Plasmin Converts Factor X from Coagulation Zymogen to Fibrinolysis Cofactor*

(Received for publication, July 28, 1998, and in revised form, December 22, 1998)

Edward L. G. Pryzdial‡, Nadine Lavigne, Nicolas Dupuis, and Garry E. Kessler
From the Research and Development Department, Canadian Blood Services and Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario K1G 4J5, Canada

Known anticoagulant pathways have been shown to exclusively inhibit blood coagulation cofactors and enzymes. In the current work, we first investigated the possibility of a novel anticoagulant mechanism that functions at the level of zymogen inactivation. Utilizing both clotting and chromogenic assays, the fibrinolysis protease plasmin was found to irreversibly inhibit the pivotal function of factor X (FX) in coagulation. This was due to cleavage at several sites, the location of which were altered by association of FX with procoagulant phospholipid (proPL). The final products were ~28 and ~47 kDa for proPL-bound and unbound FX, respectively, which did not have analogues when activated FX (FXa) was cleaved instead. We next investigated whether the FX derivatives could interact with the plasmin precursor plasminogen, and we found that plasmin exposed a binding site only on proPL-bound FX. The highest apparent affinity was for the 28-kDa fragment, which was identified as the light subunit disulfide linked to a small fragment of the heavy subunit (Met-296 to ~Lys-330). After cleavage by plasmin, proPL-bound FX furthermore was observed to accelerate plasmin generation by tissue plasminogen activator. Thus, a feedback mechanism localized by proPL is suggested in which plasmin simultaneously inhibits FX clotting function and converts proPL-bound FX into a fibrinolysis cofactor. These data also provide the first evidence for an anticoagulant mechanism aimed directly at the zymogen FX.

Blood coagulation and fibrinolysis are opposing hemostatic processes. Whereas the coagulation pathway produces insoluble fibrin clot to seal vascular leaks, the fibrinolysis pathway solubilizes the clot to restore normal blood flow. It is obvious that for either of these pathways to be effective, their amplification must be sequential. Several coordinating mechanisms have been identified that control the availability of fibrinolysis cofactors. The best described of these is the production of fibrin (1), which, unlike its precursor fibrinogen, is a fibrinolysis cofactor that colocalizes the zymogen plasminogen and an activating protease, tissue plasminogen activator (tPA), for subsequent plasmin generation. Plasmin directly mediates fibrin solubilization by proteolysis. In order for plasminogen to assemble into the ternary activation complex, the cofactor molecule must contain a COOH-terminal Lys, which is an essential constituent of the plasminogen binding site (2–10). Carboxypeptidases in plasma have been identified that excise COOH-terminal Lys and thereby limit the generation of plasmin (11–13) by restricting fibrinolysis cofactor function. In particular, an important regulatory role has been demonstrated for one of these carboxypeptidases, TAFI, which has been shown to first become activated and then later inactivated by the final coagulation enzyme, thrombin (12–14). Thus, thrombin orchestrates a lag between coagulation and the amplification of fibrinolysis to ensure sufficient clot production.

An additional mechanism of communication between coagulation and fibrinolysis has recently been reported. This involves the enzyme complex responsible for physiological thrombin production, prothrombinase (15, 16). Prothrombinase consists of the serine protease factor Xa (FXa), and the non-enzymatic cofactor Va (FVa), which in the presence of Ca2+ associate with each other and a procoagulant phospholipid (proPL)-containing surface. Together the constituents of prothrombinase enhance the FXa-mediated activation of prothrombin to thrombin by 5 orders of magnitude (17). Previous studies have shown that plasmin can inhibit the prothrombinase cofactor function of FVa (18) and the procofactor V (19). We have extended these findings to show that plasmin also proteolytically inhibits the coagulation activity of FXa, and furthermore converts both FXa and FVa into fibrinolysis cofactors (9, 20). Interestingly, the acquisition of fibrinolysis function was dependent on the prior association of FXa or FVa with proPL. This may provide a means to accelerate plasmin generation specifically at sites of vascular damage, where the exposure of proPL is selectively triggered.

The physiological generation of FXa requires recruitment of its inactive precursor, factor X (FX), to sites of vascular damage through an association with proPL. Therefore, following sufficient fibrin formation to initiate fibrinolysis, it is conceivable that localized plasmin production may modulate FX function. We now report that FX is a substrate for plasmin, but the final proteolytic products differ from that of FXa. The cleavage of proPL-bound FX by plasmin results in a diametric functional change that inhibits participation in coagulation and simultaneously induces the expression of fibrinolysis cofactor activity.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—HEPES, EDTA, (Sigma), 2-guanidino-ethylmercaptosuccinic acid (GEMSA), aprotonin (Calbiochem), N-α-Z-n-Arg-Gly-Arg-p-nitroanilide (S2655), and benzoyl-ile-Glu-(piperidyl)-Gly-Arg-p-nitroanilide (S2366) (Helena) were purchased. As a source of proPL, small unilamellar phospholipid vesicles consisting of 75% phosphatidylcholine and 25% of the procoagulant lipid, phosphatidylserine, 2-guanidino-ethylmercaptosuccinic acid; HBS, HEPES plus NaCl; PVDF, polyvinylidene difluoride.

* This work was supported by the Canadian Blood Services Research and Development Grant HO10 928. 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 and Development Dept., Canadian Blood Services, 1800 Alta Vista Dr., Ottawa, Ontario K1G 4J5, Canada. Tel.: 613-739-2462; Fax: 613-739-2426; E-mail ed.pryzdial@bloodservices.ca.
‡ The abbreviations used are: tPA, tissue plasminogen activator; FX, factor X; FXa, activated FX; FVa, factor Va; proPL, procoagulant phospholipid; PAGE, polyacrylamide gel electrophoresis; KIU, kalikrein inhibitory unit(s); RVV, Russell’s viper venom FX activator; GEMSA, 2-guanidino-ethylmercaptosuccinic acid; HBS, HEPES plus NaCl; PVDF, polyvinylidene difluoride.
Proteins—Human coagulation factor X (FX) was purified from fresh frozen plasma (21) or from prothrombin complex concentrate diluted to 1 unit/ml factor X clotting activity obtained as generous gifts from the Canadian Red Cross Society, London Collection Center, and from Bayer, Inc., respectively. For comparison to in-house preparations, FX was also purchased (Hematologic Technologies, Inc.). Human FXα (22, 23) and Lys-plasminogen were produced, as described (25). Lys-plasminogen was radiiodinated (50,000–150,000 dpm/μg) using Iodogen (Pierce) and chromatographically desalted (Excellulose 5, Pierce) to remove unincorporated 125I. Purified human plasmin was purchased (Hematologic Technologies, Inc.).

Proteolytic Time Courses—FX (9 μg) was treated with plasmin (0.1 μg) in 20 mM HEPES, 150 mM NaCl, pH 7.2 (HBS), in the presence of various combinations of Ca2+ (2 mM), EDTA (5 μM), or proPL (300 μg) at 21°C. Each digest was sampled over time, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) (26) and silver-stained. For electrophoretic comparison to the FXα fragments generated by plasmin, at each time point, aprotinin (50 kalikrein inhibitory units (KIU/ml)) was added to the plasmin-digested FX followed by treatment with RVV (10 μg/ml) at 37°C for 1 h in 10 mM CaCl2.

FX Enzymatic Activity Assays—The effect of plasmin treatment of FX on the generation of FXα coagulation and amidolytic activity was evaluated in plasmin digestion time courses identical to those described above. At each time point, aprotinin (50 KIU/ml) was added to stop further effects of plasmin. Clotting activity was measured by thromboplastin clotting time assays using FX-deficient plasma (Sigma) reconstituted with 5 units/ml purified FX treated with plasmin as a substrate. The amount of FX activation was determined by comparison to a standard curve obtained by titrating purified FXα in the presence of precomplexed plasmin and aprotinin to duplicate the conditions of the time course samples. The generation of FXα amidolytic activity at 22°C in HBS using S2765 (200 μM) as a chromogenic substrate was followed after treatment of plasmin-digested FX with RVV as described above. The rate of color development was monitored in a kinetic multwell plate reader (Vmax, Molecular Devices).

Ligand Blots—To identify FX-derived species that interact with plasminogen, ligand blotting experiments were performed according to methods published by several laboratories (5, 3, 2). In our studies, FX fragments were separated by SDS-PAGE and electrotransferred to PVDF (27, 28). The PVDF was blocked overnight at 4°C in bovine serum albumin (Sigma, 10 mg/ml) and then incubated with 125I-Lys-plasminogen (0.1 μg) for 1 h at 22°C in the presence of the protease inhibitors GEMSA (50 nM) and aprotinin (50 KIU/ml, Calbiochem). Following extensive washing with HBS, the location of bound 125I-plasminogen was determined by autoradiography and compared with the electrophoretic patterns made visible by staining the PVDF with Coomassie Brilliant Blue R-250.

Effect of Plasmin-treated FX on tPA Activity—The activation of plasminogen (1 μg) to plasmin by single-chain tPA (10 nM) was followed as a time course utilizing the chromogenic substrate S2366. To determine whether plasmin-treated FX affects tPA function, FXα was treated with plasmin (or left untreated) exactly as described above. At each time point, aprotonin (50 KIU/ml) was added to inhibit the plasmin used in this step prior to the addition of tPA and plasminogen. To control for the possible effects of added aprotinin and plasmin, reactions that did not involve a plasmin treatment step contained aprotinin and plasmin that had been preincubated.

Amino Acid Sequencing—The subunit composition of plasmin-digested FX products was investigated both by nonreducing SDS-PAGE and by two-dimensional SDS-PAGE involving a first dimension under nonreducing conditions and a second dimension under reducing conditions (29, 20). To identify proteolytic cleavage sites, electrophoretic patterns were transferred to PVDF, and bands were excised for sequence analysis (27, 28).

RESULTS

Inactivation of FX Coagulation Activity by Plasmin—To determine whether FX coagulation activity is altered upon pretreatment with plasmin, Fig. 1A shows FXα-dependent thromboplastin clotting (panel A) and RVV-initiated amidolytic (panel B) assays. Both methods demonstrated that plasmin inhibits the coagulation enzyme function of FX. This was observed regardless of whether binding of FX to proPL was facilitated by Ca2+.

Cleavage of FX by Plasmin—The demonstration that FX enzymatic activity is lost due to plasmin pretreatment strongly indicates that FX is cleaved by plasmin. To provide direct evidence, changes in plasmin-treated FX electrophoretic mobility were followed using the same digestion conditions and pretreatment times as in Fig. 1. As demonstrated by the SDS-PAGE shown in Fig. 2, plasmin sequentially cleaves FX at several sites. Interestingly, the proteolytic sites recognized by plasmin depended on whether Ca2+ was present to facilitate FX-proPL interactions. It should be noted that minor amounts of the alternate proteolytic products were detected under both reaction conditions, indicating that one pathway is favored over the other but is not exclusive. The nomenclature adopted previously for Pn-mediated FXα fragments (20) was used for the analogous FX fragments observed here. However, two additional species were observed for FX that had apparent molecular masses of 28 kDa (Fig. 2A) and 47 kDa (Fig. 2B) and resulted from cleavage by plasmin in the presence of Ca2+ and EDTA, respectively.

To determine whether only binding of FX to Ca2+ was the determinant in altering the cleavage sites recognized by plasmin, the 30-min time points shown in Fig. 2 were compared with identically treated FX in the presence of Ca2+ but no proPL. These data are shown in Fig. 3 and demonstrate that Ca2+ alone is insufficient to generate the fragmentation profile that resulted in the presence of Ca2+ and proPL. Thus, binding to proPL and not just to Ca2+ is suggested to alter the cleavage of FX by plasmin.

To further compare the cleavage of FX and FXα by plasmin, the same fragmentation time courses were conducted as in Fig. 2 except that prior to electrophoresis, the FX-derived fragments generated by plasmin were treated with RVV to remove the activation domain. The resulting SDS-PAGE profiles are shown in Fig. 4, with the mobility of plasmin-mediated species generated directly from purified FXα indicated. As shown in Fig. 4A, when FXαPn,Ca and FXαPn, Ca were treated with RVV under conditions that favored binding to proPL, the resulting species had electrophoretic migrations identical to FXαPn,Ca and FXαPn, Ca respectively. This suggests that plasmin cleaves the same sites in FX and FXα to generate these fragments. However, the 28-kDa product was unique to FX bound to proPL, and RVV had no effect on the mobility of this fragment.

In contrast to FXαPn, Ca, but similar to the further proteolyzed 28-kDa species, the data presented in Fig. 4B show that RVV does not cleave FXαPn, EDTA over the indicated time. This is additional evidence that two pathways of FX proteolysis by
EDTA and a species that migrated with the dye front. The pretreatment. No interaction was detectable between 125I-plasmin former appeared to be present as a minor constituent of our Silver-stained nonreduced proPL during the plasmin pretreatment step (Fig. 5). Binding of 125I-plasminogen to untreated FX was not detectable. However, under conditions that facilitated proPL binding (Fig. 5A), plasmin induced the expression of a receptor site(s) in FX\(\beta_{\text{Pn, Ca}}\), FX\(\gamma_{\text{Pn, Ca}}\), the 28-kDa fragment, and the \(-13\)-kDa fragment. Using this ligand blotting method, the species clearly having the highest apparent affinity for 125I-plasminogen was the 28-kDa fragment for as yet unknown reasons. These observations suggested that plasmin liberates a COOH-terminal Lys on each of these FX fragments.

Under conditions that did not facilitate binding of FX to proPL during the plasmin pretreatment step (Fig. 5B), we observed 125I-plasminogen binding only marginally to FX\(\beta_{\text{Pn}}\), EDTA and a species that migrated with the dye front. The former appeared to be present as a minor constituent of our starting FX preparation and is likely independent of plasmin pretreatment. No interaction was detectable between 125I-plasminogen and the other major cleavage products, FX\(\gamma_{\text{Pn, EDTA}}\) and the \(-47\)-kDa fragment, suggesting that a COOH-terminal Lys is not made available by proteolysis unless binding to proPL is facilitated.

Based on our ligand blotting experiments, the 28-kDa fragment in particular was suggested to have potential as a fibrinolytic cofactor. Therefore, we followed the effect of plasmin-cleaved FX on the activation of plasminogen by tPA using a chromogenic assay for plasmin (Fig. 6). As shown, a mixture of FX\(\gamma_{\text{Pn, Ca}}\) and the 28-kDa species produced by pretreatment of proPL-bound FX with plasmin for 30 min (final concentration, 0.5 \(\mu\)M FX) resulted in a 27-fold enhancement of tPA activity (Fig. 6A). In contrast, when either native FX or FX pretreated with plasmin to produce FX\(\gamma_{\text{Pn, EDTA}}\) and the \(-47\)-kDa fragment (Fig. 6B) were used, insignificant effects on tPA activity were observed. As shown in Fig. 6C, acceleration of tPA by the FX\(\gamma_{\text{Pn, Ca}}\) and the 28-kDa species mixture was titratable to levels below the circulating concentration of FX (0.17 \(\mu\)M).

Identity of FX-derived Fibrinolysis Cofactor(s)—Because the cleavage sites recognized by plasmin to produce the 28-kDa fragment are unique to FX-bound to proPL, and because this species was found to be a good tPA accelerator and plasminogen-binder, we conducted amino acid sequence analyses to locate the specific cleavage sites. Table I summarizes these data. When the nonreduced FX\(\gamma_{\text{Pn, Ca}}\) band was analyzed, two equimolar sequences were revealed that corresponded to the NH2 terminus of the heavy and light subunits of FX. Determination of the \(-13\)-kDa species amino acid sequence, which appeared concomitant with FX\(\beta_{\text{Pn, Ca}}\), revealed that FX\(\gamma_{\text{Pn, Ca}}\) is generated due to cleavage of the heavy subunit at Gly-331 (based on amino acid numbering adopted previously (30)).

When the nonreduced 28-kDa species was excised from PVDF and sequenced, three equimolar residues per cycle were observed. These corresponded to the NH2 terminus of the heavy and light subunits, in addition to an internal heavy chain sequence beginning at Met-296. To further investigate this mixture of fragments, two-dimensional SDS-PAGE was conducted in which the 28-kDa species was first electrophoresed nonreduced and then subjected to a second dimension under reducing conditions (not shown). Two species were identified after transfer to PVDF and were sequenced. The mobility of the first was not affected by reduction and yielded a sequence identical to the NH2 terminus of the FX heavy subunit. The second reduced fragment had an apparent molecular mass of approximately 20 kDa and migrated exactly to the position of the native FX light subunit. NH2-terminal amino acid sequence analysis confirmed its identity as the light subunit. These data cumulatively suggested that the nonreduced 28-kDa band was a co-migrating mixture of an NH2 terminus-derived heavy subunit fragment ending at Arg-295 and a heterodimer consisting of the intact light subunit disulfide-linked to a small heavy chain fragment. Under reducing conditions, the small heavy chain fragment was not detectable after transfer to PVDF, and consequently only the amino acid sequence under nonreducing conditions was obtained.

**DISCUSSION**

Prothrombinase is the final enzyme complex generated within the coagulation cascade and functions directly to generate thrombin. Consequently, numerous modes of regulation of prothrombinase assembly and activity have evolved to enable fine-tuning of thrombin production (31). These include anticoagulant mechanisms that exclusively target the prothrombinase constituent cofactor FV\(\alpha\) (32) and enzyme FX\(\alpha\) (33), as
knowledge this is the first evidence for an anticoagulant mechanism that operates directly at the level of FX inactivation.

Because proteolysis by plasmin is capable of producing COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

To understand the structural basis by which FX function is altered by plasmin, Fig. 7 shows a fragmentation map of FX bound to proPL. The first plasmin-mediated cleavage produces FXbPn, which has the same electrophoretic mobility as previously documented FXb generated by treatment of FX with FXa (35). The location of the cleavage site that excises the COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

To understand the structural basis by which FX function is altered by plasmin, Fig. 7 shows a fragmentation map of FX bound to proPL. The first plasmin-mediated cleavage produces FXbPn, which has the same electrophoretic mobility as previously documented FXb generated by treatment of FX with FXa (35). The location of the cleavage site that excises the COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

To understand the structural basis by which FX function is altered by plasmin, Fig. 7 shows a fragmentation map of FX bound to proPL. The first plasmin-mediated cleavage produces FXbPn, which has the same electrophoretic mobility as previously documented FXb generated by treatment of FX with FXa (35). The location of the cleavage site that excises the COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

To understand the structural basis by which FX function is altered by plasmin, Fig. 7 shows a fragmentation map of FX bound to proPL. The first plasmin-mediated cleavage produces FXbPn, which has the same electrophoretic mobility as previously documented FXb generated by treatment of FX with FXa (35). The location of the cleavage site that excises the COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

To understand the structural basis by which FX function is altered by plasmin, Fig. 7 shows a fragmentation map of FX bound to proPL. The first plasmin-mediated cleavage produces FXbPn, which has the same electrophoretic mobility as previously documented FXb generated by treatment of FX with FXa (35). The location of the cleavage site that excises the COOH-terminal Lys, which is an essential feature of known fibrinolysis cofactors (2–10), the possibility that plasmin-cleaved FX acquires fibrinolytic activity was investigated. We found that in addition to having an anticoagulant effect on FX, plasmin converted the FX into a fibrinolysis cofactor. This conclusion is based on experiments that showed plasmin induces the expression of a plasminogen binding site on FX and the ability to accelerate the rate of tPA-dependent plasmin generation by approximately 30-fold. The latter effect was observed below the circulating concentration of FX. This supports the hypothesis that at sites of clot formation, where proPL increases the local concentration of coagulation proteins (15), the in vitro effects of Pn-cleaved FX on tPA reported here may have a physiological function in concert with other tPA accelerators, notably fibrin. Because the expression of FX-derived tPA cofactor activity was observed only under conditions that facilitated FX-proPL interactions, the proposed pathway would be advantageous localized to sites of clot formation.

Fig. 5. Plasminogen binding to plasmin-cleaved FX. A, autoradiograph of ligand blot showing 125I-plasminogen (0.1 μg) binding toFX species formed in the presence of plasmin, proPL (300 μg), and Ca2+ (2 mM) (corresponds to stained gel in Fig. 2A); B, autoradiograph of ligand blot showing 125I-plasminogen (0.1 μg) binding to FX species formed in the presence of plasmin, proPL (300 μg), and EDTA (5 mM) (corresponds to stained gel in Fig. 2B).

Fig. 6. Effect of plasmin-cleaved FX on tissue plasminogen activator function. A and B, the activation of plasminogen (1 μg) to plasmin by tPA (10 nM) was followed as a time course utilizing the chromogenic substrate, S2366, in the absence () or presence () of FX (final concentration, 0.5 μg) or the same amount of plasmin-treated FX ( ). The FX was pretreated with plasmin exactly as described in Fig. 2 for 30 min. Just enough aprotinin (9.5 KIU/ml) was added to inhibit the plasmin used in this step prior to the addition of tPA and plasminogen. A, conducted in the presence of Ca2+ (2 mM) and proPL (300 μg), as in Fig. 2A. B, conducted in the presence of EDTA (5 mM) and proPL (300 μg), as in Fig. 2B. C, FX was plasmin-pretreated as in A for 30 min, and the concentration dependence of tPA activity was monitored. The data were corrected for endogenous tPA activity. (n = 3; mean ± S.D.).

| Species | Sequence | Identity | Alignment* |
|---------|----------|----------|------------|
| FXbPn, Ca | ANSLF-MKK | FXb, N terminus | Residues 1–10 |
| 28 kDa | ANSLF-MKK | FXb, N terminus | Residues 143–152 |
| 13 kDa | ANSLF-MKK | FXb, internal | Residues 296–305 |

* Numbering based on the amino acid sequence predicted from FX cDNA (30).

The second cleavage of proPL-bound FX by plasmin produced FXbPn, Ca and a 13-kDa species. Amino acid sequence analysis of the former under nonreducing conditions produced two sequences corresponding to the NH2-terminus of FX heavy and light subunits. Sequence analysis of the latter indicated cleavage of the heavy subunit within the "autolytic loop" between Lys-330 and Gly-331, which is the same as that previously reported for FXa cleaved under identical conditions (20).
FX cleavage by plasmin. A scale model based on the number of amino acids shows the plasmin-mediated fragmentation pattern of FX bound to proPL. Species observed to bind plasminogen (Pg) and accelerate tPA are indicated. Plasminogen binding sites have been inferred by predicted creation of COOH-terminal Lys due to plasmin cleavage. Act, FX activation peptide; protease, protease domain; β, β-domain or β-peptide. ●, NH₂-terminal amino acid sequences derived in the current work (Table I). *, inferred cleavage position based on plasminogen binding and previously reported sequence analysis due to cleavage by FXa (36).

Unlike the recently reported proteolysis of FX by FXa (37), cleavage in the autolysis loop by plasmin was not observed by us to be protected by Ca²⁺. The exposure of a new COOH-terminal Lys at position 330 is consistent with our finding that plasminogen interacts with FXYPn, Ca in ligand blotting experiments. Furthermore, cleavage at this site explains the loss of FX light subunit by plasmin in the absence of both Ca²⁺ and proPL, indicating that proPL binding and not just divalent cation binding was necessary. The Glu domain has been shown to be involved in activation of FX by tissue factor/FVIIa or RvV FX activator (24), which provides an explanation for inhibition of FX coagulation activity by plasmin under conditions that did not facilitate proPL binding. Further evidence for differences in the sites recognized by plasmin due to binding of FX to proPL comes from plasminogen binding and tPA activity studies. Whereas plasmin cleavage was found to convert proPL-bound FX into a plasminogen receptor and tPA accelerator, neither activity was detectable when FX was cleaved by plasmin in the absence of proPL and/or Ca²⁺.

The FX data presented here, together with our earlier reports that showed that plasmin has similar modulatory effects on proPL-bound FXa and FVa (9, 20), support a model in which plasmin functions to create a profibrinolytic milieu through direct conversion of specific proteins into tPA cofactors. We have found that neither FX, FXa, nor FVa expresses tPA cofactor function when not bound to proPL. Although the plasmin-mediated cleavage patterns differ, both proPL-bound and unbound FX, FXa, and FVa are inhibited from participating in any further clot formation. In this way, plasmin may participate in a feedback mechanism because both plasminogen binding and tPA activity studies. Whereas plasmin cleavage was found to convert proPL-bound FX into a plasminogen receptor and tPA accelerator, neither activity was detectable when FX was cleaved by plasmin in the absence of proPL and/or Ca²⁺.

Acknowledgments—We acknowledge Dr. Alex Kurosky and Steve Smith (Protein Chemistry Laboratory, University of Texas at Galveston, Medical Branch) for expert amino acid sequence analysis. We also thank Dr. Réal Lemieux, Tina Raynor, and Abed Zeidabawi for helpful suggestions regarding manuscript preparation.

REFERENCES
1. Lijnen, H. R., and Collen, D. (1994) Methods Enzymol. 223, 197–206
2. Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K., and Plow, E. F. (1991) J. Biol. Chem. 266, 1682–1691
3. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) J. Biol. Chem. 269, 21191–21197
4. Cesarmann, G. M., Guevara, C. A., and Hajjar, K. A. (1994) J. Biol. Chem. 269, 21196–21203
5. Dudani, A. K., Cummings, C., Hashemi, S., and Ganz, P. R. (1993) Thromb. Res. 69, 185–196
6. Dudani, A. K., Pluskota, A., and Ganz, P. R. (1994) Biochem. Cell Biol. 72, 129–131
7. Kelm, R. J., Jr., Swords, N. A., Orfeo, T., and Mann, K. G. (1994) J. Biol. Chem. 269, 30147–30153
8. Reimartz, J., Hansch, G. M., and Kramer, M. D. (1995) J. Immunol. 154, 844–850
9. Pryzdzial, E. L. G., Biazarz, L., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 17871–17877
10. Kassam, G., Choi, S.-S., Ghuman, J., Kang, H.-M., Fitzpatrick, S. L., Zackson, T., Zackson, S., Toba, M., Shinomiya, A., and Waisman, D. M. (1998) J. Biol. Chem. 273, 4790–4799
11. Skidgel, R. A. (1986) Trends Pharmacol. Sci. 9, 299–304
Modulation of FX by Plasmin

12. Redlitz, A., Tan, A. K., Eaton, D. L., and Plow, E. F. (1995) J. Clin. Invest. 96, 2534–2538
13. Bajzar, L., Manuel, R., and Nesheim, M. E. (1995) J. Biol. Chem. 270, 14477–14484
14. Nesheim, M. E., Wang, W., Boffa, M., Nagashima, M., Morser, J., and Bajzar, L. (1997) Thromb. Haemostasis 78, 386–391
15. Mann, R. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) Blood 76, 10952–10962
16. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) J. Biol. Chem. 254, 10952–10962
17. Omar, M. N., and Mann, K. G. (1987) J. Biol. Chem. 262, 9750–9755
18. Lee, C. D., and Mann, K. G. (1989) Blood 73, 185–190
19. Pryzdial, E. L. G., and Kessler, G. E. (1996) J. Biol. Chem. 271, 16614–16620
20. Jesty, J., and Nemerson, Y. (1976) Methods Enzymol. 45, 95–107
21. Krishnaswamy, S., Church, W. R., Nesheim, M. E., and Mann, K. G. (1987) J. Biol. Chem. 262, 3291–3299
22. Rezaie, A. R., Neuschwander, P. W., Morrissey, J. H., and Esmon, C. T. (1993) J. Biol. Chem. 268, 8176–8180
23. Nesheim, M., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541–21548
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038
26. Yuen, S. W., Chui, A. H., Wilson, K. J., and Yuan, P. M. (1989) BioTechniques 7, 74–82
27. Matsudaira, 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. Pryzdial, E. L. G., and Isenman, D. E. (1997) J. Biol. Chem. 262, 1519–1525
30. Messier, T. L., Pittman, D. D., Long, G. L., Kaufman, R. J., and Church, W. R. (1991) Gene 99, 291–294
31. Kalafatis, M., Egan, J. O., van’t Veer, C., and Mann, K. G. (1997) Crit. Rev. Eukaryotic Gene Expression 7, 241–180
32. Esmon, C. T., and Fukudome, K. (1995) Semin. Cell Biol. 6, 259–268
33. Olson, S. T., Bjork, I., and Shore, J. D. (1994) Methods Enzymol. 222, 525–559
34. Broze, G. J. (1995) Thromb. Haemostasis 74, 90–93
35. Jesty, J., Spence, A. K., and Nemerson, Y. (1974) J. Biol. Chem. 249, 5614–5622
36. Eby, C. S., Mullane, M. P., Porche-Sorbet, R. M., and Miletić, J. P. (1992) Blood 80, Suppl. 1, 306a
37. Sabharwal, A. K., Padmasabban, K., Tulinsky, A., Mathur, A., Gorka, J., and Bajaj, S. P. (1997) J. Biol. Chem. 272, 22037–22045
38. Hojrup, P., and Magnunssen, S. (1987) Biochem. J. 245, 887–892
39. Church, W. R., Messier, T., Howard, P. R., Amirah, J., Meyer, D., and Mann, K. G. (1988) J. Biol. Chem. 263, 6259–6267