THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 6, Issue of February 25, pp. 3993-4000, 1993
Printed in U.S.A.

Protein S Binding to Human Endothelial Cells Is Required for Expression of Cofactor Activity for Activated Protein C*

(Received for publication, July 14, 1992)

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An important feedback mechanism in blood coagulation is supplied by the protein C/protein S anticoagulant pathway. In this study we demonstrate that the binding of human protein S to cultured human umbilical vein endothelial cells (HUVECs) is required for the expression of cofactor activity of protein S toward factor Va inactivation by activated protein C (APC). The initial rate of endothelial cell-mediated factor Va inactivation was 21.7 pM factor Va/50 pM APC min^{-1}, which could be enhanced twice at a protein S concentration of 5 nM. This increase appeared to be specific for protein S because it could be inhibited by C4b-binding protein and polyclonal antibodies against protein S. Furthermore, thrombin-cleaved protein S did not accelerate factor Va inactivation by APC on endothelial cells.

The binding of ^{125}I-protein S to endothelial cells was time-dependent, specific, saturable, and required the presence of calcium ions. Scatchard analysis revealed $(8.0 \pm 0.3) \times 10^5$ binding sites per cell with an apparent K_d of 24.4 ± 2.2 nM. To study the physiological importance of the binding of protein S to human endothelial cells, seven monoclonal antibodies were examined for their ability to influence the protein S cofactor activity and binding capacity. Monoclonal antibodies directed against the γ-carboxyglutamic acid domain and the thrombin-sensitive region of protein S completely inhibited the protein S cofactor function in factor Va inactivation by APC on HUVECs. These monoclonal antibodies also inhibited ^{125}I-protein S binding to HUVECs. Another monoclonal antibody, directed against an epitope on the third and/or fourth epidermal growth factor-like region, did not influence either protein S cofactor activity or binding of protein S to HUVECs. We conclude that binding of protein S to HUVECs is essential for the expression of its cofactor activity for APC. At least two regions in protein S, the γ-carboxyglutamic acid domain and the thrombin-sensitive region, are involved in the expression of cofactor activity.

Protein S is a vitamin K-dependent coagulation factor involved in the regulation of the anticoagulant activity of activated protein C (APC)[1] (reviewed by Dahlbäck, 1991; Hessing, 1991; Esmon, 1992). Protein C is activated by thrombomodulin-bound thrombin on the surface of endothelial cells (Esmon and Owen, 1981; Esmon *et al.*, 1983). APC is able to inactivate two important cofactors of blood coagulation, factors Va and VIIIa. In this reaction protein S serves as a nonenzymatic cofactor to APC (Walker, 1980, 1981a, 1981b; Walker *et al.*, 1979; Gardiner *et al.*, 1984; Fulcher *et al.*, 1984; Koedam *et al.*, 1988). Protein S promotes the binding of APC to membrane surfaces (Walker, 1981b, 1984a, 1988), and it is suggested that APC and protein S form a complex on the surface of endothelial cells and platelets (Suzuki *et al.*, 1984; Harris and Esmon, 1985; Stern *et al.*, 1986a; Brett, 1988). However, no direct interaction between APC and protein S has been demonstrated. Another function of protein S was proposed by Solymoss *et al.* (1988) in which protein S inhibited the protective effect of factor Xa to factor Va in the inactivation of factor Va by APC.

Both human and bovine endothelial cells have been shown to synthesize and release functional protein S (Fair *et al.*, 1986; Stern *et al.*, 1986b). The physiological importance of protein C and S in the regulation of hemostasis is clearly demonstrated by the observation that a deficiency in either protein C or protein S is associated with a high risk of recurrent venous thromboembolism (Griffin *et al.*, 1981; Comp *et al.*, 1984; Comp and Esmon, 1984; Schwarz *et al.*, 1984; Engesser *et al.*, 1987). Complicating the diagnosis of protein S deficiency is the presence of protein S in plasma as both free protein and in a complex with C4b-binding protein (C4BP), an inhibitor of the complement system (Dahlbäck and Stenflo, 1981). Only free protein S functions as cofactor to APC, and the cofactor function is lost when protein S binds to C4BP (Comp and Esmon, 1984; Dahlbäck, 1986).

The amino acid sequence of protein S demonstrates that the protein is not a serine protease (Dahlbäck *et al.*, 1986a, 1986b). Protein S contains 11 Gla residues located in the amino-terminal region. In addition, it contains one β-hydroxyaspartic acid residue in the first epidermal growth factor (EGF)-like region and three β-hydroxyasparagine residues located in the following three EGF-like regions (Dahlbäck *et al.*, 1986a; Stenflo *et al.*, 1987).

Protein S binds to negatively charged phospholipids in the presence of calcium (Nelsestuen *et al.*, 1978; Walker, 1984b). Protein S contains Gla-dependent calcium binding sites but has also Gla-independent calcium binding sites, which seem

* This work was supported in part by Grant 900-512-130 from the Foundation for Medical Research (to T. M. H.) and by a fellowship from the Royal Netherlands Academy of Arts and Sciences (to J. C. M. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: APC, activated protein C; C4BP, C4b-binding protein; EGF, epidermal growth factor; Gla, γ-carboxyglutamic acid; HUVEC, human umbilical vein endothelial cell; PAGE, polyacrylamide gel electrophoresis; TBS Tris-buffered saline.

to be located in the EGF-like regions (Sugo *et al.*, 1986; Stenflo *et al.*, 1987; Dahlbäck *et al.*, 1990a). Between the Gla region and the EGF-like regions, there is a region containing thrombin cleavage sites (Dahlbäck *et al.*, 1986b; Malm *et al.*, 1987). After cleavage of protein S by thrombin, the Gla-dependent calcium binding and the APC cofactor activity is lost, although the Gla region is still connected to the molecule via a disulfide bond (Dahlbäck, 1983; Dahlbäck *et al.*, 1986b; Sugo *et al.*, 1986). We reported recently that a monoclonal antibody directed against the thrombin-sensitive region could inhibit the cofactor activity of protein S for the factor Va inactivation by APC on cultured human endothelial cells (Hackeng *et al.*, 1991). Dahlbäck and co-workers reported (1990a) that the Gla domain, the thrombin-sensitive region and the first EGF-like domain of protein S are important for the expression of protein S cofactor activity. In this report we demonstrated that binding of protein S to cultured human endothelial cells is required for the expression of cofactor activity to APC in inactivating factor Va.

EXPERIMENTAL PROCEDURES

Materials—Human prothrombin complex concentrate and fibronectin were generous gifts of Dr. J. Over from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam). Highly purified human α-thrombin was a generous gift from Dr. J. W. Fenton II (New York State Department of Health, Albany, NY) Recombinant desulfatohirudin (12,000 units/mg) was a generous gift of Dr. R. Wallis, Ciba-Geigy, Horsham, United Kingdom. CNBr-activated Sepharose CL-4B and PD-10 disposable desalting columns were purchased from Pharmacia (Uppsala, Sweden). Chromogenic substrate S2366 was purchased from KabiVitrum (Stockholm, Sweden). Immobilon-P polyvinylidene difluoride membrane was obtained from Millipore Corporation (Bedford, MA). Bovine serum albumin and 3,3-diaminobenzidine tetrahydrochloride were obtained from Sigma. Phospholipid preparations (Cephalin) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Culture plastics were obtained from Nunc (Rashide, Denmark), except 96-well strip plates which were purchased from Costar (Cambridge, MA). The other tissue culture supplies (media, antibiotics, and trypsin) were purchased from Gibco Biocult (Paisley, Scotland). Rabbit antiserum to protein S was prepared as described previously (Huisveld *et al.*, 1987). Polyclonal antibodies against factor V were obtained from DAKO (Denmark). Other chemicals obtained were the best grade available.

Purification of Proteins—Prothrombin, protein C, and protein S were prepared as described previously (Koedam *et al.*, 1988). Human factor X was purified as described by Miletich *et al.* (1978) with some modifications (Meijers *et al.*, 1987). For factor X, protein C, and protein S an additional Q-Sepharose Fast Flow purification step was included as described below. Factor V was purified as described by Kane and Majerus (1981). C4BP was immunopurified using immobilized monoclonal antibody chromatography as described (Hessing *et al.*, 1990a). For protein C and factor X subsequent purifications were performed using monoclonal antibody affinity chromatography. Protein G-purified monoclonal anti-protein C (RU-PC4D1) and anti-factor X (RU-FX10F1) IgG were coupled to CNBr-activated Sepharose CL-4B in a concentration of 4 mg of IgG/ml of Sepharose according to the manufacturer's instructions. Prothrombin complex concentrate was dissolved in water containing 25 mM benzamidine HCl and applied to the columns. After washing with Tris buffer with a high NaCl concentration (50 mM Tris-HCl, 500 mM NaCl, pH 7.4), protein C and factor X were eluted with 3 M potassium thiocyanate (KSCN) in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and the eluted protein fractions were dialyzed against TBS. Protein C and factor X identity was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting analysis under nonreducing and reducing conditions. Minor contaminations in the factor X, protein C, and protein S preparations were removed using Q-Sepharose Fast Flow column chromatography. The proteins were applied to Q-Sepharose Fast Flow columns in TBS, washed extensively with TBS, and were eluted with TBS containing different concentrations of $CaCl_2$. Protein S eluted in TBS containing 10 mM $CaCl_2$, 130 mM NaCl, and protein C and factor X were eluted in TBS containing 20 mM $CaCl_2$ and 110 mM NaCl. Proteins were dialyzed against TBS. With this method, highly purified protein preparations were obtained. The protein S preparation contained 30–40% cleaved protein S as estimated by visual inspection of a silver-stained SDS-PAGE gel under reducing conditions. Protein concentrations were based on $A_{280\ nm}$ readings using the following extinction coefficients ($E^{[1\%]}_{1cm}$): protein S, 9.5 (DiScipio and Davie, 1979); protein C, 14.5 (Kisiel, 1979); factor X, 11.6 (DiScipio *et al.*, 1977); prothrombin, 14.2 (Bajaj *et al.*, 1981); factor V, 9.6 (Nesheim *et al.*, 1979); C4BP, 14.1 (Perkins *et al.*, 1986).

Factor V Activation—Factor V was diluted in 10 mM Hepes, 135 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, pH 7.4, and activated by the addition of 1:20 (w/w) thrombin at 37 °C. After 20 min a 40-fold increase in clotting activity was obtained in a factor V clotting assay using factor V-deficient plasma (Diamed AG, Murten, Switzerland) in a KC-10 coagulometer (Amelung, Lemgo, Germany). The activation was terminated by the addition of a 10-fold excess of recombinant desulfatohirudin over thrombin. Factor Va was isolated using an immobilized monoclonal antibody (RU-FV3B1) directed against the heavy chain of factor Va. After elution with Hepes buffer containing 1.5 M NaCl, pH 7.4, factor Va was dialyzed and stored at −70 °C until use.

Protein C Activation—Protein C was activated essentially as described by Koedam *et al.* (1988) with some minor modifications. Protein C was incubated with thrombin (1:20 w/w) at 37 °C. After 2 h the maximal activity of APC was obtained as measured with chromogenic substrate S2366, and the activation was terminated by the addition of recombinant desulfatohirudin. APC was separated from full-length protein C and traces of thrombin/desulfatohirudin with fast protein liquid chromatography using a Mono Q column (1 ml) in TBS with a linear gradient (20 ml) of NaCl (150–500 mM) at pH 7.4. APC was homogeneous as judged by SDS-PAGE and Western blotting analysis. Amido blotting analysis according to Rosing *et al.* (1987) revealed an active, double band of APC at approximately 60 kDa, whereas no activity was observed for full-length protein C (62 kDa). Aliquots were stored at −70 °C until use.

Production of Monoclonal Antibodies—BALB/c mice were immunized by subcutaneous injection of 10 μg of either purified human protein S, protein C, factor X, or factor V in Freund's complete adjuvant; after 2, 4, and 6 weeks they were boosted with the same amount of antigen in Freund's incomplete adjuvant. Three days after the final injection, spleen cells were fused with Ag 8.653 myeloma cells. Fusion and hybridoma selection were performed according to standard procedures. Culture supernatants were screened for the presence of specific antibodies by an enzyme-linked immunosorbent assay, in which purified proteins were used as antigen. Bound antibodies were detected with peroxidase-conjugated rabbit antibodies against mouse immunoglobulins (DAKO). Positive clones were subcloned by limiting dilution, expanded, and injected intraperitoneally into Freund's incomplete adjuvant-primed BALB/c mice for the production of antibody-rich ascites fluid. The murine immunoglobulins were isolated by protein G-Sepharose chromatography (Pharmacia) according to the instructions of the manufacturer. The isotypes of the anti-protein S antibodies were determined by double-antibody sandwich enzyme-linked immunosorbent assay, using isotype-specific goat anti-mouse antisera and peroxidase-conjugated rabbit anti-mouse IgG. All monoclonal antibodies were of the IgG1 type except RU-PS3D9 (previously designated S9; Chang *et al.*, 1992), which was of the IgG2a type.

The seven monoclonal antibodies against protein S were divided into four groups based on competition experiments with antibodies whose epitopes had previously been localized on the protein S molecule (Dahlbäck *et al.*, 1990). All monoclonal antibodies had different epitopes on the protein S molecule. The monoclonal antibodies CLB-PS13 and CLB-PS64 were directed against the Gla domain of protein S, RU-PS1G7 and RU-PS8F9 (previously designated S11; Hessing *et al.*, 1990b) against the thrombin-sensitive region and RU-PS7E9 against a protein S fragment comprising the third and fourth EGF-like region. The fourth group, containing the monoclonal antibodies RU-PS3D9 and CLB-PS18 (previously designated S3; Chang *et al.*, 1992) had epitopes which could not be discerned with the use of the described competition experiments. The dissociation constants for the monoclonal antibodies for protein S were determined using the following procedure. Microtiter well strips were coated overnight at 4 °C with the various antibodies in a concentration of 5 μg/ml in a 50 mM carbonate buffer at pH 9.6. The wells were washed five times with TBS containing 3 mM $CaCl_2$ and incubated for 2 h at room temperature with a solution of 3% bovine serum albumin in TBS with 3 mM $CaCl_2$. After the wells were washed with TBS containing

0.5% Tween, they were incubated with increasing concentrations of radiolabeled protein S for 1 h. The wells were washed five times with TBS with 3 mM $CaCl_2$, and the binding of ^{125}I-protein S to the wells was measured in a γ-counter. Data were analyzed using the nonlinear data-fitting computer program Enzfitter. Calcium dependence of the interaction between monoclonal antibodies and protein S was investigated without added calcium in the presence of 1 mM EDTA.

The epitopes of the anti-protein C antibodies RU-PC4D1 and RU-PC4H5 appeared to be located on the heavy chain of protein C according to Western blotting analysis after SDS-PAGE under reducing conditions. The epitopes of RU-PC4D1 and RU-PC4H5 were not identical as was determined by competition experiments.

Electrophoretic and Immunochemical Techniques—SDS-PAGE was performed on 8 or 10% polyacrylamide slab gels according to Laemmli (1970). After electrophoresis the gels were immunoblotted on polyvinylidene difluoride membranes essentially as described by Towbin *et al.* (1979). Membranes were blocked with 10 mM Tris-HCl, 150 mM NaCl, 5% (w/v) nonfat dry milk, pH 7.4, incubated with monoclonal antibodies against protein S, protein C (RU-PC4D1 or RU-PC4H5), factor X (RU-FX10F1 or RU-FX10A6), and factor V (rabbit polyclonal anti-factor V or RU-FV3B1) in a concentration of 2.5 μg/ml. Antigen-antibody complexes were visualized with peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO) using 3,3-diaminobenzidine tetrahydrochloride as a substrate.

Autoradiography of dried gels was performed at −70 °C using Kodak X-Omat AR5 x-ray film and Du Pont Cronex Lightning Plus intensifying screens.

Cell Cultures—Human umbilical vein endothelial cells were isolated according to the method of Jaffe *et al.* (1973) with some minor modifications (Willems *et al.*, 1982). The cells were identified as endothelial cells by their typical characteristics, such as the formation of a cobblestone-like monolayer with contact inhibition at confluence and the presence of von Willebrand factor antigen. Cells were cultured in 75-cm^2 plastic flasks precoated with fibronectin. Culture medium consisted of RPMI 1640, 20% (v/v) normal human serum (pool of 20 healthy donors), 2 mM glutamine, and the antibiotics penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (5 μg/ml). When the cells reached confluence in the second passage they were subcultured on fibronectin coated 96-well tissue culture strip plates (0.4 cm^2/well, 2×10^4 cells/well) for binding and inactivation studies and 24-well plastic tissue culture plates (2.0 cm^2/well, 10^5 cells/well) for inactivation studies. After confluence the culture medium was replaced, and the cells were used within the next 24 h.

Factor Va Inactivation Assay—All studies were performed in 24-well plastic tissue culture plates at 37 °C. The endothelial cells were washed three times with a Hepes buffer containing 10 mM Hepes, 135 mM NaCl, 4 mM KCl, 15 mM glucose, pH 7.4, and then twice with the same buffer containing 3 mM $CaCl_2$ and 3 mg/ml bovine serum albumin. In this buffer, 300 pM factor Va was preincubated on the endothelial cells for 5 min together with possible ligands in a total volume of 500 μl, and factor Va inactivation was started by the addition of 50 pM APC under continuous shaking. Samples of endothelial cell supernatants were collected during 15 min of reaction, and factor Va activity was determined with a factor V clotting assay using a KC-10 coagulometer. Dilutions of purified factor Va were used as a calibration curve ranging between 10 and 500 pM factor Va. For the determination of the initial rate of factor Va inactivation, four samples for the clotting assay were drawn during the first 2 min of inactivation, and the data were analyzed according to a single exponential decay model using the Enzfitter computer program (Leatherbarrow, 1987).

Radiolabeling of Protein S—Protein S was radiolabeled with Na^{125}I (Amersham, Buckinghamshire, United Kingdom) to a specific radioactivity of 2–4 μCi/μg using the IODO-GEN procedure according to Pierce Chemical Co. Radiolabeled protein S was gel filtered on a PD-10 disposable column equilibrated with TBS containing 0.1 mg/ml bovine serum albumin to remove free iodine. Radiolabeled protein S retained its functional cofactor activity as measured in a functional protein S assay described by Chang *et al.* (1992). Autoradiography of labeled protein S after SDS-PAGE under reducing and nonreducing conditions revealed that ^{125}I-protein S was identical to unlabeled protein S.

Binding Studies—Binding studies were performed in 96-well plastic tissue culture plates at 4 °C in a final volume of 50 μl. Binding was performed at 4 °C to avoid internalization of protein S and deterioration of the cells over longer time periods. Prior to binding studies, the endothelial cells were washed as described above for the factor Va inactivation assay. Then radiolabeled protein S in the presence or absence of unlabeled protein S or other proteins was added to the cells and incubated for the times indicated. After the incubation mixtures were removed and the cells were washed rapidly five times, bound ^{125}I-protein S was counted in a γ-counter. Nonspecific binding was determined in the presence of a 200-fold molar excess of unlabeled protein S. Specific binding was obtained after subtraction of the nonspecific binding from the total binding. The nonspecific binding varied between 15 and 35% of the total radioactivity bound to the cells. No specific binding was observed to fibronectin-coated wells in the absence of cells. Each value was the average of triplicate determinations. Apparent dissociation constants and number of binding sites were estimated by the Enzfitter computer program (Leatherbarrow, 1987).

Relationship between Protein S Binding and Expression of Cofactor Activity—Binding studies at 37 °C to 0.4-cm^2 wells (96-well plates) were performed as described at 4 °C except that during binding the wells were under continuous shaking. At indicated incubation time points, the wells were washed rapidly five times with ice-cold Hepes buffer containing 3 mM calcium to remove unbound ligands. The wells were counted and represented total binding and internalized protein S. Internalized protein S was determined by counting wells which were washed five times with ice-cold Hepes buffer containing 2 mM EDTA. Internalized protein S ranged between 16 and 26% of the total binding after 30 min at the various protein S concentrations. Experiments performed at 4 °C showed that EDTA removed specific bound protein S for more than 90% without damaging the HUVEC monolayers. Bound protein S was calculated by subtracting internalized protein S from the total binding. Nonspecific binding was determined in the presence of a 200-fold molar excess of unlabeled protein S.

In two parallel experiments, 0.4-cm^2 wells were incubated with 50 μl either of various concentrations of ^{125}I-protein S or of unlabeled protein S at 37 °C (500 pM, 1 nM, 2, 5 nM, 5 nM, 10 nM, and 20 nM protein S). Under continuous shaking, binding was allowed for 5 min at 37 °C. The binding of ^{125}I-protein S was determined as described. The unlabeled protein S supernatant was aspirated after which 100 μl of a mixture of 300 pM factor Va and 50 pM APC was added to the wells. After 2 min at 37 °C the amount of factor Va activity was determined with use of a factor V clotting assay in a KC-10 coagulometer.

RESULTS

Inactivation of Factor Va on Endothelial Cells—When 300 pM factor Va was incubated with 50 pM APC on HUVECs (2.0 cm^2, 10^5 cells), a decrease in factor Va activity was observed that resulted in a 35% factor Va inactivation after 15 min (Fig. 1). The inactivation rates were half-maximal at the APC concentration used (results not shown). To exclude a possible role of endogenous protein S synthesized by the endothelial cells or of protein S derived from the culture medium, the experiments were repeated on endothelial cells which were either cultured under serum-free conditions or washed extensively with 1 mM EDTA to remove all possible surface-bound protein S. In both cases inactivation rates were identical to normal treated endothelial cells. The addition of an excess of a crude preparation of phospholipids (50 μM) resulted in a 50-fold increase in the factor Va inactivation rate by APC (results not shown).

Effect of Protein S on the Inactivation of Factor Va on Endothelial Cells—The addition of protein S to the wells resulted in a dose-dependent increase in inactivation rates reaching a maximum at 5 nM protein S at which it enhanced the factor Va inactivation twice (Fig. 1). Incubation of factor Va, APC and protein S in fibronectin-coated wells did not result in any apparent factor Va inactivation. On HUVECs in the presence of protein S, the addition of exogenous phospholipids also resulted in a 50-fold increase in the factor Va inactivation by APC, without an increase of the 2-fold stimulating effect of protein S. On HUVEC monolayers (2.0 cm^2), the rates of initial factor Va inactivation were 21.7 ± 4.6 pM/min factor Va inactivated by 50 pM APC and 45.3 ± 3.2 pM/min in the presence of 5 nM protein S. When protein S was

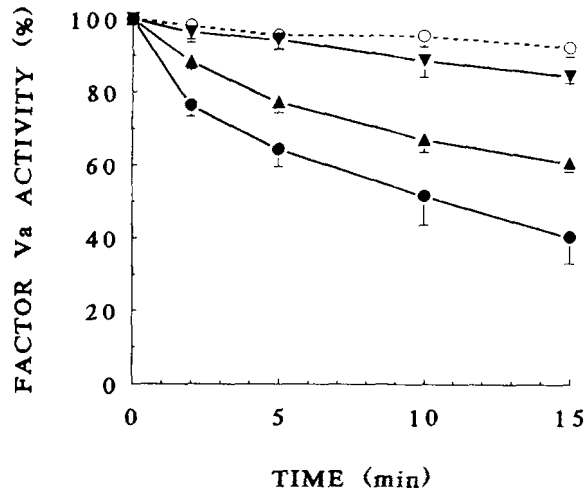

FIG. 1. **Time course of factor Va inactivation by APC on HUVEC monolayers.** 300 pM factor Va was incubated on HUVECs during a 15-min period (▼). When 50 pM APC was added, the inactivation of factor Va on HUVECs was observed (▲). This inactivation by APC was enhanced twice in the presence of 5 nM protein S (●). In fibronectin-coated wells, no inactivation of factor Va by APC in presence of 5 nM protein S could be detected (○). Factor Va activity was determined with use of a factor V clotting assay using factor V-deficient plasma. Inactivation assays were performed as described under "Experimental Procedures." The means of three measurements ± the S.D. are given.

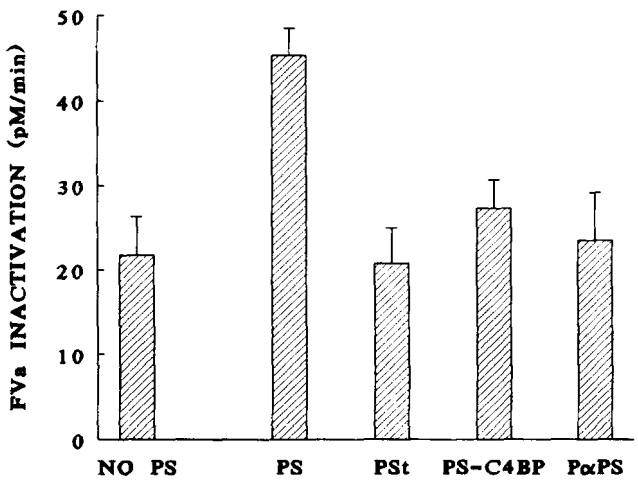

FIG. 2. **Initial factor Va inactivation rates by APC on HUVEC monolayers (pM factor Va/min).** *No PS*, no protein S present; *PS*, in the presence of 5 nM protein S; *PSt*, in the presence of 5 nM of thrombin-cleaved protein S; *PS-C4BP*, in the presence of 5 nM protein S preincubated with 50 nM C4BP; *PαPS*, in the presence of protein S preincubated with 500 nM polyclonal antibodies against protein S. Initial inactivation rate assays were performed according to "Experimental Procedures." Each *bar* represents a mean of three measurements ± S.D.

preincubated with C4BP (50 nM) or polyclonal antibodies against protein S (500 nM) for 1 h at 37 °C, protein S cofactor function was completely inhibited (Fig. 2). When thrombin-cleaved protein S was substituted for protein S, no stimulation of factor Va inactivation could be observed (Fig. 2).

Binding of ^{125}I-Protein S to Endothelial Cells—To investigate whether the binding affinity of radiolabeled protein S was the same as that of native protein S, endothelial cells were incubated for 1 h with a mixture of labeled and unlabeled protein S at 4 °C. The ratio of labeled and unlabeled protein S was varied, keeping the total concentration constant (2.5 nM). Fig. 3 shows that labeled and unlabeled protein S bound

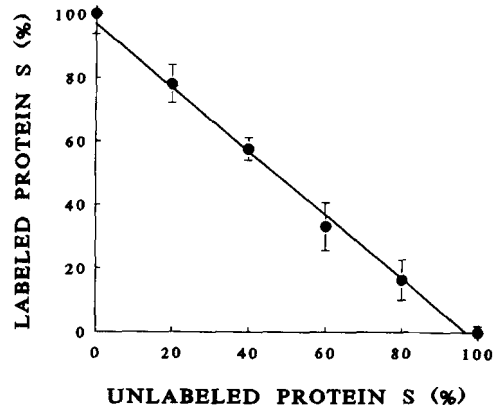

FIG. 3. **Relative binding of ^{125}I-labeled protein S and nonlabeled protein S to HUVEC monolayers.** Endothelial cells were incubated for 1 h at 4 °C with various proportions of labeled and nonlabeled protein S, keeping the total concentration of protein S constant at 2.5 nM. On the *y* axis, 100% represents the binding when 2.5 nM ^{125}I-protein S was added to the wells. Each point represents the mean of three observations ± S.D.

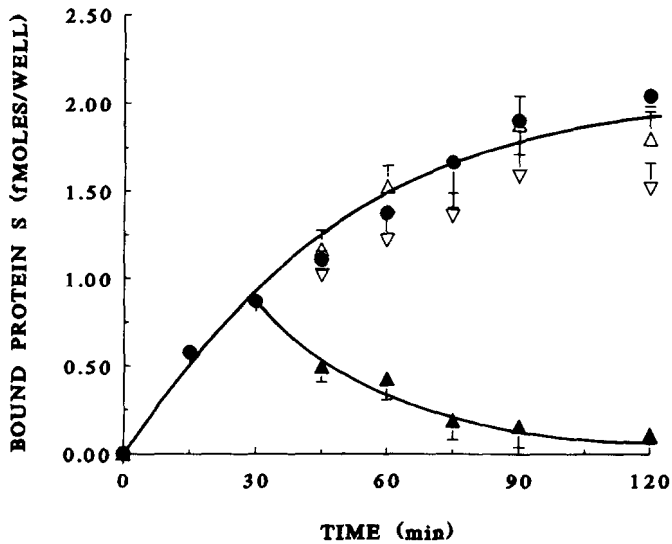

FIG. 4. **Reversibility and specificity of ^{125}I-protein S binding to HUVEC monolayers.** Endothelial cell monolayers were incubated with ^{125}I-protein S (2.5 nM) at 4 °C for the times indicated. Specific binding (●) was determined as described under "Experimental Procedures." After a 30-min incubation, a 250-fold molar excess of unlabeled protein S (▲), factor X (▽), or prothrombin (△) was added to the reaction mixture, and protein S binding was determined during the next 90 min. The means of three measurements ± S.D. are shown.

with the same affinity to endothelial cells.

The binding of protein S to endothelial cells was calcium-dependent and could be inhibited by either EDTA or EGTA. The binding of ^{125}I-protein S reached a maximum at a calcium concentration of 3–5 mM (results not shown).

The binding of ^{125}I-protein S to endothelial cells at 4 °C occurred in a time-dependent manner, and equilibrium was reached after about 2 h (Fig. 4).

The reversibility and specificity of the binding of protein S to its binding sites on the endothelial cells in the presence of 3 mM $CaCl_2$ was examined by measuring the dissociation of bound ^{125}I-protein S after 30 min of incubation. The addition of a 200-fold molar excess of unlabeled protein S induced a dissociation of approximately 90% of the specific bound ^{125}I-protein S, whereas a 200-fold molar excess of factor X or prothrombin showed no effect on binding of protein S to

endothelial cells. This suggests that the binding is specific and does not become irreversible in time.

The number and affinity of protein S binding sites on cultured human endothelial cells was determined by examining the specific binding of ^{125}I-protein S in the presence of 3 mM $CaCl_2$ as a function of the protein S concentration. To achieve equilibrium conditions, binding was allowed for 2.5 h at 4 °C. To measure specific protein S binding for the determination of the dissociation constant, specific bound ^{125}I-protein S was removed from the endothelial cell monolayer by a 90-min incubation with 400 nM unlabeled protein S. The binding of ^{125}I-protein S to the endothelial cell monolayer was observed to be saturable (Fig. 5). Analysis of the specific binding with the Enzfitter computer program revealed (8.0 ± 0.3) × 10^5 binding sites/endothelial cell with a K_d of 24.4 ± 2.2 nM. When the data were modeled for two classes of binding sites, the results essentially converged to the one-site model. Binding studies of ^{125}I-protein S in the presence of varying concentrations of APC (0.5–15 nM) gave identical results as obtained in the absence of APC. Thrombin-cleaved ^{125}I-protein S gave less than 20% binding compared with ^{125}I-protein S. ^{125}I-protein S preincubated with a 10-fold molar excess of C4BP for 1 h bound equally well to endothelial cells as ^{125}I-protein S (see Fig. 7). No effect on ^{125}I-protein S binding was detected when experiments were performed in the presence of 300 pM of factor Va (results not shown).

Effect of Monoclonal Antibodies on the Protein S Cofactor Function—Seven monoclonal antibodies against protein S were examined for their ability to influence the stimulatory effect of protein S on the rate of factor Va inactivation by APC. Mapping data revealed that CLB-PS13 and CLB-PS64 were directed against the Gla domain, RU-PS1G7 and RU-PS8F9 against the thrombin-sensitive region, and RU-PS7E9 against the third or fourth EGF-like region. The epitopes of RU-PS3D9 and CLB-PS18 could not be identified by this procedure. Western blotting revealed that all monoclonal antibodies recognized noncleaved nonreduced protein S (Table I). Western blotting analysis of a partially thrombin-cleaved protein S preparation after SDS-PAGE under reducing conditions showed that all monoclonal antibodies recognized native protein S (upper band of reduced protein S), whereas only RU-PS7E9 and CLB-PS18 recognized thrombin cleaved protein S (lower band of reduced protein S). None of these antibodies effected the interaction of protein S with C4BP (results not shown) as examined in a previously described C4BP-protein S binding assay (Hessing *et al.*, 1990b).

The effect of the anti-protein S monoclonal antibodies on the cofactor activity of protein S in the factor Va inactivation by APC on endothelial cells was investigated. The cofactor activity of protein S preincubated for 1 h with a 5-fold molar excess of these monoclonal antibodies is shown in Fig. 6. Antibodies directed against the Gla domain, the thrombin-sensitive region, and antibody RU-PS3D9 blocked protein S cofactor activity. Antibodies RU-PS7E9 and CLB-PS18 demonstrated minor inhibition of the protein S cofactor function. The addition of monoclonal antibodies against C4BP (RU-

TABLE I

Properties of monoclonal antibodies against protein S

MAb	Subtype	Calcium[a]	PSn[b]	PSn,r[b]	PSt[b]	PSt,r[b]	K_d
							M
CLB-PS13	IgG1	y	++	++	−	−	3.2 ± 0.6 × 10^{-9}
CLB-PS64	IgG1	y	++	++	±	−	0.9 ± 0.1 × 10^{-9}
RU-PS1G7	IgG1	y	++	++	−	−	3.1 ± 0.01 × 10^{-9}
RU-PS8F9	IgG1	n	++	++	−	−	7.4 ± 0.7 × 10^{-9}
RU-PS7E9	IgG1	n	+	++	±	++	1.8 ± 0.2 × 10^{-8}
RU-PS3D9	IgG2a	y	++	++	±	−	1.9 ± 0.3 × 10^{-8}
CLB-PS18	IgG1	n	++	++	++	++	2.5 ± 0.1 × 10^{-7}

[a] y, calcium-dependent epitope; n, calcium-independent epitope.
[b] Reactivity against protein S using immunoblotting techniques: PSn, native nonreduced protein S; PSn,r, native reduced protein S; PSt, thrombin-cleaved, nonreduced protein S; PSt,r, thrombin cleaved, reduced protein S. Reactivity of the monoclonal antibody in western blot: −, none; ±, weak; +, strong; and ++, very strong.

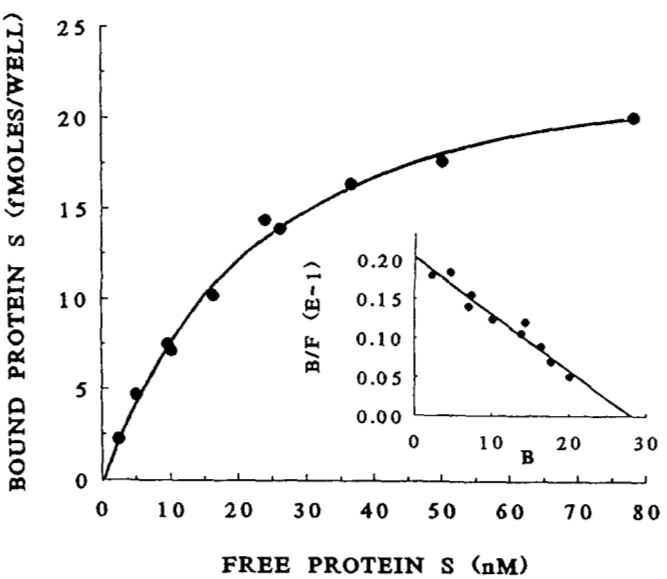

FIG. 5. **Binding isotherm of ^{125}I-protein S to HUVEC monolayers.** Endothelial cell monolayers were incubated for 2.5 h at 4 °C with the indicated concentrations of ^{125}I-protein S. Nonspecific binding of ^{125}I-protein S was determined in presence of a 250-fold molar excess of unlabeled protein S. The binding assay was carried out as described under "Experimental Procedures." Specific binding is plotted *versus* free ^{125}I-protein S, with the Scatchard plot in the *inset*. *B*, bound; *B/F*, bound/free. The means of three measurements are shown.

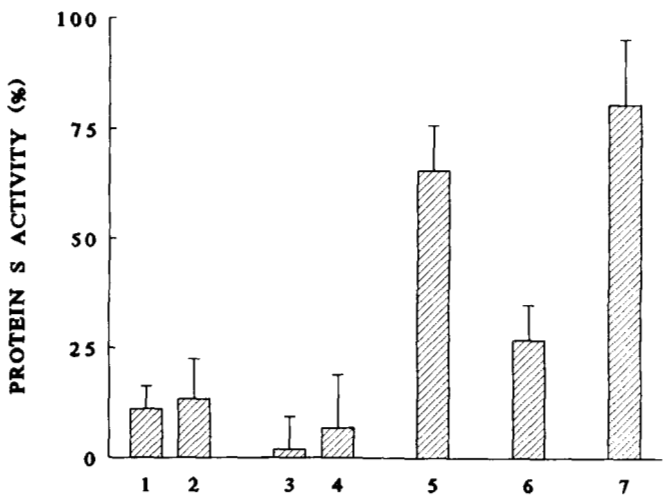

FIG. 6. **Inhibition of the cofactor activity of protein S on HUVEC monolayers by monoclonal antibodies against protein S.** 5 nM protein S was preincubated with a 5-fold molar excess of monoclonal antibodies for 1 h before addition to the factor Va inactivation assay. The 100% level represents the increase in factor Va inactivation caused by the presence of 5 nM protein S. The ability of the monoclonal antibodies to inhibit the protein S cofactor function is shown as a percentage of the maximal protein S cofactor function. Monoclonal antibodies used were CLB-PS13 (*bar 1*), CLB-PS64 (*bar 2*), RU-PS1G7 (*bar 3*), RU-PS8F9 (*bar 4*), RU-PS7E9 (*bar 5*), RU-PS3D9 (*bar 6*), and CLB-PS18 (*bar 7*). See "Experimental Procedures" for details. The means of three measurements ± S.D. are shown.

BP9H10) (Hessing *et al.*, 1991) and factor X (RU-FX10F1) had no effect on the protein S-enhanced factor Va inactivation by APC (not shown).

Effect of Monoclonal Antibodies on the Binding of ^{125}I-Protein S to Endothelial Cells—To investigate the effect of the monoclonal antibodies on the binding of protein S to endothelial cells, 2.5 nM radiolabeled protein S was preincubated for 1 h with a 5-fold molar excess of monoclonal antibodies prior to the presentation to endothelial cell monolayers. The anti-protein S monoclonal antibodies CLB-PS13, CLB-PS64, RU-PS1G7, RU-PS8F9, and RU-PS3D9 showed significant inhibition of protein S binding to endothelial cells. Monoclonal antibodies RU-PS7E9 and CLB-PS18 showed no apparent effect on protein S binding to endothelial cells (Fig. 7).

Relationship between Binding and Expression of Cofactor Activity of Protein S—The binding of ^{125}I-protein S to 0.4-cm^2 HUVEC monolayers (96-well plates) at a concentration of 500 pM, 5 nM, and 20 nM was studied during a 30-min period under continuous shaking. Maximal binding is obtained within 5 min. When monoclonal antibody CLB-PS13 was preincubated for 1 h in a 5-fold molar excess to 5 and 20 nM protein S, binding of protein S to HUVEC monolayers was completely inhibited (Fig. 8*A*).

To study a direct relationship between protein S binding and the expression of cofactor activity we determined the binding of ^{125}I-protein S at various concentrations after 5 min. In a parallel experiment using duplicate plates the same concentrations of unlabeled protein S were allowed to bind to HUVECs under the same conditions, and the cofactor activity was determined. Without HUVECs, no factor Va inactivation could be detected within 10 min. A direct relationship between the binding of protein S and the cofactor activity is observed between 0 and 2 pM protein S (Fig. 8*B*).

DISCUSSION

In this study we have demonstrated that binding of protein S to human endothelial cells is required for the expression of protein S cofactor function to APC in the inactivation of factor Va. The rate of inactivation of factor Va on HUVEC monolayers (2.0 cm^2, 10^5 cells) was found to be 21.7 ± 4.6 pM/min in the presence of 50 pM APC, which is lower than the values reported by Solymoss *et al.* (1988) on phospholipid surfaces and on platelets. This might be explained by the low amount of phospholipid surface offered by endothelial cell monolayers, because the addition of phospholipids to the incubation mixture resulted in a 50-fold increase in the rate of inactivation.

Protein S increased the APC-mediated factor Va inactivation 2-fold, and the relative effect was not enhanced by the addition of exogenous phospholipids. This stimulatory effect is comparable with previous results reported for inactivation of factor Va by APC on phospholipid surfaces (Solymoss *et al.*, 1988; Bakker *et al.*, 1991) and on human platelets (Solymoss *et al.*, 1988) but is low compared with the 9-fold maximal stimulation by protein S of the inactivation of factor VIIIa by APC (Koedam *et al.* 1988). The possibility that the moderate effect of protein S is caused by the presence of endogenous protein S synthesized by endothelial cells is unlikely because the amount of protein S synthesized and released by the endothelial cells is too small to interfere significantly with the effect of exogenous protein S (Fair *et al.*, 1986). Moreover,

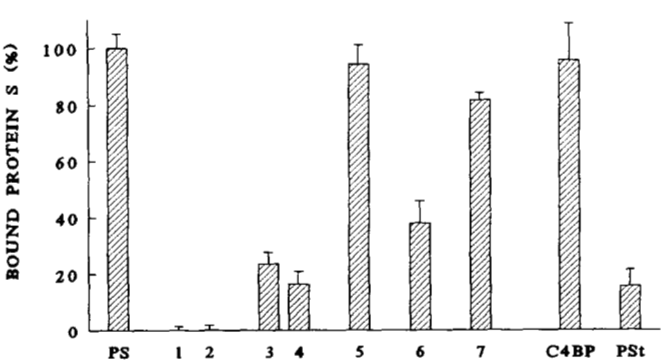

FIG. 7. **Effect of monoclonal antibodies on the binding of ^{125}I-protein S to HUVEC monolayers.** Endothelial cells were incubated for 1 h with ^{125}I-protein S (2.5 nM) preincubated with a 5-fold molar excess of different monoclonal antibodies to protein S or with a 10-fold molar excess of C4BP. Also shown is the binding of thrombin-cleaved ^{125}I-protein S (*PSt*). The 100% value (*PS*) represents the binding when 2.5 nM ^{125}I-protein S was offered to the HUVEC monolayer. Monoclonal antibodies used were: CLB-PS13 (*bar 1*), CLB-PS64 (*bar 2*), RU-PS1G7 (*bar 3*), RU-PS8F9 (*bar 4*), RU-PS7E9 (*bar 5*), RU-PS3D9 (*bar 6*), and CLB-PS18 (*bar 7*). The binding of ^{125}I-protein S-C4BP complex (*C4BP*) and the binding of thrombin-cleaved ^{125}I-protein S (*PSt*) are indicated. Specific binding was determined as described under "Experimental Procedures." Each *bar* represents the mean of three observations ± S.D.

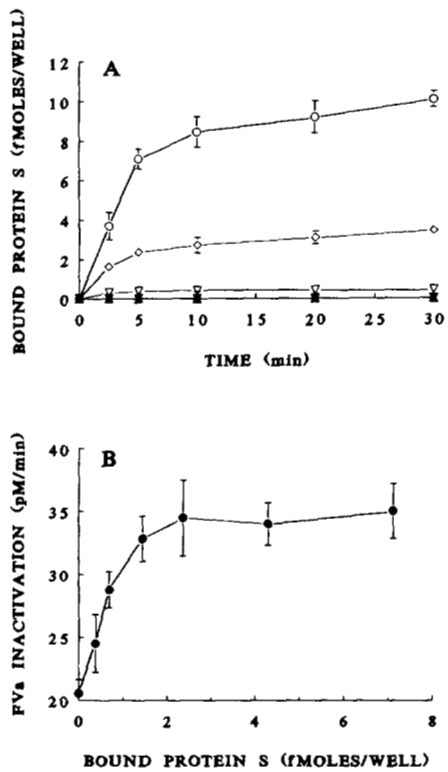

FIG. 8. **Time courses of ^{125}I-protein S binding at 37 °C (*panel A*) and the relationship between protein S binding to HUVEC monolayers and expression of cofactor activity (*panel B*).** The binding of ^{125}I-protein S to HUVEC monolayers (0.4 cm^2) was studied during a 30-min period at 37 °C. Specific binding of 500 pM (▽), 5 nM (◇), and 20 nM (○) ^{125}I-protein S is indicated. Specific binding of 5 and 20 nM ^{125}I-protein S in the presence of a 5-fold molar excess of monoclonal antibody CLB-PS13 is indicated by ▲ and ▼, respectively (*panel A*). In two parallel experiments, first the binding of 500 pM, 1 nM, 2.5 nM, 5 nM, 10 nM, and 20 nM ^{125}I-protein S after 5 min at 37 °C to HUVEC monolayers was determined. The same amounts of unlabeled protein S were allowed to bind to HUVEC monolayers, and after 5 min the wells were aspirated whereafter the wells were used for a factor Va inactivation assay. Plotted are the amounts of ^{125}I-protein S bound to the HUVEC monolayers *versus* the inactivation rates of factor Va by APC at the amounts of bound protein S (*panel B*). Each point represents the mean of three measurements ± S.D.

we showed that protein S-deficient serum-free culturing conditions as well as EDTA treatment of normal cultured cells did not affect the rate of factor Va inactivation on endothelial cells by APC. The moderate protein S cofactor effect might be because of the nature of the inactivation assay. Under the static conditions in which the factor Va inactivation is studied, APC does not necessarily need protein S for attachment to the phospholipid surface to inactivate factor Va. It could be feasible that for factor Va inactivation studies under flow conditions, the protein S cofactor function would be more pronounced.

We demonstrated that binding of protein S to endothelial cells was time-dependent and reversible as was concluded from direct displacement of ^{125}I-protein S from its endothelial cell binding site upon the addition of a 200-fold molar excess of unlabeled protein S. The binding affinities of native and radiolabeled protein S to endothelial cells were shown to be similar. Binding of protein S to cultured human endothelial cells required the presence of calcium ions as was also observed for binding of protein S to human platelets (Schwarz *et al.*, 1985: Mitchell and Salem, 1987), cultured bovine aortic endothelial cells (Stern *et al.*, 1986a), and neutrophils (Oates and Salem, 1991).

Stern *et al.* (1986a) reported the binding of bovine ^{125}I-protein S to bovine aortic endothelial cells with an apparent K_d of 11 nM and 85,000 receptors/cell. The addition of APC decreased the K_d of protein S to 0.2 nM. On the other hand, Schwartz *et al.* (1985) and Mitchell and Salem (1987) reported that the binding of human protein S to human platelets is not dependent on APC or factor Va. The results of the present study indicate that HUVECs expose approximately (8.0 ± 0.3) $\times 10^5$ protein S binding sites/cell with an apparent K_d of 24.4 ± 2.2 nM and that the binding of protein S is not affected by the addition of APC. The difference in the numbers of binding sites reported for bovine aortic endothelial cells and HUVECs may be caused by their difference in size (Stern *et al.* 1986a) and the species and tissue specificity. Nevertheless at physiological protein S concentrations of 330 nM (Dahlbäck, 1983) the protein S binding sites on the endothelium of all species tested would be saturated. The nature of these protein S binding sites is still unclear and remains to be investigated.

A direct relationship between the binding of ^{125}I-protein S to HUVEC monolayers and the expression of protein S cofactor activity was demonstrated (Fig. 8). A maximal cofactor effect was observed at concentrations of 2.5 fmol of bound protein S/well. This is most likely because at higher concentrations of bound protein S the concentration of APC becomes rate-limiting (5 fmol/well).

The Gla residues have been shown to be involved in the binding of protein S to phospholipid surfaces in the presence of calcium (Nelsestuen *et al.*, 1978; Walker, 1981b; Dahlbäck *et al.*, 1990a). Because calcium is needed for the binding of protein S to endothelial cells, the Gla residues are most likely involved, as was demonstrated by our binding studies in the presence of the monoclonal antibodies against the Gla domain. Monoclonal antibodies directed against either the Gla domain or the thrombin-sensitive region inhibited the binding of protein S to endothelial cells. The inhibition of the binding was paralleled by a loss of cofactor activity of protein S for APC in the inactivation of factor Va on endothelial cells (Fig. 6). Since the interaction of protein S and APC on phospholipid surfaces seems to be essential for their anticoagulant activity (Walker, 1981b; Stern *et al.*, 1986a, 1986b), the loss of protein S cofactor activity is most likely explained by a decreased protein S binding.

The anti-protein S monoclonal antibody RU-PS7E9 directed against the third or fourth EGF-like domain had little effect on protein S binding to endothelial cells and had little effect on protein S cofactor function in APC-catalyzed factor Va inactivation. This suggests that the third or fourth EGF-like domain is of less importance for the expression of protein S cofactor activity.

Thrombin-cleaved protein S has no cofactor activity and has reduced calcium binding and phospholipid binding properties (Dahlbäck, 1983; Walker, 1984b). In addition, we found that thrombin-cleaved protein S showed a markedly decreased binding to endothelial cells compared with native protein S. Thrombin cleaves protein S in the thrombin-sensitive loop resulting in a two-chain molecule with the Gla region linked to the rest of the molecule by a disulfide bond. The majority of the monoclonal antibodies against the Gla domain or thrombin-sensitive region of protein S did not recognize thrombin-cleaved protein S. This indicates that protein S undergoes a conformational change after cleavage, destroying one or more epitopes located in or close to the thrombin-sensitive region (Malm *et al.*, 1987). That monoclonal antibody RU-PS3D9 does not recognize thrombin-cleaved protein S under reducing conditions and it inhibits binding of protein S to HUVECs as well as protein S cofactor activity might imply that its epitope is located on or in the vicinity of the thrombin-sensitive region.

When protein S is complexed to C4BP in the presence of calcium ($K_d \sim 5.10^{-10}$) (Dahlbäck *et al.*, 1990b), the APC cofactor activity of protein S is inhibited, although protein S in complex with C4BP is still able to bind to phospholipid surfaces (Schwalbe *et al.*, 1990).

In agreement with these latter observations we showed that protein S and the protein S-C4BP complex bound equally well to endothelial cells. This suggests that the binding site for C4BP is probably not located toward the amino terminus of protein S but rather is more likely located at the carboxyl terminus of the sexual hormone-binding globulin homology region of protein S (Walker, 1989; Weinstein and Walker, 1990; Nelson *et al.*, 1991, 1992).[2] It also indicates that the inhibition of the protein S cofactor activity by C4BP is not caused by the inhibition of protein S binding to endothelial cells. For expression of cofactor activity a direct interaction of protein S with APC and/or possible interactions with factors Va and VIIIa would be of importance. Öhlin and co-workers (1988, 1990) reported that the interaction of protein S and APC may take place at the amino termini via the EGF regions. Dahlbäck and co-workers (1990a) suggested that the thrombin-sensitive region as well as the first EGF-like domain are important for protein S cofactor activity for APC by allowing protein-protein interactions on the phospholipid surface. The inhibition by C4BP of the protein S cofactor activity by hindering the interaction of APC with protein S is less likely because a ternary complex between protein S, APC, and C4BP is described by Nishioka and Suzuki (1990). The inhibition by C4BP of a possible direct interaction between the EGF-like regions or regions located toward the carboxyl terminus of protein S with factor Va and factor VIIIa remains to be elucidated.

In conclusion, our results indicate that protein S can bind to human endothelial cells in a specific, reversible way, and this binding is directly related to the expression of protein S cofactor activity for APC in the inactivation of factor Va on endothelial cells.

[2] G. T. G. Chang, B. H. A. Maas, H. K. Ploos van Amstel, P. H. Reitsma, R. M. Bertina, and B. N. Bouma, manuscript submitted for publication.

Acknowledgments—We thank Dr. B. Dahlbäck for making the mapping of the anti-protein S monoclonal antibodies available. We thank Annemarie van der Hoeven for the dedicated isolation and culturing of HUVECs and Patrick Aelmans for the preparation of the monoclonal anti-factor V antibody.

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