# Binding of Anticoagulant Vitamin K-Dependent Protein S to Platelet-Derived Microparticles†

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ABSTRACT: Vitamin K-dependent protein S is an anticoagulant plasma protein serving as cofactor to activated protein C in degradation of coagulation factors Va and VIIIa on membrane surfaces. In addition, it forms a noncovalent complex with complement regulatory protein C4b-binding protein (C4BP), a reaction which inhibits its anticoagulant function. Both forms of protein S have affinity for negatively charged phospholipids, and the purpose of the present study was to elucidate whether they bind to the surface of activated platelets or to platelet-derived microparticles. Binding of protein S to human platelets stimulated with various agonists was examined with FITC-labeled monoclonal antibodies and fluorescence-gated flow cytometry. Protein S was found to bind to membrane microparticles which formed during platelet activation but not to the remnant activated platelets. Binding to microparticles was saturable and maximum binding was seen at approximately 0.4 $\mu$M protein S. It was calcium-dependent and reversed after the addition of EDTA. Inhibition experiments with monoclonal antibodies suggested the $\gamma$-carboxyglutamic acid containing module of protein S to be involved in the binding reaction. An intact thrombin-sensitive region of protein S was not required for binding. The protein S–C4BP complex did not bind to microparticles or activated platelets even though it bound to negatively charged phospholipid vesicles. Intact protein S supported binding of both protein C and activated protein C to microparticles. Protein S-dependent binding of protein C/activated protein C was blocked by those monoclonal antibodies against protein S that inhibited its cofactor function. In conclusion, we have found that free protein S binds to platelet-derived microparticles and stimulates binding of protein C/activated protein C. The specific assembly of protein S and activated protein C on platelet-derived microparticles is an event which could contribute to the regulation of blood coagulation.

Activation of platelets by various agonists is accompanied by formation of microparticles, i.e., small platelet-derived vesicles with an average diameter of 0.1 $\mu$m that are shed from the platelet surface (Bode et al., 1985; Sandberg et al., 1985; Wiedmer & Sims, 1986, 1991; Sims et al., 1988, 1989). Agents eliciting this response include thrombin, a combination of thrombin and collagen, complement proteins C5b–9, and the calcium ionophore A 23187. Microparticles provide binding sites for coagulation factors Va and VIIIa (Sims et al., 1989; Gilbert et al., 1991), which are important cofactors in activation of prothrombin and factor X, respectively (Furie & Furie, 1988; Mann et al., 1988; Kane & Davie, 1988). Shedding of microparticles requires membrane fusion, a process suggested to produce transient flip-flop sites for membrane phospholipid (Sims et al., 1989; Cullis & DeKruijff, 1979). This results in exposure of negatively charged phospholipid on the surface of microparticles, which is crucial for assembly of the prothrombinase and the Xase complexes. It has been suggested that microparticles form most of the platelet-derived catalytic surface for these enzyme–cofactor complexes during blood coagulation (Sims et al., 1988, 1989; Gilbert et al., 1991).

Protein S and protein C are vitamin K-dependent plasma proteins involved in regulation of blood coagulation (Clouse & Comp, 1986; Esmon, 1989; Stenflo, 1988; Walker, 1988). Their physiological importance is illustrated by a high incidence of thromboembolic diseases in individuals with hereditary deficiency of either protein (Clouse & Comp, 1986; Engesser et al., 1987). Protein C is activated on the surface of endothelial cells by the thrombin–thrombomodulin complex. Activated protein C (APC)[1] proteolytically degrades phospholipid-bound coagulation factors Va and VIIIa and protein S may function as cofactor. The physiological role of protein S is, however, incompletely understood; e.g., protein S only increases the rate of APC-mediated factor Va degradation 2-fold in systems with purified human components (Dahlbäck, 1986; Walker, 1988; Solymoss et al., 1988; Tans et al., 1991; Bakker et al., 1992).

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[1] Abbreviations: APC, activated protein C; APTT, activated partial thromboplastin time; C4BP, C4b-binding protein; DiIC16(3), 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocyanide perchlorate; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; FL1 and FL2, fluorescence channel 1 and 2; Gla, $\gamma$-carboxyglutamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPS, human protein S; MOPS, 4-morpholinepropanesulfonic acid; PAPMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; PC, protein C; PIPES, 1,4-piperazinediethanesulfonic acid; TBS, 50 mM Tris-HCl and 0.15 M NaCl, pH 7.4.

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Protein S is multimodular, containing a γ-carboxyglutamic acid (Gla) rich module, a thrombin-sensitive region, four epidermal growth factor (EGF) like modules, and a C-terminal module homologous to androgen binding proteins (Dahlbäck et al., 1986; Lundwall et al., 1986; Hoskins et al., 1987; Ploos van Amstel, 1987; Gershagen et al., 1987; Baker et al., 1987). Protein S has affinity for negatively charged phospholipid vesicles (Nelsestuen et al., 1978), and it has been suggested that protein S and APC form a membrane-bound complex (Walker, 1981, 1988). The Gla module of protein S contains the phospholipid binding site, whereas the thrombin-sensitive region and the first EGF-like module have been proposed to interact with APC (Dahlbäck et al., 1990).

In plasma, protein S exists as free protein and bound to complement regulatory protein C4b-binding protein (C4BP), suggesting a role for protein S also in complement (Dahlbäck, 1991). Both forms of protein S bind negatively charged phospholipid (Nelsestuen et al., 1978; Schwalbe et al., 1990) but only free protein S (i.e., not complexed to C4BP) functions as cofactor to APC (Comp et al., 1984; Bertina et al., 1985; Dahlbäck, 1986).

APC degrades platelet factor Va, thereby regulating platelet prothrombinase activity, and protein S may function as cofactor (Comp & Esmon, 1979; Dahlbäck & Stenflo, 1980; Suzuki et al., 1984; Harris & Esmon, 1985; Solymoss et al., 1988; Tans et al., 1991). To date, most studies related to the effects of APC and protein S on platelet prothrombinase activity have been done without analyzing the contribution of microparticles. Recently, Tans et al. (1991) reported that platelet microparticles express catalytic activity for APC and that approximately 25% of total APC-mediated inactivation of factor Va catalyzed by activated platelets was attributed to the microparticle fraction. However, only half of the APC activity depended on protein S.

Although the potentiating effect of protein S on APC-mediated factor Va degradation in the presence of platelets is small, it was of interest to investigate whether protein S interacts with microparticles and/or with the platelet surface. We have examined binding of protein S to platelets using fluorescence-gated flow cytometry and FITC-labeled monoclonal antibodies and found protein S to bind specifically to microparticles but not to the remnant activated platelets. Moreover, protein S potentiated binding of both protein C and activated protein C to microparticles.

## MATERIALS AND METHODS

Bovine serum albumin (globin and fatty acid free), apyrase, and *N*-hydroxysuccinimide biotin ester were obtained from Sigma; A 23187 was from Behring Diagnostics; fluorescein 5-isothiocyanate (FITC) on Celite (10% FITC) and 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocyanide perchlorate [Di-IC16(3)] were from Molecular Probes; phycoerythrin–streptavidin conjugate was from Southern Biotechnology Associates (Birmingham, AL). Bovine brain L-α-phosphatidylserine and egg L-α-phosphatidylcholine were purchased from Avanti Polar Lipids (Pelham, AL). Collagen (equine tendon) was from Hormon-Chemie (Munich, Germany). APTT reagent was from IL Instrumentation Laboratory. (*p*-Amidinophenyl)methanesulfonyl fluoride (PAPMSF) was from Med Cal (San Francisco, CA).

*Solutions.* Solution I contained 145 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.1% (w/v) glucose, 0.1% BSA, and 5 mM PIPES, pH 6.8. Solution II contained 137 mM NaCl, 4 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM sodium phosphate, 0.1% glucose, 0.1% BSA, 2 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. Solution III contained 50 mM Tris-HCl, 0.15 M NaCl, 0.1% glucose, and 0.1% BSA, pH 7.4, TBS buffer was 50 mM Tris-HCl and 0.15 M NaCl, pH 7.4.

*Monoclonal Antibodies.* The monoclonal antibody W5, specific for membrane glycoprotein GPIb, was a kind gift of Dr. Rodger P. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK). The monoclonal antibodies HPS 21, 67, 54, 56, and 34 against human protein S have been described previously (Dahlbäck et al., 1990). A monoclonal antibody against human protein C, HPC 8, was a kind gift of Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). It recognized an epitope in one of the EGF-like domains of protein C.[2] Three monoclonal antibodies against C4BP (denoted C4BP 70, 102, and 104) reacted with epitopes located in the N-terminal half of the C4BP α-chain and did not influence binding of protein S to C4BP.[3]

*Proteins.* Human protein S was purified as previously described (Dahlbäck, 1983) with the following modification: after the blue-Sepharose chromatography, protein S was purified on an affinity column using monoclonal antibody HPS 21 (Dahlbäck et al., 1990). The proteins were loaded in the presence of TBS containing 2 mM calcium. After the column was washed with the same buffer, protein S was eluted with 50 mM Tris-HCl and 0.5 M NaCl, pH 7.5, containing 5 mM EDTA. Purified protein S was dialyzed against TBS and stored in aliquots at −70 °C. To obtain thrombin-cleaved protein S, protein S (1.3 mg/mL) in TBS containing 10 mM EDTA was incubated with thrombin (approximately 6 μg/mL final concentration, which equals 20 units/mL) for 1 h at 37 °C. To stop the reaction, antithrombin III was added to a final concentration of 20 μg/mL. Intact and cleaved protein S were analyzed on SDS–polyacrylamide gel electrophoresis (7.5% gels) after reduction with 2% β-mercaptoethanol, using the PHAST system from Pharmacia. Bovine protein S (Stenflo & Jönsson, 1979), bovine prothrombin (Owen et al., 1974), bovine factor X (Owen et al., 1974), bovine protein Z (Broze & Miletich, 1984), bovine thrombin (Owen et al., 1974), human antithrombin III (Walker & Esmon, 1979), and human C4BP (Hillarp & Dahlbäck, 1988) were prepared as described in the respective references. Human protein C (kindly provided by Dr. C. T. Esmon) was purified and activated with thrombomobulin–thrombin Affigel as previously outlined (D'Angelo et al., 1986). Human complement proteins C5b6, C7, C8, and C9 were purified as reported (Wiedmer & Sims, 1985).

*Fluorescence Labeling of Proteins and Antibodies and Preparation of Phospholipid Vesicles.* Monoclonal antibodies and Fab′ fragments were conjugated with FITC as described previously (Sims et al., 1988). FITC/IgG molar ratios of approximately 1.5 were usually obtained. Human protein S (1 mL, 0.3 mg/mL) in 5 mM MOPS and 0.15 M NaCl, pH 7.5, containing 1 mM calcium was mixed with 100 μL of 1 M sodium carbonate and 50 μL of FITC–Celite slurry (10 mg/mL). After 30 min of incubation at room temperature, the reaction was stopped by the addition of 50 μL of 1 M ammonium carbonate, pH 9.0, and the reaction mixture was separated on a PD 10 column. The molar ratio of FITC to protein S was 1.7:1. Human C4BP was FITC-labeled using

[2] C. T. Esmon, personal communication.

[3] B. Dahlbäck and A. Hillarp, unpublished observation.

the following procedure: 4 mL of C4BP (0.31 mg/mL) in 5 mM MOPS and 0.15 M NaCl, pH 7.5, was mixed with 0.4 mL of bovine protein S (1.3 mg/mL in the same buffer). The solution was made 1 mM in calcium and incubated for 10 min at room temperature to allow protein S–C4BP complex formation. Sodium carbonate, pH 9.0 (400 μL of a 1 M solution), and 200 μL of FITC–Celite slurry (10 mg/mL in water) were added. After 30 min of incubation at room temperature, the proteins were separated from the FITC on a PD 10 column (Pharmacia) in TBS. The protein solution was concentrated using a Centricon (Millipore) and applied to a column (0.9 × 55 cm) with Sephacryl S-400 in 50 mM Tris-HCl and 3 M guanidine hydrochloride, pH 7.5, to separate C4BP from protein S. The column was eluted at room temperature with a flow rate of 20 mL/h and 0.5-mL fractions were collected. The pool containing FITC-labeled C4BP was dialyzed against TBS, stored at +4 °C, and used within a few days. The labeled protein contained approximately 8 mol of FITC/mol of C4BP. The rationale for labeling C4BP in the presence of bovine protein S was to protect the protein S binding site on C4BP during labeling. This approached has previously been successfully used to protect the protein S binding subunit during proteolytic digestion (Hillarp & Dahlbäck, 1988). The antibody W5 was conjugated with *N*-hydroxysuccinimide biotin ester as described (Shattil et al., 1987). Fluorescence-labeled multilamellar phospholipid vesicles containing 75% phosphatidylcholine, 25% phosphatidylserine, and 0.1 mole % 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocyanide perchlorate [DiIC16(3)] were prepared as previously described (Sims et al., 1974).

*Activation of Platelets.* Gel-filtered human platelets were prepared as described previously (Wiedmer et al., 1986). They were collected at a concentration of (1 – 2) × $10^9$/mL in solution I. Before activation, they were diluted to 1 × $10^8$/mL in solution II. They were activated at 27 °C for 10 min with thrombin (1 unit/mL unless otherwise stated) or with the combination of thrombin (0.5 unit/mL) and collagen (10 μg/mL). In addition, other agonists, C5b–9 and the calcium ionophore A 23187 (1 μM), were used as previously described (Sims et al., 1989). The input per $10^8$ platelets of C5b–6 was 3.5 μg; C7, 1 μg; C8 and C9, each 4 μg. After activation with thrombin (alone or in combination with collagen), PAPMSF (10 μM final concentration) was added to inhibit thrombin. In the calcium-titration experiments, the platelets were diluted in solution III with 1 mM $CaCl_2$ and activated with thrombin plus collagen. After activation, EDTA was added to a final concentration of 1 mM. The dilution buffer in the calcium-titration experiment was solution III also containing 50 μM EDTA. In the calcium-titration experiment using multilamellar phospholipid vesicles, TBS containing 10 μM EDTA was used.

*Binding Experiments*: (*A*) *Platelets.* Biotinylated W5 (directed against the membrane protein GPIb) (5 μL of a solution containing 20 μg/mL) was mixed with the protein or the protein mixture to be studied, e.g., human protein S (5 μL containing various concentrations of protein S) and 25 μL of platelets (1 × $10^8$/mL). Solution II was used as diluent unless otherwise stated. The final volume of the incubation mixture was adjusted to 50 μL. After 10–15 min of incubation at room temperature, phycoerythrin–streptavidin conjugate (5 μL of a 1:20 dilution) and 5 μL of FITC-labeled monoclonal antibody (or Fab′ fragment) were added. The final concentration of the FITC-labeled reagent in all cases exceeded that of the protein to be studied. The concentration of intact antibody was sufficiently high to give at least a 2-fold molar excess over the protein S concentration (even at the highest protein S concentration used) to minimize the risk of one antibody cross-linking two protein S molecules. After 15–60 min of incubation in the dark, at room temperature, the samples were diluted with 300 μL of ice-cold solution II and immediately analyzed by flow cytometry. To follow the time course of binding, protein S (5 μL of a solution containing 200 μg/mL) was preincubated at room temperature for 15 min with FITC-labeled Fab′ fragments of the monoclonal antibody HPS 56 (10 μL of a solution containing 0.54 mg/mL) and 15 μL of solution II. This mixture was then added to a solution containing thrombin-activated platelets (100 μL, 1 × $10^8$/mL), biotinylated W5 (20 μL of a solution containing 20 μg/mL), phycoerythrin–streptavidin conjugate (20 μL of a 1:20 dilution), and 35 μL of solution II. At intervals 15-μL aliquots were drawn, diluted with 300 μL of ice-cold solution II, and immediately analyzed by flow cytometry.

(*B*) *Multilamellar Phospholipid Vesicles.* Five microliters of the phospholipid preparation (20 μg/mL final concentration) was mixed with 5 μL of each of the proteins to be tested and solution III to a final volume of 50 μL. After 15–60 min of incubation at room temperature, in the dark, 5 μL of FITC-labeled monoclonal antibody was added and after another 15 min of incubation, the samples were diluted with 300 μL of ice-cold solution II and immediately analyzed by flow cytometry.

*Flow Cytometry.* Samples were analyzed in a Becton-Dickinson FACSCAN flow cytometer formatted for two-color analysis as previously described in detail (Shattil et al., 1987; Sims et al., 1988; Abrams & Shattil, 1991). The light scattering and fluorescence channels were set at logarithmic gain. In order to resolve microparticles from background scatter, the FL2 (phycoerythrin fluorescence measured at 585 nm) threshold was set at 400. Thus, only particles distinctly positive for GPIb (the FL2 threshold signal derived from phycoerythin–streptavidin bound to the biotinylated antibody against GPIb) were included in the analysis. The use of a fluorescence-based acquisition threshold enabled small scattering particles expressing GPIb to be fully resolved from machine electronic noise and nonspecific particle scatter that would normally obscure the microparticle signal when data are accumulated in a forward scatter-based acquisition mode. Once the FL2 threshold is applied to select out nonspecific scatter, a highly fluorescent platelet and vesicle population (FL2 signal was generally >400 arbitrary fluorescence units/particle versus <20 units for background scatter) was readily discerned. Side and forward scatters were used to discriminate microparticles from platelets as described (Sims et al., 1988; Abrams & Shattil, 1991). Gates defining the two particle populations were set so as to optimize discrimination between platelets and microparticles and then maintained constant for all samples analyzed in a particular experiment. To compensate for day-to-day variability in both shape and size of washed platelets, gates were reset each day, and although we applied the same criteria for setting the gates, the exact settings did slightly differ from one day to the next. Figure 1 depicts an example of forward and side scatter gates employed to discriminate platelets and microparticles. Discrimination required (i) acquisition of all GPIb-positive events (using the FL2 acquisition threshold) regardless of their light scattering characteristics, (ii) setting optimal forward and side scatter gates for unstimulated platelets (i.e., "platelet gate"), and (iii) optimizing forward and side scatter gates for microparticles so as to maximally discriminate between the two populations of GPIb-positive particles. There was an overlap

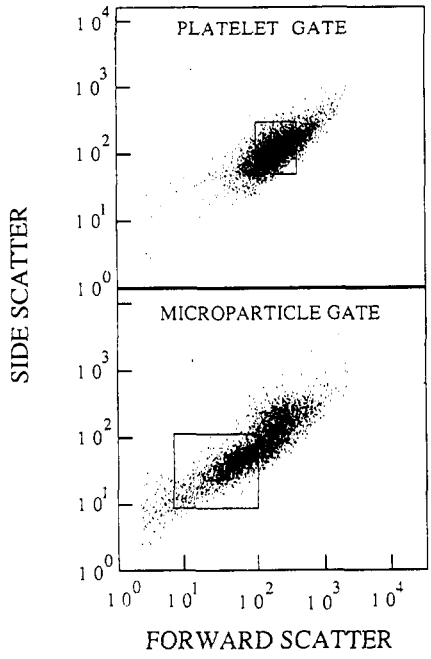


FIGURE 1: Dot plots of light scattering profiles of unactivated (upper panel) and C5b-9 activated (lower panel) platelets. Forward and side scatters are plotted on logarithmic scales (arbitrary units). The quadrangles denote how gates defining platelet and microparticle populations were set in this particular experiment.

between the two particle populations and a certain degree of cross-contamination between platelet and microparticle gates was inevitable. This did not, however, significantly influence interpretation of results. In each sample, 5000 GPIb-positive particles were analyzed for forward and right-angle light scattering and for FITC fluorescence intensity (FL1 channel at 520 nm). The percentage of events that represented microparticles, i.e., those signals that fell within the microparticle gate, was calculated. We chose to utilize the arithmetic mean FL1 signal calculated for all gated particles (both FL1 positive and negative) as a binding parameter. Although use of this parameter tended to obscure the large increase in protein S binding attributable to only the newly-formed microparticle fraction (the highly fluorescent events in the upper left quadrant of Figure 2), it had the advantage of providing an index of total protein S binding that was sensitive to both small increases above background for the quiescent platelets (anticipated to be an upward shift in a normally-distributed population) and the appearance of a new subset of distinctly FL1-positive particles (i.e., conversion to a bimodal distribution of fluorescent and nonfluorescent particles). The data obtained by flow cytometry were highly reproducible, with an analytical variance for multiple measurements on the same sample of <5% for all parameters, including the reported mean FL1 signal. The number of particles analyzed depended on the number included within each gate. In no case was mean FL1 derived from fewer than 500 particles. In experiments using phospholipid vesicles, the FL2 fluorescence threshold was set at 250 as the fluorescent lipophilic dye DiIC16(3) was found to yield a smaller FL2 signal than the phycoerythrin.

## RESULTS

*Binding of Protein S to Platelet-Derived Microparticles.* Binding of human protein S to platelets was studied either with FITC-labeled protein S or with an indirect detection system using FITC-labeled monoclonal antibodies. Similar

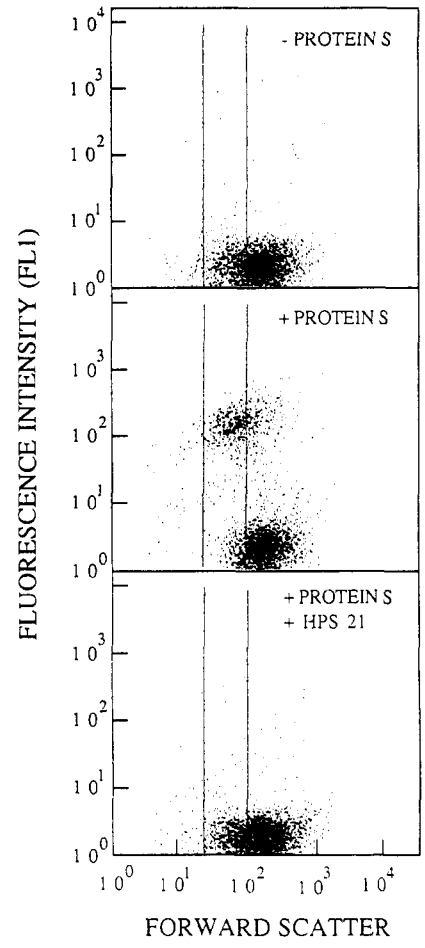


FIGURE 2: Protein S binding to platelet microparticles. Platelets were activated with thrombin (1 unit/mL) and incubated with protein S (2 μg/mL) as described in Materials and Methods. Protein S binding was detected with FITC-labeled HPS 34. The fluorescence intensity is plotted on a logarithmic scale. Each dot represents one particle. The microparticle gate was defined as outlined in Materials and Methods; the two vertical lines represent the forward scatter gate. The upper panel is a control incubation without added protein S (no binding of FITC-labeled HPS 34 observed), the middle panel represents a sample with protein S (note the appearance of the highly fluorescent events in the upper left quadrant), and the lower panel is the result of a simultaneous incubation with protein S and HPS 21 (binding of protein S was completely blocked).

results were obtained with the two approaches. Two different FITC-labeled monoclonal antibodies against protein S, HPS 34 and 56, were used. HPS 56 reacts with an epitope in the third or fourth EGF-like module, whereas the HPS 34 epitope is located in the C-terminal module.[4] Results obtained with the two antibodies were in good agreement when used as both intact antibodies and Fab' fragments. Protein S was found to bind specifically to a population of particles formed during platelet activation (Figure 2), which demonstrated a scatter characteristic of platelet microparticles (Sims et al., 1989; Abrams & Shattil, 1991). The FL1 signal associated with the exposure of protein S binding sites on platelet microparticles represented an increase in the fluorescence signal per particle that was orders of magnitude above the background signal observed for quiescent platelets. Protein S binding to the remnant activated platelet was not detected. When included in the reaction mixture, monoclonal antibody HPS 21 (against the Gla module of protein S) completely inhibited protein S binding to microparticles, demonstrating the specificity of

[4] B. Dahlbäck, unpublished observation.

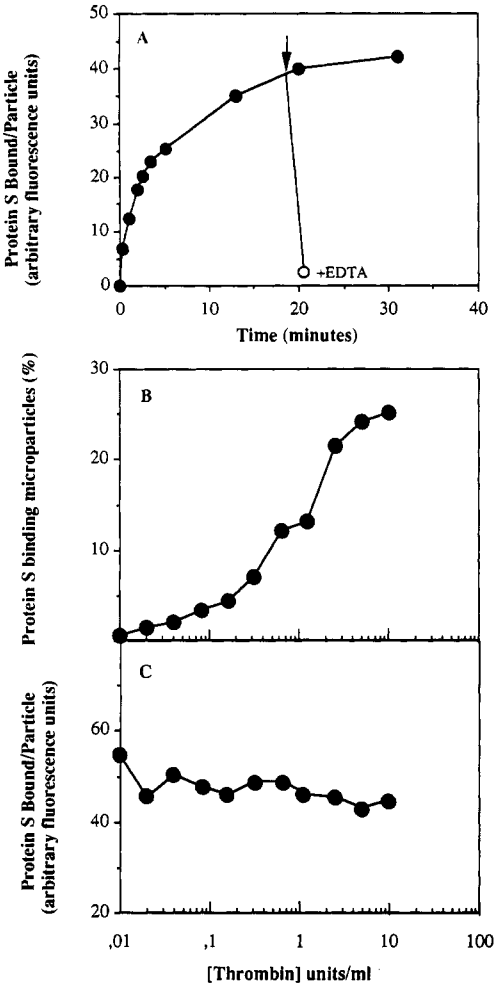


FIGURE 3: Time and thrombin dependence of protein S binding to microparticles. (A) A preincubated mixture of protein S and FITC-labeled Fab' fragments (HPS 56) was added to activated platelets as described in Materials and Methods. At intervals, aliquots were drawn and analyzed in the flow cytometer. The plot represents protein S bound per microparticle (measured as mean fluorescence per particle). The arrow indicate the addition of EDTA (10 mM final concentration) to an aliquot of the reaction mixture. (B and C) Thrombin dependence of microparticle formation and protein S binding. Platelets were incubated with increasing concentrations of thrombin for 10 min at 37 °C and then with protein S (10 μg/mL). Protein S binding to microparticles was monitored with FITC-labeled Fab' fragments of HPS 34. The microparticle gate was defined as detailed in Materials and Methods. In addition, a FL1 gate of 10–150 was used to exclude small particles that did not show any protein S binding. (B) Formation of protein S binding microparticles in relation to thrombin concentration. (C) Protein S binding per particle in relation to thrombin concentration.

the binding. In the absence of added protein S, FITC-labeled monoclonal antibodies HPS 56 and 34 did not bind to microparticles or platelets, demonstrating platelet-derived protein S not to contribute significantly to the observed binding.

The time dependence of protein S binding to the microparticles is illustrated in Figure 3A. At the protein S concentration used, binding reached its maximum after 20–30 min. There was no detectable dissociation of bound protein S when fluorescence was followed after a 100-fold dilution (dilution buffer at room temperature) of the sample (not shown). This was true even when monoclonal antibody HPS 21 was included in the dilution buffer to trap dissociated protein S molecules. This suggested binding of protein S to microparticles not to be a freely reversible equilibrium reaction and that a secondary event occurred after protein S binding, which prevented protein S from dissociating from the membrane.

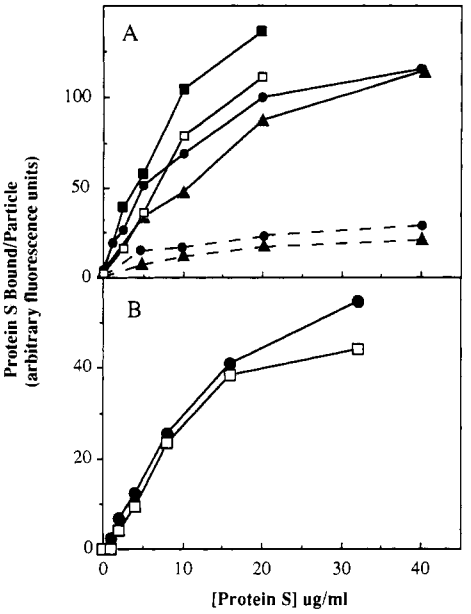


FIGURE 4: Binding of protein S to microparticles and phospholipid vesicles. (A) Platelets were activated with thrombin (■, □, ▲) or with thrombin plus collagen (●) and incubated with increasing concentrations of intact (filled symbols) or thrombin-cleaved (open symbol) protein S followed by FITC-labeled Fab'-HPS 56. Protein S binding was measured in the flow cytometer and expressed as mean fluorescence per particle. Symbols connected with solid lines represent data collected in the microparticle gate, whereas those connected with broken lines were data from the platelet gate. The three experiments using intact protein S were performed on different days, whereas (□), using thrombin-cleaved protein S, was performed in parallel with (■), an experiment using intact protein S. (B) Increasing concentrations of (●) intact or (□) thrombin-cleaved protein S were incubated with multilamellar phospholipid vesicles (20 μg/mL) in solution III containing 1 mM $CaCl_2$ (final volume 50 μL). After 15 min of incubation at room temperature, FITC-labeled Fab'-HPS 56 (5 μL giving a final concentration of 54 μg/mL) was added and after another 15–30 min of incubation, samples were analyzed with flow cytometry.

Rapid (within minutes) dissociation of protein S from the microparticle membrane was observed after addition of EDTA. To determine whether microparticles formed at low and high thrombin concentrations bound protein S with similar characteristics, increasing concentrations of thrombin (0.01–10 units/mL) were used to activate the platelets. With increasing thrombin concentrations, the number of microparticles formed increased and maximum was reached at 5–10 units of thrombin/mL (Figure 3B,C). Particles formed at low and high thrombin concentrations were equally efficient in binding protein S. At none of the thrombin concentrations was protein S binding to the platelet surface observed.

Protein S binding to platelet-derived microparticles approached saturation and maximum binding was reached at approximately 0.4 μM protein S (20–40 μg/mL) (Figure 4A). Thrombin-cleaved protein S bound equally as well as intact protein S. Binding required platelet activation and unstimulated platelets did not bind protein S. Protein S binding was confined to the microparticle population and visual inspection of the primary dot plots (like the one presented in Figure 2) revealed that the small increase in mean FL1 observed in the platelet gate (Figure 4) was due to protein S binding microparticles contaminating the platelet gate. For comparative purposes, binding of intact and thrombin-cleaved protein S to multilamellar phospholipid vesicles (75% phosphatidylcholine, 25% phosphatidylserine) was measured in the flow cytometer (Figure 4B). Intact and thrombin-cleaved protein S bound with characteristics similar to those observed

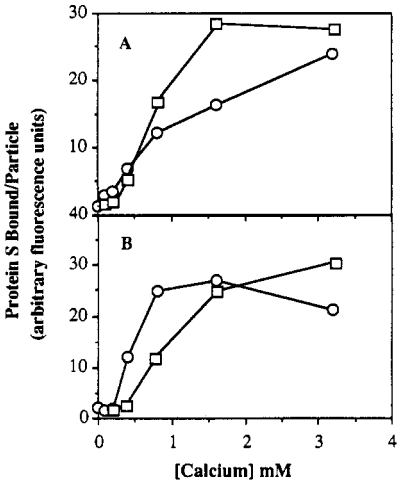


FIGURE 5: Calcium dependence of protein S binding to microparticles and to phospholipid vesicles. (A) Isolated platelets ($1 \times 10^8$/mL) in solution III containing 1 mM calcium were activated with thrombin plus collagen. EDTA was added to a final concentration of 1 mM (dilution buffer was solution III containing 50 $\mu$M EDTA). Binding of intact or thrombin-cleaved protein S (both at final concentrations of 10 $\mu$g/mL) was measured in the presence of increasing concentrations of calcium. FITC-labeled Fab' fragments of HPS 56 were used to monitor protein S binding. (○), intact protein S; (□), thrombin-cleaved protein S. (B) Multilamellar phospholipid vesicles (20 $\mu$g/mL final concentration) were incubated with intact (○) or thrombin-cleaved (□) protein S (10 $\mu$g/mL final concentration) in 50 mM Tris-HCl, 0.15 M NaCl, and 10 $\mu$M EDTA, pH 7.5, containing increasing concentrations of $CaCl_2$. Protein S binding was monitored with FITC-labeled Fab' fragments of HPS 56. Although results presented in this figure were from a single experiment, they were representative of results obtained in several independent experiments.

for protein S binding to microparticles. The binding curves suggested binding to be saturable and maximum binding was seen at approximately 0.4 $\mu$M protein S.

Binding of intact and thrombin-cleaved protein S to microparticles/phospholipid vesicles showed dependence of calcium, and thrombin-cleaved protein S consistently required slightly higher concentrations of the ion (Figure 5). At 2–3 mM calcium, both forms of protein S demonstrated maximum binding.

Most binding experiments were performed using platelets activated by thrombin or by thrombin plus collagen, and results obtained with these two platelet agonists were in good agreement. Due to thrombin's specific proteolytic action on protein S, we considered it essential to compare results obtained with thrombin to platelet activators without such proteolytic activity, and agonists like C5b–9 and the calcium ionophore A 23187 were therefore tested. Although the amount of protein S associated with each microparticle was approximately the same regardless of which agent was used to stimulate the platelets (Figure 6), the various platelet agonists demonstrated different capacities to generate microparticles. After stimulation with thrombin, 25–30% of the particles were gated as microparticles and thrombin plus collagen resulted in 30–35% of the particles in the microparticle gate, whereas C5b–9 and the calcium ionophore were the most potent microparticle inducers and 50–55% of the particles were recovered in the microparticle gate. Thus, even though the number of protein S binding sites per particles appeared to be similar with the different agonists, C5b–9 and the calcium ionophore expressed the greatest total number of binding sites. In a solution of unstimulated platelets, approximately 10% of the particles were found in the microparticle gate. These particles, which probably represented small platelets or platelet fragments, demonstrated no protein S binding ability.

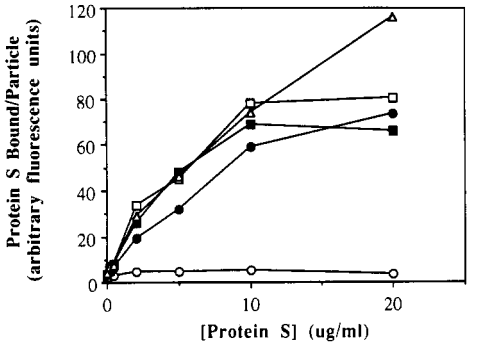


FIGURE 6: Protein S binding to microparticles formed by different agonists. Platelets were activated with the indicated agonist and incubated with increasing concentrations of protein S, followed by FITC-labeled HPS 34. Protein S binding was measured in the flow cytometer. The plot represents protein S bound per microparticle (measured as mean fluorescence per particle). In the control with unstimulated platelets (○), protein S binding was measured using gate settings defining the platelet population. The following agonists were used: (△) C5b-9 and (□) thrombin (1 unit/mL); (●) calcium ionophore A 23187 (1 $\mu$M); (■) thrombin (0.5 unit/mL) plus collagen (10 $\mu$g/mL).

Table I: Protein S Binding to Microparticles in the Presence of Monoclonal Antibodies against Protein S[a]

| | platelet agonist[b] | | | | |
|---|---|---|---|---|---|
| antibody | I A 23187 | II thrombin | III thrombin + collagen | IV C5b-9 | mean (I–IV) |
| HPS 21 | 13 | 6 | 8 | 12 | 10 |
| HPS 67 | 22 | 13 | nd | 25 | 20 |
| HPS 54 | 110 | 69 | 75 | 115 | 92 |

[a] Binding is expressed as percent of binding of controls without monoclonal antibody. [b] Platelets were activated with different agonists and incubated with protein S ($2\ \mu g/mL \approx 2.7 \times 10^{-8}$ M final concentration) and monoclonal antibodies HPS 21, HPS 67, or HPS 54 (each at a concentration of $20\ \mu g/mL \approx 1.3 \times 10^{-7}$ M). The $K_d$'s for the interactions between HPS 21, HPS 67, and HPS 54 and protein S were previously determined to be $8 \times 10^{-10}$ M, $1.3 \times 10^{-10}$ M, and $5.9 \times 10^{-11}$ M, respectively (Dahlbäck et al., 1990). Thus, antibodies were in 5-fold molar excess over protein S and antibody saturation was ensured as the $K_d$ for the protein S–antibody interaction in each case was much lower than the protein S concentration. FITC-labeled HPS 34, which was used to measure protein S binding, did not compete with any of the other monoclonal antibodies for binding to protein S (Dahlbäck et al., 1990).

To test whether other vitamin K-dependent proteins competed with protein S for binding to microparticles, they were included at 10–100-fold molar excess of human protein S. The following proteins were used: bovine prothrombin, bovine factor X, bovine protein S, and bovine protein Z (results not shown). At high concentrations of bovine protein S and protein Z (>10-fold higher than the human protein S concentration) a maximum inhibition of 50% of protein S binding was observed, whereas the other proteins demonstrated no or very little competition with protein S.

*Effects of Monoclonal Antibodies on Protein S Binding.* HPS 21, reacting with a calcium-dependent epitope in the Gla module of protein S, completely inhibited binding of protein S to microparticles (Table I). HPS 67, recognizing a calcium-dependent epitope in the thrombin-sensitive region, was also inhibitory. HPS 54 (epitope in the first or second EGF-like module of protein S) did not affect protein S binding to microparticles formed by C5b–9 or the calcium ionophore, whereas a slight inhibition (around 25–30%) was observed when thrombin or thrombin plus collagen was used as platelet agonist.

*Protein S-Dependent Binding of C4b-Binding Protein to Phospholipid Vesicles but Not to Platelet Microparticles.*

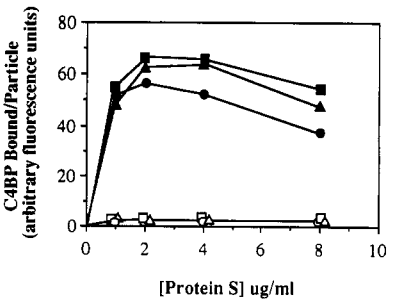


FIGURE 7: Protein S dependent binding of C4BP to phospholipid vesicles but not to platelet microparticles. Three different concentrations of FITC-labeled C4BP were incubated for approximately 15 min with increasing concentrations of protein S, in the presence of multilamellar phospholipid vesicles or thrombin plus collagen-stimulated platelets. The C4BP binding to microparticles and phospholipid vesicles was estimated in the flow cytometer. The C4BP concentrations were (○, ●) 7 μg/mL, (△, ▲), 14 μg/mL, and (□, ■) 28 μg/mL. (○), (△), and (□) represent data obtained in the platelet incubations and (●) (▲), and (■) incubation mixtures with phospholipid vesicles.

Different approaches to demonstrate binding of the protein S–C4BP complex to platelet microparticles and to phospholipid vesicles were tried. These included FITC-labeled monoclonal antibodies (or their Fab′ fragments) against C4BP and FITC labeling of C4BP. Consistently, the protein S–C4BP complex bound to phospholipid vesicles but not to microparticles or platelets (Figure 7). Binding to phospholipid was calcium-dependent and reversed after the addition of EDTA. The lower C4BP binding seen at highest protein S concentrations suggested competition by free and membrane-bound protein S for binding to C4BP. In the absence of protein S, no C4BP binding was observed.

*Protein S-Dependent Binding of Protein C and Activated Protein C to Microparticles.* A monoclonal antibody against human protein C, denoted HPC 8, was labeled with FITC and used to probe the interaction between protein C/APC and platelets. Binding of protein C and APC to platelet microparticles was observed although the mean FL1 signal was lower than that observed for protein S (Figure 8A,B). Protein C/APC binding, which was reversible after addition of EDTA, was confined to microparticles and no interaction with platelets was observed. Binding of protein C/APC depended on the presence of intact protein S as thrombin-cleaved protein S did not support binding. The phospholipid system was also used to study protein C/APC binding. Protein C and APC demonstrated some binding in the absence of protein S but addition of intact protein S potentiated binding and results were similar to those observed for microparticles (results not shown).

Protein S-dependent binding of protein C/APC to microparticles was inhibited by several monoclonal antibodies against human protein S (Figure 8C). Efficient inhibitors included antibodies against the thrombin-sensitive region and the EGF-like module. The effect of antibody HPS 54 (against the first or second EGF-like module) suggested the antibody to interfere with the protein S–APC/protein C interaction on the microparticle surface, as the antibody did not inhibit binding of protein S to any major extent (see above). HPS 34, an antibody which did not significantly inhibit the cofactor function of protein S, demonstrated no inhibitory effect on protein S-dependent binding of protein C or APC.

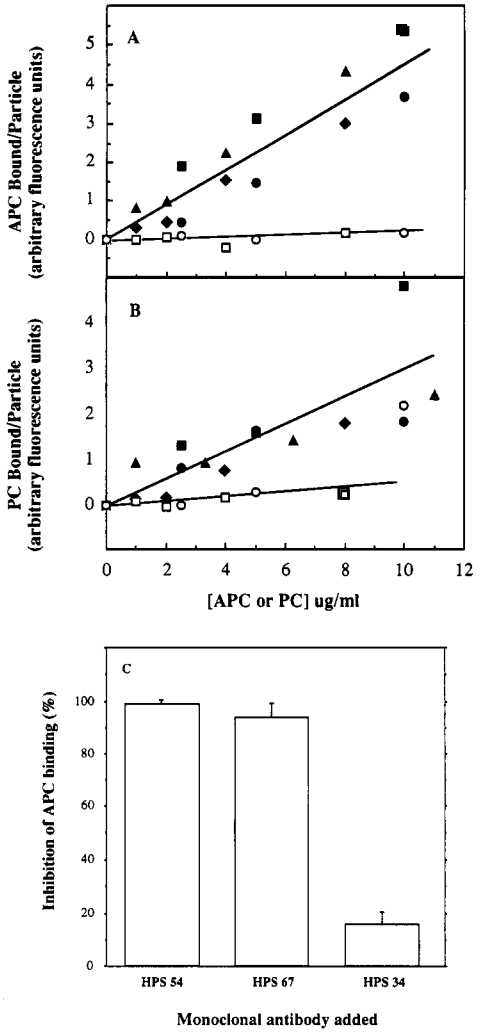


FIGURE 8: Binding of APC and protein C to platelet microparticles. (A and B) Platelets were activated with (○, ●) thrombin or (■, ▲, ◆, □) thrombin plus collagen and incubated with intact (filled symbols) or thrombin-cleaved (open symbols) human protein S (20 μg/mL) and increasing concentrations of APC (A) or protein C (PC) (B). PC and APC binding were measured with FITC-labeled HPC 8. The data were from experiments performed on four different days. (○) and (□) represent experiments using thrombin-cleaved protein S which were done in parallel with experiments using intact protein S, (●) and (■), respectively. (C) Inhibition of binding of APC by monoclonal antibodies against protein S. Activated platelets were incubated with protein S (20 μg/mL), APC (10–20 μg/mL), and the indicated monoclonal antibody against protein S (40 μg/mL). Binding of APC was measured with FITC-labeled HPC 8 and compared with control incubations without monoclonal antibody. To allow comparison of results obtained in separate experiments, data were normalized and presented as percent inhibition of APC binding. The figure represents the mean ± SD of three experiments performed on different days.

## DISCUSSION

APC regulates the platelet-associated prothrombinase complex through degradation of factor Va (Comp & Esmon, 1979; Dahlbäck & Stenflo, 1980) and several reports suggest protein S to play a role as cofactor in this reaction (Suzuki et al., 1984; Harris & Esmon, 1985; Solymoss et al., 1988; Tans et al., 1991). However, in experimental systems using human platelets and purified human proteins, protein S is not strictly required for APC-mediated degradation of factor Va and the addition of protein S only yields a 2-fold increase of the reaction rate (Solymoss et al., 1988; Tans et al., 1991). This contrasts to the distinct APC cofactor effect of protein S observed in plasma coagulation systems and indicates that studies of factor Va degradation in purified systems poorly reflect the physiological situation. A possible alternative mechanism by which protein S elicits its anticoagulant effect

may be through inhibition of the protection exerted by factor Xa on factor Va degradation (Solymoss et al. 1988), but the physiological significance of this remains to be elucidated. Even though the mechanism of action of protein S is unresolved, the importance of protein S as a natural anticoagulant is underscored by the association of thromboembolic disease and protein S deficiency (Clouse & Comp, 1986; Engesser et al., 1987).

Although the potentiating effect of protein S on APC-mediated factor Va degradation in the presence of platelets is small, it was of interest to investigate whether protein S interacts with platelet-derived microparticles and/or with the platelet surface. Our results demonstrate that human protein S binds specifically to microparticles shed from the platelet surface during platelet activation. No binding of protein S to the surface of activated or unactivated platelets could be detected. Despite the fact that protein S has only a small effect on APC-mediated factor Va degradation (Tans et al., 1991; Bakker et al., 1992), it is noteworthy that protein S potentiated binding of APC to microparticles. Apparently, functional assays focusing on factor Va degradation do not reflect the biological significance of protein S binding to microparticles. Our failure to detect protein S and protein C binding to intact platelets, and to detect protein S-independent binding of protein C/APC, may indicate that the number of binding sites for these ligands is below the level of detection by fluorescence flow cytometry. Alternatively, the affinity of the membrane sites expressed for these ligands on the platelet surface may not allow detection of binding by this technique. Thus, due to the necessity for dilution of the sample upon aspiration of particles into the flow cell of the cytometer, our methods may fail to detect bound ligands that exhibit a rapid off rate from the membrane. This conclusion is supported by results of previous studies in which binding of protein C/APC to phospholipid was characterized in the presence and absence of protein S or factor V/Va (Walker, 1988; Krishnaswamy et al., 1986).

The quantitative differences between our data relating to the distribution of binding sites for protein S and APC versus the distribution of APC enzyme activity reported by Tans et al. (1991) may relate to differences in the efficiency of recovery inherent to the methods employed to isolate a "microparticle fraction" for analysis. As Tans et al. (1991) do not report the percent of total microparticles recovered by their methods [differential centrifugation; see discussion in Sims et al. (1988)], it does not seem relevant to compare the results in detail. The distinct and specific binding of protein S to microparticles suggests a biological function and the now-described observations should facilitate elucidation of the biological activity of protein S. Once appropriate functional tests for protein S are available, it will be of interest to compare the effects of microparticles and platelets. However, such studies are complicated not only by the lack of appropriate functional tests but also by the lack of techniques to isolate activated platelets completely devoid of microparticles.

Platelets have been reported to contain protein S (Schwartz et al. 1985), which may be functionally important, although available results are conflicting (Harris & Esmon, 1985; Solymoss et al., 1988; Tans et al. 1991). In a bovine system, platelet protein S is reported to have a distinct effect (Harris & Esmon, 1985), whereas in a human system the function of platelet protein S is still unresolved (Solymoss et al., 1988; Tans et al., 1991). We were unable to detect binding to platelet or microparticles of FITC-labeled monoclonal antibodies against protein S in the absence of exogenous protein S, which suggests that little or no platelet-derived protein S is present on the platelet or microparticle surface after platelet activation.

It was noteworthy that microparticles formed by different agonists expressed similar number of protein S binding sites per particle even though the various agonists demonstrated different capacities to generate microparticles. Each of the agonists used has a fundamentally different effect on the target platelet. Neither thrombin nor collagen is considered to *directly* alter the configuration of membrane phospholipids nor to *directly* increase the permeability of the platelet plasma membrane to $Ca^{2+}$. The effects of these agonists are mediated secondarily to metabolic changes induced through stimulation of specific cell-surface receptors. A $Ca^{2+}$ ionophore, by contrast, is both lipophilic (and, potentially lipolytic) and directly increases the permeability of $Ca^{2+}$ across all platelet membranes, internal and external. By contrast, the C5b–9 complex—which is also lipophilic, potentially lipolytic, and membrane pore-forming in its properties—has its site of action restricted exclusively to the plasma membrane. These complement proteins have also been shown to directly promote transbilayer exchange of membrane phospholipids (Van der Meer et al., 1989).

The nature of the protein S binding sites on microparticles is unknown. In the case of prothrombinase and Xase complexes, it has been proposed that negatively charged phospholipids on the surface of microparticles may serve as binding sites for these enzyme complexes (Sims et al., 1989; Gilbert et al., 1991). In the now-presented study, protein S was found to bind to multilamellar phospholipid vesicles with characteristics similar to those observed for platelet microparticles, suggesting that negatively charged phospholipid constitutes an important component of the protein S binding site on microparticles. However, with regard to binding of the C4BP–protein S complex, different results were obtained with microparticles and phospholipid vesicles. Even though binding of the protein S–C4BP complex to phospholipid vesicles could easily be demonstrated, binding of the complex to microparticles was not observed. The binding of the protein S–C4BP complex to phospholipid vesicles agreed with results on record (Schwalbe et al., 1990). One possible explanation for the inability of the protein S–C4BP complex to bind to microparticles may be that microparticles contain multiple bulky membrane components which may interfere with binding of the protein S–C4BP complex but not with protein S. The difference between microparticles and phospholipid vesicles, with respect to binding of the protein S–C4BP complex, may have functional implications. It was recently suggested that the protein S–C4BP complex competitively inhibits the APC cofactor function of free protein S, which may contribute to low protein S activity in patients with low levels of free protein S (Nishioka & Suzuki, 1990). Phospholipid vesicles were used in those experiments and it is possible that, on the microparticle surface, the protein S–C4BP complex does not have a similar competitive function.

Thrombin-cleaved protein S did not support the binding of APC/protein C even though thrombin-cleaved protein S bound to microparticles under the conditions used. This suggested the thrombin-sensitive region of protein S to be crucial for the protein S–APC interaction. Inhibition of APC/protein C binding by monoclonal antibodies to the thrombin-sensitive region and to the EGF-like modules of protein S supports this concept. The first EGF-like module of APC has been suggested to interact with protein S (Öhlin et al., 1988). Protein S is an asymmetricl molecule and binds perpendicular to the phospholipid surface (Schwalbe et al., 1990). If this is also

true for APC/protein C, the regions in protein S and APC/protein C that are suggested to be involved in direct protein–protein interactions are located at similar distances from the membrane. Protein S supported binding of the zymogen form of protein C to microparticles, suggesting the regions in protein C which interact with protein S not to change conformation upon activation.

Several coagulation proteins, including factors Va and VIIIa, have been shown to bind to microparticles, and it has been suggested that microparticles from the major catalytic surface for platelet prothrombinase and Xase (Sims et al., 1988, 1989; Gilbert et al., 1991; Comfurius et al., 1990). However, factors Va and VIIIa also bind to the surface of the remnant activated platelets. Protein S is unique in specifically binding to microparticles. What structures on microparticles determine the specificity of protein S binding remains to be determined. The now-described binding of protein S to the microparticle surface will help provide the basis for future elucidation of the physiological role of microparticles and help determine whether they primarily have anti- or procoagulant functions.

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**Registry No.** APC, 42617-41-4; $Ca^{2+}$, 7440-70-2; C5b-9, 82986-89-8; protein C, 60202-16-6; thrombin, 9002-04-4.