The Lipoprotein-Associated Coagulation Inhibitor That Inhibits the Factor VII–Tissue Factor Complex Also Inhibits Factor Xa: Insight Into Its Possible Mechanism of Action

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Blood coagulation is initiated when plasma factor VII(a) binds to its essential cofactor tissue factor (TF) and proteolytically activates factors X and IX. Progressive inhibition of TF activity occurs upon its addition to plasma. This process is reversible and requires the presence of VII(a), catalytically active Xa, Ca2+, and another component that appears to be associated with the lipoproteins in plasma, a lipoprotein-associated coagulation inhibitor (LACI). A protein, LACI(HG2), possessing the same inhibitory properties as LACI, has recently been isolated from the conditioned media of cultured human liver cells (HepG2). Rabbit antiserum raised against a synthetic peptide based on the N-terminal sequence of LACI(HG2) and purified IgG from a rabbit immunized with intact LACI(HG2) inhibit the LACI activity in human serum. In a reaction mixture containing VIIa, Xa, Ca2+, and purified LACI(HG2), the apparent half-life (t1/2) for TF activity was 20 seconds. The presence of heparin accelerated the initial rate of inhibition threefold. Antithrombin III alone had no effect, but antithrombin III with heparin abrogated the TF inhibition. LACI(HG2) also inhibited Xa with an apparent t1/2 of 50 seconds. Heparin enhanced the rate of Xa inhibition 2.5-fold, whereas phospholipids and Ca2+ slowed the reaction 2.5-fold. Xa inhibition was demonstrable with both chromogenic substrate (S-2222) and bioassays, but no complex between Xa and LACI(HG2) could be visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Nondenaturing PAGE, however, showed that LACI(HG2) bound to Xa but not to X or Xa inactivated by diisopropyl fluorophosphatase. Thus, LACI(HG2) appears to bind to Xa at or near its active site. Bovine factor Xa lacking its γ-carboxyglutamatic acid–containing domain, BXa(-GD), through treatment with α-chymotrypsin, was used to further investigate the Xa requirement for VIIa/TF inhibition by LACI(HG2). LACI(HG2) bound to BXa(-GD) and inhibited its catalytic activity against a small molecular substrate (Spectrozyme Xa), though at a rate approximately sevenfold slower than native BXa. Preincubation of LACI(HG2) with saturating concentrations of BXa(-GD) markedly retarded the subsequent inhibition of BXa. The VII(a)/TF complex was not inhibited by LACI(HG2) in the presence of BXa(-GD), and further, preincubation of LACI(HG2) with BXa(-GD) slowed the inhibition of VIIa/TF after the addition of native Xa. The results are consistent with the hypothesis that inhibition of VII(a)/TF involves the formation of a VIIa-TF-Xa-LACI complex that requires the GD of Xa. Because the GD contains the α-carboxyglutamatic acids required for the Ca2+-dependent binding of factor Xa to phospholipid surfaces, the results also suggest that Ca2+ may be required for the native Xa-LACI complex to bind to and inhibit VII(a)/TF. LACI is a novel inhibitor that can rapidly affect feedback inhibition of the VIIa-Ca2+-TF enzymatic complex after the generation of small amounts of Xa and probably plays an important role in the regulation of in vivo coagulation.

Tissue factor (TF, coagulation factor III) is an integral membrane protein that functions as an essential cofactor for the expression of factor VII's proteolytic activity toward its substrates factors IX and X.1,2 Progressive inhibition of TF activity occurs upon its addition to human serum or plasma.3,9 This inhibition requires the presence of factor VII(a), factor X(a), Ca2+, and another factor present in the lipoprotein fraction of serum—a lipoprotein-associated coagulation inhibitor (LACI).6-12 It has been suggested that the inhibitory effect is directed against the VIIa-Ca2+-TF complex, thus explaining the need for VII and Ca2+ for TF inhibition to occur.6,9 The requirement for the presence of catalytically active X(a) was not immediately apparent, however, although indirect evidence suggested that it might be involved stoichiometrically in a VIIa-Ca2+-TF-Xa-LACI complex.6,13 HepG2 cells (human hepatoma cell line) secrete an activity, LACI(HG2), with the same characteristics as the inhibitor in serum.5 LACI(HG2) has recently been isolated, and one step used in the purification procedure was factor Xa–agarose affinity chromatography.11 There we report that purified LACI(HG2) directly inhibits factor Xa and suggest a possible mechanism to explain the inhibition of the VIIa-Ca2+-TF complex as well.

Materials and Methods

Sodium 125I-iodide, carrier free, and sodium [3H]borohydride were purchased from New England Nuclear (Boston) and iodogen was obtained from Pierce Chemical Co (Rockford, IL). Low-molecular weight (mol wt) standards for PAGE were from Bio-Rad Laboratories (Richmond, CA), S-2222 (CO-Ile-Glu-[γ-OR]-Gly-Arg-NH-NO2) from Helena Laboratories (Beauumont, TX), Spectrozyme Xa (MeO-CO-D-CHG-Gly-Arg-NH-NO2) from American Diagnostica (Greenwich, CT), and dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone (dansyl-GGACK) from Calbiochem (La Jolla, CA). Soybean trypsin inhibitor agarose, bovine serum albumin (BSA), acrylamide, bis-acrylamide, rabbit brain cephalin, Lubrol PX, Trizma-base, and bovine VIIa/X-deficient plasma were obtained from Sigma Chemical Co (St Louis).

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Submitted June 29, 1987; accepted September 23, 1987.

Supported in part by Grants HL34462 and HL14147 from the National Institutes of Health, Bethesda, MD, and the Monsanto Chemical Corporation, St Louis.

Presented in part at the annual meeting of the American Society for Clinical Investigation, May 2, 1987.

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0006-4971/88/7102-0004$3.00/0

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All other chemicals were of reagent grade or better and came from Fisher Scientific Co (Pittsburgh) or from Sigma. Factor X-deficient human plasma was obtained from George King Biomedical (Overland Park, KS). Serum samples from healthy blood donors were provided by the American Red Cross (St Louis).

**Proteins.** A crude preparation of TF was prepared as previously described and washed extensively with EDTA. The X coagulant protein from Russell's viper venom (XCP),13 antithrombin IIIa,16 factor VIIa,9 human factor X,17 thrombin,18 and fibrinogen19 were purified as previously described. Bovine factor X was purified from the barium sulfate eluate of bovine plasma (Sigma). Forty units (each from 1 L plasma) of eluate were resuspended in 1.6 L of H2O and the pH adjusted to 6.0 with HCI. Dry benzamidine was added to a final concentration of 1 mmol/L and the mixture centrifuged at 10,000 g and 4°C to remove insoluble material. The sample was then applied (100 mL/h) to a 5 x 95-cm column of diethylaminoethyl-Sepharose that had been equilibrated in 0.02 mol/L sodium citrate, 1 mmol/L benzamidine, and 0.02% NaN3, pH 6.0. At 4°C. After washing the column with 2 L start buffer, it was developed with a 15-L gradient from start buffer to 0.8 mol/L NaCl, 0.02 mol/L sodium citrate, 1 mmol/L benzamidine, and 0.02% NaN3, pH 6.0. Fractions containing bovine factor X1 and X2, which eluted after protein C but before protein Z, were pooled, concentrated to ~40 mL (PM10, Amicon Corp, Lexington, MA), and dialyzed into 0.1 mol/L MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.5. The yield was 68 mg purified bovine factor X. Bovine γ-carboxyglutamatic acid–domainless factor X, B(γ-DX), was prepared by using α-chymotrypsin as described by Morita and Jackson and isolated by chromatography upon a mono Q column.20 Human factor Xa, bovine factor Xa (B(Xa)), and bovine γ-carboxyglutamic acid–domainless factor Xa (B(Xa−GD)) were produced from their respectivezymogen forms by treatment with insolubilized XCP.21 The B(Xa) and B(Xa−GD) were further isolated by chromatography upon soybean trypsin inhibitor agarose as will be described later. The extinction coefficients at 280 nmol/L of B(Xa) and B(Xa−GD) were assumed to be 10.0.

LACI(HG2) was isolated from HepG2 cell serum–free conditioned media as previously described22 by using a modification of this procedure. It has an apparent mol wt of 38,000 by SDS-PAGE and a specific activity of 9,800 to 10,500 TF inhibitory U/mg protein where 1 unit of activity was defined as that present in 1 mL normal pooled human serum and the extinction coefficient at 280 nmol/L for LACI was assumed to be E (extinction coefficient) 1%/1 cm − 1.10 Unless otherwise noted, the LACI(HG2) used in the following experiments was diluted to a concentration of 1 μg/mL in 0.1 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.5, 0.02% NaN3, and 1 mg/mL BSA (TBSA) and dialyzed overnight against 4,000 vol of 0.1 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.5, and 0.02% NaN3.

**125I-labeled proteins.** Human factor X was labeled with 125I by using iodogen and retained 90% of its initial clotting activity.23 Its specific radioactivity was 1,340 dpm/ng (670 cpm/ng), and > 98% of the radioactivity was precipitable with trichloroacetic acid (TCA) (10% wt/vol). The method of Craig et al24 was used to isolate 125I-Xa and DIP(diisopropylphosphoryl)-125I-Xa. The 125I-X was diluted into TBSA and passed through a 1-mL column of soybean trypsin inhibitor agarose and the fractions pooled by radioactivity. The 125I-X that flowed through the column (40 μg/mL) was activated by adding a 0.1 vol of a 50% slurry of XCP-agarose (1 mg XCP/mL packed gel) and CaCl2 (final concentration, 6 mmol/L). After rocking for 30 minutes at room temperature, the sample was separated from the XCP-agarose by centrifugation through a 0.2-μm filter (Centrex disposable microfilter, Schleicher and Schuell, Inc, Keene, NH). The recovered solution was then applied to a 1-mL column of soybean trypsin inhibitor agarose, and after washing with TBSA, the 125I-Xa was eluted with 0.5 mol/L benzamidine in TBSA.

The fractions were pooled on the basis of radioactivity and dialyzed extensively against 0.1 mol/L NaCl and 0.1 mol/L Tris-HCl, pH 8.1 (T/S). After dialysis half of the 125I-Xa preparation was treated with DFP (10 mmol/L) for 48 hours at 4°C. This fraction was then dialyzed once again against T/S and subsequently passed through a 1-mL soybean trypsin inhibitor agarose column equilibrated in TBSA. The DIP,125I-Xa that flowed through the column had < 1% of the activity of the 125I-Xa by bioassay.

BXa and BXa(GD) was radiolabeled with 125I by using iodogen to a specific activity of 2,000 and 1,500 dpm/ng, respectively. Greater than 97% of the radioactivity was precipitable with 10% TCA. The iodinated proteins retained > 80% of their catalytic activity against Spectrozyme Xa. XaB was the predominant form of Xa in both radiolabeled preparations (>90% in 125I-Xa and 70% in 125I-Xa[GD]). In addition, an apparent further degradation product was present in the 125I-BXa sample (see later sections). Dansyl-GGACK (0.5 mmol/L) was used to inactivate both 125I-BXa and 125I-BXa(GD) (< 1% of the original activity as determined by cleavage of Spectrozyme Xa).

**Coagulation assays.** Factor X and factor Xa bioassays were performed as previously described25 by using human X-deficient plasma for human Xa and bovine VII/X-deficient plasma for BX/BXa.

**Xa inhibition by LACI(HG2).** Reaction mixtures containing purified LACI(HG2) (250 ng/mL), human Xa (25 ng/mL), and either EDTA (1 mmol/L) or CaCl2 (5 mmol/L) and a 1:40 dilution of stock rabbit brain cephalin (prepared as described by Sigma) in TBSA were incubated at room temperature. At various times, a 100-μL subsample was added to a fibrometer cup already containing 50 μL CaCl2 (25 mmol/L) and 50 μL rabbit brain cephalin (1:10 dilution of stock) at 37°C. Fifty microliters of factor X-deficient plasma was then added immediately and the time to clot formation determined on a fibrometer (BBL, Cockeysville, MD). Factor Xa activity was determined by comparing the clotting times with a standard curve constructed by using dilutions of Xa in the same incubation mixtures lacking LACI.

**Thrombin inhibition by LACI(HG2).** Reaction mixtures containing LACI(HG2) (400 ng/mL) and thrombin (0.5 U/mL), 180 ng/mL) were incubated at room temperature in TBSA. At various time points a subsample was added to 100 μL of 0.15 mol/L NaCl, 6.6 g/L polyethylene glycol 6000, 10 mmol/L imidazole, and 10 mmol/L CaCl2, pH 7.4, warmed to 37°C. Fifty microliters of fibrinogen (2 mg/mL) was then added immediately and the time to clot formation determined on a fibrometer (BBL, Cockeysville, MD). Factor Xa activity was determined by comparing the clotting times with a standard curve constructed with dilutions of thrombin.

**Inhibition of TF by LACI(HG2).** One hundred microliters of a mixture containing VIIa (200 ng/mL), Xa (200 ng/mL), CaCl2 (8 mmol/L), and TF (2% vol/vol) that had been incubated at room temperature for one minute was added to 100 μL TBSA containing LACI(HG2) (600 ng/mL) with or without antithrombin IIIa (130 μg/mL), and heparin (1 U/mL). At specified time points thereafter, a 10-μL sample was removed and diluted 100-fold into TBSA with 5 mol/L CaCl2. Due to practical considerations, the diluted samples made from earlier time points were held until the final, one-minute, sample had been obtained, and then all were assayed for residual TF activity by using a two-stage assay. Fifty microliters factor VIIa (1 μg/mL), 50 μL CaCl2 (25 mmol/L), 50 μL diluted sample, and 50 μL human factor X (10 μg/mL) were incubated at 37°C. After one minute, 50 μL of a mixture containing ten parts factor X-deficient plasma and one part rabbit brain cephalin stock reagent (prepared as described by Sigma) was added, and the time to clot formation was determined with a fibrometer.6

TF inhibition in mixtures containing BXa and/or BXa(GD) was determined as follows: 50 μL LACI(HG2) (30 ng/mL) and 10 μL
Bxa (1 μg/mL) and/or BXa(-GD) (1 μg/mL) were mixed. After incubation for 15 minutes at 20°C, 40 μL of a mixture containing VIIa (250 ng/mL), CaCl2 (10 mmol/L), and TF (2.5%, vol/vol) was added. In one case, 1 μL BXa (10 μg/mL) was added to the 60-μL sample containing LACI(HG2) and BXa(-GD) just before the addition of the VIIa-TF-CaCl2 mixture. At various times thereafter, a 10-μL sample was removed from the reaction mixtures and diluted 100-fold in TBSA plus 5 mol/L CaCl2 and assayed for residual TF activity as described earlier.

Because this TF assay involves a dilution of a sample from the original incubation mixture and a further one- to two-minute incubation, the derived inhibition rates must be viewed cautiously and will be referred to in the text as "apparent" rates.

**Chromogenic assays.** Reaction mixtures containing human Xa (25 ng/mL) and LACI(HG2) (250 ng/mL) in TBSA were incubated at room temperature. At specific times a 200-μL subsample was removed and placed in a cuvette containing 100 μL S-2222 (10 mmol/L) and the initial rate of increase in optical density at 405 nm (OD405) determined. A standard curve relating ΔOD405/min to the Xa concentration was constructed by using dilutions of Xa. Studies in which rabbit brain cephalin was used in the initial reaction mixture were handled slightly differently. In these cases, 50 μL of 5% Lubrol PX was included in the cuvette. The presence of Lubrol PX decreased the rate of ΔOD405 for a given concentration of Xa by ~50%.^36^ One-milliliter samples containing 20 ng/mL BXa or BXa(-GD) and 0.1 mmol/L Spectrozyme Xa were incubated at room temperature and the ΔOD405 monitored. After stable, baseline hydrolysis of the chromogenic substrate was established (about two minutes), 3.3 μL LACI activity measured in the first stage of the assay were also performed to determine the anti-LACI effect of the rabbit sera. First-stage mixtures contained 25 μL human serum, 25 μL rabbit serum, and 50 μL of a mixture containing VIIa (200 ng/mL), X (2 μg/mL), CaCl2 (8 mmol/L) and TF (2% vol/vol). After ten minutes a sample was removed and diluted 100-fold in TBSA plus 5 mmol/L CaCl2. Fifty microliters of the diluted sample was then incubated with 50 μL VIIa (1 μg/mL), 50 μL X (10 μg/mL), and 50 μL CaCl2 (25 mmol/L) at 37°C. After one minute, 50 μL of a mixture containing nine parts human factor X-deficient plasma and one part stock rabbit brain cephalin (prepared as described by Sigma) was added and the time to clot formation determined. A standard curve for LACI activity was constructed by using dilutions of human serum in TBSA and plotting on log-log paper the prolongation of the clotting time above the control (TBSA) level against the concentration of human serum in the first stage of the assay. Because rabbit sera also contains LACI activity that reacts modestly in the human system, additional assays in which rabbit serum without human serum was incubated in the first stage of the assay were also performed to determine the contribution of endogenous rabbit LACI to the total LACI activity measured (~10%). Preimmune rabbit sera served as negative controls. Intact LACI(HG2), 100 μg, in complete Freund’s adjuvant was also used to immunize a rabbit. After 3 weeks, blood was withdrawn from the central artery of an ear and allowed to clot for one hour at 37°C and the serum collected after centrifugation. The IgG fraction from this serum was isolated by chromatography on Protein A agarose (Repligen, Cambridge, MA). Its anti-LACI activity was determined as described earlier except that 25 μL IgG (6 mg/mL) was used instead of rabbit antiserum. Preimmune rabbit IgG isolated in the same manner served as a negative control.

**PAGE.** SDS-PAGE was performed by using the method of Laemmli^38^ with a 10% acrylamide separating gel and 4% stacking gel or by the method of Weber and Osborn^39^ with a 10% acrylamide separating gel. The method of Davis^40^ was used for nondenaturing gel electrophoresis in a 7.5% acrylamide gel. The samples were not boiled before electrophoresis in either PAGE system. The gels were fixed and stained, dried, and autoradiographed as previously described.^41^

**Activation peptide release assay for activation of factor X in serum.** Human [Siayl-3H]X was prepared by using the method described by Walsh et al for the tritium labeling of factor IX. The [Siayl-3H]X had a specific activity of 1.55 x 10^6 dpm/μg and retained >90% of its functional activity by bioassay. To determine the effect of anti-LACI IgG and BXa(-GD) upon the activation of [Siayl-3H]X, 150 μL serum and 150 μL rabbit anti-LACI (6 mg/mL) or 150 μL BXa(-GD) (10 μg/mL) or 150 μL TBSA were incubated for 15 minutes at room temperature, and then 228 μL TBSA, 60 μL CaCl2 (25 mmol/L), 6 μL [Siayl-3H]X (316 μg/mL), and 6 μL TF were added in succession. At specific times after the addition of TF, 50-μL samples from the reaction mixture were mixed with 150 μL TBSA containing 50 mmol/L EDTA and placed on ice. Fifty microliters iced-cold TCA (25% wt/vol) was added, and after an additional two minutes on ice, the samples were centrifuged three minutes at 12,000 g. Two hundred microliters of the resultant supernatant was added to 5 mL 3a70 (Research Products International Corp, Mount Prospect, IL) for counting in a Tracer Analytic Mark III liquid scintillation system. The total radioactivity in a sample was determined in the same fashion except that the samples were not centrifuged after the addition of TCA. Background 3H disintegrations per minute soluble in 5% TCA were 0.5% of the total [Siayl-3H]X disintegrations per minute. Maximal releasable 3H disintegrations per minute were determined by treatment of [Siayl-3H]X in TBSA with XCP (1/10, wt/wt) in the presence of rabbit brain cephalin (1:10 dilution of stock) and CaCl2 (6 mmol/L, final concentration) for 30 minutes and were 34% of the total 3H disintegrations per minute.

**RESULTS**

**Inhibition of Xa by LACI.** Incubation of purified LACI(HG2) (250 ng/mL) with human Xa (25 ng/mL) led to a loss of human Xa coagulation activity in a functional bioassay (Fig 1). In the presence of EDTA (1 mmol/L) the inhibition was rapid, with 50% of the initial human Xa activity remaining at 50 seconds. The presence of saturating phospholipids and Ca2+ (5 mmol/L) protected human Xa modestly (t1/2, 140 seconds). This inhibitory effect of LACI(HG2) was not a general property directed against all serine proteases because thrombin was completely unaffected by incubation with LACI(HG2) (Fig 1). The inhibition of Xa by LACI(HG2) was also demonstrable in assays using small-mol wt chromogenic substrates. In an incubation mixture containing 25 ng/mL Xa and 250 ng/mL LACI(HG2), the Xa activity as determined by cleavage of S-2222 was progressively inhibited such that <2% of the initial activity remained at ten minutes. As in the bioassay, the inclusion of saturating phospholipids and Ca2+ (5 mol/L) slowed the inhibition of Xa by LACI(HG2) approximately threefold as determined by chromogenic assay.
Fig 1. Effect of LACI (HG2) upon human factor Xa and thrombin. Reaction mixtures containing purified LACI(HG2) and either Xa or thrombin were constructed as described in Materials and Methods. At specified times, samples were removed and assayed for residual enzyme activity by bioassay. □—□, thrombin; ○—○, Xa; ●—●, Xa with phospholipids and Ca\(^{2+}\). In control mixtures lacking LACI there was no loss of HXa or thrombin activity (not shown).

Although LACI(HG2) inhibited Xa's activity in both a functional bioassay and an assay using small-mol wt substrates, a complex between LACI(HG2) and \(^{125}\)I-Xa could not be demonstrated by SDS-PAGE and autoradiography when either the buffer system of Laemmli\(^{26}\) (Tris/glycine, pH 8.9, Fig 2) or of Weber and Osborn\(^{29}\) (sodium phosphate, pH 7.0, not shown) were used. This suggests that a covalent complex between LACI(HG2) and Xa is not formed during the inhibition process and is consistent with the fact that LACI(HG2) could be eluted from the Xa affinity column used in its purification.\(^{11,22}\) When similar reaction mixtures of \(^{125}\)I-Xa and LACI(HG2) were electrophoresed under nondenaturing conditions\(^{30}\) (Fig 2), however, an apparent complex could be detected. Although LACI(HG2) was in excess over Xa in this experiment, only a portion of the \(^{125}\)I-Xa was present in the complex. This is most likely due to the reversible nature of the Xa-LACI(HG2) interaction (see Discussion), with dissociation of the complex occurring during electrophoresis. Notable is the fact that no complex formation appeared to occur with \(^{125}\)I-X or \(^{125}\)I-DIP-Xa.

Inhibition of TF by LACI(HG2). In the presence of VIIa, Xa, and Ca\(^{2+}\), LACI(HG2) produced very rapid inhibition of TF activity with an apparent \(t_{1/2}\) of 20 seconds (Fig 3). The inclusion of heparin (0.5 U/mL) in the mixtures
accelerated the initial rate of inhibition threefold (t₁/₂, 6 seconds). The rate of inhibition of Xa by LACI(HG2) determined by chromogenic substrate assay was also enhanced to a similar degree (two- to threefold) by heparin (data not shown). Inhibition of apparent TF activity by LACI(HG2) was not affected by the inclusion of antithrombin IIIa (65 µg/mL) in the reaction, but the presence of both heparin and antithrombin IIIa markedly abrogated LACI(HG2)'s inhibitory effect (Fig 3).

BXa(-GD). BXa(-GD), produced by treatment with chymotrypsin, was used to investigate the requirement of the γ-carboxyglutamic acid domain of factor Xa for the Xa-LACI interaction and the ultimate formation of the putative VII-Ca²⁺-TF-Xa-LACI inhibitory complex. Initial attempts to prepare human Xa(-GD) were unsuccessful due to our inability to activate human X(-GD) with XCP and because treatment of preformed human Xa with chymotrypsin produced more than a single cleavage. Therefore BXa(-GD) was used in the following studies.

BXa contains 12 γ-carboxyglutamic acid residues near its NH₂ terminus that are required for the binding of BXa to phospholipid surfaces via Ca²⁺ bridges and the expression of its biologic enzymatic activity. Limited proteolytic treatment of bovine X with α-chymotrypsin removes a 44–amino acid peptide from the NH₂ terminus of the molecule that contains all the γ-carboxyglutamic acids. Subsequent activation of this modified X with XCP produces Xa lacking the γ-carboxyglutamic acid–containing domain (-GD). This BXa(-GD) has equivalent catalytic activity against small-mol wt, chromogenic substrates such as native BXa but essentially lacks functional activity in bioassays.

Interaction between LACI(HG2) and BXa or BXa(-GD). The effect of LACI(HG2) on the catalytic activity of BXa and BXa(-GD) against Spectrozyme Xa is shown in Fig 4A and B. Under the conditions of this experiment the apparent t₁/₂ of BXa activity was 30 seconds and that of BXa(-GD), 3.5 minutes. The companion experiment shown in Fig 5B confirms that LACI forms apparent complex(es) with both BXa and BXa(-GD) that are retarded on non-denaturing PAGE. As previously shown for human Xa, these complexes do not withstand SDS-PAGE (Fig 5A), and their formation requires the active catalytic site of both BXa and

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**Fig 5.** Interaction between BXa and BXa(-GD) and LACI(HG2) determined by PAGE. In A, B, and C, mixtures containing ¹²⁵I–BXa or ¹²⁵I–BXa(-GD) (100 ng/mL) with or without LACI(HG2) (300 ng/mL) final concentration obtained by direct 100-fold dilution of LACI from stock in 1 mol/L NaSCN, 0.05 mol/L Tris-HCl, pH 7.5, and 0.5% Lubrol PX were incubated at 20°C for 20 minutes before electrophoresis. Lane 1, BXa; lane 2, BXa plus LACI(HG2); lane 3, BXa(-GD); lane 4, BXa(-GD) plus LACI(HG2). (A) SDS-PAGE by the method of Laemmli. (B) Native gel electrophoresis using the method of Davis with a 7.5% acrylamide gel. (C) Native gel electrophoresis using Xa and Xa(-GD) inactivated by treatment with dansyl-GGACK. (D) Mixtures containing LACI(HG2) (300 ng/mL) that had been diluted and dialyzed before use and ¹²⁵I–BXa (100 ng/mL) with (lane 1) or without (lane 2) unlabeled BXa (10 µg/mL) were incubated at 20°C for 20 minutes before native gel electrophoresis. The samples were neither boiled nor reduced, before electrophoresis. Degr, degradation product.
BXa(-GD) (Fig 5C). As expected, the addition of excess unlabeled BXa or BXa(-GD) prevented the association of \(^{125}\)-BXa and \(^{125}\)-BXa(-GD) with LACI(HG2), respectively (eg, Fig 5D), on nondenaturing PAGE. Note that in Fig 5B, as opposed to Figs 2B and 5D, a significant portion of the putative \(^{125}\)-BXa-LACI(HG2) and \(^{125}\)-BXa(-GD)-LACI(HG2) complexes migrate only a short distance into the gel. In the experiments depicted in Figs 2B and 5D, the LACI(HG2) was diluted in TBSA and dialyzed extensively against T/S before incubation with the labeled Xa preparation, whereas in Fig 5B a 1:100 dilution of the LACI(HG2) preparation in 1.0 mol/L NaSCN, 0.5% Lubrol PX, and 0.05 mol/L Tris-HCl, pH 7.5, was used directly. Thus, we suspect the very slow migrating radioactive active bands in Fig 5B are labeled Xa-LACI(HG2)-detergent complexes.

Because BXa(-GD) binds to LACI(HG2) and yet has little if any biologic activity, it seemed likely that it would retard the inhibition of native BXa by LACI(HG2) as determined by bioassay. Figure 6 shows the results of such an experiment. Preincubation of LACI(HG2) (100 ng/mL) with BXa(-GD) (800 ng/mL) for 60 minutes abrogated the subsequent inhibition of BXa (20 ng/mL).

Effect of BXa(-GD) on TF inhibition by LACI(HG2). Reaction mixtures containing VIIa, TF, CaCl₂, LACI(HG2), and BXa and/or BXa(-GD) were constructed as described in Materials and Methods. The LACI(HG2) and BXa (or BXa(-GD)) were incubated for 15 minutes before the addition of the remaining components. At specific times thereafter, the TF activity remaining in each mixture was determined and is shown in Fig 7. In the presence of BXa there was a rapid loss of TF activity, whereas no significant reduction in TF activity could be detected in the presence of BXa(-GD). The simultaneous addition of both BXa and BXa(-GD) led to TF inhibition that was similar to that of Xa alone. When BXa(-GD) was preincubated with LACI for 15 minutes before the addition of BXa and the other components of the reaction mixture, however, the degree of TF inhibition was considerably reduced.

Inhibition of human serum LACI activity by anti-LACI(HG2) antisera. LACI(HG2) isolated from HepG2 cell-conditioned media was used in the previous experiments.

The functional characteristics of this purified protein are the same as the inhibitor present in human serum. They both require the presence of VIIa, Ca²⁺, and X(a) to induce reversible (EDTA) TF inhibition. To document antigenic similarity between the two moieties as well, two rabbits were immunized with a synthetic peptide based on the N-terminal sequence of purified LACI(HG2), and one was immunized with intact LACI(HG2). When compared with preimmune sera, the antisera from the rabbits immunized with the N-terminal peptide inhibited the apparent LACI activity in human sera 96% and 60%, respectively. Isolated IgG (1.5 mg/mL) from the rabbit injected with intact LACI(HG2) inhibited the serum LACI activity >97%.

Effect of Anti-LACI IgG and BXa(-GD) on TF-initiated X activation in serum. [Siayl⁺⁻H]X was used to investigate the effects of rabbit anti-LACI IgG and BXa(-GD) on the activation of X in serum after the addition of TF. Mixtures were constructed as described in Materials and Methods, and at various time points a sample was removed and the degree of conversion of X to Xa determined by the release of the tritiated activation peptide (Fig 8). As compared with maximum \(^3\)H-peptide release produced by treatment of \(^3\)H-X with XCP, defined as 100% activation, at 30 minutes 90% of the X was activated in the presence of anti-LACI IgG, 63% in the presence of BXa(-GD), and 33% in the presence of serum without additions.

DISCUSSION

The fact that serum contains an apparent inhibitor of TF activity has been known for many years. This was most dramatically demonstrated by experiments that showed that preincubation of tissue thromboplastin preparations with serum prevented their lethal effect upon subsequent infusion into mice. Indirect experiments have shown, as originally proposed by Hjort, that the inhibition is directed against the VII-Ca²⁺-TF complex (rather than TF per se) and, further, that catalytically active Xa is required for the inhibition to occur. Although the role of Xa in the inhibitory process could be multiple (including, for instance, the proteolytic activation of an inactive LACI precursor), one explanation...
that has been suggested is that Xa was involved stoichiometrically in a VIIa-\(\text{Ca}^{2+}\)-TF-Xa-LACI inhibitory complex.\(^9\) This supposition led to our use of DIP-Xa-agarose chromatography in the purification of LACI(HG2) secreted by HepG2 cells.\(^11\) Although a critical step in the isolation of LACI(HG2), the capacity of the DIP-Xa-agarose column was so low that it suggested that trace amounts of active Xa in the gel were actually responsible for the binding of LACI(HG2). Further investigation of the interaction between LACI(HG2) and Xa led to the experiments presented here.

Purified LACI(HG2) rapidly inhibited Xa when tested in not only a bioassay but a chromogenic substrate assay as well, which suggests that it bound at or near the active site of Xa. This was confirmed by nondenaturing PAGE that showed that Xa but notzymogen X or DIP-Xa formed a complex with LACI(HG2). A similar complex between LACI(HG2) and Xa could not be detected after SDS-PAGE, however, thus implying that a covalent bond between LACI(HG2) and Xa is not formed during the inhibition process. Preliminary experiments suggest that in kinetic terms the binding of LACI(HG2) to Xa is “slow,” “tight,” reversible, and competitive in nature.\(^15\) In addition, they show that LACI(HG2) appears to bind more tightly to bovine Xa than to human Xa. This latter fact probably explains in part the qualitative differences between the relative amounts of BXa and HXa that can be detected complexed with LACI(HG2) after native PAGE (Fig 2B v Figs 5B and 5D).

Fig 8. Effect of anti-LACI IgG and BXa(-GD) on TF-induced activation of \([\text{Sialy}-\text{H}]\)X in serum. Reaction mixtures were constructed as described in Materials and Methods. \(\bullet\) — \(\bullet\), serum (25% vol/vol); \(\text{II}, \text{serum plus BXa(-GD)}(2.5 \mu\text{g/mL, final concentration); } \Delta \) — \(\Delta\), serum plus anti-LACI IgG (1.5 mg/mL, final concentration). The y-axis represents \(^3\text{H}\)-activation peptide release as determined by \(^3\text{H}\) disintegrations per minute soluble in 5% wt/vol TCA. Maximal activation by XCP yielded 67 \(\times\) 10\(^4\) dpm. In similar reaction mixtures lacking TF, no significant release of \(^3\text{H}\)-activation peptide occurred.

On the basis of experiments using purified proteins, LACI(HG2) appears to be at least as potent as antithrombin III, \(\alpha_1\)-protease inhibitor, and \(\alpha_2\)-macroglobulin in its ability to inactivate Xa.\(^38\) Its possible contribution to the overall inhibition of Xa in plasma is not known. Previous studies in this regard may have missed an effect of LACI because they relied on the formation of \(^125\text{I}\)-Xa-inhibitor complexes that were stable to SDS-PAGE and used relatively high concentrations of exogenously added Xa.\(^39\)\(^40\)

In 1972, Marciniak and Tsukamura demonstrated the presence of an Xa inhibitor in the macroglobulin fraction of serum, and later studies by Barrowcliffe et al showed that this “fast-acting Xa inhibitor” appeared to be associated with the serum lipoproteins.\(^41\)\(^42\) Interestingly, this inhibitor also appears to be more potent against BXa than human Xa.\(^43\)\(^44\) Reportedly, however, it does not affect the catalytic activity of Xa against chromogenic substrates (S-2222), and thus its relationship to LACI is unclear.\(^45\)

At concentrations of VIIa and Xa presumed to be saturating relative to TF, LACI produced a rapid inhibition of TF activity (apparent \(t_{1/2}, 20\) seconds) that was not affected by the addition of antithrombin III\(\alpha\) and appeared to be enhanced approximately threefold in the presence of heparin. The simultaneous presence of both heparin and antithrombin III\(\alpha\), however, markedly reduced the degree of TF inhibition. Because the effect of antithrombin III and heparin on VIIa under these circumstances should be minimal,\(^14\) this latter phenomenon is most likely due to the rapid, covalent binding of Xa to antithrombin III in the presence of heparin, thereby preventing its participation with LACI in the inhibition of the VIIa-\(\text{Ca}^{2+}\)-TF complex.

The rate of inactivation of BXa(-GD) was slower than that of native BXa thus implying that the GD (amino acids 1 to 44) is required for the optimal binding of LACI(HG2) to Xa. It is unlikely that this difference is related only to conformational changes associated with \(\text{Ca}^{2+}\) binding by the GD because EDTA actually enhances the inhibition of native Xa by LACI (Fig 1 and unpublished observations). Although BXa(-GD) interacts with LACI(HG2), albeit more slowly than does native BXa, it apparently fails to participate in the VII-TF-Xa-LACI(HG2) complex required for the inhibition of TF activity (Fig 4). This suggests that the GD is necessary for the binding of the Xa/ LACI(HG2) complex to VII/TF. Because it is the GD of factor Xa that is needed for the \(\text{Ca}^{2+}\)-dependent binding of Xa to phospholipid surfaces, this result also implies that \(\text{Ca}^{2+}\) may be required for the binding of the native Xa-LACI complex to VII/TF.

Figure 9 is a schematic representation of a possible mechanism of inhibition of the VIIa-\(\text{Ca}^{2+}\)-TF complex by LACI and the effect LACI might have on the generation of thrombin in plasma. Gel filtration and ultracentrifugation experiments suggest that LACI is associated with plasma lipoproteins.\(^9\)\(^12\) The nature of the LACI-lipoprotein interaction and whether LACI must be “activated” (proteolytic or otherwise) and released from a lipoprotein particle to express functional activity is not known. In any event, to simplify the figure, LACI’s possible continued association with a lipoprotein particle is omitted. On the left, LACI is shown complexing with Xa near its active serine site and thus inhibiting Xa’s functional activity. Two pathways are shown for the formation of a final quaternary complex associated with the inhibition of VIIa-\(\text{Ca}^{2+}\)-TF at the bottom of the figure. In one (on the left), a Xa-LACI complex binds to the VII-TF complex, whereas in the other (on the right) LACI binds to a preexisting VII-TF-Xa complex. Though included in the figure for completeness, there are no direct data at present to
show that a VII-TF-Xa complex exists. Interestingly, however, Jesty has noted that the combination of both VII and TF (but not TF alone) markedly slows the inhibition of Xa by antithrombin III, thereby suggesting that some interaction between VII-TF-Xa may occur. The theorized, direct participation of Xa in the final complex, however, is consistent with a number of facts: (a) catalytically active Xa is required for inhibition of VIIIa-TF to occur; and LACI(HG2) will only bind to catalytically active Xa; (b) when recalcified plasma is treated with Xa and then the Xa neutralized with specific antibodies, no subsequent inhibition of VIIa-TF occurs; (c) the presence of heparin and antithrombin III abrogates the inhibitory effect; and (d) BXa(GD) binds to LACI(HG2) but fails to support the inhibition of VII-TF.

Other investigators have described additional possible mechanisms for the inhibition of TF-initiated coagulation. Carson has recently shown that purified apolipoprotein AII (apo-AII) inhibits the activation of X by VIIa-TF. His kinetic experiments suggested that this effect was due to apo-AII’s prevention of the association between VIIa and TF. Kondo and Kissel have reported that a lipoprotein that copurifies with X also inhibits VIIa-TF activation of X. This lipoprotein contained several proteins by SDS-PAGE. Their kinetic experiments showed that the inhibition was noncompetitive in nature, which suggested to them that the lipoprotein’s mechanism of action involved sequestration of either VIIa or X. Notably, this inhibitory lipoprotein did not affect the amidolytic activity of Xa. Funakoshi et al have isolated an anticoagulant protein from placenta that, based upon amino acid sequence data, appears to be a member of the lipocortin family. Its anticoagulant effect in plasma induced to clot by either kaolin or TF was related to its binding of phospholipids. This inhibitor neither bound to nor affected the amidolytic activity of Xa. Whether a form of this inhibitor is also present in plasma is not known. The characteristics of these inhibitors are sufficiently disparate from that of LACI to suggest that more than one means of inhibiting TF initiated coagulation may exist in plasma.

Thus, LACI appears to play a role in a novel feedback inhibition system (see Fig 9B). When plasma is exposed to TF, at the site of a wound, for instance, VII(a) binds to TF and can activate factor X and IX. After the generation of small amounts of Xa and IXa, the VII(a)-Ca²⁺-TF complex would be inhibited through the action of LACI. Further generation of Xa would then proceed through an alternative pathway involving IXa and factor VIIIc. This feedback inhibition of TF-initiated coagulation likely explains the results of Marlar et al who found that the final extent of Xa generation was markedly depressed in hemophiliac (VIII or IX deficient) plasmas as compared with normal plasma when clotting was initiated by small quantities of TF. If the defect in Xa generation in hemophiliac plasma is critical to the bleeding diathesis in these people, an agent that inhibits LACI activity might conceivably be useful in the treatment of hemophiliacs with inhibitors. In sum, the results of in vitro studies suggest that LACI could be an important regulator of vivo hemostasis. To confirm this, investigations concerning hemostasis in animal models and a search for an individual with LACI deficiency who might theoretically have an increased risk for thrombosis have been initiated.

ACKNOWLEDGMENT

The authors would like to thank Drs Craig, Bach, and Olson for sharing with us their unique method for the isolation of factor Xa before its publication and Betty Greene for preparing this manuscript.

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The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor Xa: insight into its possible mechanism of action

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