Inhibition of Intrinsic Xase by Protein S
A Novel Regulatory Role of Protein S Independent of Activated Protein C

Rima Chattopadhyay,* Tanusree Sengupta,* Rinku Majumder

Objective—Protein S is a vitamin K–dependent plasma protein that functions in the feedback regulation of thrombin generation. Our goal was to determine how protein S regulates the intrinsic pathway of blood coagulation.

Methods and Results—We used plasma, including platelet-rich plasma, and in vitro methods to determine how the intrinsic pathway of blood coagulation is regulated by protein S. We obtained the following results: (1) activated partial thromboplastin time assays with protein S–supplemented plasma confirmed that protein S prolongs clotting time; (2) a modified activated partial thromboplastin time assay with factor IX (fIX)–deficient plasma confirmed that protein S affects fIX-initiated clotting; (3) a fIXa/factor VIIIa (fVIIIa)–mediated thrombin generation assay with either platelet-rich plasma or factor-deficient plasma, initiated with a limiting amount of tissue factor, was regulated by protein S; (4) in the presence of phosphatidylserine vesicles, protein S inhibited fIXa in the absence and presence of fVIIIa; and (5) protein S altered only the $K_m$ for factor X activation by fIXa in the absence of fVIIIa and both $k_{cat}$ and $K_m$ in the presence of fVIIIa.

Conclusion—From our findings, it can be concluded that protein S inhibits fIXa in the presence or absence of fVIIIa in an activated protein C–independent way. (Arterioscler Thromb Vasc Biol. 2012;32:2387-2393.)

Key Words: anticoagulants ■ blood coagulation ■ thrombosis ■ factor IXa ■ protein S

Blood coagulation occurs through a cascade of enzymatic reactions, resulting in fibrin formation.1,2 Central to this process are complexes of a vitamin K–dependent protease, factor IXa (fIXa), and an activated protein cofactor, factor VIIIa (fVIIIa), assembled on a phospholipid-containing membrane; the fIXa/fVIIIa complex (intrinsic Xase complex) is the kinetically significant activator of factor X (fX).3,4

Coagulation is a finely tuned process. During thrombin formation by fX, several anticoagulant reactions prevent systemic activation of coagulation, and impairment of anticoagulant activity increases the risk of venous thrombosis.5 A common cause of high-risk venous thrombosis is deficiency of the anticoagulant protein S; arterial thrombosis (stroke, heart attack) may also be linked to protein S deficiency.6

Protein S was initially identified as a cofactor for activated protein C (APC)6,9 but was later found to stimulate APC activity by only a factor of 3 to 10.10,11 Plasma coagulation assays in the absence of APC suggest that protein S has other anticoagulant role(s). Indeed, protein S acts as an APC-independent anticoagulant factor by directly inhibiting prothrombin activation via interactions with fXa, fVa, and phospholipids12–14; however, the APC-independent function of protein S is highly dependent on its ability to bind to phospholipids.14,15 For example, at constant fXa and fVa concentrations, preincubation of the prothrombinase components with protein S at 100 µmol/L phosphatidylcholine-phosphatidylserine vesicles failed to inhibit the initial rate of thrombin generation.16 However, 50% inhibition of thrombin formation by protein S was observed at 1 µmol/L phosphatidylcholine-phosphatidylserine vesicles, and nearly 100% inhibition was observed at 0.1 µmol/L phosphatidylcholine-phosphatidylserine.15 The profound effect of protein S at low phospholipid concentration was explained by the fact that protein S multimers form under such a condition, and because protein S multimers have the highest prothrombinase inhibitory activity, the multimers interfere with activity measurements.17,18 Therefore, in binding and kinetic studies of purified coagulation proteins, high phospholipid concentrations are required to avoid artifacts caused by protein S multimers.17–19

Koppelman et al20 demonstrated another APC-independent protein S anticoagulant function, inhibiting activation of FX possibly by direct interaction between protein S and factor VIII.20 However, their observations were similarly skewed by the presence of protein S multimers at low phospholipid concentrations. Takeyama et al21 reported that protein S binds to fVIIIa and directly modulates Xase activity by competing with fIXa in the formation of enzyme complex,21 but their study was also performed in the presence of low membrane concentration (10 µmol/L). Protein S has also been suggested to function in an APC-independent manner as a stimulator of fXa–tissue factor pathway inhibitor (TFPI) complex formation.22,23

In view of the shortcomings of previous studies, here we determine the anticoagulant mechanism of protein S on intrinsic Xase in the presence of sufficient phospholipids to prevent artifacts from protein S multimerization. Formation of the intrinsic Xase complex by the 2 crucial clotting proteins factors IXa and VIIIa is the rate-limiting step in thrombin
generation, and shutting down thrombin generation by the Xase complex prevents thrombosis. Therefore, determining the mechanism of protein S function on intrinsic Xase will guide strategies to prevent thrombosis. Using both plasma and purified proteins, we show that the inhibitory effect of protein S is mediated through its interaction with fIXa (with and without fVIIIa), observations that are both novel and significant. By acting as a cofactor for TFPI, protein S controls the initiation of coagulation. However, our findings establish a role for protein S in regulating the propagation step, thus establishing the importance of protein S in the entire coagulation process.

Materials and Methods

Materials
Human protein S, fIX, fIXa, α-thrombin, and active site of factor IXa blocked with Dansyl-Glu-Gly-Arg chloromethyl ketone (DEGR-IXa) were purchased from Hematologic Technologies Inc (Essex Junction, VT). Human protein S was also purchased from Enzyme Research Laboratories (South Bend, IN). Human factor VIII was a gift from Dr Rodney Camire (CHOP, Philadelphia, PA). The fXa-specific substrate N-2-benzoxycarbonyl-α-arginyl-l-arginine p-nitroanilide dihydrochloride (S-2765) was purchased from DiaPharma (West Chester, OH). 1,2-dioleoyl-3-sn-phosphatidylcholine and porcine brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Concentrations of the phospholipids were established by inorganic phosphate determination. Hirudin was purchased from Calbiochem (San Diego, CA). Normal pooled plasma (NPP) was from George King Bio-Laboratories (South Bend, IN). Human factor VIII was a gift from Dr George King. Bio-medicals Inc (Overland Park, KA), and protein S– and protein C–deficient plasmas were obtained from DiaPharma. Platelet-rich plasma (PRP), isolated as described, was provided by Dr Leslie Parise (University of North Carolina). The platelet count in PRP was 2×10⁸/mL. Anti–human protein S antibody (sheep polyclonal, pure IgG fraction) was obtained from Hematologic Technologies Inc (Essex Junction, VT). Human anti-TFPI monoclonal antibody (2B12, raised in mouse) was a gift from Dr George Broze (Washington University School of Medicine). Kontact reagent for the activated partial thromboplastin time (aPTT) assay was from Pacific Hemostasis (Middletown, VT), and thromboplastin C plus for partial thromboplastin time (PT) assays was purchased from Dade International Inc (Miami, FL). Technothrombin thrombin generation assay calibrator and thrombin generation assay reagent technothrombin TGA reagent B. (containing low amounts of tissue factor [TF] and phospholipid) and thrombin generation assay reagent technothrombin TGA reagent C (containing high TF and phospholipid) were purchased from DiaPharma. The fluorogenic substrate Z-Gly-Gly-Arg-AMC, for thrombin generation assays, was from Bachem Americas Inc (Torrance, CA). All other chemicals were ACS reagent grade or the best available; all solvents were high-performance liquid chromatography grade.

Methods

Additional Methods can be found in the online-only Data Supplement.

Preparation of Phospholipid Vesicles
Small unilamellar vesicles composed of 1,2-dioleoyl-3-sn-phosphatidylcholine and bovine brain PS in the molar ratio 75:25 were prepared at 37°C using a microtiter plate reader as described. Briefly, 20 mmol/L fIXa was incubated with 200 mmol/L vesicle ±150 mmol/L protein S in 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca²⁺, 175 mmol/L NaCl, and 0.6% polyethylene glycol. After a 10-minute incubation in a 96-well assay plate, the conversion of fIX to fIXa was initiated by addition of varying concentrations of fIX (50 mmol/L to 200 mmol/L) and 1 mmol/L of chromogenic substrate S-2765. From the absorbance change at 405 nm, the amount of fIXa was calculated from a calibration curve determined under the assay conditions and made with known amounts of fIXa. To determine the effect of protein S on the activity of fIXa in the presence of its cofactor fVIIIa, the same assay with some modifications was performed in the presence of 0.5 mmol/L fIXa and 0.4 mmol/L fVIIa. After an initial incubation of 10 minutes, freshly prepared fVIIIa (fVIIIa is labile) was added, and after 1 minute, generation of fIXa from fIX was initiated by addition of varying concentrations of fVIII (50 mmol/L to 2 μmol/L).

Results

Effect of Protein S on the Clotting Activity of Intrinsic Pathway
To investigate the anticoagulant effect of protein S on the intrinsic Xase complex, an aPTT assay was performed with NPP in the presence of anti–protein S antibody. In the aPTT assay, clotting was initiated by activation of contact factors following the traditional intrinsic pathway of blood coagulation. When NPP was incubated with increasing concentrations of anti–protein S antibody, a gradual shortening of clotting time was observed (Figure 1A). Clotting time was reduced by 50% with 300 mmol/L anti–protein S antibody, and the effect was almost saturated at that concentration. These data suggested that endogenous protein S acted as an anticoagulant of the intrinsic blood coagulation pathway. Furthermore, addition of protein S to protein S–deficient plasma prolonged clotting time, and normal clotting time was

Modified aPTT Assay
To determine the effect of protein S on the intrinsic pathway of coagulation, a modified aPTT assay (reaction initiated with 40 mmol/L CaCl₂ and different concentrations of fIXa or fVIIa instead of Kontakt reagent) was performed with fIX-deficient plasma and increasing concentrations of fIXa or fVIIa, ±anti–protein S antibody and 50 μmol/L phospholipid.

Thrombin Generation Assay
The amount of thrombin generated in protein S–deficient plasma at different protein S concentrations was measured as described. To monitor the effect of protein S exclusively on the fVIIa/TF-dependent pathway, protein S–deficient plasma (40 μL) was incubated with Technothrombin RC reagent (10 μL), having a high TF concentration (5 pmol/L TF/80 μmol/L phospholipid) and different concentrations of protein S. To determine the effect of protein S on the fIXa/fVIIa pathway, a thrombin generation assay was performed using Technothrombin RB reagent with a low TF concentration (0.4 pmol/L TF and 8 μmol/L phospholipid) instead of RC reagent, in the absence and presence of anti-TFPI inhibitor, keeping all other conditions unchanged. Thrombin generation curves were analyzed to determine peak thrombin generated and total thrombin generated, as described. Thrombin generation was also measured with 0.4 pmol/L TF and 30 μmol/L phospholipid to eliminate effects of protein S multimers. Results obtained in both cases (8 and 30 μmol/L phospholipids) were the same, thus ruling out artifacts because of protein S multimers. Finally, a thrombin generation assay was performed in PRP using RB reagent (0.4 pmol/L TF and 8 μmol/L phospholipid) in the presence of increasing amounts of anti–protein S antibody. All plasma assays (aPTT, modified aPTT, PT, and thrombin generation assays) used 40 μL of plasma in a total reaction volume of 120 μL.

Inhibition of fIXa-Mediated Activation of fX by Protein S, ±fVIIIa
The initial rate of fX activation by fIXa was measured on PS/PC vesicles (bovine brain PS: 1.2-dioleoyl-3-sn-phosphatidylcholine =25:75) at 37°C using a microtiter plate reader as described. Briefly, 20 mmol/L fIXa was incubated with 200 mmol/L vesicle ±150 mmol/L protein S in 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca²⁺, 175 mmol/L NaCl, and 0.6% polyethylene glycol. After a 10-minute incubation in a 96-well assay plate, the conversion of fIX to fIXa was initiated by addition of varying concentrations of fIX (50 mmol/L to 200 mmol/L) and 1 mmol/L of chromogenic substrate S-2765. From the absorbance change at 405 nm, the amount of fIXa was calculated from a calibration curve determined under the assay conditions and made with known amounts of fIXa. To determine the effect of protein S on the activity of fIXa in the presence of its cofactor fVIIIa, the same assay with some modifications was performed in the presence of 0.5 mmol/L fIXa and 0.4 mmol/L fVIIa. After an initial incubation of 10 minutes, freshly prepared fVIIIa (fVIIIa is labile) was added, and after 1 minute, generation of fIXa from fIX was initiated by addition of varying concentrations of fIX (50 mmol/L to 2 μmol/L).
restored with anti–protein S antibody (Figure 1B); addition of anti–protein S antibody alone to protein S–deficient plasma did not affect clotting time. These latter results confirmed that protein S acted as an anticoagulant of the intrinsic coagulation pathway under our experimental conditions.

To further support our findings, we also performed a PT assay (clotting initiated by FVIIa-TF) with protein S–deficient plasma and increasing concentrations of protein S. No significant changes in clotting times were observed compared with control (data not shown), indicating that protein S could not affect clotting initiated by FVIIa.

Effect of Protein S on fIXa Assayed by a Modified aPTT Assay

To identify the specific target of protein S in the intrinsic coagulation pathway, we measured clotting time in fIX-depleted plasma–anti–protein S antibody. Clotting was initiated by adding different concentrations of either fIXa (Figure 2A) or FVIIa (Figure 2B). When clotting was initiated by fIXa, there was a significant difference in clotting time in the presence of anti–protein S antibody compared with no antibody. In summary, our results indicate that, in fIXa-deficient plasma, clotting initiated with fIXa is greatly affected by protein S, and blocking protein S with its antibody prevents inhibition. However, clotting initiated with FVIIa was unaltered in the presence of protein S–protein S antibody.

Effect of Protein S on Thrombin Generation Initiated by Limited (Intrinsic Pathway) or Excess (Extrinsic Pathway) TF

To assess the efficacy of protein S as an inhibitor of intrinsic coagulation, plasma thrombin generation assays were performed under various conditions. To determine whether the intrinsic or extrinsic coagulation pathway was affected, thrombin generation was performed with TF at low (0.4 pmol/L) and high (5 pmol/L) concentrations. The magnitude of plasma thrombin generation is highly dependent on the factor IX pathway, is practically independent of TFPI (only 10% increase in thrombin generation), which is highly dependent on the contact pathway. No difference in thrombin generation in the presence of anti-TFPI antibody compared with no antibody. In summary, our results indicate that, in fIXa-deficient plasma, clotting initiated with fIXa is greatly affected by protein S, and blocking protein S with its antibody prevents inhibition. However, clotting initiated with FVIIa was unaltered in the presence of protein S–protein S antibody.

in the presence of TFPI antibody (which inhibits TFPI), thrombin generation decreased by 80% with increasing concentrations of protein S. The curve in the inset (solid circles) shows that thrombin generation in protein S–deficient plasma, in the absence of anti-TFPI antibody, decreased by 90% with addition of protein S. A marginal decrease in thrombin generation in the presence of anti-TFPI antibody indicated that TFPI had only a slight effect on fXa generation in the initial phase of thrombin generation (lags shown in Figure 3A). Under these conditions, the rate of fXa generation was slow, and TFPI, at least in part (≈10%) more thrombin generation compared with that in the absence of anti-TFPI antibody, regulates thrombin generation. This experiment demonstrated that thrombin generation initiated with low TF, which is highly dependent on the factor IX pathway, is practically independent of TFPI (only 10% increase in thrombin generation in the presence of anti-TFPI antibody).

To determine whether protein S has any inhibitory effect on the contact pathway, thrombin generation was measured in a control experiment, with protein S–depleted plasma having limiting TF concentrations and in the presence of corn trypsin inhibitor, a potent inhibitor of the contact pathway. No difference in thrombin generation was observed (data not shown). As protein S concentration was increased, gradual prolongations of the lag time and the time to peak thrombin generation were observed (Figure 3A). However, these inhibitory effects were completely eliminated when protein S was incubated with human anti–protein S polyclonal (pure IgG fraction) antibody (Figure 3B). Controls were performed measuring thrombin generation with anti–protein S antibody but without addition of protein S (the antibody was preheated at 56°C for 1 hour to avoid antibody-induced contact activation). The initial lag period of thrombin generation was also diminished in the presence of the antibody. However, when thrombin generation was initiated by excess TF (5 pmol/L) in
the addition of increasing amounts of anti–protein S antibody deficient plasma, and thrombin generation was initiated with different concentrations of factor IXa (fIXa) (frame A) or different concentrations of fVIIa (frame B) in the absence (black bar) and presence (checked bar) of anti–protein S antibody. fIXa in the presence of anti–protein S antibody significantly shortened the clotting time (frame A), whereas clotting was unaffected in the presence of anti–protein S antibody when the clotting was initiated with fVIIa (frame B).

However, to ensure accuracy and precision, increasing concentrations of anti–protein S antibody were added to protein C–depleted plasma supplemented with varying amounts of protein S, thrombin generation was unaffected by protein S (Figure 3C; inset shows similar amount of thrombin generation at different protein S concentrations). To assess the physiological relevance of our hypothesis that protein S inhibits fIXa/fVIIa, we also measured thrombin generation in PRP in the presence of a low amount of TF (0.4 pmol/L). With increasing amount of anti–protein S antibody, the amount of thrombin generation increased from 135 nmol/L (no antibody) to 227 nmol/L (200 nmol/L anti–protein S antibody; Figure 4).

Protein S Inhibits Intrinsic Pathway Independent of Protein C

Plasma thrombin generation was initiated either with low (0.4 pmol/L) or high (5 pmol/L) TF concentrations in fIX-deficient plasma supplemented with varying amounts of protein S. The rotational mobility of DEGR-IXa is restricted; this restricted rotational mobility is reflected in the polarization of the DEGR signal. As a result, an increase in anisotropy was observed with a gradual increase in protein S concentration and protein S was blocked with anti–protein S antibody (gray solid bar). In contrast, thrombin generation remained unchanged without (checked bar) or with anti–protein S antibody (open bar) in the presence of high TF. As discussed before, thrombin generation is largely independent of fIX in the presence of excess TF (4–5 pmol/L) but highly dependent on fIX in the presence of limiting TF. Our studies further confirmed that protein S inhibits thrombin generation by inhibiting the fIX pathway of fX generation. Thrombin generation that does not involve the fIXa–fVIIIa pathway is unaffected by protein S. Inset I shows a plot of peak thrombin generated as a function of increasing protein S concentration in the presence of a low TF concentration (0.4 pmol/L) as described in the Methods section. Strong inhibition by protein S was observed with increasing protein S concentrations: no protein S, open circles; protein S at 25 nmol/L, hexagon dotted; 50 nmol/L, open square; 100 nmol/L, triangle down; 150 nmol/L, hexagon; 150 nmol/L, triangle up; 175 nmol/L, diamond; and 200 nmol/L, star. Inset I shows a plot of peak thrombin generated as a function of increasing protein S concentration in the presence of high TF concentration. Thrombin formation was measured in the presence of increasing concentrations of protein S in the presence of high TF concentrations. Thrombin formation was measured in the presence of high TF concentrations (5× the concentration of protein S used) and 0.4 pmol/L TF. When thrombin generation was measured in the presence of high TF concentration and protein S was blocked with anti–protein S antibody, no inhibition of thrombin formation was observed. Symbols for different protein S concentrations are same as frame A. C, Effect of protein S on plasma thrombin generation in protein S–deficient plasma in the presence of a high TF concentration. Thrombin formation was measured in the presence of high TF concentrations (5× the concentration of protein S used) and 0.4 pmol/L TF. When thrombin generation was measured in the presence of high TF concentration and protein S was blocked with anti–protein S antibody, no inhibition of thrombin formation was observed. Symbols for different protein S concentrations are same as frame A.

To determine whether protein S inhibition of the fIXa–fVIIIa pathway is influenced by protein C, thrombin formation was measured in protein C–depleted plasma. In the absence of thrombomodulin, APC generation in plasma is negligible. However, to ensure accuracy and precision, increasing concentrations of anti–protein S antibody were added to protein C–deficient plasma, and thrombin generation was initiated with limiting TF (0.4 pmol/L). Thrombin generation increased with the addition of increasing amounts of anti–protein S antibody (Figure 5B, filled circles). Addition of anti–protein C antibody (without addition of fIXa) to fIX-deficient plasma produced a low level of thrombin generation (Figure 5B, open circles, inset) that was affected by anti–protein S antibody (0.7 nmol/L thrombin without anti–protein S antibody versus 12 nmol/L thrombin at 400 nmol/L anti–protein S antibody). In fIX-deficient plasma with limiting TF, a significantly lower amount of thrombin was generated in the absence of fIX, indicating that protein S has a strong inhibitory effect on fIX independent of protein C.

Binding of Protein S to fIXa

Binding of protein S to fIXa was measured by the steady-state fluorescence anisotropy of DEGR-fIXa in the presence of 50 pmol/L PS/PC vesicles. Because protein S binds to DEGR-fIXa, the rotational mobility of DEGR-fIXa is restricted; this restricted rotational mobility is reflected in the polarization of the DEGR signal. As a result, an increase in anisotropy was observed with a gradual increase in protein S concentration.
of 200 nmol/L protein S. The $K_i$ was 50±8 nmol/L, close to the $K_d$ (40 nmol/L) obtained from anisotropy measurements.

**Mechanism of Protein S Regulation of fIXa- and fIXa/fVIIIa-Catalyzed fX Activation**

The effect of protein S on catalytic activation of fX by fIXa in the intrinsic complex was evaluated by a proteolytic activity assay. fIXa-mediated conversion of fX to fXa was inhibited by protein S (Figure 7). The activity assay was performed in the presence of a high concentration (200 pmol/L) of membrane, which binds protein S multimers that otherwise interfere in the assay.4,15,17,18,22 Maximum inhibition ($≈$40%) occurred at 150 nmol/L fX. Different kinetic parameters of fIXa activation by protein S are presented in the Table. Protein S did not alter the $k_{cat}$ of fIXa-activated generation of fXa from fX. However, the $K_m$ was ≈3-fold larger in the presence of protein S. The inhibitory role of protein S, therefore, may be attributed to the decrease in affinity of fIXa toward the substrate, fX. The $k_{cat}/K_m$ of the reaction decreased slightly in the presence of protein S. We also followed the generation of fXa from fX activated by fIXa in the presence of its cofactor fVIIIa and determined the effect of protein S on Xase complex formation by fIXa-fVIIIa (Figure 7B). As expected, there was a 66-fold enhancement of the substrate affinity of fIXa in the presence of fVIIIa (Table) without protein S; however, protein S reduced substrate affinity by ≈3-fold. Protein S also lowered the $k_{cat}$ of fIXa/fVIIIa-mediated formation of fXa by ≈5-fold. The $k_{cat}/K_m$ values were 43±26 (nmol/L)$^{-1}$ min$^{-1}$ and 2.3±0.9 (nmol/L)$^{-1}$ min$^{-1}$ without and with protein S. Overall, protein S reduced the $k_{cat}/K_m$ by nearly 20-fold. Assays of fIXa with fVIIIa performed in the presence of varying protein S concentrations showed a gradual decrease in fIXa activity, becoming saturated at ≈200 to 250 nmol/L protein S (data not shown). The corresponding inhibition was ≈65%. The data, fitted to a single site–binding model, gave a $K_i$ of 38±6 nmol/L.

**Discussion**

Mis-regulation of coagulation is an important factor in thrombotic disease. Thrombosis—localized intravascular blood clotting—can occur in the arterial or venous circulation and has a major medical impact.5,6,23 The importance of protein S as a natural anticoagulant is validated by the occurrence of severe thrombotic complications in infants with a homozygous hereditary protein S deficiency and by the mild thrombotic tendency in heterozygous subjects.5,6 To understand how thrombosis occurs due to protein S deficiency, its physiological function and regulatory mechanism as an anticoagulant must be known. However, the function of protein S has been the least understood among the coagulation proteins; hence, diagnosis of protein S deficiency and assessment of the associated thrombotic risk have not been straightforward.

Recent data suggest that protein S plays an important role in the initiation of coagulation by stimulating TFPI activity.22 These data (collected in the presence of high concentration of membranes to prevent interference by protein S multimers) suggest that protein S deficiency increases the risk of thrombosis by impairing the protein C system and by reducing the ability of TFPI to downregulate the extrinsic coagulation pathway.22 On the basis of these observations, it was speculated that increased risk of venous thrombosis associated with
protein S deficiency may, in part, be explained by an impaired downregulation of the extrinsic coagulation pathway.

However, our data indicate a completely unexpected role of protein S in binding to and directly inhibiting fIXa. The schematic (online-only Data Supplement) describes the 3 reported functions of protein S: (1) cofactor for APC, (2) cofactor for TFPI, and (3) inhibitor of fIXa (this report). Here we show that protein S inhibits fIXa formation by fIXa in the absence and presence of fVIIIa. The use of a high amount of sonicated lipids in our activity assay and binding experiments prevented interference by protein S multimers. Multimeric protein S could not be identified in normal human plasma; therefore, it is unlikely that this form of protein S contributed to its APC-independent anticoagulant activity observed in plasma. Furthermore, to ensure that the protein S was free from proteolytic products, we performed gel electrophoresis on 2 different protein S preparations.

In addition to the aforementioned precautions, our study is unique for the following reasons: (1) To characterize the regulatory role of protein S, independent of APC, we used a multifaceted approach, including detailed plasma studies, isolated factor studies, and biophysical and biochemical assays. (2) We determined the physiological relevance of fIXa regulation by protein S using physiological conditions (clotting and thrombin generation) and performing in vitro studies to determine the amount of fX generation by fIXa/fVIIIa at physiological concentrations in the presence of protein S. (3) Most importantly, we used PRP to measure thrombin generation in the presence of low (0.4 pmol/L) TF and observed that thrombin generation predominately through factors fIXa/fVIIIa was inhibited by protein S. (4) We used increasing concentrations of anti–protein S antibody and found that thrombin generation initiated with limited TF increased proportionally, suggesting that protein S inhibits the fIXa/fVIIIa pathway of thrombin generation in PRP.

It is well established that protein S stimulates TFPI and inhibits its free fIXa, thus accelerating feedback inhibition of the initiation of coagulation by the extrinsic pathway. However, our data show for the first time that protein S is an inhibitor of the intrinsic Xase complex as it inhibits fIXa in the presence or absence of fVIIIa.

Our data clearly indicate that thrombin generation in different plasmas (protein S-deficient, fIXa-deficient, PRP) initiated with a low concentration of TF occurs exclusively via the intrinsic (fIXa/fVIIIa) pathway. We did not observe any inhibitory effect of protein S on the extrinsic pathway because the TF concentration was too high for TFPI to control excess fIXa generation. It has been reported that TFPI can act as a cofactor of protein S to inhibit fIXa generation through the extrinsic coagulation pathway only if the TF concentration does not exceed ≈2 pmol/L. At high TF concentrations, protein S hardly displayed any APC-independent anticoagulant activity in plasma. Thus, thrombin generation assays with protein S–depleted plasma under various conditions confirmed that the inhibitory effect of protein S persists solely to the intrinsic fIXa/fVIIIa pathway. Therefore, all

Table. Kinetics Parameters of Intrinsic Xase Complex in the Presence and Absence of Protein S

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$k_{cat}$, min$^{-1}$</th>
<th>$K_{m}$, nmol/L</th>
<th>$k_{cat}/K_{m}$, min$^{-1}$ (nmol/L)$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>fIXa without S</td>
<td>0.069±0.003</td>
<td>278±25</td>
<td>2.5×10$^{4}$±0.3×10$^{-4}$</td>
</tr>
<tr>
<td>fIXa with S</td>
<td>0.068±0.002</td>
<td>796±75</td>
<td>8.5×10$^{3}$±1.4×10$^{-5}$</td>
</tr>
<tr>
<td>fIXa–fVIIIa without S</td>
<td>518±29</td>
<td>12±4</td>
<td>43±26</td>
</tr>
<tr>
<td>fIXa–fVIIIa with S</td>
<td>159±10</td>
<td>69±12</td>
<td>2.3±0.9</td>
</tr>
</tbody>
</table>

fIXa indicates factor IXa; fVIIIa, factor VIIIa.
of our plasma studies were consistent with one other, and they confirmed that protein S is indeed an anticoagulant in the fiXa-fiVIIa pathway. Our findings identify novel and intriguing facts about the mechanisms of the anticoagulant activity of protein S in the intrinsic fiXa-fiVIIa pathway.

Our studies with isolated factors also verified our observations with plasma. In the presence of its cofactor fiVIIa, fiXa was more effectively inhibited by protein S. Possible reasons for this stronger inhibitory effect of protein S are (1) protein S changes the conformation of fiVIIa, altering binding of fiVIIa to fiXa, an explanation consistent with other reports\(^{20,21}\) showing that protein S binds to fiVIIa, (2) the affinity of fiXa for fiX is reduced but still further in the presence of fiVIIa, thus increasing the \(K_M\) of the intrinsic Xase for fiX, and (3) protein S binding to fiXa in the presence of fiVIIa confers a different conformational change on fiXa favorable for inhibition by protein S.

Our work now explains the anticoagulant mechanism of protein S in inhibiting one of the most important complexes in blood coagulation, intrinsic Xase, which is formed by the 2 crucial proteins of blood clotting, fiIXa and VIIIa. Intrinsic Xase activity is vital in determining the balance between coagulation and anticoagulation. Thus, factors such as protein S that affect Xase activity serve as gatekeepers that modulate this critical balance.

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Disclosures
None.

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Rima Chattopadhyay, Tanusree Sengupta and Rinku Majumder

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Schematic Representation of Various Roles of Protein S
**Activated partial thromboplastin time (aPTT) assay**

To measure the effect of protein S on the intrinsic pathway of blood coagulation, an aPTT assay was performed with normal pooled plasma (NPP) and increasing concentrations of anti-protein S antibody as described \(^\text{29}\). Briefly, NPP was incubated with different concentrations of anti-protein S antibody and Kontact aPTT reagent in a 96 well polypropylene plate at 37 °C for 3 minutes. Clotting was initiated by addition of 40 µL of 25 mM CaCl\(_2\) and monitored at 405 nm in a SoftMax plate reader (Molecular Devices, Sunnyvale, CA). The aPTT assays were also performed with protein S-deficient plasma with increasing concentrations of protein S and protein S + anti-protein S antibody. Protein S was incubated with 50 µM vesicle (PS:PC =25:75) in the absence and presence of anti-protein S antibody in 50 mM Tris-HCl (pH 7.4), 5 mM Ca\(^{2+}\), 175 mM NaCl, and 0.6% PEG at room temperature for 10 min followed by addition of protein S-deficient plasma and Kontact reagent. The mixture was assayed in a 96 well plate as described above.

**Prothrombin time (PT) assay**

To study the effect of protein S on the extrinsic pathway, a PT assay was performed with protein S-deficient plasma supplemented with increasing concentrations of protein S, using thromboplastin C plus as the PT reagent, as described \(^\text{29}\).

**Anisotropy and fluorescence of DEGR-IXa**

To study the interaction of fIXa with protein S, the steady state fluorescence anisotropy of active state-labeled fIXa was measured in the presence of increasing concentrations of
protein S on PS/PC membranes. FIXa was labeled with a dansyl dye via a Glu-Gly-Arg (EGR) to yield DEGR-fIXa. Anisotropy was measured at 23 °C with a Spex® FluoroLog-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ), using an excitation wavelength of 340 nm (band pass 2.5 nm) and emission wavelength of 520 nm (band pass 2.5 nm). Each point in the titration curve was derived from an average of 5 anisotropy measurements. The same experiment was repeated with DEGR-VIIa to confirm its binding with protein S.