Protease Nexin II Interactions with Coagulation Factor XIa Are Contained within the Kunitz Protease Inhibitor Domain of Protease Nexin II and the Factor XIa Catalytic Domain†

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Received November 4, 1999; Revised Manuscript Received February 8, 2000

ABSTRACT: Protease nexin II, a platelet-secreted protein containing a Kunitz-type domain, is a potent inhibitor of factor XIa with an inhibition constant of 250−400 pM. The present study examined the protein interactions responsible for this inhibition. The isolated catalytic domain of factor XIa is inhibited by protease nexin II with an inhibition constant of 437 ± 62 pM, compared to 229 ± 40 pM for the intact protein. Factor XIa is inhibited by a recombinant Kunitz domain with an inhibition constant of 344 ± 37 pM versus 422 ± 33 pM for the catalytic domain. Kinetic rate constants were determined by progress curve analysis. The association rate constants for inhibition of factor XIa by protease nexin II [(3.35 ± 0.35) × 10^6 M^{-1} s^{-1}] and catalytic domain [(2.27 ± 0.25) × 10^6 M^{-1} s^{-1}] are nearly identical. The dissociation rate constants are very similar, (9.17 ± 0.71) × 10^{-4} and (7.97 ± 1.1) × 10^{-4} s^{-1}, respectively. The rate constants for factor XIa and catalytic domain inhibition by recombinant Kunitz domain are also very similar: association constants of (3.19 ± 0.29) × 10^6 and (3.25 ± 0.44) × 10^6 M^{-1} s^{-1}, respectively; dissociation constants of (10.73 ± 0.84) × 10^{-4} and (10.36 ± 1.3) × 10^{-4} s^{-1}. The inhibition constant (K_i) values calculated from these kinetic parameters are in close agreement with those measured from equilibrium binding experiments. These results suggest that the major interactions required for factor XIa inhibition by protease nexin II are localized to the catalytic domain of factor XIa and the Kunitz domain of protease nexin II.

Recent studies focused on the initiation of blood coagulation have suggested that factor XI has an essential function as an amplifier of the coagulation response (*1*−*5*). Factor XI is a 160 000 dalton serine protease zymogen composed of two identical 80 kDa chains, each containing an active site. Evidence for the physiological relevance of factor XI lies in the hemostatic defects that occur in individuals with factor XI deficiency (*6*−*8*), whereas factor XII deficiency is not associated with abnormal bleeding (*9*). It is, therefore, unlikely that factor XI is activated under normal physiological conditions by factor XIIa through the contact activation pathway (*2*). Factor XI can be activated by thrombin in the presence of negatively charged surfaces such as heparin, other glycosaminoglycans (*1, 2*) and on the activated platelet surface (*5*). This observation is particularly relevant to the revised theory of blood coagulation (*1, 2*), which suggests that the amount of thrombin activated by the tissue factor: factor VIIa pathway is not sufficient to produce an adequate fibrin clot, since this pathway is rapidly inactivated by tissue factor pathway inhibitor (*9, 10*). The need for factor XIa to activate factor IX is shown by the bleeding problems faced by individuals with factor XI deficiency. Factor XIa, activated by thrombin, is essential for activation of factor IX and the propagation of the coagulation response.

Regulation of factor XIa is, therefore, crucial to the control of the coagulation process. Factor XIa is regulated by two classes of inhibitors, serine protease inhibitors (*3, 11*−*14*) and Kunitz-type inhibitors (*15*−*18*). The serine protease inhibitors shown to be most effective in the inhibition of factor XIa are α_1-antiprotease (*11*), C_1-inhibitor (*12, 13*), and antithrombin III (*14*). These inhibitors are present in plasma in micromolar concentrations, the most abundant being α_1-antiprotease, at a concentration of approximately 45.5 μM. They are characterized by association rate constants of (1.3−4) × 10^4 M^{-1} s^{-1} (*13*).

In contrast, protease nexin II is a Kunitz-type protease inhibitor released from activated platelets (*15*−*17, 19*). Protease nexin II refers to forms of the amyloid precursor protein that contain the Kunitz domain. It is a 100−120 kDa protein which contains one inhibitory domain, the Kunitz protease inhibitor domain (KPI)[1] (*20*). This domain contains 56 amino acids and 3 intrachain disulfide bonds (*21*) known to be conserved in Kunitz-type inhibitors. Amyloid precursor protein is encoded on chromosome 21 (*21*) with the predominant isoforms consisting of 695, 714, 751, and 770 amino acids expressed through differentially expressed

† This study was supported by research grants from the National Institutes of Health (HL46213, HL56153, and HL56914, and by NRSA1 F31 NR07022).

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10.1021/bi9925468 CCC: $19.00
Published on Web 03/28/2000

mRNA. The isoforms of 751 (*21, 22*) and 770 (*15*) amino acids contain the Kunitz domain. The form of protease nexin II secreted from platelets has been shown to be proteolytically cleaved at a site amino terminal to the transmembrane domain (*23, 24*).

Protease nexin II has been shown to inhibit factor IXa (*25, 26*), factor Xa (*27*), and factor XIa (*16, 18*). The inhibition constant (K_i) for factor IXa by protease nexin II, in the presence of factor VIIIa and platelets, was reported to be 120 nM (*26*). The K_i for factor Xa inhibition by protease nexin II in the presence of the prothrombinase complex was reported to be 19 nM (*27*). In contrast, the inhibition of factor XIa by protease nexin II is characterized by a K_i of 300−450 pM (*15, 16, 18*). Van Nostrand et al. (*28*) quantified the amount of protease nexin II released by activated platelets to be 27 nM with a plasma platelet concentration of 3×10^8 platelets per milliliter. This suggests that it is unlikely that protease nexin II is a physiologically relevant inhibitor of either factor IXa or factor Xa, given their relatively high K_i values. It also suggests that protease nexin II is, more specifically, a physiologically relevant inhibitor of factor XIa.

Scandura et al. (*17*) further characterized the inhibition of factor XIa by protease nexin II by analyzing progress curves and determining kinetic rate constants. The association rate constant, $(2.1 \pm 0.2) \times 10^6$ M^{-1} s^{-1}, and dissociation rate constant, $(8.5 \pm 0.8) \times 10^{-4}$ s^{-1}, gave a calculated K_i of 400 pM, in good agreement with previous reports. The goal of the present work is to localize the interaction between factor XIa and protease nexin II. Using both equilibrium methods and analysis of progress curves, the inhibition of the isolated factor XIa catalytic domain by protease nexin II versus a recombinant Kunitz protease inhibitor (rKPI) domain were compared to inhibition of intact factor XIa.

MATERIALS AND METHODS

Materials

Human factor XIa was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Protease nexin II was purified from stably transfected 293-human embryonic kidney cells (a gift from W. Van Nostrand). Recombinant Kunitz protease inhibitor domain was also a kind gift from W. Van Nostrand (*29*). The chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline hydrochloride (S2366) was purchased from Chromogenix (Mölndal, Sweden). The fluorogenic substrate Boc-Glu(O-bzl)-Ala-Arg-methylcoumaryl-7-amide (Boc-EAR-MCA) was obtained from Peptides International (Louisville, KY). The monoclonal antibody 22C11 was purchased from Boehringer Mannheim (Indianapolis, IN). The monoclonal factor XI antibody 5F7 (*30*) was expanded as ascites in mice in the hybridoma facility of the Temple University Thrombosis Center. Alkaline phosphatase conjugated anti-mouse IgG, horseradish peroxidase conjugated anti-mouse IgG, *p*-nitrophenyl phosphate tablets, fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB), fluorescein, glutamine, penicillin/streptomycin, Sepharose CL-6B, DEAE-Sepharose, Sephacryl S-400, dithiothreitol, iodoacetamide, and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech (Herndon, VA.). Newborn calf serum and Geneticin were obtained from Gibco (Grand Island, NY). Microtiter plates were purchased from Becton Dickinson Labware (Lincoln Park, NJ). SuperSignal chemiluminescence substrate was purchased from Pierce (Rockford, IL). All other reagents were analytical grade or the best quality commercially available.

The compositions of buffers utilized are as follows: phosphate-buffered saline (PBS), 20 mM sodium phosphate, 150 mM NaCl, pH 7.4; Hepes-buffered saline (HBS), 0.01 M Hepes, 150 mM NaCl, pH 7.4; Tris-buffered saline (TBS), 0.1 M Tris−OH, 150 mM NaCl, pH 7.4.

Methods

Preparation of Isolated Factor XIa Catalytic Domain. The catalytic domain of factor XIa was isolated by a method using gentle reduction and alkylation shown to result in a fully functional light chain as previously described (*30*). Factor XIa, 200 μg, was placed in 0.5 mL of Tris/succinate buffer, pH 8.3 (40 mM Tris, 10 mM succinic acid, 1 mM benzamidine, 1 mM EDTA, 50 μg/mL Polybrene). Dithiothreitol (0.1 mM) was added, and the reduction was allowed to proceed for 1 h at room temperature under nitrogen in the dark. Iodoacetamide (0.5 mM) was added, and the reaction was allowed to continue for 1 h under the same conditions. After dialysis against phosphate-buffered saline, the reduced and alkylated material was applied to a heavy chain-specific monoclonal antibody (5F7) column. The flow-through fraction was run on 10% SDS−PAGE, and the active-site concentration was determined using fluorescein mono-*p*-guanidinobenzoate hydrochloride (FMGB).

Preparation of Dextran Sulfate−Sepharose Column. A dextran sulfate−Sepharose column was prepared essentially as described (*31*). Sepharose CL-6B, 250 mL, was washed with 2 L of distilled water. The Sepharose was then activated by adding 125 mL of 1 M NaOH, 2 mg/mL $NaBH_4$, and 125 mL of diepoxide 1,4-butanediol diglycidyl ether, and gently rocking for 8 h at room temperature. The activated Sepharose was washed with 3 L of distilled water, and then mixed with 250 mL of 10 G dextran sulfate in 0.2 M sodium bicarbonate. The coupling reaction was maintained overnight at 37 °C. Ethanolamine, 1 M final concentration, was added to block remaining reactive epoxide groups and mixed for 24 h. The coupled dextran sulfate−Sepharose was then washed with 2 L of 0.2 M sodium bicarbonate (pH 11), followed by 2 L of 0.1 M sodium acetate (pH 4) and 2 L of PBS. The matrix was then stored in PBS, 0.02% sodium azide, at 4 °C until use.

Purification of Protease Nexin II. Protease nexin II was purified from transfected 293-human embryonal kidney cells as described (*31*). Cells were grown to confluency in DMEM containing 200 mM glutamine, 10% newborn calf serum, 100 units penicillin/mL, 100 μg of streptomycin/mL, and 50 μg/mL Geneticin in a 5% CO_2, 37 °C incubator. The cells were then removed with trypsin/EDTA and separated into

[1] Abbreviations: KPI, Kunitz protease inhibitor; rKPI, recombinant Kunitz protease inhibitor; K_i, inhibition constant; K_m, Michaelis constant; V_{max}, maximum rate of substrate hydrolysis; FMGB, fluorescein mono-*p*-guanidinobenzoate hydrochloride; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HBS, Hepes-buffered saline; TBS, Tris-buffered saline; IC_{50}, 50% activity remained.

roller bottles. After 3 days, the medium was removed and replaced with serum-free DMEM, 0.1% BSA, and the antibiotics. After 24 h, this medium was replaced with serum-containing medium. The serum-free medium was filtered and chilled to 4 °C, and 200 μM phenylmethylsulfonyl fluoride was added. The serum-free medium was applied to a dextran sulfate−Sepharose column equilibrated in phosphate-buffered saline (PBS). The column was washed with PBS until the A_{280} returned to base line. Adsorbed protein was eluted with a 0.15−1.5 M NaCl in PBS linear gradient, and 20 mL fractions were collected. Fractions containing protease nexin II were detected by dot-blot using the monoclonal antibody 22C11 and horseradish peroxidase conjugated anti-mouse IgG and detected using chemiluminescent substrate. Positive fractions were diluted with 20 mM potassium phosphate, pH 7.4, to reduce the conductivity, and applied to a DEAE-Sepharose column equilibrated in 20 mM K_2HPO_4, 0.2 M NaCl, pH 7.4. After washing the column with 4 column volumes, bound protein was eluted with 0.75 M NaCl in 20 mM K_2HPO_4. Two milliliter fractions were collected and analyzed by dot-blot as described above. Protease nexin II-positive fractions were pooled, concentrated to 1 mL, and applied to a Sephacryl S-400 column (Sigma Chemical Co., St. Louis, MO) equilibrated in 20 mM K_2HPO_4, 0.5 M NaCl. One milliliter fractions were collected and analyzed by SDS−PAGE. Positive fractions were tested for activity and combined, and the active-site concentration was determined using active-site titrated factor XIa, as previously described (*17*). The active-site concentration was calculated at 2.33 μM, a value which was 99% of the protein concentration as determined by A_{280}.

Determination of Protein Concentration by Active-Site Titration. Factor XIa and the isolated catalytic domain were assayed for active-site concentration using the method of Melhado et al. (*32*). The release of fluorescein from 10 μM FMGB was monitored in a Bowman Series 2 spectrofluorimeter (SLM Aminco, Urbana, IL) both before and after the addition of enzyme. The excitation wavelength was 500 nm, emission 520 nm, with 4 nm slit widths on both sides. After correction for spontaneous hydrolysis, the burst hydrolysis was converted to a molar concentration of enzyme by comparison to a standard curve made with known concentrations of fluorescein. The active-site concentration of factor XIa was essentially identical to the protein concentration calculated using the published extinction coefficient of factor XIa, 1.34 at 0.1%, and the absorbance at 280 nm.

Inhibition Kinetics. Either factor XIa or isolated catalytic domain was diluted in 0.05 M HEPES, 0.15 M NaCl, 0.1% BSA to a final concentration of 0.1 nM, calculated by active-site titration. Samples were incubated with increasing concentrations of protease nexin II or rKPI for 1 h at 37 °C. Residual amidolytic activity was measured under pseudo-first-order kinetic conditions for 15 min on a Hewlett-Packard, model 8452A, diode array spectrophotometer (Waldbronn, Germany). Results were then converted to the fraction of amidolytic activity remaining, and the concentration at which 50% activity remained (IC_{50}) was determined using Kaleidograph v3.05 (Abelbeck Software, PCS, Inc., Reading, PA) nonlinear least-squares regression software. The IC_{50} was converted to an inhibition constant (K_i) using eq 1:

$$K_i = IC_{50}/(1 + S/K_m) \quad (1)$$

where S is the substrate concentration and K_m is the Michaelis constant (K_m) of factor XIa for S2366, determined to be approximately 250 ± 20 μM in previous work in this laboratory (*17*).

Progress Curve Analysis of Inhibition Kinetics. To compare the kinetics of inhibition by protease nexin II of isolated factor XIa light chain to factor XIa, progress curves of product formation were analyzed as described by Scandura et al. (*17*). The fluorogenic substrate Boc-EAR-MCA (25 μM) was incubated with 10 pM factor XIa or factor XIa catalytic domain. After steady-state substrate hydrolysis was achieved (10 min), protease nexin II was added and the change in the rate of product formation measured for an additional 50 min, giving a 1 h total observation time. The resulting progress curve can then be analyzed according to eq 2 (*33*):

$$P = V_s t + (V_o - V_s)(1 - e^{-k_{obs}t})/k_{obs} \quad (2)$$

where V_o is the velocity of product formation at the time of addition of the inhibitor, V_s is the velocity after a steady state has been reached between inhibitor and enzyme, k_{obs} is the observed first-order rate constant that characterizes the transition from V_o to V_s, and P is the concentration of product formed at any time, t.

The association rate was determined by substituting the measured parameters into eq 3:

$$k_{association} = (V_o - V_s)k_{obs}(1 + S/K_m)/(V_o[\text{protease nexin II}]) \quad (3)$$

The dissociation rate was calculated using eq 4:

$$k_{dissociation} = V_s k_{obs}/V_o \quad (4)$$

The inhibition constant (K_i) was calculated using eq 5:

$$K_i = k_{dissociation}/k_{association} \quad (5)$$

Calculation of Binding Energies. Binding energies were calculated using eq 6:

$$\Delta G = -RT \ln K_i \quad (6)$$

where R = the gas constant (1.987 cal mol^{-1} K^{-1}) and T = degrees Kelvin (273 + °C) (*34*).

Molecular Modeling of the Factor XIa Catalytic Domain. The crystal structure of bovine pancreatic trypsinogen has been refined to 1.9 Å (*35*). This was displayed using Sybyl 6.4 molecular modeling software (Tripos, Inc., St. Louis, MO) on a Silicon Graphics, model 6.5.4, computer (Silicon Graphics, Inc., Mountain View, CA). The catalytic domain of factor XIa was homology-modeled, utilizing Sybyl 6.4, using the crystal structure of bovine pancreatic trypsinogen, since the two protein structures have approximately 49% sequence identity. Amino acids for the factor XIa light chain were substituted, as necessary, in the trypsinogen sequence. Only one gap occurred, one amino acid in size, which was closed by joining of amino acids in the factor XIa sequence. Cysteine bonds were established, charges were assigned using the Gasteiger−Hückel method (*36*), and the energy

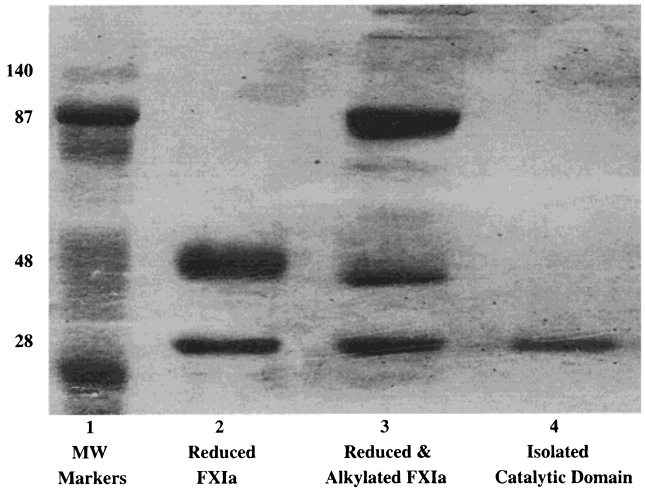

FIGURE 1: SDS−PAGE of factor XIa and the isolated catalytic domain. The factor XIa catalytic domain was isolated by gentle reduction with dithiothreitol and alkylation with iodoacetamide, under oxygen-free conditions, as described under Materials and Methods. The factor XIa heavy chain was then separated from the light chain, or catalytic domain, by immunoaffinity chromatography on a 5F7 monoclonal antibody column which is specific for the factor XI heavy chain. The isolated catalytic domain migrates with the same molecular weight as that which had been reduced using β-mercaptoethanol (lane 2).

was minimized using a combination of the Simplex method and MAXIMIN2 procedure. The electrostatic potential was displayed on a Connolly surface using the program MOLCAD.

RESULTS

Isolation and Characterization of Factor XIa Catalytic Domain. The catalytic domain, or light chain, of factor XIa was gently reduced, alkylated, and separated by immunoaffinity chromatography on a 5F7, heavy-chain-specific, monoclonal antibody column. As shown in Figure 1, the isolated catalytic domain migrated at the same molecular mass as the 34 kDa catalytic domain in the reduced factor XIa samples in lanes 2 and 3. The reduction and alkylation procedure did not completely reduce all of the factor XIa in the reaction mixture, as seen by the nonreduced factor XIa at 160 kDa and the partially reduced monomer at 80 kDa in lane 3. The isolated catalytic domain is seen in lane 4. The fractions were pooled and tested for active-site concentration by hydrolysis of FMGB compared to a fluorescein standard curve. The active-site concentration of the catalytic domain was approximately 580 nM. The protein concentration was determined by A_{280} and an extinction coefficient, $E^{1\%}{}_{1\text{ cm, }280\text{ nm}} = 0.65$, calculated according to the method of Gill and von Hippel (*37*). This concentration, approximately 0.02 mg/mL, gives a specific activity of 0.87 mol of active site/mol of catalytic domain monomer.

Determination of Michaelis Constant (K_m) and Maximum Rate of Substrate Hydrolysis (V_{max}) of the Factor XIa Catalytic Domain versus Intact Factor XIa. To ensure that the factor XIa catalytic domain had the same ability to cleave substrate as factor XIa, the K_m and the V_{max} were compared to those of factor XIa (shown in Figure 2). The value of the K_m of the catalytic domain was 174 ± 20 μM, and was not significantly different from that of intact factor XIa, 147 ± 13 μM. Similarly, the V_{max} of the catalytic domain, 3.16 ± 0.17 pmol/min, is nearly identical to that of factor XIa (calculated as monomer), 3.21 ± 0.14 pmol/min.

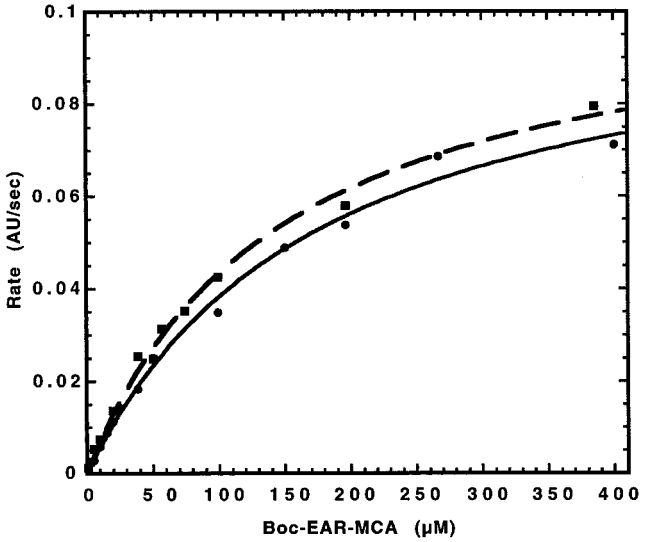

FIGURE 2: Catalytic domain and factor XIa amidolytic activity. Either factor XIa or catalytic domain, 10 pM, was added to a cuvette. Concentrations of the substrate, Boc-EAR-MCA, from 1 to 400 μM were added at 10 min intervals, and a steady state of hydrolysis was established. (●) Factor XIa catalytic domain; (■) factor XIa. Graphs represent the means of two determinations.

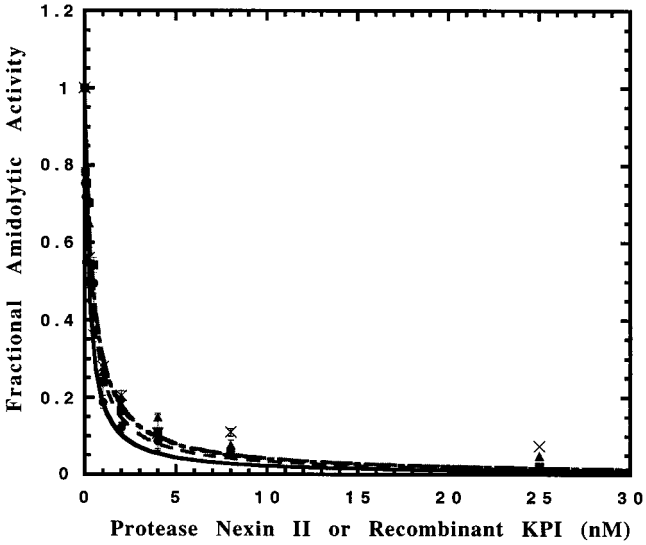

FIGURE 3: Inhibition of factor XIa and factor XIa catalytic domain by PNII and rKPI domain. Either factor XIa or factor XIa catalytic domain, 0.1 nM by active-site titration, was incubated with increasing concentrations of either PNII or rKPI for 1 h at 37 °C. The residual amidolytic activity was measured with S2366, and then divided by the hydrolysis rate in the absence of inhibitor. (●) Factor XIa + PNII; (■) catalytic domain + PNII; (×) factor XIa + KPI; (▲) catalytic domain + KPI. Each data point = mean ± SE for nine determinations.

Comparison of Factor XIa and Factor XIa Catalytic Domain Inhibition by Protease Nexin II and Kunitz Domain. Increasing concentrations of either protease nexin II or rKPI domain were incubated with 0.1 nM active-site-titrated factor XIa or factor XIa catalytic domain. A graph of factor XIa and catalytic domain inhibition by protease nexin II and KPI is shown in Figure 3, with the fractional amidolytic activity versus the inhibitor concentration. The K_i values are displayed in Table 1 and represent the mean ± standard deviation of nine separate determinations. The difference in

Table 1: Comparison of Inhibition Constants Derived from Kinetic Rate Constants or Equilibrium Methods[a]

	$k_{association}$ ($\times 10^6$ M^{-1} s^{-1})	$k_{dissociation}$ ($\times 10^{-4}$ s^{-1})	$K_{i(calcd)}$ (pM)	calculated binding energy (kcal/mol)	measured K_i (pM)	calculated binding energy (kcal/mol)
factor XIa/ PNII	3.35 ± 0.35	9.17 ± 0.71	274 ± 16	13.56	229 ± 40	13.54
catalytic domain/PNII	2.27 ± 0.25	7.97 ± 1.1	351 ± 20.5	13.41	437 ± 62	13.23
factor XIa/ KPI	3.19 ± 0.29	10.73 ± 0.84	354 ± 34	13.40	344 ± 37	13.42
catalytic domain/KPI	3.25 ± 0.44	10.36 ± 1.3	399 ± 50	13.33	422 ± 33	13.3

[a] Kinetic rate constants were obtained from analysis of the progress curves in Figure 4 using the method of Cha et al. (*33*), with K_i values calculated according to eq 5 under Materials and Methods. Calculated K_i values are the mean ± SD of 15 determinations at different inhibitor concentrations. K_i values determined by equilibrium techniques, as shown in Figure 2, are the mean ± SD for nine separate determinations.

K_i values between factor XIa (229 ± 40 pM) and catalytic domain (437 ± 62 pM) inhibition by protease nexin II was statistically significant ($p < 0.001$). The binding energies were, therefore, calculated, and the difference was determined. The calculated binding energy characterizing factor XIa inhibition by protease nexin II is approximately 13.54 kcal mol^{-1}, and that of the catalytic domain is 13.23 kcal mol^{-1}, i.e., a difference of 0.31 kcal mol^{-1}, or 2.3% of the binding energy. The inhibition constants for either factor XIa (K_i = 344 ± 37 pM) or catalytic domain (K_i = 422 ± 33 pM) inhibition by KPI domain were not significantly different, nor was there a significant difference in binding energy.

Progress Curve Determination of the Kinetics of Factor XIa and Catalytic Domain Inhibition by Protease Nexin II and Recombinant Kunitz Protease Inhibitor (rKPI). To further compare the inhibition of intact factor XIa by protease nexin II or rKPI to that of factor XIa catalytic domain, the respective interactions were analyzed by examining progress curves of inhibition as described under Methods. A steady state of hydrolysis of the fluorogenic substrate (Boc-EAR-MCA) was established in the presence of either factor XIa or the catalytic domain. Varying concentrations of either protease nexin II or rKPI were added, and the change in the rate of substrate hydrolysis was observed during the time course of binding of the inhibitor (either protease nexin II or rKPI) and the enzyme (either factor XIa or catalytic domain). The reaction was allowed to continue for 1 h total, during which time a steady state of inhibition was established. Representative progress curves are shown in Figure 4.

The association and dissociation rate constants for these interactions were calculated and are summarized in Table 1. Results are the means ± SD of 15 determinations. The association rate constants for inhibition of either factor XIa or the catalytic domain by either protease nexin II or rKPI domain are nearly identical. The dissociation rate constants for factor XIa and catalytic domain inhibition by protease nexin II are also very similar. The dissociation rate constants for factor XIa and catalytic domain inhibition by rKPI are also identical, but slightly faster than those describing protease nexin II inhibition. These rate constants are also in good agreement with previously published rate constants for the interaction of protease nexin II and factor XIa (*17*).

Molecular Model of Factor XIa Catalytic Domain. Using the Sybyl molecular modeling program, the structure of bovine trypsinogen, as determined by X-ray crystallography (*35*), was displayed and is shown in Figure 5. A molecular model of the factor XIa catalytic domain was created by making appropriate substitutions in the trypsinogen sequence and is also shown in Figure 5. A comparison was made between the residues of porcine pancreatic kallikrein A, identified by Hynes et al. (*38*) as interacting with the protease nexin II KPI domain, and the factor XIa catalytic domain sequence. A similar comparison was made between the factor XIa catalytic domain sequence and the residues of trypsin found to interact with the KPI domain in crystal structures (*39*). In both the trypsinogen and factor XIa catalytic domain structures shown in Figure 5, the catalytic triad and the residues which may bind to the KPI domain are labeled. The catalytic triad of trypsin includes residues H^{57}, D^{102}, and S^{195}. The catalytic triad of the factor XIa catalytic domain includes residues H^{413}, D^{462}, and S^{557}. A comparison of the amino acids, in trypsin and in porcine pancreatic kallikrein A, that are involved in their inhibition by the KPI domain and the residues in the same sequence position in the factor XIa catalytic domain demonstrated high homology between the three proteins in the residues surrounding the catalytic triad. In both trypsin and porcine kallikrein A, a salt bridge is formed between the enzyme catalytic serine and the P1 arginine in the protease nexin II KPI domain. Of the 17 residues in trypsin identified as interacting by hydrogen bond, salt bridge, or hydrophobic interaction, 10 are identical in the factor XIa catalytic domain.

DISCUSSION

The first major finding of the present work is that the interactions necessary for factor XIa inhibition by protease nexin II are localized to the catalytic domain of factor XIa and the KPI domain of protease nexin II. As shown in Figure 2 and summarized in Table 1, the inhibition constants characterizing the inhibition of factor XIa versus catalytic domain by protease nexin II, although significantly different ($p < 0.001$), are quantitatively similar. There is a difference of only 2.3% in the binding energy between the inhibition of factor XIa and catalytic domain inhibition by protease nexin II. This small difference may have been a result of small changes in the conformation of the catalytic domain resulting from the process of reduction and alkylation used to separate it from the heavy chain of factor XIa. It is also possible that interactions between the factor XIa catalytic domain and the heavy chain in intact factor XIa produce a slightly different conformation in the catalytic domain. However, no significant difference was observed in K_i values for factor XIa versus catalytic domain inhibition by the rKPI domain (Table 1). This may mean that there are some interactions, albeit relatively weak, between the FXIa heavy chain and residues in protease nexin II outside of the KPI domain.

The conclusion that the interaction between factor XIa and protease nexin II is localized to the catalytic domain of factor

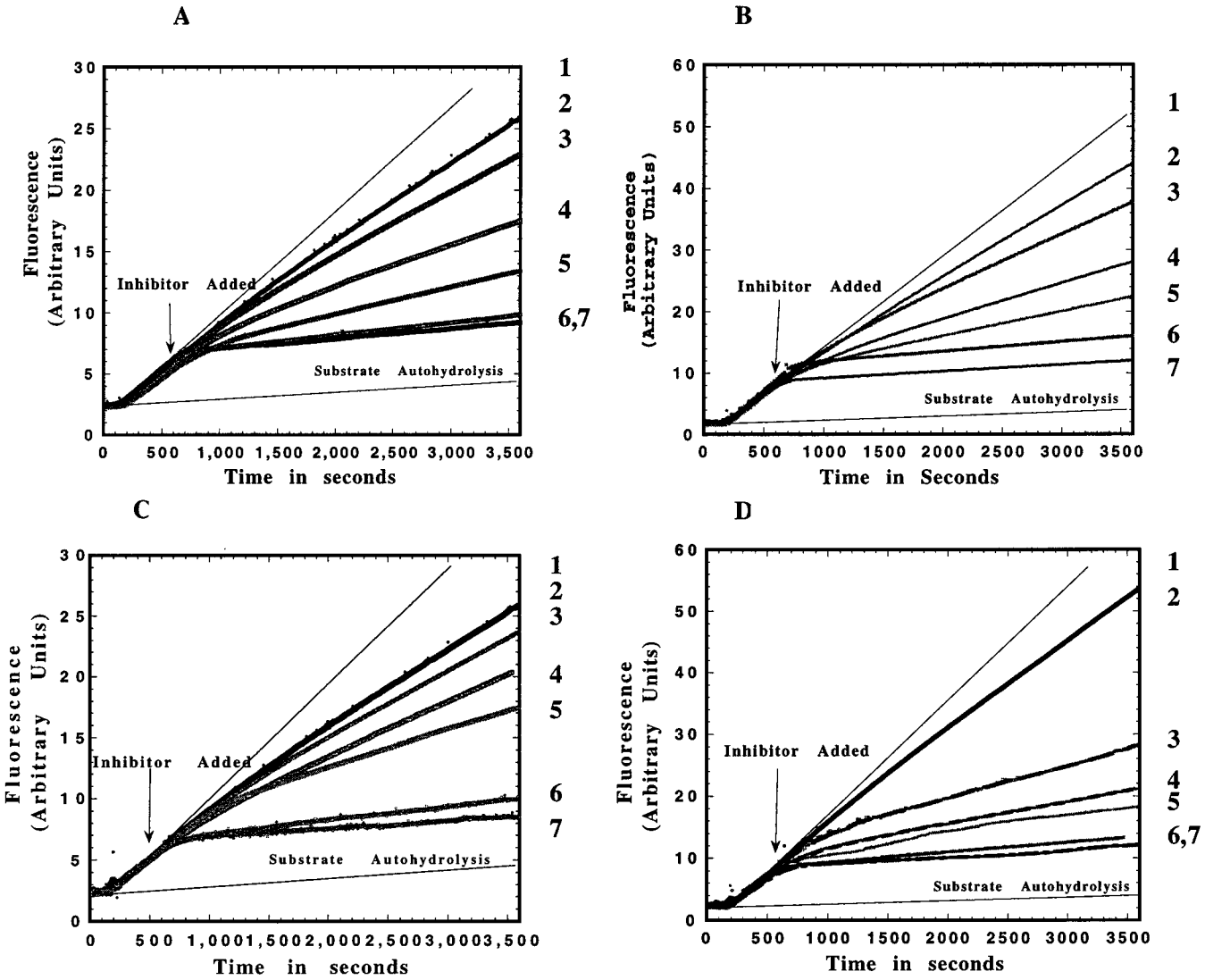

FIGURE 4: Representative progress curves of either factor XIa or factor XIa catalytic domain inhibition by either PNII or rKPI domain. Factor XIa, 10 pM, or catalytic domain, 20 pM, was added to a stirred solution of Boc-EAR-MCA, 25 μM. After a steady state of substrate hydrolysis was achieved, inhibitor was added, and the change in the rate of hydrolysis was followed for 1 h total. 1 = uninhibited rate (extrapolated); 2 = 100 pM inhibitor; 3 = 250 pM; 4 = 500 pM; 5 = 1 nM; 6 = 5 nM; 7 = 10 nM. The substrate autohydrolysis curve is extrapolated from the rate over the first 3−5 min. Panel A: factor XIa + PNII; panel B: catalytic domain + PNII; panel C: factor XIa + KPI; panel D: catalytic domain + KPI.

XIa and the KPI domain of protease nexin II is further supported by the similarities in the rate constants which describe the inhibition. As shown in Table 1, the association rate constant of factor XIa with protease nexin II, $(3.35 \pm 0.35) \times 10^6$ M^{-1} s^{-1}, is nearly identical to that of the catalytic domain, $(2.27 \pm 0.25) \times 10^6$ M^{-1} s^{-1}. The association rate constant of rKPI domain and factor XIa, $(3.19 \pm 0.29) \times 10^6$ M^{-1} s^{-1}, and that of rKPI and catalytic domain, $(3.25 \pm 0.44) \times 10^6$ M^{-1} s^{-1}, were also essentially identical. The dissociation rate constants of inhibition of both factor XIa and catalytic domain by rKPI were slightly higher than the respective rate constants observed with full-length protease nexin II inhibition. These differences are, most likely, explained by the smaller size (56 amino acids) and greater diffusion coefficient of the KPI domain. It is reasonable to conclude that factor XIa inhibition is a result of residues within the 56 amino acid KPI domain of protease nexin II binding to residues within the catalytic domain of factor XIa.

Several groups of investigators (*29*, *40*−*42*) have studied the inhibitory properties of recombinant forms of the protease nexin II Kunitz domain. The results of these studies, summarized in Table 2, disagree as to the inhibition constants describing the inhibition of different proteases by rKPI and raise questions about whether the KPI domain contains all of the inhibitory activity of protease nexin II. Both Kido et al. (*40*) and Kitaguchi et al. (*41*) expressed 60 amino acid rKPI domains. Sinha et al. (*42*) purified a 194 amino acid KPI fusion protein and compared it to a recombinant full-length protease nexin II purified from 293-human embryonic kidney cells. Wagner et al. (*29*) expressed a 62 amino acid KPI construct in *Pichia pastoris* and compared it to full-length protease nexin II. As seen in Table 2, there is as much as a 57-fold difference in the reported K_i's for trypsin inhibition by rKPI, from 300 pM reported by Sinha et al. (*42*) to 2.7 nM reported by Kido et al. (*40*). There is an even more dramatic difference, greater than 1,000-fold, in the K_i's of plasmin inhibition by rKPI, from 75 pM reported by Kido et al. (*40*) to 81 nM reported by Kitaguchi et al. (*41*). All of these studies determined K_i's by measurement of fractional protease activity, using either methylcoumaryl amide or

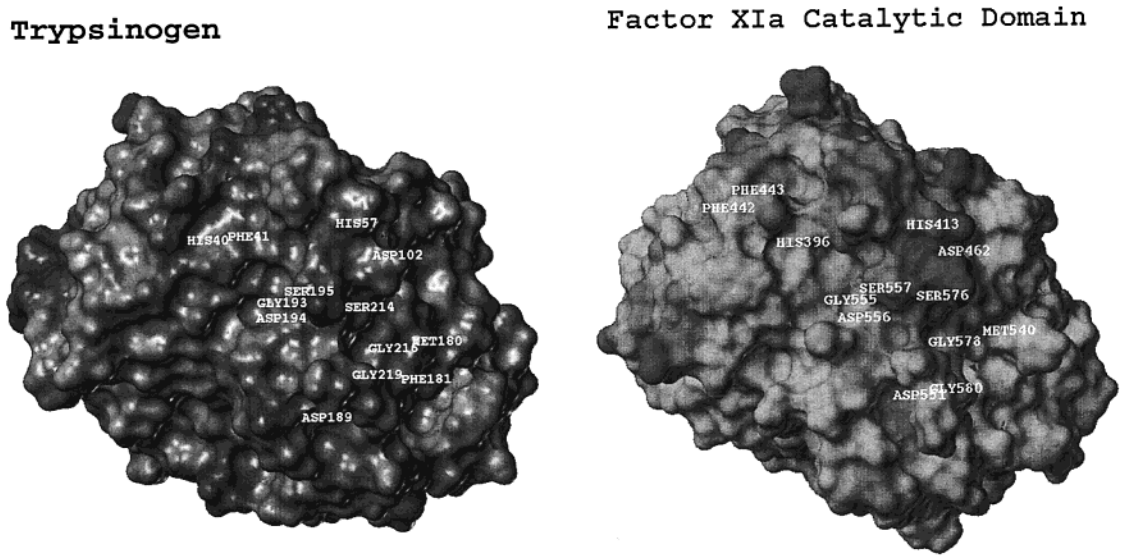

FIGURE 5: Comparison of the molecular model of trypsinogen, based on the crystal structure published by Kossiakoff et al. (*35*), and the molecular model of the catalytic domain of factor XIa, developed using the Sybyl 6.4 program from Tripos, Inc. The majority of the trypsinogen residues involved in interactions with the Kunitz domain of PNII, labeled in the model above, are also identically placed in the catalytic domain structure.

Table 2: Inhibition Studies Comparing Recombinant Kunitz Protease Inhibitor (rKPI) Domain to Protease Nexin II[a]

investigators	inhibitor	enzyme	inhibition constant (nM)
Kido et al. *(40)*	60 amino acid	trypsin	2.7
	KPI domain	plasmin	0.075
Kitaguchi et al. (*41*)	60 amino acid	trypsin	17.0
	KPI domain	plasmin	81.0
Sinha et al. (*42*)	194 amino acid	trypsin	0.3
	fusion KPI domain	plasmin	60.0
Wagner et al. (*29*)	62 amino acid	trypsin	0.31
	KPI domain	factor XIa	0.45
	recombinant protease	trypsin	0.83
	nexin II	factor XIa	0.4

[a] Inhibition studies were previously published as referenced. Using the equilibrium method, as described under Materials and Methods, investigators determined inhibition constants for trypsin, plasmin, and/or factor XIa inhibition by either a recombinant Kunitz protease inhibitor domain or a full-length protease nexin II.

p-nitroaniline substrates, in the presence of increasing concentrations of inhibitor. The incubation times used in these studies, which varied from 5 to 20 min, may have either allowed or disallowed the establishment of equilibrium between the enzyme and the inhibitor. This may, at least partially, explain the as much as 1000-fold discrepancy in the K_i values for plasmin and trypsin found in these reports.

The reports of Sinha et al. (*42*) and Wagner et al. (*29*) do support the conclusion that all of the amino acids in protease nexin II involved in enzyme inhibition are located in the KPI domain. The results reported here in Figures 2 and 4, and summarized in Table 1, more specifically confirm that this conclusion is valid for factor XIa inhibition. They further suggest that the amino acids in factor XIa involved in its inhibition by protease nexin II are all contained in the factor XIa catalytic domain.

The protease nexin II KPI domain has been crystallized with other serine proteases. It is possible, therefore, to examine the residues mediating the interaction of these enzymes with the Kunitz domain of protease nexin II and predict which residues within factor XIa may be involved. Two loop structures in the KPI domain were shown to interact with porcine kallikrein A (*38*) and rat trypsin (*39*). The residues are numbered from 1, beginning with the first residue of the Kunitz domain (residue 290 of the 751 isoform of protease nexin II), to 56. Following this numbering sequence, the loops are ^{11}TGPCRAMIS^{19} and ^{34}FYGGCGGN^{41}. Arginine^{15} within the inhibitor is at the P1 position and forms a number of interactions with the enzyme. A salt bridge is formed by the guanidinium group of R^{15} with an aspartic acid in the enzyme. The same inhibitor arginine interacts with the amine nitrogen of the active-site serine and G^{193}, G^{219}, D^{194}, and S^{214} in trypsin (*39*). There are several other hydrogen bonds formed between inhibitor and amino acids in trypsin: P^{13} in KPI with G^{216}, M^{17} with H^{40}, and I^{18} with H^{57} and D^{102}. Methionine^{17} and phenylalanine^{34} in the inhibitor form a hydrophobic patch on the surface of the KPI domain which interacts with three phenylalanines and a methionine on kallikrein A. The primary determinant of affinity between inhibitor and enzyme is the KPI domain, N^{41}, which determines the backbone conformation of the last three residues in the loop (*38*). This conformation either allows or disallows interactions between R^{39} and a protease residue. The corresponding amino acids in either trypsin or porcine kallikrein A were not identified in the reports (*38, 39*) of the crystal structures with KPI domain.

An examination of the molecular model of both trypsin and the isolated factor XIa catalytic domain (Figure 5) shows that, with the exception of the histidine and phenylalanine residues, the amino acids predicted to bind to the KPI domain form a discreet surface adjacent to the catalytic triad in trypsin. Corresponding residues in the factor XIa catalytic domain, as displayed in the model in Figure 5, also present a surface which may represent the region of interaction between factor XIa and protease nexin II. Although the similarity of structure between the catalytic domains of factor XIa, trypsin, and porcine kallikrein supports the possibility that these residues are important in the interaction between

Table 3: Comparison of Inhibition Half-Times of Factor XIa Inhibitors[a]

protease inhibitor	association rate ($M^{-1}s^{-1}$)	plasma concn	inhibition half-time (s)
α_1-protease inhibitor	1.0×10^2	45.5 μM	150.7
C1 inhibitor	1.8×10^3	2.0 μM	192.5
antithrombin III	3.2×10^2	2.7 μM	770
protease nexin II	3.0×10^6	27.5 nM	8.4

[a] Association rate constants for the serpins were reported by Minnema (*3*). Plasma concentrations were obtained from reports cited in the introduction. Inhibition half-times were calculated according to the method of Bieth (*44*), as described under Discussion.

factor XIa and protease nexin II, structural studies will be necessary to confirm these predictions. Through the use of site-directed mutagenesis, it will be possible to identify which specific residues within factor XIa are important for protease nexin II inhibition. The results of such studies would improve understanding of the differences in affinity of protease nexin II for its three target coagulation factors (IXa, Xa, and XIa) as well as the mechanism of inhibition of other coagulation proteins by Kunitz-type inhibitors. Van Nostrand et al. (*43*) mutated the arginine in the protease nexin II Kunitz domain to a lysine. This mutation resulted in a 10-fold increase in affinity of the KPI domain for plasmin and a 22-fold decrease in the affinity for factor XIa inhibition by the KPI domain. These findings support the significance of the residues surrounding the enzyme catalytic triad in contributing to the binding energy of the interaction between the KPI domain and factor XIa.

The very tight affinity of the KPI domain for the factor XIa catalytic domain is important when considering the relevance of protease nexin II as a factor XIa inhibitor. Several serpins are also known factor XIa inhibitors with much higher plasma concentrations. A comparison of the half-time ($t_{1/2}$) of inhibition can be calculated by the equation:

$$t_{1/2} = 0.693/(k_{\text{association}} \times I)$$

where $k_{\text{association}}$ is the second-order rate constant for the enzyme/inhibitor reaction and I is the plasma concentration of inhibitor (*44*).

Protease nexin II is present in picomolar amounts in plasma (*28*). The concentration of protease nexin II in platelets has been estimated to be 1.1 μg/10^8 platelets (*28*). Assuming a platelet count of 3×10^8 platelets/mL of plasma, the local concentration of protease nexin II could realistically reach 27.5 nM. Using this estimated concentration and the published concentrations of the serpins, estimates of the inhibition half-times can be made. The second-order rate constants for inhibition of factor XIa by the serpins were published by Wuillemin et al. (*13*). As shown in Table 3, the half-time of protease nexin II inhibition of factor XIa is 18 times faster than that of the most abundant inhibitor, α_1-protease inhibitor, 92 times faster than antithrombin III, and 23 times faster than C_1-inhibitor. Protease nexin II, by these calculations, would seem to be the most important factor XIa inhibitor in the local clot environment of activated platelets, which also provide a surface that vigorously promotes factor XI activation by thrombin (*5*).

The importance of protease nexin II as a localized inhibitor of factor XIa was also suggested by Minnema et al. (*3*). This group examined factor XI activation after infusion of endotoxin to a group of healthy human volunteers. The amount of factor XIa generated, using this sepsis model, was measured by ELISA of factor XIa−serpin inhibitor complexes. In fitting their data to a model, they found that the time course and quantity of factor XIa−factor XIa inhibitor complexes fit best to a model in which factor XIa is inhibited by a surface-bound inhibitor and then is gradually released into the plasma compartment. Bornebroek et al. (*45*) detected protease nexin II−factor XIa complexes in plasma samples of normal individuals. This finding supports the view that protease nexin II is an important physiologic inhibitor of factor XIa. Protease nexin II has been shown by a number of investigators to bind to glycosaminoglycans (*46*−*48*). Once released from the activated platelet, it may bind to endothelial and subendothelial glycosaminoglycans. Therefore, it may be the most relevant inhibitor within the proximity of the endothelial wall-bound platelet thrombus. It has also been shown that binding of factor XIa to the platelet surface protects it from inactivation by protease nexin II (*17*). It can be concluded that the major function of platelet-bound factor XIa is procoagulant. Factor XIa that is not bound to the platelet surface is vigorously inhibited by protease nexin II released from platelets.

ACKNOWLEDGMENT

We are grateful to Dr. Yan Stephanie Zhang (University of Cincinnati, Cincinnati, OH) for her helpful guidance and to Patricia Pileggi for her expertise in manuscript preparation.

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BI9925468