The autolysis loop (residues 143–154 in chymotrypsinogen numbering) plays a pivotal role in determining the macromolecular substrate and inhibitor specificity of coagulation proteases. This loop in factor IXa (FIXa) has 3 basic residues (Arg143, Lys147, and Arg150) whose contribution to the protease specificity of factor IXa has not been studied. Here, we substituted these residues individually with Ala in Gla-domainless forms of recombinant factor IX expressed in mammalian cells. All mutants exhibited normal amidolytic activities toward a FIXa-specific chromogenic substrate. However, Arg143 and Lys147 mutants showed a 3- to 6-fold impairment in FX activation, whereas the Arg150 mutant activated factor X normally both in the absence and presence of factor VIIIa. By contrast, Arg143 and Lys147 mutants reacted normally with antithrombin (AT) in both the absence and presence of the cofactor, heparin. However, the reactivity of the Arg150 mutant with AT was impaired 6.6-fold in the absence of heparin and 33- to 70-fold in the presence of pentasaccharide and full-length heparins. These results suggest that Arg143 and Lys147 of the autolysis loop are recognition sites for FX and its deficiency is associated with the life-threatening disease, hemophilia B (6). FIX circulates in plasma as a single-chain inactivezymogen with 415 amino acids (6). Proteolytic cleavage at two sites (Arg145-Ala, and Arg150-Val) by either one of two physiological activators, the factor VIIa-tissue factor complex or factor XIa, releases an activation peptide that converts the zymogen to an active enzyme consisting of a light and a heavy chain held together by a single disulfide bond (7–9). The N-terminal light chain of FIXa contains the non-catalytic Gla and two epidermal growth factor (EGF)-like domains, whereas the C-terminal heavy chain contains the trypsin-like catalytic domain of the molecule (10). The Gla and the N-terminal EGF domains are involved in the Ca2+-dependent assembly of the protease with FVIIIa on membrane surfaces (10, 11). It is believed that the protease domain of FIXa also interacts with FVIIIa (12). The role of the C-terminal EGF domain of FIXa is not known. Crystallographic and mutagenesis data suggest that it makes extensive interactions with the catalytic domain and thus may be critical for the structural integrity and proper orientation of the catalytic domain for interaction with either substrate and/or cofactor on membrane surfaces (13, 14). In the absence of a cofactor, FIXa exhibits very low catalytic activity toward both small synthetic and natural macromolecular substrates and inhibitors. Although FVIIIa enhances the proteolytic activity of FIXa toward its natural substrate FX by four or five orders of magnitude, the cofactor has no effect on the catalytic activity of FIXa toward small synthetic substrates (1, 11, 15). The exact mechanism by which FVIIIa improves the catalytic competency of FIXa toward FX is not known. Recent structural and mutagenesis data have indicated that the “99-loop” (per nomenclature of Ref. 25) of FIXa traps the protease in an inactive conformation by blocking the entrance of FX into the catalytic pocket. Thus, one mechanism by which FVIIIa may activate FIXa involves conformational rearrangement of the 99-loop (16).

Similar to its reaction with FX, the reactivity of FIXa with the target serpin inhibitor antithrombin (AT) is very poor in plasma (17, 18). In this case, heparin functions as a cofactor to accelerate the rate of FIXa inhibition by the serpin by several orders of magnitude (18, 19). Heparin has more recently been shown to accelerate the inhibition of FIXa and FXa by AT by similar mechanisms (20–22). For both enzymes, the rate-accelerating effect of heparin is believed to arise from 1) the ability of heparin to change the conformation of the serpin to facilitate its optimal recognition by the enzyme (allosteric activation mechanism) and 2) the ability of a full-length heparin to bridge the inhibitor and enzyme in a ternary complex, thereby promoting the initial interaction between the two proteins (template mechanism). With respect to the latter template mechanism, recent mutagenesis data have indicated that both FXa (20) and FXa (21) have a cluster of basic residues in their
catalytic domains that can interact with heparin when physiological levels of Ca\(^{2+}\) are present. Thus, the simultaneous binding of a full-length heparin to the basic site of either protease in the presence of Ca\(^{2+}\) and to a basic site of AT improves the \(K_{d}\) for the interaction of the two proteins, leading to a 100- to 1000-fold acceleration of the reaction (20–22). In the case of the allosteric activation mechanism, the binding of a distinct pentasaccharide sequence of the full-length heparin to AT allosterically changes the conformation of the serpin, leading to an additional 200- to 500-fold improvement in reactivity of the serpin with both FIXa and FXa (20, 22, 23). Allosteric activation of AT by heparin has minimal effect on its reactivity with thrombin (23). It is not known how allosteric activation of AT by heparin specifically improves the reactivity of the serpin with the coagulation proteases, FIXa and FXa.

Structural data suggest that the catalytic domain of FIXa, similar to other coagulation proteases, has several solvent-exposed surface loops that surround the substrate-binding pocket of the enzyme (13, 24). Although these surface loops are generally conserved at similar three-dimensional locations on all coagulation proteases, the length and amino acid residues forming the loops are not conserved in the members of the family. One of these surface loops consisting of residues 143–154 (chymotrypsinogen numbering (25)), referred to as the autolysis loop, has been demonstrated to play a crucial role in determining the substrate and inhibitor specificity of coagulation proteases (26–29). In the case of FIXa, we recently demonstrated that the basic residues of the autolysis loop minimally contribute to recognition of the substrate prothrombin in either the absence or presence of factor Va on negatively charged phospholipid vesicles (30). In its reaction with AT, however, we discovered that basic residues of this loop play distinct roles in FIXa recognition of AT. Thus, neutralization of charges of both Arg\(^{43}\) and Lys\(^{47}\) by their substitution with Ala improved the reactivity of the mutant proteases with AT in the absence of heparin, whereas the substitution of Arg\(^{150}\) with Ala led to an order of magnitude impairment in the reactivity of the mutant with AT specifically in the presence of heparin pentasaccharide (30). These results suggested that Arg\(^{150}\) of factor Xa makes a specific interaction with AT when the serpin is in an activated conformation, presumably involving a recently proposed serpin exosite (31). Interestingly, similar to FXa and unlike thrombin, the autolysis loop of FIXa contains three basic residues that are conserved at the same three-dimensional locations (13, 32). To understand the contribution of these basic residues to determining the substrate and inhibitor specificity of FIXa, we prepared three Gla-domainless mutants of FIX (GD-FIX) in which these residues were substituted with Ala. The mutants, GD-FIX R143A, K147A, and R150A together with the wild-type GD-FIX, were expressed in mammalian cells and purified to homogeneity (20). Following activation by Russell’s viper venom protease, the catalytic properties of the mutants were characterized with respect to their ability to hydrolyze a FIXa-specific chromogenic substrate, activate the natural substrate FX in both the absence and presence of FXVIIIa, and react with AT in either the absence or presence of pentasaccharide and full-length heparins. The results indicate that the basic residues Arg\(^{43}\) and Lys\(^{47}\) are critical for interaction of FIXa with FX independent of FXVIIIa. Moreover, Arg\(^{150}\) is a recognition site on FIXa for AT that can effectively interact with AT only if the serpin is in the heparin-activated conformation.

EXPERIMENTAL PROCEDURES

Mutagenesis and Expression of Recombinant Proteins—The wild-type and FIX mutants, Arg\(^{43}\) → Ala (R143A), Lys\(^{47}\) → Ala (K147A), and Arg\(^{150}\) → Ala (R150A) (chymotrypsinogen numbering (25)) corresponding to residues Arg\(^{43}\), Lys\(^{47}\), and Arg\(^{150}\) in the FIX cDNA numbering (6), were generated in the RSV-FLA expression/purification vector system in Gla-domainless forms (GD-FIX) by standard PCR mutagenesis methods used previously (20). After confirmation of the accuracy of the mutagenesis by DNA sequencing, the constructs were introduced into HEK293 cells, and the mutant proteins were isolated from 20 liters of cell culture supernatants by an immunoaffinity chromatography using the HPC4 monoclonal antibody as described previously (20).

Human plasma FIXa, FXa, and FX activating enzyme from Russell’s viper venom (RVV-X) were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Human recombinant AT was prepared as described previously (33). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described previously (34). Human recombinant FVIIIa was a generous gift from Dr. Philip Fay (University of Rochester, Rochester, NY). Unfractionated heparin (heparin sodium injection, USP, 10,000 units/ml) from beef lung and the active AT-binding pentasaccharide fragment of heparin (fondaparinux sodium) were purchased from Quintiles Clinical Supplies (Mt. Laurel, NJ). The concentration of pentasaccharide, used in molar excess over AT in all reactions, was calculated based on its weight assuming a molecular mass of 1728 Da (C\(_{31}\)H\(_{43}\)N\(_{3}\)Na\(_{10}\)O\(_{49}\)S\(_{8}\)). The concentration of the unfractionated heparin was based on antithrombin binding sites and was determined by stoichiometric titrations of antithrombin with the polysaccharide, with monitoring of the interaction by changes in protein fluorescence as described previously (35). Heparin-Sepharose was purchased from Amersham Biosciences (Piscataway, NJ), the chromogenic substrates, Spectrozyme FXa (SpFXa) and CH\(_{2}\)SO\(_{4}\)-d-Leu-Gly-Arg-pNA (LGR-pNA) were purchased from American Diagnostica (Greenwich, CT) and Midwest Bio-Tech Inc. (Fishers, IN), respectively. Polybrene and paminobenzamidine were purchased from Sigma (St. Louis, MO).

Activation of FIX Mutants—FIX derivatives (0.5 mg) in 0.02 M Tris-HCl (pH 7.5), 0.1 mM NaCl (TBS buffer) containing 5 mM Ca\(^{2+}\) were converted to their active forms with 50 pM of RVV-X for 3 h at 37 °C as described previously (20). FIXa derivatives were separated from the snake venom by applying the activation mixtures on a 2-ml heparin-Sepharose column pre-equilibrated with the same buffer as described previously (20). The column was extensively washed with the same buffer containing 0.15 M NaCl followed by elution with a linear gradient of NaCl (0 to 1 M). The active fractions were identified by an amidolytic activity assay, pooled, and active-size-titrated with known concentrations of AT in the presence of pentasaccharide as described (20).

Cleavage of Chromogenic Substrates—The steady-state kinetics of hydrolysis of LGR-pNA (0.04–5 nm) by FIXa derivatives (20 nm) were studied in TBS containing 0.1 mg/ml bovine serum albumin (BSA), 33% ethylene glycol, and 5 mM Ca\(^{2+}\). In some experiments ethylene glycol was omitted from the buffer. The initial rate of hydrolysis was measured at 405 nm at room temperature in 96-well plates by a V\(_{max}\) Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described (20). The apparent \(K_{m}\) and \(k_{cat}\) values for substrate hydrolysis were obtained by linear regression of the results of double-reciprocal equation, and the catalytic efficiencies were expressed as the ratio of \(k_{cat}/K_{m}\).

Factor X Activation by Factor IXa Mutants—The initial rate of FX activation by the wild-type and mutant GD-FIXa derivatives was measured in both the absence and presence of FVIIIa. In the absence of the cofactor, the time course of the initial rate of activation was measured by incubating each FIXa derivative (50 nm) with human FX (2 µM) on PC/PS vesicles (50 µM) in TBS containing 0.1 mg/ml BSA, 0.1% polyethylene glycol (PEG) 8000 and 5 mM Ca\(^{2+}\) (TBS/ Ca\(^{2+}\)) at room temperature. At different time intervals, small aliquots of the activation reactions were transferred into a 96-well assay plate containing Spectrozyme FXa (200 µM) in TBS containing 30 mM EDTA and in a 100-µl final volume). The initial rate of activation was determined by measuring the increase in amidolytic activity of the samples toward SpFXa as monitored at 405 nm by a V\(_{max}\) Kinetic Microplate Reader. The concentrations of FXa generated in the activation reactions were determined from a standard curve prepared from the cleavage rate of SpFXa by known concentrations of plasma FXa under exactly the same conditions (20).

In the presence of factor VIIIa, the activation of factor X by the FIXa derivatives on phospholipid vesicles was evaluated by incubating each FIXa (20 nm) with varying concentrations of human FVIIIa (7.5–500 nm) on 50 µM PC/PS vesicles in TBS/Ca\(^{2+}\) at room temperature. The activation reactions were incubated by adjusting the pH to 7.5 and allowed to proceed for 60 min, following which they were terminated by addition of EDTA to a final concentration of 20 mM. The rate of FIXa generation was measured from the cleavage rate of SpFXa as described above. The apparent \(K_{d}\) for the factor VIIIa-FIXa interaction and maximum
inal FX activation rate for wild-type and variant FIXas were determined by non-linear regression fits of data by a hyperbolic kinetic equation (35).

**Reaction with Antithrombin**—The rate of inactivation of FIXa derivatives by AT in both the absence and presence of unfractonated full-length heparin and the pentasaccharide fragment of heparin were measured under pseudo-first-order conditions by a discontinuous assay as described previously (20). In the absence of a cofactor, each FIXa derivative (10 nM) was incubated with AT (0.5–2.0 μM) in TBS containing 0.1 mg/ml BSA, 33% ethylene glycol, and 5 mM CaCl2 for 30–120 min. All reactions were carried out in 50-μl volumes in 96-well polystyrene assay plates at room temperature. In the presence of pentasaccharide, the reaction conditions were the same except that FIXa derivatives were incubated with AT (50–400 nM) in complex with a saturating concentration of pentasaccharide (1 μM) for 0.5–120 min. In the presence of full-length heparin, each FIXa derivative (10 nM) was incubated with 200 nM AT in the presence of catalytic levels of heparin (0.6–10 nM) for 10 s to 50 min in the same TBS buffer system. All reactions were stopped by addition of 50 μl of LGR-pNA (final concentration of 1.0 mM) in TBS containing 1 mg/ml Polybrene. The remaining enzyme activity was measured with a Vmax Kinetics Microplate Reader at 405 nm as described above. The observed pseudo-first-order inactivation rate constants (kobs) were determined by fitting the time-dependent change of the protease activity to a first-order rate equation. Second-order rate constants (kcat) were obtained from the slopes of linear plots of kobs versus the concentration of AT or AT-heparin complex as described (20). The AT-heparin complex concentrations were calculated from the dissociation constant for the AT-heparin interaction and total concentrations of AT and heparin using the quadratic equation as described (35).

**Inhibition by α-Aminobenzamidine**—The K<sub>i</sub> value for α-aminobenzamidine interaction with the active-site pocket of wild-type and mutant GD-FIXa derivatives was evaluated. In all cases, GD-FIXa (20 nM) was incubated with increasing concentrations of the inhibitor (0–320 μM) in the presence of different fixed concentrations of LGR-pNA (312–2500 μM) in TBS containing 0.1 mg/ml BSA, 33% ethylene glycol, and 5 mM Ca<sup>2+</sup>. The enzyme activity was measured from the initial cleavage rate of the chromogenic substrate as described above and the K<sub>i</sub> values were determined by global fitting of data to a kinetic equation for competitive inhibition as described (36).

**RESULTS**

**Expression, Purification, and Activation of Recombinant Proteins**—Wild-type and Ala substitution mutants of FIX in Gla-domainless forms were expressed in HEK293 cells and purified by immunoaffinity chromatography as in previous studies (20). SDS-PAGE analysis of the purified proteins under non-reducing conditions indicated that the isolated proteins are relatively pure and that they all migrate with similar expected molecular masses of ~60 kDa suggesting that 293 cells properly processed the recombinant proteins (Fig. 1). All GD-FIX derivatives were activated by RVV-X and, following their purification on a heparin-Sepharose column as described (20), were found to be fully active based on active-site tetrations with AT in the presence of pentasaccharide.

**Amidolytic Activity**—Kinetic parameters for the hydrolysis of the chromogenic substrate LGR-pNA by FIXa derivatives are presented in Table I. Wild-type and mutant GD-FIXa derivatives exhibited similar or slightly improved k<sub>cat</sub> and K<sub>m</sub> values toward LGR-pNA in both the absence (data not shown) and presence of the activator, ethylene glycol (Table I). The mutagenesis of basic residues of the autolysis loop thus has no apparent adverse effect on the S3–S1 substrate binding pockets of FIXa mutants. In agreement with this finding, K<sub>i</sub> values for the interaction of all mutants with the S1 site-specific competitive inhibitor of serine proteases, p-aminobenzamidine, were comparable to or slightly improved relative to the wild-type enzyme (Table I). Previous mutagenesis studies of basic residues of the autolysis loop of FIXa yielded similar results (30). Because the residues under study are solvent-exposed in the crystal structure of human FIXas (13), their mutagenesis is not expected to affect the catalytic pocket of FIXa.

**Factor X Activation**—The catalytic properties of FIXa derivatives toward activation of the natural substrate FX was studied in both the absence and presence of FVIIIa and Ca<sup>2+</sup> on PC/PS vesicles. The time course of FX activation in the absence of the cofactor indicated that GD-FIXa can convert FX to Fxa at a very slow rate. Under the experimental conditions described under "Experimental Procedures" (2 μM FX and 50 nM FIXa), similar initial rates of ~0.03 nM FXa/min were found for the wild-type and the R150A mutant of GD-FIXa. However, under the same conditions, the initial rate of FX generation by both R143A and K147A mutants (~0.005 nM/min) was impaired ~6-fold. The initial rates of FX activation by all GD-FIXa derivatives were 7- to 8-fold slower in the absence than in the presence of PC/PS vesicles. The ability of GD-FIXa derivatives to activate FX in the presence of FVIIIa was also evaluated. To determine the effect of deleting the Gla domain on the high affinity interaction of FIXa with FVIIIa, the rate of FX activation by plasma FIXa and recombinant GD-FIXa derivatives were compared in the presence of increasing concentrations of FVIIIa on PC/PS vesicles. Interestingly, relative to full-length plasma FIXa, the apparent K<sub>i</sub> for the interaction of wild-type GD-FIXa with FVIIIa (82.7 nM) was weakened only 3.5-fold (Fig. 2 and Table II). However, the rate of FXa generation by GD-FIXa was dramatically impaired (Fig. 2). In the presence of a saturating concentration of FVIIIa, the maximum rate of activation of the plasma-derived FIXa (211.8 nM/min/nM) was 20,000-fold higher than the corresponding value with wild-type GD-FIXa (10.6 nM/min/nM, Table II). The R150A mutant exhibited a similar maximal rate of FXa generation and K<sub>i</sub>(app) for FVIIIa interaction, suggesting that Arg<sup>150</sup> does not play a role in the macromolecular recognition of factor X by the FIXa-FVIIIa complex. In the case of the R143A and K147A mutants, the maximal FX activation rates were impaired 3- to 4-fold and the K<sub>i</sub>(app) for interaction with FVIIIa was unaffected for R143A, but impaired ~3-fold for the K147A mutant (Table II).

**Reaction with Antithrombin**—We previously demonstrated that removal of the Gla domain from FIXa does not affect its reactivity with AT in either the absence or presence of heparin cofactors when physiological levels of Ca<sup>2+</sup> are present (20). Thus, the reactivities of only GD-FIXa derivatives with AT were evaluated under these conditions. Relative to the wild-type enzyme, there was minimal change in the reactivity of either R143A or K147A mutant enzymes with AT both in the absence and presence of pentasaccharide or full-length heparins (Table III). On the other hand, the R150A mutant reacted with AT with 6.6-, 70-, and 33-fold slower second-order association rate constants (k<sub>on</sub>) than the wild-type enzyme when no heparin, pentasaccharide heparin, or full-length heparin was present, respectively (Fig. 3 and Table III). The rate enhancement due to conformational activation of AT is given by the ratio of k<sub>on</sub> values in the presence and absence of pentasaccharide (Table III). The conformational activation rate enhancement was minimally affected by the R143A and K147A mutations, whereas it was reduced 10-fold by the R150A mutation.
TABLE I
Kinetic constants for the cleavage of LGR-pNA by FIXa derivatives and for competitive inhibition of the enzymes by p-aminobenzamidine

The kinetic constants were calculated from the initial cleavage rates measured at increasing concentrations of LGR-pNA (0.04-5 mM) by each FIXa derivative (20 nM) in TBS buffer containing 5 mM Ca2+, 0.1 mg/ml BSA, and 33% ethylene glycol. The \( K_i \) values for p-aminobenzamidine (PAB) were measured from the decrease in the initial rates of LGR-pNA hydrolysis produced by the competitive inhibitor in the same TBS buffer system. See “Experimental Procedures” for further details. Kinetic values are the average of three experiments ± SD.

| Derivative | \( K_{\text{in}} \) (nM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_{\text{in}} \) (\( \mu \text{M} \cdot \text{s}^{-1} \)) | \( K_i \) (PAB) (\( \mu \text{M} \)) |
|------------|----------------|----------------|-----------------|----------------|
| pFIXa      | 2.3 ± 0.1      | 13.2 ± 0.4     | 5.7 ± 0.4       | ND             |
| GD-FIXa    | 2.0 ± 0.2      | 13.2 ± 0.4     | 6.6 ± 0.9       | 53 ± 2         |
| R143A      | 1.2 ± 0.1      | 10.7 ± 0.2     | 8.9 ± 0.9       | 40 ± 1         |
| K147A      | 2.2 ± 0.1      | 15.6 ± 0.5     | 7.1 ± 0.5       | 38 ± 1         |
| R150A      | 2.0 ± 0.2      | 13.4 ± 0.5     | 6.7 ± 0.9       | 44 ± 3         |

\( ^{a} \) ND, not determined.

The apparent \( K_i \) for interaction with FVIIIa and the maximum rate of FX activation (\( k_{\text{cat}} \)) by FIXa derivatives on PC/PS vesicles

The \( K_{\text{app}} \) and \( k_{\text{cat}} \) values were determined by fitting the initial rates of FX activation by plasma and recombinant GD-FIXa derivatives as a function of the FVIIIa concentration shown in Fig. 2 to a hyperbolic equation. See the legend of Fig. 2 for further experimental details. Values are the average of two or three experiments ± S.D.

| Derivative | \( K_{\text{app}} \) (nM) | \( k_{\text{cat}} \) (pM/min/nM) |
|------------|----------------|----------------|
| pFIXa      | 23.3 ± 2.8    | (212 ± 10) × 10\(^3\) |
| GD-FIXa    | 82.7 ± 6.0    | 10.6 ± 0.3     |
| R143A      | 65.5 ± 11.2   | 2.8 ± 0.1      |
| R147A      | 246 ± 24      | 3.7 ± 0.2      |
| R150A      | 115 ± 10      | 9.2 ± 0.3      |

FIG. 2. The cofactor concentration dependence of factor X activation by plasma-derived FIXa and GD-FIXa derivatives on PC/PS vesicles. A, plasma FIXa (100 nM) was incubated with FX (1 \( \mu \text{M} \)) in the presence of increasing concentrations of FVIIIa (1.5–100 nM) on PC/PS vesicles (50 \( \mu \text{M} \)) in TBS containing 0.1 mg/ml BSA, 0.1% PEG 8000, and 5 mM Ca\(^{2+}\). Following 1–2 min of activation at room temperature the reaction was terminated by addition of 20 mM EDTA and the rate of FXa generation was measured by an amidolytic activity assay as described under “Experimental Procedures.” B, the same as A except that FIXa mutants (20 nM) each were incubated with FX in the presence of increasing concentrations of FVIIIa as indicated on x-axis. The symbols in panel B are: wild-type GD-FIXa (○) and GD-FIXa mutants R143A (●), K147A (□), and R150A ( ■).

The rate enhancement due to the template effect of the full-length heparin can be obtained from the ratio of \( k_{\text{cat}} \) values in the presence of heparin to those in the presence of the pentasaccharide (Table III). None of the mutants were found to be impaired with respect to the template effect of heparin.

DISCUSSION

It has become apparent in recent years that residues on exposed surface loops, near or remote from the catalytic pocket, play key roles in determining the macromolecular substrate and inhibitor specificity of coagulation proteases. It is believed that these loops either directly interact with target substrate or inhibitor molecules or provide recognition sites for interaction with specific cofactors of coagulation proteases on membrane surfaces. In this study, we demonstrated that the basic residues of the autolysis loop (Fig. 4) play specific roles in the catalytic function of FIXa in its reaction with the physiological substrate, FX, and the serpin inhibitor, AT. Thus, the two residues Arg\(^{143}\) and Lys\(^{147}\) of the loop are likely cofactor-independent recognition sites for FX based on our observation that the rate of FX activation was impaired to similar extents by mutating either residue in both the absence and presence of FVIIIa. On the other hand, Arg\(^{150}\) does not play a significant role in the protease interaction with the substrate, because mutation of this residue was found to minimally alter the rate of FX activation. Conversely, both Arg\(^{143}\) and Lys\(^{147}\) were dispensable for FIXa recognition of AT in either the absence or presence of heparin cofactors, whereas Arg\(^{150}\) was discovered to be an essential residue for the protease interaction with the serpin. The observation that the reactivity of the R150A mutant with AT is impaired in both the absence and presence of pentasaccharide suggests that Arg\(^{150}\) interacts with both the native and activated conformations of the serpin. Interestingly, however, the impairment was 10-fold greater in the presence than in the absence of pentasaccharide, indicating that this residue interacts effectively with AT only when the serpin is in its activated conformation.

Similar to FIXa, the autolysis loop of FXa is basic with four residues Arg\(^{143}\), Lys\(^{147}\), Arg\(^{150}\) and Arg\(^{154}\) conserved at the same three-dimensional locations (32, 37). We recently demonstrated that Arg\(^{150}\) of FXa plays a similar role in the protease reaction with AT. This was evidenced by the observation that the reactivity of the Arg\(^{150}\) mutant of FXa with AT was impaired by an order of magnitude specifically in the presence of the pentasaccharide (30). Unlike FIXa and FXa, Arg\(^{150}\) is not conserved in the autolysis loop of thrombin, which does not differentiate between the native and activated conformations of AT (23, 24). These results suggest that structural differences in the autolysis loops of coagulation proteases are partly responsible for their differential reactivity with the native and the heparin-activated conformations of AT. The mechanism by which heparin activates AT is not completely understood. It was initially believed that the residues of the reactive-site loop of AT may not have a proper conformation to fit the catalytic pocket of coagulation proteases and that activation by heparin...
Kinetic constants for AT inhibition of FIXa derivatives in the absence and presence of pentasaccharide and full-length heparins

The inactivation rate constants in the absence of cofactor (k_{2,uncat}) were determined from the residual activities of the FIXa derivative (20 nM) after incubation at room temperature with AT (0.5-2.0 μM) for 30-120 min in TBS buffer, containing 5 mM CaCl₂, 0.1 mg/ml BSA, and 0.1% PEG 8000, as described under “Experimental Procedures.” The k_{2,penta} values were determined by the same procedures except that 10 nM enzyme was incubated with 50-400 nM AT for 0.5-120 min in the presence of 1 μM pentasaccharide. The k_{2,H} values were determined by the same procedures except that the FIXa derivatives were incubated with AT (200 nM) in the presence of heparin (0.6-10 nM) for 10 s to 50 min. k_{2,uncat}, k_{2,penta}, and k_{2,H} values were determined from the slopes of linear plots of k_{obs} versus the concentration of AT-heparin complexes shown in Fig. 3. Values are the average of at least three experiments ± S.D.

|        | k_{2,uncat} | k_{2,penta} | k_{2,H} | k_{2,penta}/k_{2,uncat} | k_{2,H}/k_{2,uncat} |
|--------|-------------|-------------|--------|-------------------------|---------------------|
| GD-FIXa| 7.3 ± 0.3   | 4.9 ± 0.1   | 29.7 ± 0.3 | 670 ± 40               | 606 ± 18            |
| R143A  | 6.6 ± 0.6   | 2.6 ± 0.1   | 20.1 ± 0.6 | 390 ± 50               | 773 ± 53            |
| R147A  | 6.2 ± 0.4   | 2.9 ± 0.1   | 17.7 ± 0.5 | 470 ± 47               | 610 ± 38            |
| R150A  | 1.1 ± 0.1   | 0.07 ± 0.01 | 0.9 ± 0.005 | 64 ± 15               | 1290 ± 190          |

FIG. 3. Dependence of the pseudo-first-order rate constant (k_{obs}) for AT-heparin inhibition of GD-FIXa derivatives on AT-heparin concentration. A, the k_{obs} values for the inactivation of wild-type GD-FIXa (○) and R143A (●), R147A (□), and R150A (■) GD-FIXa mutants (10 nM each) by AT (50–400 nM) in the presence of pentasaccharide (1 μM) were determined and plotted versus the AT-pentasaccharide (HS) complex concentration as described under “Experimental Procedures.” B, the same as A except that k_{obs} values for inactivation of 10 nM GD-FIXa derivatives by AT (200 nM) were determined in the presence of different concentrations of heparin (0.6–10 nM) and plotted versus the AT-heparin complex concentration. The k_{obs} values were determined from slopes of the fitted straight lines and presented in Table III.

The autolysis loop of FIXa from -aminobenzamidine. The autolysis loop of FIXa from residues 143–154 is shown in green. The catalytic Ser145 is shown in red. The three basic residues, which were mutated in this study are shown in purple. The coordinates (Protein Data Bank code 1RFN) of the C-terminal EGF and catalytic domains of FIXa were used to prepare the figure (14).

FIG. 4. Crystal structure of the catalytic domain of FIXa in complex with p-aminobenzamidine. The autolysis loop of FIXa from residues 143–154 is shown in green. The catalytic Ser145 is shown in red. The three basic residues, which were mutated in this study are shown in purple. The coordinates (Protein Data Bank code 1RFN) of the C-terminal EGF and catalytic domains of FIXa were used to prepare the figure (14).
have both membrane and Gla-independent recognition sites for interaction with the coagulation proteases. In the case of FIXa, basic residues of the C-terminal 162-helix in the catalytic domain have been proposed to constitute a recognition site for interaction with FVIIia (12). The same basic helix of FXa has also been demonstrated to be critical for interaction with factor Va and/or prothrombin in the prothrombinase complex (21).

Both EGF-like domains of FXa have also been implicated in binding to FVIIia (43). However, it is not known if the Gla domain of FIXa interacts with the cofactor. The observation that FVIIIia functioned as a cofactor to enhance the rate of FX activation by GD-FIXa supports the proposal that the EGF and/or protease domains of FXa have one or more binding sites for the cofactor. Moreover, the finding that the apparent $K_d$ for the interaction of GD-FIXa with FVIIia was only impaired 3.5-fold relative to that with full-length FXa suggests that the Gla domain of FIXa may not significantly contribute to the high affinity of the protease-cofactor interaction. However, the observation that the catalytic efficiency of GD-FIXa toward FX at a saturating concentration of FVIIia was impaired more than four orders of magnitude suggests that the Gla domain of FIXa plays a pivotal role in the cofactor-dependent activation of FX by the activation complex. The binding of the Gla domain to membrane surfaces may be required for an appropriate topographical arrangement of the protease domain in the activation complex for proper interaction with the substrate. Although GD-FIXa cannot interact with the membrane surface, its ability to activate FX was stimulated 7- to 8-fold by PC/PS vesicles. Thus, the binding of FX to the phospholipid surface through the Gla domain may be associated with a conformational change in the structure of the substrate that facilitates its optimal recognition by the protease. The Gla domain may also play a similar role in the function of FIXa. It is worth noting that previous results have indicated that the apparent $K_d$ for the interaction of Gla-domainless FXa with factor Va is three order of magnitude worse than that of wild-type FXa as determined by the activation complex. 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