Blood coagulation factor Xa (FXa) has recently been shown to function as a plasminogen receptor in the presence of procoagulant phospholipid (phosphatidylserine; PS) and Ca\(^{2+}\). In the current work, the possible effect of autoproteolytic and plasmin-mediated cleavage of FXa on complex formation with plasminogen was investigated. \(^{1,2}\)Plasminogen binding to derivatives of FXa electrotransferred to polyvinylidene difluoride revealed that the autoproteolytic conversion of FXa\(^\alpha\) to FXa\(^\beta\) was required for the expression of a plasminogen binding site. In the presence of PS and Ca\(^{2+}\), plasmin was shown to convert FXa\(^\alpha\) to a FXa\(^\beta\)-like species at least 3 orders of magnitude faster than the autoproteolytic mechanism. This also resulted in the exposure of a plasminogen binding site. Further processing by plasmin generated a fragment (33 kDa) due to cleavage at Gly\(^{133}\) in the FXa heavy chain. Production of this species enhanced apparent plasminogen binding compared with FXa\(^\beta\) and resulted in the loss of FXa amidolytic and clotting activity. In the absence of either PS or Ca\(^{2+}\), the plasmin-mediated fragmentation of FXa\(^\alpha\) was altered to include a FXa\(^\beta\)-like molecule and a species (40 kDa) with intact \(\beta\)-heavy chain disulfide linked to a COOH-terminal fragment of the light chain starting at Tyr\(^{44}\). Neither of these products was observed to interact with plasminogen. The 40-kDa species had amidolytic activity comparable with FXa\(^\alpha\) but inhibited clotting activity. Cumulatively, the data provide the first evidence for a functional difference between the FXa subforms and suggest a mechanism where autoproteolysis and plasmin-mediated cleavage modulate the function of FXa\(^\alpha\) from a procoagulant enzyme to a profibrinolytic plasminogen receptor.

The serine protease factor Xa (FXa)\(^\dagger\) functions to generate the principal biological effectors of blood coagulation, thrombin (1). To avoid thrombosis, the expression of FXa activity is strictly limited. One level of regulation is the requirement that FXa associate with the nonenzymatic cofactor Va (FVa) and procoagulant phospholipid (e.g. phosphatidylserine (PS)) in the presence of Ca\(^{2+}\) to form prothrombinase. Within this complex, the catalytic rate of FXa toward the thrombin precursor, prothrombin, is accelerated 5 orders of magnitude and becomes biologically significant (2). The need to assemble into prothrombinase furthermore confines FXa activity to sites of vascular damage, where PS is accessible (3–6).

Although FXa biochemistry is well studied, a question that still remains concerns the functional significance of an autoproteolytic step that excises a 4-kDa glycopeptide (\(\beta\)-peptide) from the heavy subunit COOH terminus (7–9). The intact species, FXa\(^\alpha\), is converted to FXa\(^\beta\). This process is greatly accelerated by Ca\(^{2+}\)-dependent binding to vesicles containing PS (7, 9–11). FXa\(^\beta\) has been shown to undergo a second autoproteolytic step when phospholipid binding is facilitated (i.e. PS and Ca\(^{2+}\)) that produces a species referred to as FXa\(^\gamma\) (10). Since this cleavage is at a position NH\(_2\)-terminal to the active site Ser, irreversible inhibition of enzymatic function results. In the absence of PS, the second autoproteolytic cleavage appears to occur at a position on the light chain that inhibits clotting but not amidolytic activity (12).

In a recent study, FXa was observed to accelerate the generation of the fibrinolytic enzyme plasmin by tissue plasminogen activator (tPA) (13). This was found to involve an interaction with the precursor, plasminogen (13), which is known from other work to require Lys at the COOH terminus of the receptor (14, 15). The acceleration of tPA by FXa was found to be dependent on the presence of both PS and Ca\(^{2+}\) and was inhibited by the inactivation of FXa\(^\alpha\) by Glu-Gly-Arg-chloromethylketone. One explanation for these observations may be the inhibition of FXa autoproteolysis, which could be necessary for complex formation with plasminogen. Evidence for an involvement of the FXa COOH terminus was given by the finding that carboxypeptidase pretreatment of FXa inhibited the acceleration of tPA (13). Since autoproteolysis creates a new COOH terminus, FXa\(^\gamma\) subform conversion may be required to provide an accessible Lys for plasminogen binding.

In addition to the possible involvement of autoproteolysis in the tPA cofactor function of FXa, the involvement of plasmin-mediated proteolysis was suggested by the observation that plasmin treatment of FXa decreased the profibrinolytic activity by approximately 50% (13). The proteolysis of FXa by plasmin has not been previously reported, although plasmin is known to have a relatively broad specificity (16). As a pertinent example, cleavage of the FXa cofactor, FVa, by plasmin has been observed and was reported to alter function through inhibition of coagulation (17, 18) and acceleration of tPA (13). Evidence to suggest an effect of plasmin on FXa function in coagulation is not available.

The goal of the current study was to clarify the roles of autoproteolysis and plasmin-mediated cleavage in the plasminogen binding function of FXa. We now report that plasmin rapidly converts FXa\(^\alpha\) into a FXa\(^\beta\)- and FXa\(^\gamma\)-like species and that either autoproteolytic or plasmin-mediated processing of FXa\(^\alpha\) is required for expression of a plasminogen binding site.

*This work was supported by Canadian Red Cross Society Research and Development Grant HO1O 924. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Research Dept., The Canadian Red Cross Society, 1800 Alta Vista Dr., Ottawa, Ontario, Canada, K1G 4J5. Tel.: 613-739-2462; Fax: 613-739-2426.

\(^1\) The abbreviations used are: FXa, factor Xa; FX, factor X; PS, phosphatidylserine; PCPS, phosphatidylinositol-phosphatidylserine; tPA, tissue plasminogen activator; RVV, Russell's viper venom; PAGE, polyacrylamide gel electrophoresis.
Experimental Procedures

Chemicals and Reagents—HEPES, EDTA (Sigma), Glu-Gly-Argchloromethylketone, 2-guanidinoethylmercaptosuccinic acid, aprotinin (Calbiochem), and benzoyl-Ille-Glu (piperidyl)-Gly-Arg-p-nitraaldehyde (S-2337, Chromogenics) were obtained commercially. Small unilamellar phospholipid vesicles (PCPS) consisting of 75% phosphatidylcholine and 25% PS were synthesized and quantified as described (19). Proteins—Human coagulation factor X (FX) was purified from fresh frozen plasma (20) or from prothrombin complex concentrate diluted to 1 unit/ml factor X clotting activity as obtained as generous gifts from the Canadian Red Cross Society, Ottawa Collection Centre, or from Bayer, respectively. For comparison to in-house preparations, FX was purchased (Haematologic Technologies, Inc.). FXa was generated from FX (21, 22) by treatment with the purified activator from Russell’s viper venom (RVV, Haematologic Technologies) (23) followed by affinity chromatography using benzamidine-Sepharose (Pharmacia Biotech Inc.) to remove the FX activation peptide, RVV, and residual inactivated FX (22). Autoproteolysis during the course of FX activation was inhibited by conducting the reaction in a slurry of benzamidine-Sepharose (~0.3 ml of packed resin per ml). Human Glu-plasminogen was purified from fresh frozen plasma as described (24) and passed over benzamidine-Sepharose (Pierce) to remove any trace of serine protease activity. Plasminogen was radioiodinated (50,000–150,000 dpm/μg) using Iodogen (Pierce) and chromatographically desalted (Excocellulose 5, Pierce) or dialyzed to remove unincorporated 125I. The homogeneity of proteins was assessed by SDS-polyacrylamide electrophoresis (SDS-PAGE) (25) and where applicable by autoradiography using X-Omat AR film (Kodak) and QuantaIII intensifying screens (Dupont). Purified human plasmin was prepared commercially (Haematologic Technologies).

Proteolytic Time Courses—FX (9 μM) or FXα (5 μM) were treated with RVV (10 μg/ml) or plasin (0.1 μM) or allowed to autoproteolyze in 20 mM HEPES, 190 mM NaCl, pH 7.2 (HBS) in the presence of various combinations of Ca2+ (2 mM), EDTA (5 mM), or PCPS (300 μM). Each digest was sampled over time, subjected to SDS-PAGE (12% acrylamide, 2 μg of protein/lane), and stained with Coomassie Brilliant Blue G-250 or transferred to polyvinylidene difluoride (PVDF) filters and stained with Coomassie Brilliant Blue R-250.

Two-dimensional Electrophoresis—The disulfide-linked subunit compositions of plasmin-digested FXα and end products were investigated by nonconventional two-dimensional electrophoresis (two-dimensional SDS-PAGE), as described previously (26). The protocol involved excising the appropriate lane from a 0.75-mm-thick nonreduced SDS-PAGE (27, 28). The PVDF was blocked overnight at 4°C in bovine serum albumin (10 mg/ml) and Tween 20, and bands were excised for amino acid sequence analysis (Dr. A. Kurosky, Protein Chemistry Laboratory, University of Texas at Galveston, Medical Branch) (27, 28).

Ligand Blots—To identify FX- or FXα-derived species that interact with plasminogen, ligand blotting experiments were performed according to methods published by several laboratories (29–31). In our studies, FX or FXα fragments were electrophoresed to PVDF after SDS-PAGE (27, 28). The PVDF was blocked overnight at 4°C in bovine serum albumin (Sigma, 10 mg/ml) and then incubated with 125I-plasminogen (0.1 μM) for 1 h at 22°C in the presence of the indicated concentrations of purified FXa, 2-guanidinoethylmercaptosuccinic acid (50 μM) and aprotinin (Calbiochem, 50 kalikrein-inactivating units/ml). Sequencing of proteins was assessed by SDS-polyacrylamide electrophoresis (SDS-PAGE) (25) and where applicable by autoradiography using X-Omat AR film (Kodak) and QuantaIII intensifying screens (Dupont). Purified human plasmin was prepared commercially (Haematologic Technologies).

Results

Comparison of Plasminogen Binding to FXα and FXα—The binding of 125I-plasminogen to proteins electrotransferred to PVDF (or nitrocelullose) has been used extensively in the past for the identification of plasminogen receptors (29–31). To determine the effect of FXα autoproteolysis on the interaction with plasminogen, similar ligand blotting experiments were conducted. In this system ligand blotting is advantageous because the relative amount of plasminogen associated with discrete proteolytic products of FXα in a mixture can be directly analyzed due to the resolution offered by electrophoresis. Fig. 1 shows the autoradiography of FXα in the absence (panel A) and presence (panel C) of conditions that facilitate PCPS binding. The resulting autoradiograms (panels B and D, respectively), revealed that expression of new plasminogen binding sites paralleled FXα generation. The amount of plasminogen-FXα complex detected was small compared with that observed with FXα. In the absence of PCPS binding (panels A and B), subform conversion was insignificant, and as a result expression of new plasminogen binding sites was not evident. This demonstrated that structural changes in FXα over the course of the experiment that are indiscernible by SDS-PAGE do not account for the observed plasminogen association. The amount of intrinsic plasminogen binding to FXα varied somewhat between preparations but was always low compared with FXα. The batch of FXα used to conduct the experiment in Fig. 1 had the highest amount of endogenous plasminogen binding that we observed. All other preparations of FXα (or FX) bound even less plasminogen relative to FXα and were used in the remaining experiments presented here (e.g. Figs. 2, 5, and 6). Pretreatment of the FXα with carboxypeptidase B prior to conducting the ligand blotting experiment, as described before (13), or inclusion of α-aminoacapric acid (10 mM) with the 125I-plasminogen inhibited the interaction (data not shown). These observations indicated an involvement of a COOH-terminal Lys. The specificity of 125I-plasminogen binding was demonstrated by inhibition due to excess unlabeled plasminogen and the lack of binding to human prothrombin transferred to PVDF (data not shown). Furthermore, no binding was detected when the 125I-plasminogen was substituted for 125I-FXα or 125I-FXα.

Plasminogen Binding to FX Activation Products—The activation of FX requires proteolysis of only the NH2 terminus of
The heavy subunit. Therefore FX and FXα should possess the same COOH-terminal amino acid residue, which is predicted from the cDNA sequence to be Lys (32, 33). To test whether our relative inability to detect plasminogen binding to FXα was acquired during zymogen activation, FX was activated with RVV and allowed to subsequently autoproteolyze. The time course of this reaction (panel A) and corollary plasminogen blot (panel B) are presented in Fig. 2. These data showed that neither FX nor FXα interact appreciably with plasminogen in comparison with FXα.

**Cleavage of FXα by Plasmin**—To directly establish if FXα is a substrate for plasmin, as suggested by previous functional data (13), digestion time courses were carried out and are shown in Fig. 3. The data in panel B demonstrated that FXα is indeed cleaved by plasmin. For comparison, the autoproteolysis of FXα under identical conditions except in the absence of plasmin is shown in panel A. In the presence of PCPS and Ca2+, plasmin generated molecules corresponding in electrophoretic mobility under nonreducing conditions to FXα and FXα (33 kDa). The appearance of a faint band was routinely observed just above the dye front (~13 kDa) and correlated to the appearance of the 33-kDa species. Although 50-fold less plasmin was present in the reaction mixtures than FXα, the conversion of FXα generation after 1 min in the presence of plasmin (panel B) is comparable with that after 30 min by autoproteolysis (panel A). This indicated that on a molar basis FXα subform conversion is mediated more than 1500 times faster by plasmin than by FXα.

A dependence on PCPS and Ca2+ was previously observed for the participation of FXα in plasminogen activation by tPA (13). To determine whether the Ca2+-dependent binding to PCPS was necessary for fragmentation of FXα by plasmin, an identical experiment in the presence of the chelator EDTA was conducted. The fragmentation time-course shown in panel C demonstrated that inhibition of the interaction of FXα with PCPS by EDTA altered the recognition of cleavage sites by plasmin. In the presence of EDTA, production of a FXα-like species was also observed as the initial fragment. However, this migrated as an electrophoretic doublet, suggesting an additional or alternate cleavage(s). Subsequently a product with an apparent molecular mass of 40 kDa was generated. The same SDS-PAGE pattern was observed when the reaction was conducted in the presence of Ca2+ but without PCPS. This showed that binding to PCPS influenced the plasmin-dependent FXα cleavage pattern and that conformational changes in FXα that are induced by Ca2+ (34–37) were not responsible.

To obtain information regarding the location of the plasmin-mediated cleavage sites on FXα, 30-min digestions were conducted and subjected to two-dimensional SDS-PAGE (the first dimension was nonreduced with a reduced second dimension). As shown in Fig. 4A, the 33-kDa species that was produced when FXα was bound to PCPS resolved into subunits corresponding to the intact light chain of FXα and a 15-kDa fragment of the FXα heavy chain. Amino acid sequence analysis of the 15-kDa species demonstrated identity to the NH2 terminus of the FXα heavy subunit (Table I). The location of the heavy chain cleavage site was obtained by sequence analysis of the 13-kDa fragment not disulfide-linked to the light chain, which revealed a new NH2 terminus corresponding to Gly331 (Table I). A similar proteolytic pattern was observed when Ca2+ was present but PCPS was omitted (data not shown). The same 33-kDa species was produced if FXα was first allowed to convert to FXαβ by autoproteolysis, followed by the addition of plasmin (data not shown).

The 40-kDa plasmin-mediated fragment formed when FXα was not bound to PCPS was demonstrated by two-dimensional SDS-PAGE (Fig. 4, panel B) to be composed of the intact FXαβ heavy subunit and a 13-kDa fragment of the FXα light chain. The species that was detected under nonreducing conditions that migrated slightly below the FXαβ-like species to form a doublet (Fig. 3, panel C) is shown faintly in Fig. 4 to be composed of an 18-kDa light chain fragment disulfide-linked to the FXαβ heavy chain. Since this species did not accumulate, it was presumed to be an intermediate leading to the production of the 40-kDa fragment. Sequence analysis of the band corresponding to the 13-kDa fragment observed on one-dimensional SDS-PAGE under reducing conditions revealed an NH2 terminus beginning at Tyr342 (Table I). A lesser species that comigrated with the 13-kDa light subunit fragment was also identified by sequence analysis and aligned with the NH2 terminus of the FXα heavy subunit (data not shown). This may be due to production of a FXα derivative composed of the 13-kDa light subunit piece disulfide-linked to a 13-kDa fragment of the heavy chain, which we have yet to resolve.

Both panels A and B showed lower apparent molecular

---

**Fig. 2. Plasminogen binding to FX activation products.** Panel A, stained PVDF showing the nonreduced SDS-PAGE (12% acrylamide) pattern of FX (9 μg) activation by RVV (10 μg/ml) in the presence of PCPS and Ca2+, and subsequent autoproteolysis time course; panel B, autoradiograph showing [125I]plasminogen (0.1 μg/ml) binding to species in panel A. Incubation time (min) at 22 °C was as follows: 0 (lane 1), 1 (lane 2), 4 (lane 3), 7 (lane 4), 10 (lane 5), 15 (lane 6), 20 (lane 7), 30 (lane 8).

**Fig. 3. Time course of FXα cleavage by plasmin.** Panel A, stained nonreduced SDS-PAGE (12% acrylamide) showing the time course of FXα (5 μM) autoproteolysis in the presence of PCPS (300 μM) and Ca2+ (2 mM); panel B, FXα digested with plasmin in the presence of PCPS and EDTA (5 mM). Incubation time (min) at 22 °C was as follows: 0 (lane 1), 1 (lane 2), 4 (lane 3), 7 (lane 4), 10 (lane 5), 15 (lane 6), 20 (lane 7), 30 (lane 8).

---

2 Numbering based on the amino acid sequence predicted from FX cDNA (33).
weight bands that were not detected in Fig. 3 due to less protein loaded onto the gel. Their identities are currently being investigated. A small amount of the 40-kDa fragment was observed in panel A, which indicated that a fraction of the FXa was not bound to phospholipid under the conditions of this digest.

Plasminogen Binding to Plasmin-mediated FXa Products—To determine whether plasminogen binding sites are exposed when FXa is cleaved by plasmin, the electrophoretic time courses shown in Fig. 3 were transferred to PVDF and blotted with 125I-plasminogen. The resulting autoradiographs are shown in Fig. 5. When conditions were established to establish plasminogen binding initially correlated with the production of the fragments of FXa. Having demonstrated that plasmin generates a FXa fragment in the presence of PCPS and Ca\(^{2+}\) with similar apparent molecular weight as authentic FXaY, we compared the affinity of the two species for plasminogen. The stained gel in Fig. 6A shows the relative amount of protein blotted to PVDF, which was subsequently probed with 125I-plasminogen to produce the autoradiograph in panel B. Although the electrophoretic migration of FXa by autoproteolysis is the same as that produced by plasmin, the amount of plasminogen binding is clearly disproportionate and favors the latter. This experiment also confirms the inability of FX, FXa, and plasmin-mediated fragments formed in the presence of EDTA to bind plasminogen.

Effect of Plasmin on FXa Enzymatic Activity—Having established that FXa can be cleaved by plasmin, we next determined the enzymatic activity of the plasmin-processed FXa. Autoproteolytic or plasmin digestion time courses that were identical to those shown in Fig. 3 were assayed for FXa activity. The data presented in Fig. 7A showed that the cleavage of FXa by autoproteolysis or by plasmin in the presence of EDTA had little effect on amidolytic activity over the time frame of the experiment. On the other hand, cleavage of FXa by plasmin in the presence of Ca\(^{2+}\) substantially inhibited amidolytic activity. The incomplete inhibition of amidolytic activity was consistently observed, although negligible intact FXa or FXa remained after 30 min of incubation. The fragmentation of FXa by plasmin in the presence or absence of PCPS binding

![Fig. 4. Disulfide-linked subunit composition of plasmin-cleaved FXa. The nonreduced SDS-PAGE (12% acrylamide) pattern of FXa (7 μM) digested with plasmin (0.1 μM) for 30 min was excised and electroblotted onto PVDF, the various proteolytic derivatives of FXa (panel A). A, FXa digested with plasmin in the presence of PCPS (300 μM) and Ca\(^{2+}\) (2 mM); lane 1, FXa; lane 2, FXa; lane 3, FXa incubated 30 min in the presence of PCPS and Ca\(^{2+}\) to give mostly FXa; lane 4, FXa incubated 4 h in the presence of PCPS and Ca\(^{2+}\) to give FXa; lane 5, FXa treated for 30 min with plasmin in the presence of PCPS and Ca\(^{2+}\); lane 6, FXa treated for 30 min with plasmin in the presence of PCPS and EDTA; lane 7, and 30 (lane 8).

![Fig. 5. Plasminogen binding to plasmin-cleaved FXa. Panel A, autoradiograph showing 125I-plasminogen (0.1 μM) binding to FXa species formed in the presence of plasmin, PCPS (300 μM) and Ca\(^{2+}\) (2 mM) (corresponds to stained gel in Fig. 3B); panel B, autoradiograph showing 125I-plasminogen (0.1 μM) binding to FXa species formed in the presence of plasmin, PCPS (300 μM) and EDTA (5 mM) (corresponds to stained gel in Fig. 3C). Incubation time (min) at 22 °C was as follows: 0 (lane 1), 1 (lane 2), 4 (lane 3), 7 (lane 4), 10 (lane 5), 15 (lane 6), 20 (lane 7), and 30 (lane 8).

![Fig. 6. Relative binding of plasminogen to autoproteolytic and plasmin-mediated products of FXa. After SDS-PAGE and transfer to PVDF, the various proteolytic derivatives of FXa were probed with 125I-plasminogen. Panel A, stained PVDF; panel B, autoradiograph. Lane 1, FX; lane 2, FXa; lane 3, FXa incubated 30 min in the presence of PCPS and Ca\(^{2+}\) to give mostly FXa; lane 4, FXa incubated 4 h in the presence of PCPS and Ca\(^{2+}\) to give FXa; lane 5, FXa treated for 30 min with plasmin in the presence of PCPS and Ca\(^{2+}\); lane 6, FXa treated for 30 min with plasmin in the presence of PCPS and EDTA; lane 7, and 30 (lane 8).]
inhibited clotting activity (panel B). The slower loss of clotting activity in the presence of EDTA correlated with the relative rates of 33- versus 40-kDa fragment production (Fig. 3).

DISCUSSION

The studies described in this paper are an extension of a previous report that identified FXa as a plasminogen receptor and accelerator of the tPA-mediated generation of plasmin (13). Evidence is presented in the current study revealing that the expression of a plasminogen binding site on FXa requires prior conversion of FXa to the \( \beta \)-subform. This conclusion provides a possible explanation for our earlier observation that inhibition of the FXa cofactor effect on tPA by chloromethylketone (13) may have been due to blocking of the autoproteolytic production of FXa\( \beta \). To our knowledge, these are the first data that demonstrate a functional difference between FXa and FXa\( \beta \) in a well-characterized system.

Since we found in the earlier study that plasmin pretreatment of FXa alters its tPA cofactor activity (13), we investigated the ability for plasmin to cleave FXa. The current work shows that FXa and FXa\( \beta \) are previously unrecognized substrates for plasmin. A schematic summarizing the plasmin-mediated cleavage sites in FXa is shown in Fig. 8. Under conditions that facilitate binding of FXa to phospholipid vesicles, the fragmentation pattern closely resembled that of autoproteolysis, which included the sequential generation of species identical by reduced and nonreduced SDS-PAGE to FXa\( \beta \). This species was 40 kDa as determined by electrophoresis in a well-characterized system.

Combined with the conclusion that authentic FXa\( \beta \) but not FXa binds plasminogen, the finding that plasmin rapidly generates a FXa\( \beta \)-like molecule suggested a mechanism of regulation in which plasmin production could directly accelerate the conversion of FXa into a plasminogen receptor. Indeed, similar to the autoproteolytic processing of FXa, proteolysis by plasmin was shown to liberate a plasminogen binding site upon production of FXa\( \beta \). Furthermore, plasmin was observed to expose a plasminogen binding site on FXa\( \gamma \), which appeared to have an even higher affinity than the first cleavage product. Like authentic FXa\( \gamma \) (10), the production of FXa\( \gamma \) was involved cleavage at a site NH\(_2\)-terminal to the active site Ser, which we identified as Gly\(_{331}\). Consequently, inhibition of amylolytic and clotting activity resulted due to disruption of the active site catalytic triad shown in Fig. 8 (H, D, and S).

When conditions were established to block the interaction between FXa and phospholipid (e.g., chelation of Ca\(^{2+}\) by EDTA), the plasmin-mediated fragmentation pattern was altered. The first cleavage product of FXa by plasmin resulted in a species that resembled FXa\( \beta \) (FXa\( \gamma \)). Unlike the species generated by plasmin when FXa was bound to PCPS, the final digestion product in the presence of EDTA did not resemble FXa\( \gamma \). This species was 40 kDa as determined by nonreduced SDS-PAGE and consisted of the intact FXa, FXa\( \beta \), and FXa\( \gamma \). (FXa\( \beta \) and FXa\( \gamma \) were previously unrecognized substrates for plasmin).

Combined with the conclusion that authentic FXa\( \beta \) but not FXa binds plasminogen, the finding that plasmin rapidly generates a FXa\( \beta \)-like molecule suggested a mechanism of regulation in which plasmin production could directly accelerate the conversion of FXa into a plasminogen receptor. Indeed, similar to the autoproteolytic processing of FXa\( \beta \), proteolysis by plasmin was shown to liberate a plasminogen binding site upon production of FXa\( \beta \). Furthermore, plasmin was observed to expose a plasminogen binding site on FXa\( \gamma \), which appeared to have an even higher affinity than the first cleavage product. Like authentic FXa\( \gamma \) (10), the production of FXa\( \gamma \) was involved cleavage at a site NH\(_2\)-terminal to the active site Ser, which we identified as Gly\(_{331}\). Consequently, inhibition of amylolytic and clotting activity resulted due to disruption of the active site catalytic triad shown in Fig. 8 (H, D, and S).

When conditions were established to block the interaction between FXa and phospholipid (e.g., chelation of Ca\(^{2+}\) by EDTA), the plasmin-mediated fragmentation pattern was altered. The first cleavage product of FXa by plasmin resulted in a species that resembled FXa\( \beta \) (FXa\( \gamma \)). Unlike the species generated by plasmin when FXa was bound to PCPS, the final digestion product in the presence of EDTA did not resemble FXa\( \gamma \). This species was 40 kDa as determined by nonreduced SDS-PAGE and consisted of the intact FXa, FXa\( \beta \), and FXa\( \gamma \). (FXa\( \beta \) and FXa\( \gamma \) were previously unrecognized substrates for plasmin).
Considerable evidence has accumulated that suggests a COOH-terminal Lys in a receptor may be the only structural characteristic required for plasminogen binding (14, 15). Since the cDNA sequence for the FX and FXa heavy subunit predicts the COOH terminus to be Lys^348 (32, 33), it is interesting that association of plasminogen with either FX or FXa was found to be insignificant compared with FXa those. This observation can be explained by recent reports of constitutive (39) and inducible (40, 41) carboxypeptidase B activity in plasma, which excises COOH-terminal Lys and consequently attenuates plasmin production. The mechanisms by which these carboxypeptidases are inhibited when clot lysis is desirable has been tied to the control of thrombin production by activated protein C (40). At least one of the plasma carboxypeptidases is activated (40, 41) and subsequently inactivated (40) by thrombin. While direct COOH-terminal sequencing has not yet been conducted, the lack of plasminogen binding suggested that FX is a substrate for the carboxypeptidase(s). Evidence for this hypothesis was previously obtained by amino acid sequence analysis of the β-peptide excised from FX/FXa, which showed the predicted COOH-terminal Lys was missing (42). Thus, Fig. 8 shows processing of FX by a plasma carboxypeptidase, which would result in FXa^447 as a COOH terminus.

It is well known that serine proteases (e.g., FXa and plasmin) preferentially cleave at basic amino acids. To explain our observation was inconsistent with the amino acid sequence for plasminogen was not observed by ligand blotting. This observation may create new plasminogen receptors with consequent acceleration of plasmin generation (13). A feedback system is indicated when plasmin is present, which rapidly converts FXa to plasmin (13). A plasminogen receptor that resembles FXa is plasmin-mediated cleavage of FXa may create new plasminogen binding sites. Therefore, authentic FXa may be implicated as new COOH terminal due to autoproteolysis, with the latter being a minor product (42). To explain the lack of plasminogen binding to the FXa^447, it is proposed that Arg^429 may be the COOH terminus.

The exact autoproteolytic site(s) in the FXa heavy chain that produces FXa has not been determined. However, a region corresponding to the α-thrombin autolysis loop has been identified in FX, which undergoes extensive cleavage (43) and would give rise to a species consistent with the electroreptor properties of FXa. These cleavage sites could expose new COOH-terminal Lys at positions 330 and 338 or Arg at positions 326, 332, and 336 (43). By NH₂-terminal sequence analysis of the FXa, the exposure of COOH-terminal Lys^339 in FXa^447 is suggested to account for the acquisition of plasminogen binding. A proportion of authentic FXa having COOH-terminal Arg may explain why it appeared to associate with lower affinity to plasminogen than FXa^447. Based on NH₂-terminal amino acid sequence and plasminogen binding data, the predicted COOH termini exposed on FXa due to processing by plasmin are summarized in Fig. 8.

When FXa was cleaved by plasmin under conditions that did not facilitate binding to PCPS, the exposure of binding sites for plasminogen was not observed by ligand blotting. This observation was inconsistent with the amino acid sequence data that predicted the creation of COOH-terminal Lys at position 43 upon production of FXa^447, as shown in Fig. 8. One explanation for this discrepancy is that the light chain fragment containing the relevant COOH-terminal Lys^43 may be trapped poorly by PVDF and that consequently an association with plasminogen was not detectable by ligand blotting experiments.

In order to explain how phospholipid alters the observed plasmin-mediated cleavage pattern of FXa, two determining factors may be involved. The first is that the cleavage site in the Gla domain may become inaccessible to plasmin simply because it is masked when bound to PCPS. This cannot account for the absence of heavy chain cleavage when the FXa is free in solution. Therefore, the association with PCPS may also promote a conformational change in FXa that exposes the heavy chain cleavage site. Evidence for a conformational change in FXa due to PCPS binding has been previously reported (44) and may contribute to the prothrombinase cofactor effect of PCPS.

From previous reports a pathway of communication between coagulation and fibrinolysis has emerged that involves the modulation of plasminogen by plasmin. In these studies plasmin was found to 1) inhibit the procoagulant activity of FVα (17, 18), 2) convert the FVα into a plasminogen receptor and tPA accelerator (13), and 3) inhibit the attenuation of FXa as a tPA cofactor by native FVα (13). The current study adds to this model by providing evidence for a direct effect of plasmin on FXa function and a role for autoproteolytic conversion of FXa to FXa^447. In summary, the new data show that neither FX nor FXa^447 can interact appreciably with plasminogen. Autoproteolysis results in the expression of a plasminogen binding site on FXa^447, presumably by creating a COOH-terminal Lys. This would facilitate the functioning of FXa^447 as a plasminogen receptor with consequential acceleration of plasmin generation (13).

Acknowledgments—We thank Tina Raynor, Michael Sutherland, and Dr. Réal Lemieux for helpful suggestions regarding the manuscript. We also acknowledge the expertise of Steve Smith and Dr. Alex Kurosky (University of Texas at Galveston, Medical Branch, Protein Chemistry Laboratory) for amino acid sequence analyses.

REFERENCES

1. Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Blood 76, 1–16
2. Nesheim, M. E., Tawsew, J., and Mann, K. G. (1979) J. Biol. Chem. 254, 10967–10962
3. Mann, K. G., Tracey, P. B., Krishnaswamy, S., Jeny, R. J., Osdegaard, B. H., and Nesheim, M. E. (1987) Thrombosis and Haemostasis (Versteegh, M., Vermylen, J., Links, H. R., and Arnout, J., eds) pp. 505–523, International Society on Thrombosis and Haemostasis and Leiden Press, Leiden
4. Packham, M. A., and Mustard, J. F. (1984) Blood Platelet Function and Medical Chemistry (Lasala, A., ed) pp. 61–128, Elsevier Biomedical, New York
5. Bevers, E. M., Comfurius, P., van Rijn, J. L. M. L., Henker, H. C., and Zwaal, R. F. A. (1982) Eur. J. Biochem. 122, 429–436
6. Bevers, E. M., Comfurius, P., and Zwaal, R. F. A. (1983) Biochim. Biophys. Acta 736, 57–66
7. Jesty, J., Spencer, A. K., and Nemerson, Y. (1974) J. Biol. Chem. 249, 5614–5623
8. Fujikawa, K., Coan, M. H., Legaz, M. E., and Davies, E. W. (1974) Biochemistry 13, 5290–5299
9. Fujikawa, K., Tittari, K., and Davies, E. W. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3359–3363
10. Mertens, K., and Bertina, R. M. (1980) Biochem. J. 185, 647–658
11. Link, R. P., and Castellino, F. J. (1982) Arch. Biochem. Biophys. 215, 215–221
12. Bock, P. E., Craig, P. A., Olson, S. T., and Singh, P. (1989) Arch. Biochem. Biophys. 273, 375–388
13. Przyzialek, E. L. G., Bajzar, L., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 17871–17877
14. Ploix, E., Feliç, J., and Miles, L. A. (1991) Thromb. Haemostasis 66, 32–36
15. Hajjar, K. A., and Nachman, R. L. (1994) in Hemostasis and Thrombosis: Basic Principles and Clinical Practice (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 823–836, J. B. Lippincott Co., Philadelphia
16. Castellino, F. J., and Powell, J. R. (1981) Methods Enzymol. 80, 365–378
16620

Modulation of FXα by Autoproteolysis or Plasmin

17. Lee, C. D., and Mann, K. G. (1989) Blood 73, 185–190
18. Omar, M. N., and Mann, K. G. (1987) J. Biol. Chem. 262, 9750–9755
19. Krishnaswamy, S., Nesheim, M. E., Pryzdial, E. L. G., and Mann, K. G. (1994) Methods Enzymol. 222, 260–280
20. Bajaj, S. P., Rapaport, S. I., and Prodanos, C. (1981) Prep. Biochem. 11, 387–412
21. Jesty, J., and Nemerson, Y. (1976) Methods Enzymol. 45, 95–107
22. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) J. Biol. Chem. 262, 3291–3299
23. Jesty, J., and Nemerson, Y. (1976) Methods Enzymol. 45, 95–107
24. Nesheim, M., Fredenburgh, J. C., and Larson, G. R. (1990) J. Biol. Chem. 265, 21541–21548
25. Laemmli, U. K. (1970) Nature 227, 680–685
26. Pryzdial, E. L. G., and Isenman, D. E. (1987) J. Biol. Chem. 262, 1519–1525
27. Matsuda, P. (1987) J. Biol. Chem. 262, 10035–10038
28. Yuen, S. W., Chui, A. H., Wilson, K. J., and Yuan, P. M. (1989) BioTechniques 7, 74–82
29. Dudani, A. K., Cummings, C., Hashemi, S., and Ganz, P. R. (1993) Thromb. Res. 69, 185–196
30. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) J. Biol. Chem. 269, 21191–21197
31. Miles, L. A., Dahlberg, C. M., Plascia, J., Félez, J., Kato, K., and Plow, E. F. (1991) Biochemistry 30, 1682–1691
32. Fung, M. R., Hay, C. W., and MacGillivray, R. T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3591–3595
33. Messier, T. L., Pittman, D. D., Long, G. L., Kaufman, R. J., and Church, W. R. (1991) Gene (Amst.) 99, 291–294
34. Raddi, R. D., and Barton, P. G. (1972) J. Biol. Chem. 247, 7735–7742
35. Nelsestuen, G. (1976) J. Biol. Chem. 251, 5648–5656
36. Church, W. R., Boulanger, L. L., Messier, T. L., and Mann, K. G. (1989) J. Biol. Chem. 264, 17882–17887
37. Pryzdial, E. L. G., and Mann, K. G. (1991) J. Biol. Chem. 266, 8969–8977
38. Höjrup, P., and Magnusson, S. (1987) Biochem. J. 245, 887–892
39. Skidgel, R. A. (1986) Trends Pharmacol. Sci. 9, 299–304
40. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484
41. Redlitz, A., Tan, A. K., Eaton, D. L., and Plow, E. F. (1995) J. Clin. Invest. 96, 2534–2538
42. Eby, C. S., Mullane, M. P., Porche-Sorbet, R. M., and Miletich, J. P. (1992) Blood 80, Suppl. 1, 306 (abstr.)
43. Padmanabhan, K., Padmanabhan, K. P., Tulinsky, A., Park, C. H., Bode, W., Huber, R., Blankenship, D. T., Cardin, A. D., and Kissel, W. (1993) J. Mol. Biol. 232, 947–966
44. Husten, E. J., Esmon, C. T., and Johnson, A. E. (1987) J. Biol. Chem. 262, 12953–12962