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
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 IXa-specific substrate N-2-benzoylcarbonyl-l-arginyl-l-arginine p-nitroanilide dihydrochloride (S-2765) was purchased fromDiaPharma (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 Biologicals (North Carolina). The platelet count in PRP was 2×10^8/mL. Anti–human factor VIIIa), 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.

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 in 20 mmol/L Tris-HCl (pH 7.4) and 150 mmol/L NaCl, as described. 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 fVIIIa/fVIIIa-dependent pathway, protein S–deficient plasma (40 µL) was incubated with Techno thrombin RC reagent (10 µL), having a high TF concentration (5 pmol/L TF/8.0 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 Techno thrombin RB reagent with a low TF concentration (0.4 pmol/L TF and 8.0 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. Hirudin generation was also measured with 0.4 µmol/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 µmol/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 µmol/L vesicle ± 150 mmol/L protein S in 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L Ca^2+, 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 fXa 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 fXa 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 fIX 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 fVIIIa. After an initial incubation of 10 minutes, freshly prepared fVIIIa (fVIIIa is labile) was added, and after 1 minute, generation of fXa from fIX was initiated by addition of varying concentrations of fX (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, a 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...
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 fXa Assayed by a Modified aPTT Assay**

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 independent of TFPI, we measured thrombin generation with anti–protein S antibody but without addition of protein S (the antibody was used at 2× the concentration of protein S). 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 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).

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

To determine whether thrombin generation through the fIX pathway is independent of TFPI, we measured thrombin generation with 0.4 pmol/L TF but with anti-TFPI antibody. We incubated protein S–deficient plasma with an ≈5-fold excess of TFPI antibody (ie, 12 nmol/L anti-human antibody 2B12, as the free TFPI in plasma is 1 to 2.5 nmol/L) and the TFPI concentration in protein S–deficient plasma is 50% of that present in NPP) for 10 minutes and then determined thrombin generation in the presence of 0.4 pmol/L TF. The inset in Figure 3A (open circles) shows that, even
we also measured thrombin generation in PRP in the presence of varying amounts 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 in the presence of fIXa (5 nmol/L) or anti–protein S antibody (Figure 5C; 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/fVIIIa, 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).

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.
The inhibitory effect of protein S on fIXa was assessed by assaying the proteolytic activity of fIXa with increasing protein S concentrations (Figure 6B). Generation of fXa from 150 nmol/L fIX activated by fIXa was reduced by almost 40% in the presence of 200 nmol/L protein S. The $K_d$ was 50±8 nmol/L, close to the $K_i$ (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.24,15,17,18,22 Maximum inhibition (~40%) occurred at 150 nmol/L fX. Different kinetic parameters of fIXa activation±protein S are presented in the Table. Protein S did not alter the $k_{cat}$ of fIXa-activated generation of fXa from fIX. 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 fIX 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 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 studies, and biophysical and biochemical assays).

It is well established that protein S stimulates TFPI and inhibits free fIXa, thus accelerating feedback inhibition of the initiation of coagulation by the extrinsic pathway. Here we 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 fXa 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 pertains 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 protein S</td>
<td>0.069±0.003</td>
<td>278±25</td>
<td>2.5×10$^{-5}$×0.3×10$^{-4}$</td>
</tr>
<tr>
<td>fIXa with protein S</td>
<td>0.068±0.002</td>
<td>796±75</td>
<td>8.5×10$^{-5}$×1.4×10$^{-5}$</td>
</tr>
<tr>
<td>fIXa–fVIIIa without protein S</td>
<td>518±29</td>
<td>12±4</td>
<td>43±26</td>
</tr>
<tr>
<td>fIXa–fVIIIa with protein 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–FVIIa pathway. Our findings identify novel and intriguing facts about the mechanisms of the anticoagulant activity of protein S in the intrinsic FIXa–FVIIa pathway.

Our studies with isolated factors also verified our observations with plasma. In the presence of its cofactor FVIIa, 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 FVIIa, altering binding of FVIIa to FIXa, an explanation consistent with other reports20,21 showing that protein S binds to FVIIa, (2) the affinity of FIXa for FX was reduced still further in the presence of FVIIa, thus increasing the $K_m$ of the intrinsic Xase for FX, and (3) protein S binding to FVIIa in the presence of FVIIIa 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, FIXa 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.

References

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Schematic Representation of Various Roles of Protein S

1. APC (Activated Protein C) as a cofactor for Protein S.
2. TFPI (Tissue Factor Pathway Inhibitor) as a cofactor for Protein S.
3. Inhibitor binding to fXa and fVIIIa.

Thrombin generation is indicated by the downward flow of the diagram.
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 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 ⁰C for 3 minutes. Clotting was initiated by addition of 40 µL of 25 mM CaCl₂ 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 Ca²⁺, 175 mM NaCl, and 0.6% PEG at room temperature for 10 min followed by addition of protein S-deficient plasma and Kontakt 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 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.