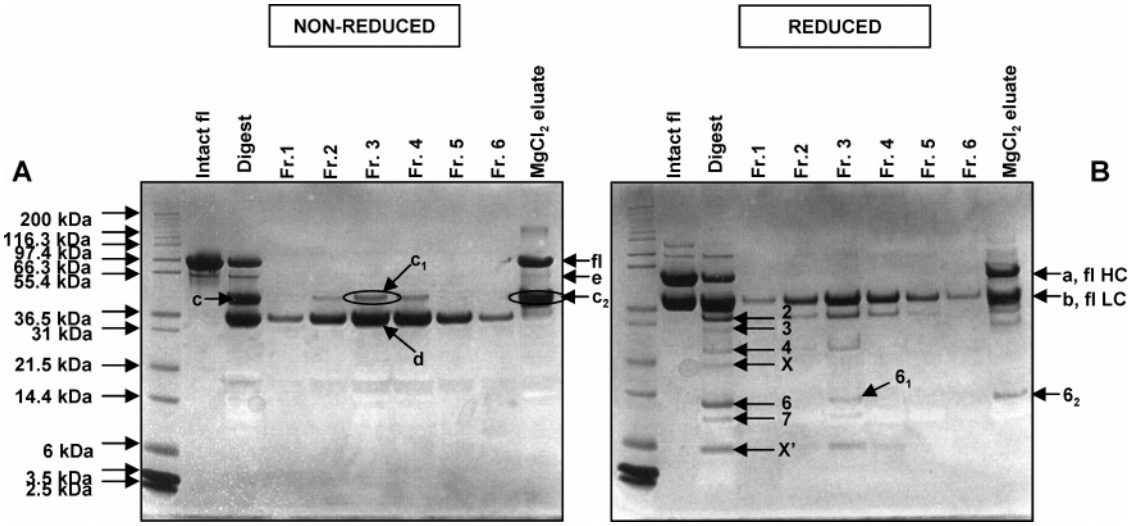

FIGURE 5: Purification of the fI SP domain by antibody affinity chromatography. A digestion of fI with plasmin was prepared under optimized conditions. One milligram (844 μL) of fI was incubated with 60 μg (120 μL) of plasmin at 37 °C for 20 min in PBS and 0.5 mM EDTA, pH 7.4. The reaction was stopped by the addition of 30 μg of SBTI (30 μL), and the digest mixture was processed further as described in the Materials and Methods section. SDS−10% PAGE (precast 10% NuPAGE Bis-Tris gel system) analysis of the material obtained shows that fractions 1−6 that contain all of the unbound material have no intact fI, while the $MgCl_2$ eluate appears to contain all of the uncleaved fI and heavy chain fragments that carry the mAb recognition epitope. N-Terminal sequencing revealed that the species in (B), indicated by the arrows (2, 3, 4, X, 6, 7, and X′; same numbering as in Figure 4 and Table 2), are fragments originating from the fI heavy chain.

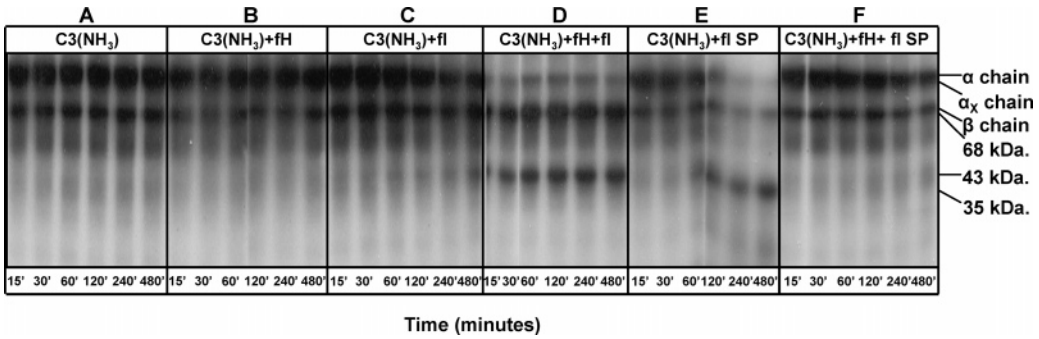

FIGURE 6: Activities of factor I or the SP domain in the proteolytic assay using ^{125}I-C3(NH_3). The autoradiograph shows the cleavage of ^{125}I-C3(NH_3) by fI or the fI SP domain in the absence or presence of fH. The procedure and C3(NH_3) fragmentation pattern are as described in the Materials and Methods section.

bridge with D^{194} (chymotrypsin numbering) (D^{506} in Figure 1) in trypsin and chymotrypsin (*24*−*27*). In the zymogens of trypsin-like proteases, the oxyanion hole [the amides of G^{193} and S^{195} (G^{505} and S^{507} in Figure 1)] and about 50% of the substrate binding pockets are not in the catalytically competent conformation seen in the activated enzyme (*28*). In contrast, the catalytic triad of the zymogens is arrayed as in the activated form. Upon zymogen activation a highly conserved new N-terminus (I-I/V-G-G) establishes a conserved and buried salt bridge between the newly formed amino terminus and D^{194}. The establishment of this salt bridge contributes to the refolding of the molecule into the conformation desired for catalysis. This information is derived mainly from crystallographic structures and can be assumed to be the case for fI, since the fI SP domain is homologous to and shares key functional conserved residues with the trypsin and chymotrypsin family (*29*). However, it is not known whether either of the detected cleavages at the SP domain causes dissociation of the peptide from the N-terminus of the SP domain, so the N-terminal Ile may still be present, and linked to D^{506} (Figure 1), even in the cleaved forms. Thus the cleaved forms of the fI SP domain may or may not be active.

Functional Activity of the Purified fI SP Domain. (A) The Proteolytic Assay: Cleavage of ^{125}I-C3(NH_3) by the fI SP Domain. The enzymic properties of the purified SP domain were tested in the proteolytic assay using C3(NH_3) or ^{125}I-C3(NH_3) as a substrate in the presence or absence of factor H. Native fI or the SP domain with or without fH was incubated with ^{125}I-C3(NH_3) for up to 480 min (8 h) at 37 °C in the presence of SBTI as described in the Materials and Methods section. Results are shown in Figure 6. In the absence of fI, ^{125}I-C3(NH_3) is stable in the presence or absence of fH during this incubation period. When ^{125}I-C3(NH_3) is incubated with fI, but no fH, there is very slight cleavage, visible at 480 min (Figure 6C). In the presence of fH, fI cleaves ^{125}I-C3(NH_3) at a high rate (at least 300 times faster than in the absence of fH), resulting in nearly complete cleavage of the α chain within 15 min (Figure 6D). The cleavage gives the well-established pattern of products (*30*, *31*). Even after 480 min, there is some residual uncleaved α chain. This is probably due to disulfide rearrangement in

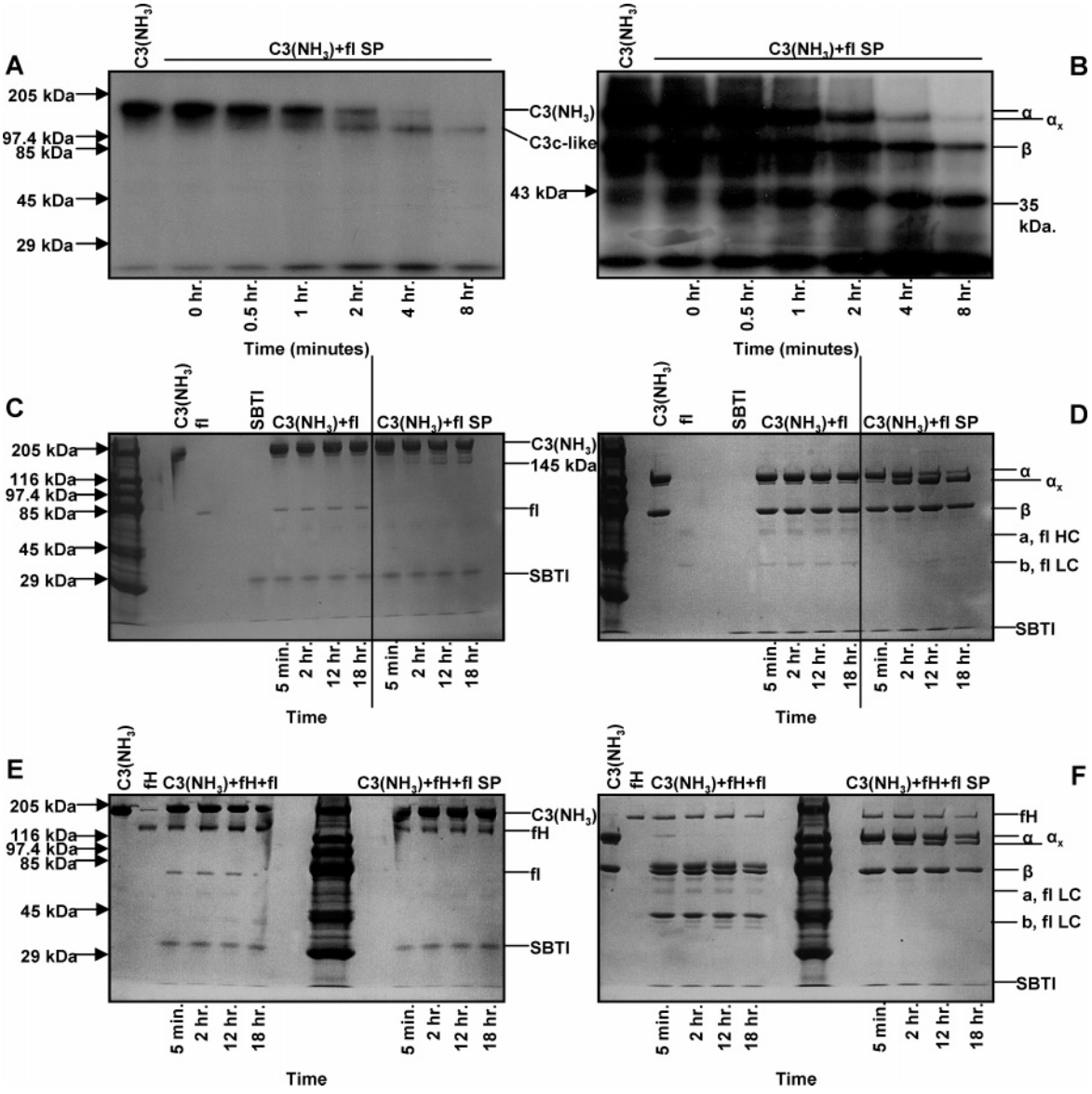

FIGURE 7: Fragmentation pattern of ^{125}I-C3(NH_3) or C3(NH_3) by fI or the fI SP domain. (A, B) Digestion of ^{125}I-C3(NH_3) by the fI SP domain. Conditions are as described in the Materials and Methods section except that the SP domain concentration was 2-fold lower than in Figure 6. The reaction mixtures that were generated were analyzed by SDS−PAGE and autoradiography both under reduced (A) and nonreduced (B) conditions. Under nonreduced conditions uncleaved C3(NH_3) runs as a single species of 185 kDa. (C−F) Digestion of unlabeled C3(NH_3). C3(NH_3) was digested with fI or the SP domain in the absence (C, D) or presence (E, F) of fH as described in the Materials and Methods section. Coomassie Blue stained SDS−PAGE gels are shown: C, D, nonreduced; D, F, reduced.

the C3(NH_3) which leads to a form of C3(NH_3) which is not cleaved by fI (*32*).

Surprisingly, the results obtained for the SP domain differ qualitatively from those for intact fI. In the presence of fH (Figure 6F), the cleavage of ^{125}I-C3(NH_3) by SP proceeds at a very low rate with barely detectable cleavage of the α chain after 480 min of incubation. This is at least 300-fold lower activity than intact fI in the presence of fH. Remarkably, however, in the absence of fH, the SP domain cleaves ^{125}I-C3(NH_3) quite rapidly, yielding initially a similar ∼43 kDa product but with further cleavage to an end product about 5−8 kDa smaller (Figure 6E). This cleavage is still about 16-fold slower than the cleavage by intact fI in the presence of fH (Figure 6D). The controls (Figure 6A−C) showed the expected response. Since there could be traces of plasmin in the SP domain material, controls of the cleavage of C3(NH_3) by plasmin were carried out. In the presence of SBTI (which is present in all of these assays), no cleavage of C3(NH_3) by plasmin was observed (data not shown). The major feature of Figure 6 is that, while the action of intact fI is barely measurable without fH, the activity of the SP domain is in contrast much greater in the absence of fH than in its presence.

The fragmentation pattern of C3(NH_3) by the SP domain was investigated more thoroughly, and the results are shown in Figure 7. In Figure 7A,B, cleavage of ^{125}I-C3(NH_3) by the SP domain in the absence of fH is shown. Figure 7B shows a pattern of cleavage similar to that in Figure 6E. The α chain of ^{125}I-C3(NH_3) is cleaved progressively, with appearance of a product at approximately 43 kDa, which is trimmed to 35 kDa. This is distinct from the pattern of C3-(NH_3) cleavage by intact fI in the presence of fH (Figure 6D or Figure 7F). In addition, it appeared that there was cleavage of the α chain to an α′-like product. Examination

of nonreduced samples (Figure 7A) confirmed that the SP domain does not produce the same cleavage pattern as is observed with fI + fH: the SP domain cleaves ^{125}I-C3(NH_3) to a C3c-like product (145 kDa) (Figure 7A), while fI + fH do not (Figure 7E). The SP domain also appears to produce a general degradation of ^{125}I-C3(NH_3), as the total material in high molecular weight bands diminishes (Figure 7A,B) with accumulation of low molecular weight fragments. This suggests that the SP domain cleaves many peptide bonds in ^{125}I-C3(NH_3).

The early stages of cleavage of C3(NH_3) by the SP domain were examined using unlabeled C3(NH_3) (Figure 7C−F) with a substrate concentration 670 times greater than in Figure 7A,B. In panels C and D, cleavage without fH is shown. fI produces no cleavage of C3(NH_3) over an 18 h period (Figure 7C,D). The SP domain, however, cleaves the α chain of C3-(NH_3) (Figure 7D) producing a fragment of similar molecular weight to the C3 α′ chain (108 kDa). Since we did not obtain sequence data to prove this is α′, it is designated a_x. Analysis of nonreduced samples (Figure 7C) shows that the SP domain produces slight fragmentation of C3(NH_3) to a fragment of ∼145 kDa.

In the presence of fH (Figure 7E,F), fI as expected rapidly (5 min) cleaves C3(NH_3) to iC3(NH_3) with no further cleavages up to 18 h. iC3(NH_3) coruns with C3(NH_3) without reduction (Figure 7E) and on reduction runs as the β chain plus 68 and 43 kDa α chain fragments. The SP domain, however, does not produce iC3(NH_3) (Figure 7F); instead, it produces the same cleavage to a_x fragments as are produced without fH (Figure 7D,F). Cleavage to a C3c-like fragment is obscured by fH on Figure 7E. As seen on Figure 6, the presence of fH slows the rate of cleavage by the SP domain. In Figure 6, where [fH] was 100-fold greater than [C3(NH_3)], the cleavage by the SP domain is almost completely stopped. In Figure 7F, where fH is present at much lower concentration than C3(NH_3) (0.15:1.0 molar ratio), cleavage does occur but is 2−3-fold slower than in the absence of fH (Figure 7D,F).

These results show that the SP domain cleaves C3(NH_3) relatively slowly at multiple sites (overall loss of high molecular weight material in Figure 7A,B) and that fH protects C3(NH_3) from this cleavage. fI, in contrast, does not cleave C3(NH_3); this may be because fI interacts via its heavy chain with C3(NH_3) in a specific orientation which predominates over random interaction. fH allows fI to cleave C3(NH_3) rapidly at only two sites to produce the stable iC3b. fH binding to C3(NH_3) may be required to expose these sites to fI in the ternary fI−fH−C3(NH_3) complex.

It seems that the conversion of C3(NH_3) to iC3(NH_3) is a highly specific process in which the heavy chain of fI must play an important role by primarily orientating the catalytic domain to the R^{1281}−S^{1282} and R^{1298}−S^{1299} cleavage sites in C3(NH_3). In the absence of the fI heavy chain, prolonged incubation results in the cleavage of bonds that are not cleaved by intact fI.

(B) The Amidolytic Assay: Cleavage of FGR-AMC by the fI SP Domain. The SP domain and native fI were compared for their amidolytic activity with the substrate FGR-AMC (Figure 8). Their activities are similar. If the cleaved forms of the SP domain present in the SP preparation are inactive, it would be expected that the SP domain material would be only 40% as active as fI on a molar basis. In fact, the SP domain activity is only 15−20% lower than fI. It can be concluded that the Kazal domain in the heavy chain does not inhibit catalytic activity, as absence of the heavy chain does not increase activity.

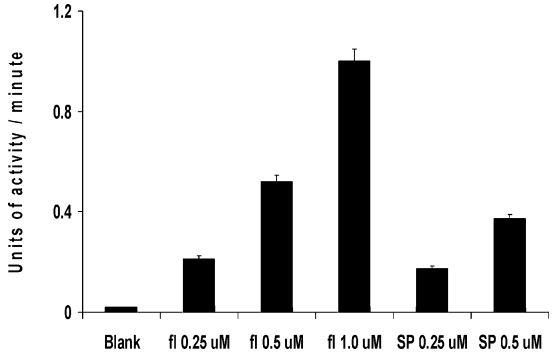

FIGURE 8: Comparison of activity of fI and the SP domain on the synthetic substrate FGR-AMC. The amidolytic assay was performed as in the Materials and Methods section. For every reaction the substrate, 0.1 mM in 100 μL of 20 mM HEPES, pH 8.5, was added to 100 μL of fI or SP solution in the same buffer in white Microfluor plate wells and incubated for 2 h at 37 °C.

Table 3: Summary of Effects of Selected Compounds on the Proteolytic Activities of fI and the SP Domain[a]

		inhibition[b]	
compound	final concn	fI	fI SP domain
Pefabloc SC	0.25 mM	**	**
suramin	1 mM	**	−
benzamidine	20 mM	−	−
antipain	0.1 mM	*	*
aprotinin	0.5 μM	*	*
leupeptin	10 μM	*	*
hirudin	5 ATU[c]	−	−
ϵACA	20 mM	−	−
1,10-phenanthroline	0.1 mM	−	−

[a] Potential inhibitors were tested in the proteolytic assay as described in the Materials and Methods section. fI and the SP domain were tested in parallel, and the inhibition was compared with untreated samples. [b] Classification of inhibition: strong inhibition (**) (60−100%); moderate inhibition (*) (10−40%); no inhibition (−) (0−10%). [c] Unit definition: 1 antithrombin unit (ATU) neutralizes 1 NIH unit of thrombin (fibrinogen assay) at 37 °C; 1 NIH unit of thrombin clots a standard fibrinogen solution in 15 s at 37 °C; 5 ATU are expected to neutralize 2.5 μg of thrombin.

Effect of Inhibitors on fI SP Domain Proteolytic Activity. The effect of compounds previously tested on the fI proteolytic activity (*10*) were also tested on the purified fI SP domain. The compounds were used at a single concentration, and the level of inhibition was estimated from end-point reactions. The results are shown in Table 3. From the compounds examined, only Pefabloc SC was found to inhibit strongly the proteolytic activity of the SP domain. This is in agreement with the result from testing on fI. Suramin, however, which strongly inhibits fI, did not inhibit the SP domain. Antipain, aprotinin, and leupeptin were all found to cause moderate inhibition of both fI and the SP domain. As expected, hirudin, ϵACA, and 1,10-phenanthroline were all found not to inhibit either. With the exception of suramin, these results are in agreement with recent extensive testing of inhibitor compounds on the proteolytic activity of fI (*10*).

DISCUSSION

The work described in this report focuses on the enzymic properties of the SP domain of fI. This was studied to

elucidate the roles of the fI heavy chain in restricting catalytic activity or substrate specificity. The SP domain of fI was purified after limited proteolysis of fI by plasmin. The final yield of the SP domain fragment was high. From 1000 μg of digested fI, approximately 430 μg of product d (Figure 3A, Table 1) was obtained after affinity chromatography. This corresponds to >90% recovery of material containing light chain disulfide bridged to a small (no more than 20 residues) heavy chain fragment. However, only 35−40% of the light chain in this material was intact: 60−65% was partially cleaved near the N-terminus (Table 1). Therefore, the yield of material with the intact SP domain was (0.35−0.45) × 90% = 32−41%. Protein sequencing required to characterize the SP domain also allowed us to locate the interchain disulfide bond formed between C^{309} and C^{435} and localize the MRC-OX21 binding site within the region S^{72}LECL-K^{152}.

The SP domain material was found to have amidolytic activity against the synthetic substrate FGR-AMC. This activity was similar in magnitude to that of intact fI (Figure 8) so the SP domain and the intact enzyme have similar affinity and catalytic activity for the synthetic substrate. In contrast, the proteolytic activities of the intact fI and the SP domain were strikingly different. Intact fI showed (almost) no cleavage of C3(NH_3) if fH was absent (Figures 6C and 7C,D). When fH was present, cleavage was rapid and specific, generating iC3(NH_3) [cleavage of C3(NH_3) at two sites (*30*, *33*, *34*)] (Figures 6D and 7E,F). The SP domain, however, can cleave C3(NH_3) in the absence of fH (Figures 6E and 7C,D). Cleavage was slow and less specific in that more sites were cleaved than with intact fI plus fH. Further, cleavage was inhibited, not stimulated, by fH (Figures 6F and 7E,F). The inhibitors Pefabloc SC, antipain, aprotinin, and leupeptin all act similarly on the proteolytic activity of intact fI or the SP domain (Table 3). Suramin, however, does not inhibit the SP domain although it is a potent inhibitor of fI. The observed lack of inhibition of the SP domain may indicate that suramin binding involves both chains of fI. Suramin has been reported to bind to proteins containing LDLRa domains (*35*, *36*) but also inhibits simple proteases, like trypsin, with only SP domains (*37*, *38*). Suramin may therefore bridge between the chains of fI, possibly with higher affinity binding to the heavy chain. Suramin has been reported to cause oligomerization of complement components C8 and C9, thus blocking membrane attack complex formation and preventing complement-mediated hemolysis (*39*). Suramin was found not to cause any aggregation of fI: using analytical size exclusion chromatography we observed that fI remains monomeric in the presence of 1 mM suramin (data not shown).

The contrast in the proteolytic activities of intact fI and the SP domain confirms that the heavy chain and cofactor have a role in contact with the substrates. The SP domain cleaves C3(NH_3) at several sites, so it is less "specific" than intact fI + cofactor. This strongly suggests that the fI heavy chain binds to substrate and orients the SP domain of intact fI toward the two cleavage sites in C3b which are cleaved to form iC3b.

Intact fI alone does not cleave C3b at all, while direct binding between them has been demonstrated (*8*). The interaction involves both chains of C3(NH_3), presumably indicating two or more sites of interaction. At least one binding interaction in the fI heavy chain may orient fI to the two specific cleavage sites. However, in the absence of fH, no cleavage occurs, indicating that fH may be required to alter substrate conformation such that these two sites are exposed. Thus, fI binds to C3b, probably via its heavy chain; this binding may be of sufficient affinity and duration to prevent the more random proteolysis seen with the SP domain. The binding of fH to both C3b and fI may enhance the binding of fI to C3b, while it may alter C3b to expose the two specific cleavage sites and also influence the orientation of fI. The activity of the SP domain is inhibited by fH (Figures 6F and 7E,F). This suggests that the conformational change in C3b induced by fH "hides" the bonds which are susceptible to cleavage by the SP domain either by steric interference or by altering C3(NH_3) conformation.

iC3b has not been reported to be generated from C3b by other proteases; several proteases, however, cleave C3b in the less specific pattern similar to that seen with the SP domain. Thus, the fI heavy chain contacts and the cofactor serve to restrict the specificity of fI to produce iC3b through a specialized catalysis event crucial for the innate immune response.

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