Role of Zymogen and Activated Factor X as Scaffolds for the Inhibition of the Blood Coagulation Factor VIIa-Tissue Factor Complex by Recombinant Nematode Anticoagulant Protein c2*

Recombinant nematode anticoagulant protein c2 (rNAPc2) is a potent, factor Xa (fXa)-dependent small protein inhibitor of factor VIIa-tissue factor (fVIIa-TF), which binds to a site on fXa that is distinct from the catalytic center (exo-site). In the present study, the role of other fX derivatives in presenting rNAPc2 to fVIIa-TF was investigated. Catalytically active and active site blocked fXa, as well as a plasma-derived and an activation-resistant mutant of zymogen fX bound to rNAPc2 with comparable affinities (Kp = 1–10 nM), and similarly supported the inhibition of fVIIa-TF (Kp = –10 µM). The roles of phospholipid membrane composition in the inhibition of fVIIa-TF by rNAPc2 were investigated using TF that was either detergent-solubilized (TF₀), or reconstituted into membranes, containing phosphatidylcholine (TFPC) or a mixture of phosphatidylcholine and phosphatidylserine (TFCPS). In the absence of the fX derivative, inhibition of fVIIa-TF was similar for all three conditions (Kp = 1–10 µM), whereas the addition of the fX derivative increased the respective inhibition by 35-, 150-, or 100,000-fold for TF₀, TFPC, and TFCPS. The removal of the γ-carboxyglutamic acid-containing domain from the fX derivative did not affect the binding to rNAPc2, but abolished the effect of factor Xa as a scaffold for the inhibition of fVIIa-TF by rNAPc2. The overall anticoagulant potency of rNAPc2, therefore, results from a coordinated recognition of an exo-site on fXa and of the active site of fVIIa, both of which are properly positioned in the ternary fVIIa-TF-fXa complex assembled on an appropriate phospholipid surface.

The blood coagulation response to vascular injury or inflammation results from a series of amplified reactions, in which several specific zymogens of serine proteases in plasma are sequentially activated by limited proteolysis (1). The serine protease factor VIIa (fVIIa) present in the blood specifically binds to tissue factor (TF), a transmembrane receptor glycoprotein bound to subendothelial structures or present on the surface of monocyte or other inflammatory cells, which accumulate at the site of injury (2). The exposure of TF to circulating blood is the triggering event that results in the formation of a catalytic complex (fVIIa-TF) that initiates the amplified cascade of proteolytic events leading to the formation of the serine protease thrombin (3). The action of thrombin coupled with the particular rheological environment found in diseased or damaged vascular beds, result in thrombi with compositions that vary from platelet-rich, a characteristic of the arterial vasculature, to fibrin-rich, platelet-poor clots, typical of the venous vasculature (4).

The pathway leading from the formation of the fVIIa-TF complex to thrombin proceeds through the serine protease factor X (fXa). Factor Xa is formed by the proteolytic activation of the zymogen factor X (fX) either by the fVIIa-TF complex or by the catalytic complex composed of the serine protease factor IXa and its nonenzymatic cofactor factor VIIa assembled on an appropriate phospholipid surface (5). Factor Xa catalyzes the formation of thrombin following assembly into a macromolecular catalytic complex (prothrombinase) with the nonenzymatic cofactor factor Va (fVa) that binds to a procoagulant phospholipid surface, such as activated platelets or inflammatory cells adhered to the site of vascular damage (6).

The regulation of the blood coagulation involves a variety of components, most of which act to down-regulate the proteolytic response initiated following vascular injury. The primary physiological inhibitor of the fVIIa-TF complex, tissue factor pathway inhibitor (TFPI), mediates one of these crucial pathways (7). The efficient inhibition of fVIIa-TF by TFPI requires binding of the inhibitor to the active site of fXa via the second of its three Kunitz-like inhibitory domains followed by the formation of the final quaternary inhibitory complex with fVIIa-TF,

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The abbreviations used are: fVIIa, factor VIIa; EGR-fXa, L-glu-tamyl-l-glycyrl-l-arginyrl chomethyl ketone-modified factor Xa; Gladomain, γ-carboxyglutamic acid-containing domain; des-Gla-Glu-fXa, L-glutamyl-l-glycyl-l-arginyl chomethyl ketone-modified factor Xa lacking the γ-carboxyglutamic acid-containing domain; PC, l-a-palmito-lyloleoyl phosphatidylcholine; PS, l-a-stearoyleoyl phosphatidyl- serine; PLV, phospholipid vesicle; TFCPS, 75% (w/w) phosphatidylcholine vesicles; TF₀, tissue factor reconstituted into 100% (w/w) phosphatidylcholine vesicles; TFCPS, tissue factor reconstituted into 75% (w/w) phosphatidylcholine, 25% (w/w) phosphatidylserine vesicles; TFPI, human tissue factor pathway inhibitor; HPLC, high performance liquid chromatography; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; nAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
which the active site of fVIIa is occupied by the first Kunitz domain of the inhibitor (8). A recent study suggested that the rate-limiting step governing the inhibition of fVIIa by TFPI is the binding to fXa, which occurs while fXa is either bound to or remains in the near vicinity of the fVIIa-TF complex following zymogen cleavage of fX (9). Therefore, it appears that the role of fXa in the inhibition of fVIIa by TFPI is that of an “inhibitory scaffold,” upon which is built the final inhibitory complex between the Kunitz-1 domain of TFPI and the active site of fVIIa. This proposed mechanism requires the ternary fVIIa-TF-fXa complex to display a limited half-life or stability. This originates, in part, from specific protein-protein interactions outside the catalytic center of fVIIa at exo-sites on fVIIa-TF, to which the substrate fX or product fXa binds (10, 11).

Previously, we described a potent 84-amino acid, non-Kunitz-like inhibitor of the fVIIa-TF complex called nematode anticoagulant protein c2 (NAPc2) that was originally isolated from the hemaphagous nematode hookworm Ancylostoma caninum (12). An 85-amino acid recombinant form of NAPc2 (rNAPc2) was shown to significantly inhibit fVIIa-TF-mediated factor IX activation, but only in the presence of fXa, or fXa that had been irreversibly inhibited with the active-site inhibitor Glu-Gly-Arg-chloromethylketone (EGR-fXa). The effectiveness of EGR-fXa as an inhibitory scaffold suggested that rNAPc2 bound to a region of fXa outside of the catalytic center. The utilization of such an exo-site by rNAPc2 distinguishes it from TFPI, which has been shown to require an unoccupied active site in fXa to allow the binding of the Kunitz 2 domain of the inhibitor (7). Therefore, we propose that, although rNAPc2 is functionally similar to TFPI with respect to the requirement of an inhibitory scaffold to mediate its inhibition of fVIIa-TF, it is mechanistically distinct based on the specific binding interaction with fXa.

In this report, we characterize the interaction of rNAPc2 with fX derivatives that support inhibition of fVIIa-TF. We demonstrate that rNAPc2 can bind with high affinity to zymogen fX, indicating that the activation status of the inhibitory scaffold is not crucial for the inhibition of fVIIa-TF by rNAPc2. The phospholipid membrane composition and the Gla-domain of the fX/fXa play critical roles in the presentation of rNAPc2 to TF by fVIIa.


tf Reconstitution

The synthetic phospholipids 1-α-palmitolyleoyl phosphatidylcholine (PC) and 1-α-stearoylreoyl phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Full-length TF apoprotein was reconstituted into phospholipid vesicles (PLV), consisting of 75% PC (w/w) and 25% PS (w/w) (TFPCPS), or 100% PC (TFPC) in the presence of detergent, as previously described (19), followed by dialysis to 50 mM NaCl, pH 6.5. TF was dialyzed immediately following dialysis. The diameter of the resulting vesicles was measured by light scattering (Fine Particle Technology, Menlo Park, CA), yielding a volume-weighted Gaussian distribution centered at a mean diameter of 83 ± 20 nm (PCPS PLV) and 112 ± 20 nm (PC PLV). The concentration of phospholipids in each preparation was determined by a colorimetric assay, using fIX deficient plasma (George King, Overland Park, KS).

Measurement of the Overall Apparent Dissociation Constant (K_i*) for the Inhibition of fVIIa-TF-mediated (3)HfIX activation by rNAPc2

The kinetic measurement of fVIIa-TF-mediated release of tritiated activation peptide from radialeolated fIX ((3)HfIX) was performed as described. (12). Briefly, a complex of fVIIa and TFPCPS was formed in 10 mM Hepes, 150 mM NaCl, 15 μM BSA, 3.0 mM CaCl2, pH 7.4 (designated assay buffer), for 10 min prior to adding increasing amounts of equimolar rNAPc2 and one of the following fX derivatives: fX, recombinant fX (rFfX), and FLAG-rFfX (FLAG-rfXa). The fX derivatives were expressed in the methylotropic yeast, Saccharomyces cerevisiae, and purified with FLAG-agarose chromatography. The molecular mass was confirmed by electrospray mass spectrometry. The C-2081 substrate was reconstituted in deionized water just prior to use.

Proteins

Recombinant human factor VIIa (fVIIa) was obtained from Novo Nordisk A/S (Gentofte, Denmark). Recombinant, human full-length tissue factor (TF) was produced in the methylotropic yeast, Saccharomyces cerevisiae, and purified with FLAG-agarose chromatography. The molecular mass was confirmed by electrospray mass spectrometry. The C-2081 fVIIa was synthesized as a trifluoroacetic acid salt, purified to homogeneity using HPLC, and lyophilized. The resulting molecular mass was confirmed by electrospray mass spectrometry. The C-2081 substrate was reconstituted in deionized water just prior to use.

Materials

Hepes and Tris buffers, bovine serum albumin (BSA), CHAPS, Tween 20 and all other reagents, not indicated otherwise, were from Sigma.
The Inhibitory Effects of rNAPc2 on the Kinetics of Peptidyl Amidoxylic Substrate Hydrolysis by fVIIa

Base-line Kinetic Constants for Substrate Hydrolysis in the Assay—All studies were performed in assay buffer at ambient temperature (23.5 ± 0.7 °C). The kinetics of hydrolysis of the chromogenic substrate C-2081 by fVIIa were measured under a number of experimental conditions, prior to examining the inhibitory effects of rNAPc2 under these conditions.

Reactions were initiated by the addition of uncomplexed fVIIa (10 nM) or fVIIa (2 nM) in complex with 5 nM TF (TF<sub>5</sub>, TF<sub>PC</sub>, TF<sub>PSC</sub>) to the individual wells of a 96-well plate (Corning), containing C-2081 (0.05–3.0 nM) in a final volume of 125 μl of assay buffer. Where indicated other reagents were added to the following final concentrations: PCPS or PC PLV (6.8 μM), EGR-fXa (20 nM with free fVIIa, or 5 nM with fVIIa-TF). Factor VIIa and TF were incubated for 10 min prior to the addition to the reaction mixture. Initial reaction velocities were measured as a linear increase in the absorbance at 405 nm (A<sub>405 nm</sub>) over 10 min at 9-s intervals, using a Thermomax kinetic microplate reader. Measurements were made under steady-state conditions, where less than 5% of the substrate was consumed. The K<sub>r</sub> was derived from the nonlinear regression fit of the averaged velocities of triplicate reactions versus the respective concentration of C-2081, using Enzfitter software (Biosoft, Cambridge, United Kingdom). Kinetic values were averaged from three independent kinetic determinations, generating the following K<sub>r</sub> values for each experimental condition: uncomplexed fVIIa (2.6 μM), uncomplexed fVIIa + EGR-fXa (1.8 μM), fVIIa-TF (all TF preparations) (range: 82–356 μM), fVIIa-TF (all TF preparations) + EGR-fXa (range: 272–345 μM).

Assessment of the Time Dependence of the Inhibition of fVIIa and fVIIa-TF by rNAPc2—The effect of incubation time on the inhibition of fVIIa and fVIIa-TF amidolytic activity by rNAPc2 varied depending on the particular reaction condition used. The extent to which the inhibition for the eight tested reaction conditions was either kinetically "fast," or time-independent, or kinetically "slow" or time-dependent determined how the corresponding dissociation constant (K<sub>d</sub>) was determined (23). The relative inhibitory potency of rNAPc2 was measured over a range of concentrations for each reaction condition by two kinetic procedures, using identical concentrations of reagents: 1) fVIIa was added to a mixture of rNAPc2 and substrate to initiate reactions (no pre-incubation between enzyme and rNAPc2), and 2) rNAPc2 was first pre-incubated with fVIIa for 30 min (inhibition in the absence of substrate), followed by addition of substrate to initiate reactions. If the apparent potency of rNAPc2, measured by each of these two procedures was equivalent, then the interaction of rNAPc2 was judged time-independent or fast. The K<sub>d</sub> was measured in subsequent experiments, as detailed below for time-independent inhibition. In contrast, if the apparent potency of rNAPc2 measured by procedure 2 was significantly greater than that measured for procedure 1, the dissociation rate constant (k<sub>d</sub>) was subsequently measured as detailed below for time-dependent inhibition.

Measurement of the Overall Equilibrium Dissociation Constant (K<sub>d</sub>) for the Time-independent Inhibition of fVIIa and fVIIa-TF by rNAPc2—The potency of rNAPc2 was measured over a range of substrate concentrations in the presence of increasing concentrations of rNAPc2, and when included, of equimolar EGR-fXa or des-Gla EGR-fXa. Reactions were initiated by the addition of either free fVIIa (10 nM), or preformed fVIIa-TF complex (2 nM fVIIa with 5 nM TF<sub>5</sub>, TF<sub>PC</sub>, or TF<sub>PSC</sub>) to pre-activated inhibitor and substrate in the wells of microtiter plates. All reactions were performed in triplicate, and contained in 125 μl of assay buffer, containing a range of six to eight inhibitor concentrations ([I]) and six substrate concentrations ([S]). For reactions with free fVIIa, the concentrations were 1–20 μM ([I]) and 0.1–3.0 μM ([S]), and those with fVIIa-TF complexes were 0.5–10 μM ([I]) and 0.1–2.0 μM ([S]). The initial velocities measured over 10 min under steady-state conditions for three separate experiments were fit by reiterative nonlinear regression to Equation 2, describing a time-independent, classical, competitive inhibitor, to derive the K<sub>d</sub> value.

\[ V = V_{max} + (K_r/([S][I])+1) [I]/K_d \]  
(Eq. 2)

Measurement of the Apparent Overall Equilibrium Dissociation Constant (K<sub>d</sub>) for the Time-dependent Inhibition of fVIIa and fVIIa-TF by rNAPc2—Varying concentrations of rNAPc2 and equimolar EGR-fXa (0.00025–1 μM) were pre-incubated with a complex of fVIIa (2 nM) and TF<sub>5</sub>, TF<sub>PC</sub>, or TF<sub>PSC</sub> (5 nM) for 30 min. Initial velocities were measured, following the addition of substrate (650 μM). Preliminary experiments showed that EGR-fXa in the absence of rNAPc2 had no effect on V<sub>o</sub>. Ratios of the inhibited reaction velocity (V<sub>i</sub>) to the uninhibited velocity (V<sub>o</sub>) for each concentration of rNAPc2 were fit to the quadratic Equation 1 for slow, tight-binding inhibitors for three separate experiments to give the apparent dissociation constant (K<sub>d</sub>).

\[ P = V_o + (V_o - V_i) (1 - e^{-kt})/k_{obs} \]  
(Eq. 3)

The derived, apparent first-order rate constants (k<sub>obs</sub>), from three separate experiments derived using Equation 3, were fit along with the corresponding (rNAPc2/EGR-fXa) by re-iterative nonlinear regression to Equation 4, which describes a one-step mechanism for slow-binding inhibitors to give the measured constants of k<sub>j</sub> and k<sub>-1</sub>. The derived dissociation inhibition constant (K<sub>j</sub>) was calculated from the ratio of k<sub>j</sub>/k<sub>-1</sub>.

\[ k_{obs} = k_j + k_1 ([I]/([S])K_j) \]  
(Eq. 4)

Determination of rNAPc2 Binding to Inhibitory Scaffolds

Surface Plasmon Resonance Analysis—Binding kinetics for derivatized of X to immobilocaptured FLAG-rNAPc2 were determined by surface plasmon resonance, using a BIAcore 2000 instrument (Pharmacia Biosensor). The mAb to the FLAG epitope (Anti-FLAG M1, Eastman Kodak Co.) was immobilized on the surface of a CM5 sensor chip by amine coupling, according to manufacturer’s recommendations. Recombinant FLAG-rNAPc2 (0.1 mg/ml) was injected onto the sensor chip to saturate the immobilized antibody in Hepes-buffered saline containing 1 mM CHAPS, 0.005% surfactant P20, 5 mM CaCl<sub>2</sub>, pH 7.4. The kinetics of binding of various derivatives of X was measured, following the injection of each protein at different concentrations (3–300 nM). Between runs, the Ca<sup>2+</sup>-dependent mAb was regenerated by eluting bound rNAPc2 with 1 M EDTA. The dissociation rate constant (k<sub>d</sub>) was determined by nonlinear regression analysis of the data from three separate experiments using software provided by the manufacturer. The association rate constant (k<sub>j</sub>) was calculated from multiple sensorsgrams, representing at least five different concentrations of ligand for each experiment. The dissociation rate constant (k<sub>d</sub>) was calculated from the initial dissociation phase of the binding curves, and the equilibrium dissociation constant (K<sub>d</sub>) equaled the ratio of k<sub>j</sub>/k<sub>d</sub> (27).

Immunocapture of rNAPc2 to Immobilized X Derivatives—The binding of FLAG-rNAPc2 to immobilocaptured X derivatives was measured by modified enzyme-linked immunosassay detection method. A murine monoclonal antibody (mAb 10<sup>7</sup>) produced against human X, which was shown to recognize all X derivatives equally well, was used for solid phase immobilization of the various X-derived inhibitory scaffolds. mAb 10 was bound to microtiter plate wells following an overnight incubation in phosphate-buffered saline (PBS) at 4 °C followed by washing with PBS containing 0.05% Tween 20 (v/v). Blocking with PBS containing 1% BSA (w/v), and 2% mannose (w/v) was followed by washing, and the addition of a saturating amount of one of the X derivatives (final concentration 50 nM) in Hepes-buffered saline containing 15 μM BSA. Following a 1-h incubation and washing, varying concentrations of FLAG-rNAPc2 (0–100 nM) were added and incubated for 1 h followed by washing and addition of the HRP-anti-FLAG mAb M1 conjugate. Following a 1-h incubation and washing, bound peroxidase activity was visualized using 3,3’,5,5-tetramethylbenzidine dihydrochloride hydrochloride and 1 M H<sub>2</sub>SO<sub>4</sub>. The end point absorbance (A<sub>405 nm</sub>) was read, and the data were analyzed by fitting A<sub>405</sub>, A<sub>500</sub> versus [X derivative] using reiterative nonlinear regression to the binding equation Y = B<sub>max</sub> + [I]/(K<sub>j</sub> + [L]), where [L] represents the concentration of X derivative, B<sub>max</sub> the maximal binding, and K<sub>j</sub> the measured dissociation constant.

<sup>27</sup> T. S. Edgington, unpublished data.
Factor X as a Scaffold for Inhibition of fVIIa-TF by rNAPc2

RESULTS

rNAPc2-mediated Inhibition of fVIIa-TF by Derivatives of Zymogen and Activated Factor X—Previously, the inhibition of fVIIa-TF by rNAPc2 was shown to be dependent on fXa that was either catalytically active or irreversibly inactivated with the peptidyl chloromethylketone EGR-cK (EGR-fXa) (12). To further define the repertoire of inhibitory scaffolds used by rNAPc2, we investigated zymogen fX, and a pre-formed complex with fVIIa that could have been formed during the course of the incubation with fVIIa-TFPCPS. To directly address this issue, a recombinant form of wild-type fX (fXa) was compared with the following recombinant mutant fX derivatives, all of which contained the S195A mutation in the catalytic triad, rendering them incapable of forming a catalytically active fXα: rFX S195A, rFX S195 (activated rFX S195A, prepared using Russel’s viper venom), and rFX R15Q/S195A (resistant to proteolytic activation by fVIIa-TFPCPS due to the mutation of the scissile bond Arg15 to Gln (Ref. 10)). As shown in Table II, recombinant fX and the active-site mutant rFX S195A and rFX S195A were indistinguishable from each other, and were only about 3-fold less potent than the corresponding plasma-derived zymogen and active enzyme counterparts. This slightly increased \( K_i \) for rNAPc2 inhibition obtained with the recombinant forms of fX may be a reflection of subtle differences in either their post-translational modifications or purification. Despite this, the proteolytically resistant form of fX (rFX R15Q/S195A) was equipotent to the other recombinant derivatives, demonstrating that proteolytic activation of zymogen fX was not required for the formation of a high affinity inhibitory complex with fVIIa-TF.

High Affinity Binding of rNAPc2 to Zymogen and Activated Forms of fX—The data in Table I and II suggest a possible direct interaction of rNAPc2 with the various fX-derived scaffolds, each of which resulted in a similarly effective bimolecular complex that then inhibited fVIIa-TF. Therefore, to quantify the energetic contribution of the protein-protein interaction between rNAPc2 and the fX derivatives to inhibitory complex formation, direct binding measurements were performed with these fX derivatives and rNAPc2 that contained the aminoterminal FLAG sequence (rFLAG-NAPc2) to facilitate its immobilization and detection. The addition of the FLAG sequence did not affect the inhibitory potency of rNAPc2 in the presence of EGR-fXa in the [\(^{3}H\)]fIX activation assay as described above (data not shown). For surface plasmon resonance (BLAcore) measurements, immobilized anti-FLAG antibody was used to immunocapture rFLAG-NAPc2 for determination of the binding parameters for the various fX derivatives (Fig. 2). Human

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**Table I**

| fX derivative | \( K_i \) pM |
|---------------|---------------|
| fX            | 11 ± 1        |
| fXa           | 13 ± 5        |
| EGR-fXa       | 4 ± 1         |
| TAP-fXa       | 6 ± 3         |

Inhibition of [\(^{3}H\)]fIX activation by rNAPc2 in the presence of various fX derivatives

The data shown in Fig. 1 were fit to Equation 1 to yield the apparent overall equilibrium dissociation constant (\( K_i \); the reported \( K_i \) value is the mean and standard deviation of the individually resolved \( K_i \) values from three to six experiments.

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FIG. 1 Inhibition of [\(^{3}H\)]fIX activation by rNAPc2: dependence on fX and its derivatives. A preformed complex between fVIIa and TFPCPS was incubated with increasing concentrations of equimolar rNAPc2 and one of the fX derivatives (0–1.0 nm) for 30 min, followed by the addition of [\(^{3}H\)]fIX to initiate the reaction. Complexes between fXa (0–1.0 nm) and rTAP (10 nm) were preformed for 30 min prior to its incubation with rNAPc2. Dependence of the ratio of the inhibited velocity (\( V_i \)) to the respective uninhibited velocity (\( V_U \)) (without rNAPc2) on the concentration of rNAPc2 was shown as the mean and S.D. of three to six separate experiments. The forms of fX included: fX (○), fXa (●), EGR-fXa (△), and TAP-fXa (□). The fX derivatives (1 nm) without rNAPc2 had negligible effects on \( V_U \). In the absence of cofactor, rNAPc2 inhibited [\(^{3}H\)]fIX activation by fVIIa-TFPCPS by \( \sim 17\% \) at \( \sim 2 \mu \text{M} \). Inset, an expanded representation of the data up to 300 pm rNAPc2.

Three separate binding experiments were performed for each fX derivative, and the reported \( K_i \) value represented the mean of the three resolved \( K_i \) values from each of those experiments.

Identification of the Cleavage Site in the Reactive Loop of rNAPc2

rNAPc2 was added to both a preformed complex of fVIIa-TFPCPS or TFPCPS alone (control), and incubated for 0–5 h at 37 °C. The final concentration of reactants in 300 μl of 25 mM Heps, 150 mM NaCl, 5 mM CaCl₂, pH 7.4, was 90 μM rNAPc2, 1.9 μM TFPCPS, 966 μM PCPS PLV, and 0.9 μM rVIIa. At various time intervals (0–5 h), aliquots were quenched with EDTA and submitted to SDS-PAGE followed by Coomassie Blue staining, which demonstrated that rNAPc2 was completely cleaved by the fVIIa-TF into two distinct bands by 5 h. The control sample ran as a single band, comparable to the starting material.

The remaining sample was reduced and carboxymethylated by adding dithiothreitol (10 mM) and heating at 100 °C for 2 min in denaturing buffer (final concentration 0.1 M Tris, 6 M guanidine HCl, pH 8.0) followed by the addition of sodium iodoacetamide (NaIOAc) in denaturing buffer (1 mM) and an additional 30-min incubation in the dark at 23 °C. Additional NaIOAc (40 mM) and dithiothreitol (43 mM) were added and incubated sequentially, followed by separation using reverse phase HPLC C18 column chromatography (Vydac). Fractions containing two distinct absorbance peaks were collected, pooled, and lyophilized. SDS-PAGE analysis confirmed that each of the two bands from the original digestion aliquot comigrated with a corresponding band from one of the eluted pools. The two individual purified pools were subjected to NH₂-terminal sequence analysis (Protein and Nucleic Acid Facility, Stanford University Medical Center, Stanford, CA).
plasma-derived IXa (Fig. 2A), EGR-IXa, and the recombinant mutant rIXS195A all bound with high affinity to rFLAG-NAPc2, with $K_D$ values ranging from 0.6 to 1.0 nM (Table III). Plasma-derived zymogen IX (Fig. 2B) and the activation-resistant recombinant mutant rIXR15Q/S195A bound to rFLAG-NAPc2 with association rates that were ~50-fold lower than those measured for the various IXa species, while the dissociation rates of IX versus IXa were <2-fold different. A possible explanation for the slower association kinetics of the zymogen forms with rNAPc2 could stem from the known partial disorder of the zymogen serine protease domain prior to its activation, and formation of the stabilizing Ile16-Asp194 salt bridge, (28, 29) requiring an induced fit during the association phase of the immunocaptured FLAG-rNAPc2 with the zymogen IX.

As also shown in Table III, these results are from an alternative ELISA-based assay that measured the interaction between rFLAG-NAPc2 and several derivatives of IXa, which were immobilized using a IX monoclonal antibody as opposed to the surface plasmon resonance measurements which were made with immobilized FLAG-rNAPc2. In this assay, rFLAG-NAPc2 bound to IXa species, including IXa that lacks the Gla-domain, with $K_D$ values of 1.5-2.8 nM, which are comparable to those measured using the BIAcore method (Table III). However, it appeared that rFLAG-NAPc2 bound to immunocaptured, plasma-derived IX with a higher affinity, as reflected in the roughly 4-fold reduction in the $K_D$ compared with the BIAcore measurement. The time scale of the individual incubation steps in the ELISA format minimize effects of different association rates and often confer sensitivity to dissociation differences. Thus, the similarity in affinity of rNAPc2 for zymogen IX compared with those for the IXa species, measured by ELISA can be considered a reflection of the similar dissociation rates, also evident from the BIACore measurements.

Nevertheless, both assays demonstrate highly stable complexes between of rNAPc2 and either the zymogen or the activated forms of IX. This finding provides a rational for the similar potency of all IX species in stabilizing the quaternary, inhibited complex, as demonstrated in Tables I and II.

### Table II

| Recombinant IX derivative | $K_a$ ($\pm$ SEM) | $K_D$ ($\pm$ SEM) |
|---------------------------|-------------------|-------------------|
| rIX                       | 30.4 ± 10.3       | 1.0 ± 0.5         |
| rIXR15Q/S195A             | 33.0 ± 0.8        | 0.6 ± 0.2         |
| rIXS195A                  | 22.4 ± 4.0        | 1.5 ± 0.8         |
| rIXR15Q/S195A             | 23.7 ± 9.7        | 0.8 ± 0.2         |

Factor X as a Scaffold for Inhibition of fVIIa-TF by rNAPc2

Inhibition of $[^3H]$fIX activation: effect of site-directed mutation of IX and IXa on the potency of rNAPc2

Site-directed mutations are shown; R15Q is the P1 Arg of the scissile bond in the activation peptide, and S195A is the active site serine. Data are an average of two to three experiments, performed and analyzed as described in Table I.

The findings provided a rationale for the similar potency of all IX species in stabilizing the quaternary, inhibited complex, as demonstrated in Tables I and II.

In the presence of EGR-IXa, the phospholipid binding of IX and specific protein-protein interaction with the fVIIa-TF complex (30, 31) were examined. The influence of the supporting phospholipid surface on the ability of "Experimental Procedures". These data demonstrate that the phospholipid surface did not directly affect the inhibition of fVIIa-TF by rNAPc2 in the absence of an appropriate inhibitory scaffold.

The addition of EGR-IXa had a profound effect on the inhibition of fVIIa-TF amidolytic activity, depending on the preparation of TF used. In the absence of a phospholipid membrane (fVIIaTFS), EGR-IXa increased inhibitory potency, as reflected in ~30-fold decrease in $K_a$ to 28.5 nM (Table IV). The inhibition of fVIIaTFS by rNAPc2 was improved by ~5-fold ($K_a$ of 6.5 nM), compared with that with TF$_{PS}$, suggesting that a neutral membrane surface could facilitate quaternary complex formation. The effect of EGR-IXa was greatest, when fVII-TF$_{PCPS}$ was used, resulting in an ~100,000-fold decrease in $K_a$ ($9.6$ pm) compared with fVII-TF$_{PCPS}$ without EGR-IXa ($K_a$ = 0.96 μM), clearly implicating the procoagulant, anionic PS component as responsible for optimizing quaternary complex formation.

The anionic Gla-domain of IX is known to play a crucial role in the interaction of this coagulation protease with a pro-coagulant membrane surface (32), and directly with the fVIIa-TF complex (15). To further demonstrate the importance of this domain for the activity of these inhibitory scaffolds, we used IX derivatives lacking the Gla-domain. The removal of the first 45 amino acids of the Gla-domain from IXa by chymotrypsin digestion resulted in a single NH$_2$ terminus and chromogenic substrate hydrolysis that was comparable to full-length IXa (data not shown). In addition, the binding of des-Gla-IXa to rFLAG-NAPc2 was indistinguishable from full-length IXa, demonstrating that this domain was not crucial for rNAPc2 binding (Table III). Active site-blocked des-Gla-EGR-IXa was a completely ineffective inhibitory scaffold for the inhibition of fVIIa-TF amidolytic activity by rNAPc2 (Table IV). Inhibition of fVIIa-TF in the presence of des-Gla-EGR-IXa was indistinguishable from the inhibition of fVIIa-TF without an added inhibitory scaffold under any of the TF reconstitution conditions. This indicates that the primary role of the Gla-domain of the inhibitory scaffold is to mediate a productive interaction with fVIIa-TF on the membrane surface.

Certain assumptions were made regarding the kinetic mechanism governing the inhibition of fVIIa amidolytic activity by rNAPc2 under the various conditions described in Table IV. These assumptions were based on the experimental observations that the inhibition by rNAPc2 of fVIIa was either kinetically fast (time-independent) or slow (time-dependent) relative to the rate of substrate hydrolysis. The experimental approach took these differences into account, and the measured $K_a$ or $K_s$ values are listed accordingly in Table IV for the respective fast or slow kinetic behavior. For most conditions, the inhibition of fVIIa-TF by rNAPc2 could be best described as fast (time-independent), reversible, competitive inhibition as shown in Fig. 3A for the inhibition of fVIIa-TF$_{PCPS}$ by rNAPc2. Only upon the addition of EGR-IXa could the kinetics of fVIIa-TF inhibition be best described as slow (time-dependent) and competitive, as shown in Fig. 3B for the inhibition of fVIIa-TF$_{PCPS}$ by rNAPc2 in the presence of EGR-IXa.

Analysis of representative progress curves for inhibition of
amidolytic substrate hydrolysis by fVIIa-TPS, fVIIa-TPPC, and fVIIa-TPPCPS using Equation 3 yielded values for $k_{obs}$ as described under “Experimental Procedures.” The linear relationship between the measured $k_{obs}$ and the concentration of rNAPc2-EGR-IXa complex shown in Fig. 4 was consistent with a slow, competitive interaction between rNAPc2-EGR-IXa and fVIIa-TP for all three conditions. The relative rates of association ($k_+$) and dissociation ($k_-$) derived from the data shown in Fig. 4, were used to calculate the respective $K_s$ values of 9.2 pm, 2.0 nm, and 8.4 nm for fVIIa-TPPC, fVIIa-TPPC, and fVIIa-TPS, which were in agreement with the $K_s$ values in Table IV, determined using a mechanism-independent approach (Table I). The calculated $k_+$ and $k_-$ values gave further insight into differences between the membrane surfaces in IXa-dependent inhibition of fVIIa-TP by rNAPc2. By assuming a first order dissociation, the half-life ($t_{1/2}$) of the inhibited complex was found to be dramatically stabilized by PS (fVIIa-TPPCPS) ($t_{1/2}$ = 4.3 ± 1 h), as compared with fVIIa-TP (t1/2 = 15 ± 4 min) or fVIIa-TPPC (t1/2 = 13 ± 4 min). Although the negatively charged PS headgroups facilitated optimal IXa-dependent association (−10-fold more rapidly than on the PC membrane), the most striking effect of PS appeared to be the stabilization of the inhibited complex.

**Confirmation of the Putative Reactive Loop of rNAPc2**—The inhibition data described above suggest that rNAPc2 either interacts directly with the active site of fVIIa, or sterically prevents access of the substrate to the active site. Previously, we proposed the putative reactive loop region of rNAPc2 (42LVR45V), based on the homology to the Ascaris family of serine protease inhibitors and to another member of the NAP family (NAP-5, formerly AcAP5) (12). To confirm a direct interaction between rNAPc2 and fVIIa, fVIIa-TPPCPS was incubated with excess rNAPc2 for 5 h at 37 °C under reaction conditions that allowed for the slow cleavage of the inhibitor. Amino-terminal sequence analysis of two resulting peptides prevented access of the substrate to the active site. Previously, evidence that rNAPc2 is proteolytically cleaved under the conditions used for determining the inhibition constants, which is described under “Experimental Procedures.”

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**Table III**

| Ligand          | $k_a$ | $k_d$ | $K_D$ | Binding ELISA$^b$ |
|-----------------|-------|-------|-------|-------------------|
|                 | $x^{-1} s^{-1} (\times 10^6)$ | $s^{-1} (\times 10^{-6})$ | $\text{nm}$ | $\text{nm}$ |
| IXa             | 0.59 ± 0.08 | 6.1 ± 0.8 | 1.0 ± 0.1 | 1.5 ± 0.8 |
| EGR-IXa         | 1.12 ± 0.18 | 6.4 ± 1.0 | 0.6 ± 0.2 | 2.1 ± 0.8 |
| IXa             | 0.11 ± 0.04 | 9.2 ± 1.0 | 8.9 ± 2.5 | 2.0 ± 0.7 |
| rIXa$^{\text{R109A}}$ | 0.82 ± 0.14 | 5.0 ± 0.2 | 0.6 ± 0.1 | 7.0 ± 1.7 |
| rIXa$^{\text{R109A,D110A}}$ | 0.06 ± 0.02 | 9.5 ± 0.4 | 16.2 ± 4.5 | 30.0 ± 6.0 |
| Des-Gla-EGR-IXa |       |       |       | 2.8 ± 0.6 |

$^a$ Binding of IX derivatives to immunocaptured FLAG-rNAPc2, as measured by surface plasmon resonance (BLAcore).

$^b$ Binding of FLAG-rNAPc2 to IX derivatives immunocaptured by a mAb to hufX(a). Experimental details are described under “Experimental Procedures.” All values represent an average of three separate experiments.

**Table IV**

| Reaction conditions | TF$_S$ | TF$_PC$ | TFPCPS |
|---------------------|--------|---------|--------|
| vIIa                | $K_s = 6.8 ± 0.7 \times 10^{-6}$ | $K_s = 8.8 ± 0.7 \times 10^{-6}$ | $K_s = 2.8 ± 0.1 \times 10^{-6}$ |
| vIIa + EGR-IXa      | $K_s = 1.0 ± 10^{-6}$ | $K_s = 1.0 ± 10^{-6}$ | $K_s = 0.90 ± 0.1 \times 10^{-6}$ |
| vIIa + TF           | $K_s = 2.8 ± 0.1 \times 10^{-6}$ | $K_s = 6.5 ± 1.4 \times 10^{-9}$ | $K_s = 9.6 ± 6.7 \times 10^{-12}$ |
| vIIa-TP + EGR-IXa   | $K_s = 0.90 ± 0.1 \times 10^{-6}$ | $K_s = 0.70 ± 0.03 \times 10^{-6}$ | $K_s = 0.50 ± 0.07 \times 10^{-6}$ |

$^a K_s = k_+$ derived from $V = V_{\text{max}} K_s/(S + 1/K_s)$ + (1 + 1/K+$^*$) describing a mixed, noncompetitive interaction; $K_s = 0.5 ± 0.1 \mu M, K_s^* = 2.3 ± 0.5 \mu M, K_m = 452 ± 42 \mu M.$

FIG. 2. Kinetics of plasma-derived IXa and zymogen FX binding to immobilized FLAG-rNAPc2 measured by surface plasmon resonance. Representative sensograms obtained from the injection of IXa (A) or FX (B) at concentrations from 6–500 nM (IXa) and 12–1000 nM (FX) over a surface containing immobilized FLAG-rNAPc2 are shown. The rates of association ($k_+$) and dissociation ($k_-$) of IXa or FX to immobilized FLAG-rNAPc2 were determined following data reduction as described under “Experimental Procedures.”
**FIG. 3.** Progress curves illustrating the inhibition of fVIIa-TF by rNAPc2 in the absence and presence of the inhibitory scaffold EGR-fXa. Panel A, the chromogenic substrate CVS-2081 (800 μM) was added to the wells of a microtiter plate containing rNAPc2 at concentrations corresponding to 0, 1, 2, 4, 6, and 10 μM (A–F, respectively). Panel B, the chromogenic substrate CVS-2081 (800 μM) was added to the wells of a microtiter plate, containing rNAPc2 at concentrations corresponding to 0, 0.4, 0.6, 1.0, 1.2, 1.6, 1.8, and 2.0 nM (A–H, respectively) and equimolar EGR-fXa in assay buffer. The reactions were initiated by the addition of a preformed complex of fVIIa (2 nM) and TF (5 nM) plus PCPS (5.0 μM) and measured as ΔA405 nm over 60 min, where less than 5% of the substrate was consumed. The solid line represents the fit of the observed data using linear regression (for panel A) or nonlinear regression to Equation 3 as described under “Experimental Procedures.”

**FIG. 4.** Kinetics of association of rNAPc2-EGR-fXa and fVIIa-TF: effect of the TF reconstitution conditions on the assembly of the quaternary complex. The chromogenic substrate CVS-2081 (800 μM) was added to the wells of a microtiter plate, containing a range of concentrations of rNAPc2 and equimolar EGR-fXa (0–100 μM) in assay buffer. The reactions were initiated by the addition of a preformed complex of fVIIa (1.5 nM) and TF (5 nM), containing, when indicated, PCPS or PC PLV (5.0 μM). Progress curves similar to those shown in Fig. 3, were analyzed using Equation 3 developed by Cha (25) and Williams and Morrison (26) and are detailed under “Experimental Procedures.” The dependence of the derived, apparent first-order rate constant (kobs) (mean and S.D. of three separate experiments) on the corresponding concentration of rNAPc2-EGR-fXa is shown for: soluble fVIIa-TF (○), fVIIa-TFPC (●), and fVIIa-TFPCPS (▲). Inset, an expansion of fVIIa-TFPCPS (●). The data from three separate experiments, represented by these plots were fit by nonlinear regression to Equation 4 as described by Morrison (20). The following kinetic parameters were derived for the association of rNAPc2-EGR-fXa and fVIIa-TF: on PCPS, k1 = 4.86 ± 0.27 × 10^5 M^-1 s^-1, k-1 = 4.46 ± 1.69 × 10^-8 s^-1, Kt = 9.2 ± 2.6 ps; on PC, k1 = 4.63 ± 0.21 × 10^5 M^-1 s^-1, k-1 = 9.2 ± 2.4 × 10^-8 s^-1, Kt = 2.0 ± 0.5 nm; detergent-solubilized, k1 = 0.93 ± 0.05 × 10^5 M^-1 s^-1, k-1 = 7.89 ± 2.11 × 10^-4 s^-1, Kt = 8.4 ± 2.2 nm.

**DISCUSSION**

This study characterizes key interactions that allow for tight (Kt = 10 ps) complex formation between the small protein inhibitor rNAPc2 and fVIIa-TF in the presence of an inhibitory scaffold derived from FX. Active site occupancy of FXa with small peptidyl (EGR-ck) or larger small protein (rTAP) inhibitors did not appreciably influence the affinity of rNAPc2 for FXa, strongly suggesting that this inhibitor is binding to a high affinity accessory binding site or exo-site. Since binding of rTAP to FXa does not influence the function of FXa as an inhibitory scaffold, it is clear that the binding site for rNAPc2 is distinct from the proposed accessory site used by rTAP (36). The high affinity binding of rNAPc2 to FX suggests that a binding site for rNAPc2 is also exposed in the zymogen, although ongoing biochemical and x-ray crystallographic structural studies are necessary to conclusively demonstrate whether the binding site on FXa and FXa are identical. However, the high affinity binding of rNAPc2 to zymogen FX is a unique characteristic of this inhibitor that should influence its biological properties. Most notably, the biochemical data predict that rNAPc2 should form a stable complex with physiologic plasma concentrations of FX zymogen. This was recently confirmed by the finding that rNAPc2 quantitatively binds to circulating FX, when administered to humans and other species such as the rat, dog, and cynomolgus monkey (37).

The formation of this stable complex of rNAPc2 with physiologic concentrations of FX strongly support a sequential inhibitory mechanism for rNAPc2 in vivo, as schematically depicted in Fig. 5. In step 1 of this mechanism, rNAPc2 binds to zymogen FX or activated FXa, either in solution or bound to an appropriate anionic phospholipid surface. Although contributions of a membrane surface to this initial interaction cannot be entirely ruled out, rNAPc2 can form a sufficiently stable and physiologically relevant complex with FX in the absence phospholipid. In step 2, the rNAPc2-FX inhibitory scaffold docks to fVIIa-TF. This docking is highly dependent on the phospholipid composition of the membrane, in which TF resides. There is a strong requirement for a pro-coagulant surface containing the anionic phospholipid PS for maximal inhibition, as evidenced by the differential rates of association of the rNAPc2-EGR-FXa complex with the fVIIa-TF between TFPCPS and TFPC.

The interaction of the rNAPc2-FX(a) complex with membrane-associated fVIIa-TF appears to be predominately mediated by the Gla-domain of the inhibitory scaffold. However, even in the absence of a phospholipid membrane, deletion of the Gla-domain in FXa resulted in an inactive scaffold, indicating that the Gla-domain is also playing a role in docking to a
binding interface formed by fVIIa and TF. A similar role of the Gla-domain in binding to the fVIIa-z TF complex has been previously suggested for the substrate fX, and the fXa-z TFPI complex (9, 15). Thus, the inhibitor appears to utilize a docking mode similar to that of the substrate (fX) for delivery to the active site of fVIIa, which is the last step of the reaction mechanism proposed in Fig. 5. Solid evidence of a fVIIa-z TF cleavage site in the reactive loop of rNAPc2 confirms docking of the inhibitor to the active site of fVIIa. Even though the reactive loop region of rNAPc2 was shown to be cleaved by fVIIa-TF under certain reaction conditions, it is likely that there is an equilibrium reached between the cleaved and un-cleaved inhibitor (rNAPc2-z fX(a)) and the enzyme (fVIIa-TF), resulting in a stable inhibitory complex, which similarly occurs with other small protein inhibitors of serine proteases (33, 34, 38).

The requirement for an inhibitory scaffold composed of a fX derivative is similar to TFPI, which, like rNAPc2, forms a stable complex with fVIIa-TF assembled on a phospholipid surface. Even though the level of potency of fVIIa-TF inhibition is quite similar between TFPI (K* = 8 pM (Ref. 9)) and rNAPc2 (K* = 10 pM), there are several significant differences between the two inhibitors that distinguish their respective mechanisms for achieving inhibition of fVIIa-TF. First and foremost is the difference between the two inhibitors with respect to the inhibitory scaffold requirement. Catalytically active fXa is exclusively required by TFPI for the inhibition of fVIIa-TF due to the requirement for an accessible catalytic center by which the second Kunitz domain of TFPI can bind to fXa (8). As described above, there is no such requirement for catalytically active fXa for rNAPc2-mediated inhibition of fVIIa-TF, as evidenced by the roughly equivalent extent of inhibition using inhibited forms of fXa (EGR-fXa and rTAP-z fXa) or the zymogen fX. Both rNAPc2 and TFPI share the same requirements of an anionic phospholipid surface as well as an intact Gla-domain on the fX(a) inhibitory scaffold for maximal inhibition of fVIIa-TF. It is likely that both the protein-lipid and to some extent the protein-protein interactions of the Gla-domain with the membrane-associated fVIIa-TF complex are the principal factors responsible for the observed specificity and potency of fVIIa-TF inhibition by rNAPc2. This is also true for the TFPI-fXa complex, based on the similar degree of inhibition observed for native TFPI and a hybrid protein composed of Kunitz domain I and the light chain of fXa (39). The function of the fX(a) derivative for both rNAPc2 and TFPI appears to be the same: to properly present a canonical loop region of each inhibitor to the active site of fVIIa.

Overall, it is remarkable that such structurally unique proteins like TFPI and rNAPc2 derived from such disparate species (vertebrates and nematode parasites, respectively) have evolved such a similar strategy of utilizing an inhibitory scaf-
fold derived from fX to block blood coagulation by inhibiting the fVIIa-TF complex. A possible explanation for this evolutionary convergence may lie in the fact that the ternary fVIIa-TF-fX(a) complex has a sufficient biological half-life on cells or procangulant surfaces to serve as a relevant target for an inhibitory mechanism that simultaneously exploits recognition determinants in both the docked fX(a) and the active site of fVIIa. To our knowledge, the binding of rNAPc2 to fX provides the first description of a substrate-mediated delivery of an inhibitor to an enzyme complex. The binding of rNAPc2 to zymogen fX may offer a uniquely effective strategy for inhibiting the initiation of blood coagulation mediated by fVIIa-TF in vivo. This is based on the expectation that circulating rNAPc2-fX complexes would rapidly inhibit fVIIa-TF prior to the initiation of coagulation, obviating the need for any fXa generation as would be required by TFPI. The relative difference in affinity of the rNAPc2-fX complex (K_a = ~10 μM) versus fX (K_m = ~100–200 nM (Refs. 9 and 11)) for fVIIa-TF would favor the formation of the quaternary complex (rNAPc2-fX-fVIIa-TF) even under conditions where fX is not saturated with rNAPc2. Therefore, in most cases of clinical thrombosis where the amount of fVIIa-TF generated at the site of vascular damage would be limited by the exposure of tissue factor, antithrombotic efficacy would be predicted at concentrations of rNAPc2 far below the plasma concentration of fX. Indeed, clinical efficacy of rNAPc2 has been demonstrated at a dose that yielded a plasma concentration of rNAPc2 far below the plasma concentration of fX. (10 pM) versus fX (5 μM) (10). Therefore, the unique properties of rNAPc2 not only offer future opportunities in gaining a better understanding of the mechanistic details of fVIIa-TF-mediated inhibition of blood coagulation, but also a possible new approach to clinical anti-coagulant therapy.

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Role of Zymogen and Activated Factor X as Scaffolds for the Inhibition of the Blood Coagulation Factor VIIa-Tissue Factor Complex by Recombinant Nematode Anticoagulant Protein c2

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