Previous studies have demonstrated that protein S is required for optimal activated protein C-mediated inactivation of Factor Va on the surface of either the platelet or phospholipid vesicles. In this report, we demonstrate assembly of the activated protein C-protein S complex on the surface of cultured bovine aortic endothelial cells. Endothelial cell surface acceleration of Factor Va inactivation by activated protein C required the presence of protein S. Kinetic studies indicated that the rate of Factor Va inactivation was half-maximal at a protein S concentration of 0.2 nM and an activated protein C concentration of 0.05 nM. Binding of 125I-activated protein C to endothelial cell monolayers was absolutely dependent on the presence of protein S. At saturating levels of protein S, activated protein C binding was saturable with $K_a = 0.04$ nM. In contrast, specific, time-dependent, and saturable binding of 125I-protein S to endothelial cell occurred in the absence of activated protein C. Addition of activated protein C increased the affinity of protein S from $K_a = 11$ nM to 0.2 nM, but did not change the number of molecules bound per cell at saturation (85,000 molecules/cell). These studies suggest that activated protein C increases the affinity of protein S for pre-existing sites on the endothelial cell surface. The close correlation between the parameters of protein S-activated protein C binding to endothelium and Factor Va inactivation supports the concept that it is bound protein S and activated protein C that are the active species. Formation of functional activated protein C-protein S complexes thus occurs effectively on the endothelial cell surface and represents a new addition to the list of vessel wall anticoagulant properties.

Protein C and protein S are two regulatory plasma coagulation proteins (reviewed in Refs. 1 and 2). After activation, protein C is a potent anticoagulant enzyme capable of inactivating factors Va and VIIIa, thereby blocking activation of the coagulation system (3-6). Activated protein C (APC)

requires the presence of negatively charged phospholipid surfaces (3, 4) and protein S (7) for optimal anticoagulant activity. Protein S has been shown to function as a nonenzymatic cofactor for the binding of APC to phospholipid surfaces by forming a complex with the enzyme (8, 9). This complex has enhanced affinity for membrane surfaces (8, 9). A recent study has demonstrated assembly of the APC-protein S complex on the platelet surface with subsequent acceleration of Factor Va inactivation (10).

As the cells forming the luminal vascular surface, endothelial cells are strategically located to play an important role in the regulation of coagulation. Anticoagulant properties of endothelium include the presence of anticoagulant heparin-like molecules (11-13) and thrombomodulin (14) on the cell surface. Elaboration of tissue plasminogen activator (15) and prostacyclin (16) also contributes to the antithrombotic nature of endothelium. In addition, endothelium has recently been shown to participate in a series of procoagulant reactions (17-21) including Factor IX, VIII-mediated Factor X activation (17-19) and Factor Xa-VIIIa-mediated prothrombin activation (18, 21). This suggests that an effective vessel wall anticoagulant mechanism would involve inactivation of the cofactors, Factors Va and VIIIa, which play an integral role in the activation of coagulation. These considerations prompted us to examine the interaction of APC and protein S with endothelium. The results indicate that cultured endothelial cells can provide a surface capable of assembling the protein S-APC complex, thereby promoting Factor Va inactivation.

**Experimental Procedures**

Protein Purification and Radiolabeling—All purified coagulation factors were of bovine origin. Purification of protein S was carried out as described (7). Protein S (0.9 mg/ml) in 2 mM Tris (pH 7.5), 0.1 M NaCl was inactivated (9) by treatment with 5% purified bovine $\alpha$-thrombin (22) (w/w) for 4 h at 37°C. The pH of the reaction mixture was then adjusted to 6.0 using MES, and thrombin was removed by chromatography on sulfopropyl-Sephadex (bed volume, 5 ml) (Pharmacia). Reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23) demonstrated complete conversion of protein S to its thrombin-cleaved form (9). Thrombin-cleaved protein S had no anticoagulant activity in the protein S functional assay described below. Radiolabeling of protein S was accomplished by the lactoperoxidase method (24), using Enzyme-Units according to the manufacturer's instructions. The reaction was carried out at room temperature for 15 min by incubating Enzyme-Units (Bio-Rad) (50 µl), protein S (40 µl; 29 µg), Na[I] (2 mCi), and glucose (2 µl). Free iodine was separated from protein S by gel filtration using a column (1 X 20 cm) of Sephadex G-25. The specific radiactivity of 125I-protein S was 5,000-12,000 cpm/µg (corresponding to approximately 0.1 mol of [125I] per mol of protein S) over five radioiodinations. Radioiodinated protein S co-migrated with unlabeled material on SDS-PAGE. Protein S anticoagulant activity (see below) was not affected by the radiolabeling procedure.

Protein S anticoagulant activity was assayed (25) using oxalated bovine plasma previously adsorbed with barium sulfate (50 mg/ml)
and supplemented with prothrombin (28) (150 µg/ml). This plasma (0.1 ml) was mixed with purified Factor Xa (27) (the concentration of Factor Xa was selected to give a clotting time of 30 s), activated protein C (2 µg/ml; 10 µl), cephalin (0.1 ml; Ortho Pharmaceutical), and varying concentrations of protein S and CaCl2 (25 µM; 0.1 ml) at 37°C.

Protein C was purified as described previously (4) and was activated by incubation with 5% thrombin (w/w) for 3 h at 37°C in 1 mM Tris (pH 7.4), 0.1 M NaCl. The reaction mixture was adjusted to pH 6.5 with MES and chromatographed on QAE-Sephadex (0.6 x 5 cm) equilibrated with MES, 0.1 M NaCl. Activated protein C was eluted using a 0.1-0.6 M linear salt gradient (5 ml/reservoir). Radio labeling of protein C was accomplished by the method of Bolton and Hunter (28) using N-succinimidyl-3-(4-hydroxy-5-['~I]iodophenylpropionate (Amersham Corp.) with a specific activity of 1950 Ci/mmol. After evaporation to dryness, Bolton-Hunter reagent (2 ml) was incubated with protein C (50 µg) in borate buffer (0.1 M, pH 8.8) for 20 min at 4°C. The reaction mixture was chromatographed on a Sephadex G-25 column (0.9 x 20 cm) (1 mM Tris (pH 7.5), 0.1 M NaCl, 0.2% gelatin) to remove excess reagent. The radiolabeled preparation was activated as described for unlabeled protein C. The pass-through fractions containing APC from the sulfopropyl-Sepharose column were pooled and applied to a 6-aminobenzamidine Affigel column (bed volume, 2 ml) prepared by the method of Guinto (29). After washing the column with 10 bed volumes of 0.02 M Tris/HCl (pH 7.5), 0.1 M NaCl, the column was eluted with the same buffer containing 1 M benzamidine hydrochloride. The peak fractions were eluted and those fractions that were determined to be 64-65% pure were pooled and dialyzed for 3 days against three changes of 0.02 M Tris (pH 7.5), 0.1 M NaCl (11/dialysis) at 4°C. SDS-PAGE demonstrated that this material co-migrated with unlabeled APC and had a specific radioactivity of 2.2-3.5 x 10^7 cpm/ng (corresponding to approximately 0.25 mol of 131I/mol of APC). The radiolabeling procedure had no effect on APC anticoagulant activity measured in a clotting assay as described (25).

Factor Va was purified as described (30), and the preparations used in this study were recombined from isolated subunits in the presence of 10 mM CaCl2 overnight at 4°C. Factors IX (260 units/mg) and X (100 units/mg) and prothrombin (13 units/mg) were purified as described (28, 31-32). Factor IX was activated by incubation with Factor XI, bound to CH-Sepharose as described (18), and Factor X was activated by incubation with the coagulant protein from Russell’s viper venom (33) coupled to CH-Sepharose as described (18). Burro anti-bovine Factor V IgG (34) was generously provided by Dr. Tracy and Mann (University of Vermont, Burlington, VT). Nonimmune burro IgG was prepared by standard methods (35).

Protein concentrations were determined colorimetrically (36). SDS-PAGE was carried out as described (24), and slabs gels were dried (Bio-Rad slab gel drier, model 224) and processed for autoradiography using Kodak X-Omat (XAR 5) film. A Cromogen intensifying screen (DuPont) was used and exposure times were 24 h. Standard curves were constructed using purified Factor Va, and all clotting times were done in duplicate or triplicate. The rate of Factor V inactivation was determined from the slope of the linear initial portion of a plot of Factor V activity versus incubation time and generally included the 10-, 30-, 60-, and 120-s points.

Binding Studies—Binding studies were carried out after washing endothelial cells twice with calcium/magnesium-free Hanks’ balanced salt solution containing dextran sulfate. Incubation buffer containing radiolabeled proteins alone or in the presence of other proteins, as indicated, was then added. APC binding studies were carried out in a final volume of 1 ml of 3.6-cm2 wells. Protein S binding studies employed a final volume of 0.1 ml in 0.32-cm2 wells. When anti-bovine Factor V IgG or nonimmune IgG was used, endothelial cells were preincubated with the antibody preparation in incubation buffer for 45 min at 25°C and washed twice with ice-cold incubation buffer, and then coagulation proteins were added. Binding assays were carried out at 15°C for the indicated times and were terminated by three rapid washes (0.1 ml/wash) over 3 s with incubation buffer (4°C). Cells were solubilized with 0.2 N NaOH, 1% SDS, and 10 mM EDTA. No binding was observed in wells without cells. Data from binding experiments were fit to the equilibrium binding equation:

\[ B = \frac{nK}{1 + KA} \]

(40) assuming a one-site model, where \( B \) is the amount bound, \( n \) is the number of sites/cell, \( A \) is the free concentration of radioligand, and \( K \) is the association constant. A nonlinear least squares program (SAS Institute, Cary, NC) was used to obtain the best fit curve, to solve for \( n \) and \( K \), and to determine the S.E. A plot of residual versus free radioligand for the binding data shown in Figs. 5A and 7B indicated that no systematic error was involved in fitting the binding to the model used (data not shown).

Dissociation studies were carried out using the method of infinite dilution (Fig. 3B) as described by Lollar et al. (41): after endothelial cell monolayers were incubated with 125I-protein S and washed free of unbound protein, fresh incubation buffer was added (0.1 ml). At the indicated times, incubation buffer was aspirated, and the wells were washed twice (0.2 ml/wash) with the same buffer and solubilized as described above. Dissociation of cell-bound 125I-protein S was also studied by adding excess unlabeled protein S to the incubation mixture (Fig. 3B).

**RESULTS**

When Factor Va and APC are incubated with cultured bovine aortic endothelial cells, rapid Factor Va inactivation is dependent on the presence of protein S (Fig. 1). Since these concentrations of APC (1 nM) and protein S (2 nM) do not,
under comparable conditions, result in effective Factor V* (70 nM) inactivation in a test tube without cells, the results in Fig. 1 indicate that the endothelial cell surface promotes APC-protein S-mediated Factor V* inactivation. Addition of thrombin-treated protein S in place of native protein S did not promote APC-mediated Factor V* inactivation. Thus, cleaved protein S, which has been reported to have no anticoagulant activity (9), does not substitute for protein S in this system.

To better characterize the involvement of endothelial cells in this reaction, the dependence of the rate of Factor V* inactivation on the concentration of both APC and protein S was examined. Endothelial cell-dependent enhancement of Factor V* inactivation was saturable with respect to APC (Fig. 2A) with half-maximal rates at an APC concentration of 0.05 ± 0.01 nM. The enzyme system was also saturable with respect to protein S (Fig. 2B) with half-maximal Factor V* inactivation rates occurring at a protein S concentration of 0.20 ± 0.04 nM. This is considerably below the plasma concentration of protein S (approximately 100 nM).

These data suggest that a limited number of cellular binding sites might mediate the interaction of protein S and APC with endothelium. The binding of 125I-protein S to endothelial cell monolayers occurred in a time-dependent manner (Fig. 3A). The second-order rate constant for association calculated from the data in Fig. 3A, was approximately 10^6 min^−1 M^−1. The concentration of binding sites used in this calculation was taken from the data in Fig. 5A (see below). Even at the lowest concentrations of 125I-protein S employed, binding reached an apparent maximum by 45 min (Fig. 3A). Dissociation studies indicated that the interaction of 125I-protein S and endothelial cells was reversible with a first-order dissociation constant of 8.6 × 10^−3 min^−1 (Fig. 3B). A similar dissociation rate was observed whether elution of cell-bound protein S was studied by the method of infinite dilution or in the presence of excess unlabeled protein S. Since this dissociation rate is quite slow, requiring about 4 h for 90% of the specifically bound 125I-protein S to dissociate, binding studies were carried out at 15 °C to prevent internalization of surface-bound protein S. Under these conditions, addition of dextran sulfate (10 mg/ml) effected rapid elution of cell-bound 125I-protein S (Fig. 3B, ×) even at later times, indicating that protein S was present on the cell surface. Dextran sulfate did not result in endothelial cell detachment or loss of viability.

SDS-PAGE of the pool of dextran sulfate-elutable 125I-protein S indicated that it co-migrated with the initial tracer prior to incubation with endothelial cells (Fig. 4). 125I-protein S binding to endothelial monolayers was facilitated by calcium, being maximal at 2-3 mM.

Employing these conditions for equilibrium binding, the association of 125I-protein S with endothelial cell monolayers was observed to be saturable in the absence of other coagulation factors (Fig. 5A, C). Binding was half-maximal at 11 ± 1 nM, and at saturation there were 8.5 ± 1.2 × 10^4 molecules bound per cell. Although not required for protein S binding, activated protein C enhanced the affinity of protein S for the endothelial cell-binding sites (Fig. 5A, ×). Consistent with this enhanced protein S-endothelial cell interaction observed in the presence of APC, the time course of 125I-protein S-endothelial cell binding was accelerated approximately 10-fold (Fig. 3A, ×) when APC was added. Optimal 125I-protein S binding occurred at or above an activated protein C concentration of 0.5 nM (Fig. 5B). At saturating levels of activated protein C, the affinity of 125I-protein S binding increased from 11 ± 1 to 0.2 ± 0.03 nM, although the number of sites was unchanged. Scatchard analysis (Fig. 5B, insets 1 and 2) clearly demonstrated this change in the affinity of protein S binding in the presence of APC.

Binding of 125I-protein S to endothelial cells was not subject to competitive inhibition by other vitamin K-dependent coagulation factors including Factors IX, IX, and X and prothrombin (Fig. 6). Unlabeled protein S, however, was an effective inhibitor. Pretreatment of endothelial cells with antibody to bovine Factor V (10-400 µg/ml) had no effect on 125I-protein S binding, suggesting that endothelial cell Factor V (42) may not be involved in the binding site. Furthermore, addition of Factor V* (6.5-200 nM) had no effect on the
binding of 125I-protein S or 125I-APC (see below) when the experiments were not carried out as described for Figs. 5 and 7. Thus, in contrast to the substrate-dependent enhancement of enzyme binding observed in the tissue factor pathway (43) and formation of the intrinsic Factor X activation complex on the endothelial cell surface (19), the substrate did not enhance enzyme or cofactor binding in this system.

To further characterize the nature of these endothelial cell sites, APC-endothelial cell interaction was examined. Binding of 125I-APC to endothelial cell monolayer was absolutely dependent on the presence of protein S (Fig. 7A). In the absence of protein S, no specific binding was observed. In the presence of protein S, 125I-APC binding occurred, being optimal at a protein S concentration greater than 1.0 nM. The low amount of specifically bound 125I-APC precluded detailed definition of APC-endothelial cell binding parameters even in the 9.6-cm² wells. However, 125I-APC binding was observed to be reversible following the addition of a 100-fold molar excess of unlabelled APC. Furthermore, even at the lowest APC concentrations employed in these studies, 125I-APC binding reached an apparent maximum by 45 min. At saturating levels of protein S, 125I-APC bound in a high affinity fashion to the monolayers with $K_d = 0.04 \pm 0.005$ nM and 180 ± 15 molecules bound per cell at saturation (Fig. 7B). Scatchard analysis (45) (Fig. 7B, inset) of the binding data was consistent with a single class of binding sites.

It is apparent from the binding data that the observed ratio of cell-bound protein S (Fig. 5A) exceeds bound activated protein C (Fig. 7B) by approximately 400-fold. If one expects a stoichiometry of 1:1 for the protein S-activated protein C complex, as suggested by the results of experiments employing synthetic phospholipids (8), then the number of activated protein C-binding sites should be equal to the number of protein S-binding sites. The results of the cellular binding studies in Figs. 5 and 7 would then be difficult to explain. If the problem was technical and related entirely to radiolabeling, then one might expect the total number of activated protein-binding sites to equal the number of protein S sites. This led us to carry out pilot studies employing radiolabeled protein S and activated protein C prepared by different methods. Radiolabeled protein S prepared by tritiation of the sialic acid residues (44) is uniformly modified and had a similar affinity and number of cellular binding sites compared with 125I-protein S (Fig. 5). Activated protein C was also radiolabeled by additional methods. Studies with radiiodinated activated protein C prepared by the lactoperoxidase method (23) indicated an approximate $K_d$ of 0.05 nM and 120 sites/cell. Further studies with tritiated activated protein C (50–100 cpm) (10) demonstrated less than 40 cpm bound per 10⁶ cells (corresponding to less than 4,000 sites/cell). This is far less than anticipated if the number of activated protein C-binding sites equaled the number of protein S sites (85,000/cell). The latter would correspond to approximately 800 cpm of 3H-activated protein C bound per 10⁶ cells. Thus, the binding of activated protein C labeled by three different methods involving three different sites on the molecule consistently yields low numbers of cellular sites, suggesting that there are many more protein S- than activated protein C-binding sites.

**DISCUSSION**

The physiologic significance of the protein C-protein S system in vivo is implied by the thrombotic diathesis observed in kindreds with deficiencies of either protein (25, 46–48). Since formation of functional activated protein C-protein S complex requires assembly on membrane surfaces, this indicates the potentially central role that cellular surfaces can play as modulators of this anticoagulant mechanism. The results reported here suggest that the endothelial cell can provide a surface for assembly of the activated protein C-protein S complex. The relatively high affinity of activated protein C for the endothelial cell surface suggests that complex formation of activated protein C with protein S should occur on the vessel wall in response to concentrations of activated protein C formed in vivo as predicted from the clinical studies of Bauer et al. (49). Particularly in the microcirculation, where a high surface to volume ratio exists, the vessel wall protein S-activated C system should provide an effective clearance pathway for circulating Factor V, and, presumably, by analogy, Factor VIII.

Compared with previous data from studies on the platelet surface (10) and synthetic phospholipids (8), activated protein C appears to have a considerably higher affinity for the endothelial cell surface in the presence of protein S (11 nM for activated protein C and 14 nM for the activated protein C-protein S complex on the platelet surface and phospholipid, respectively, *versus* 0.05 nM on the endothelial cell surface). This indicates that the activated protein C-protein S complex
Activated Protein C-Protein S and Endothelium

FIG. 5. The binding of \(^{125}\)I-protein S to bovine aortic endothelial cells: the effect of APC. A, endothelial cell monolayers were incubated for 45 min with the indicated concentrations of \(^{125}\)I-protein S alone (total binding) or \(^{125}\)I-protein S and a 200-fold molar excess of unlabeled protein S (nonspecific binding) in the presence (•) or absence (○) of APC (2 nM). The binding assay was carried out as described under "Experimental Procedures." Specific binding (total minus nonspecific binding) is plotted versus free \(^{125}\)I-protein S. Data were analyzed by the nonlinear least squares program, and the curve (-) indicates the best fit line. Nonspecific binding accounted for 16–21% of the total binding. Insets, Scatchard analysis of the same data: inset 1, \(^{125}\)I-protein S binding in the presence of APC; inset 2, \(^{125}\)I-protein S binding in the absence of APC. B/F, bound/free; B, bound. B, endothelial cells were incubated with \(^{125}\)I-protein S (0.2 nM) in the presence of the indicated concentrations of APC. The mean and S.E. of specific \(^{125}\)I-protein S binding are plotted versus added APC.

Fig. 6. Competitive \(^{125}\)I-protein S-endothelial cell binding studies. Endothelial cell monolayers were incubated for 40 min with \(^{125}\)I-protein S (8.8 nM) and the indicated concentrations of the following unlabeled proteins: protein S (■), Factor IX (Δ), Factor IXα (▲), Factor X (○), Factor Va (■■■■), and prothrombin (○). Maximal specific binding, the difference between the binding observed in wells incubated with \(^{125}\)I-protein S alone and \(^{125}\)I-protein S in the presence of a 250-fold excess of unlabeled protein S, was 2.8 fmol/well. Per cent maximal specific binding, the mean of duplicates, is plotted versus the concentration of unlabeled protein added.

Fig. 7. The binding of \(^{125}\)I-activated protein C to bovine aortic endothelial cells: the effect of protein S. A, endothelial cells were incubated for 45 min with the indicated concentrations of protein S and \(^{125}\)I-APC alone (0.1 nM) (total binding) or \(^{125}\)I-APC in the presence of a 200-fold molar excess of unlabeled APC (nonspecific binding). The mean and S.E. of specific \(^{125}\)I-APC binding (total minus nonspecific binding) are plotted versus added protein S concentration. The protocol for binding studies is described under "Experimental Procedures." Nonspecific binding accounted for 22–25% of the total binding. B, endothelial cells were incubated for 45 min with protein S (2 nM) and the indicated concentrations of \(^{125}\)I-APC alone or \(^{125}\)I-APC in the presence of a 200-fold molar excess of unlabeled APC as described in A above. An incubation time of 45 min was sufficient to allow \(^{125}\)I-APC binding to reach an apparent maximum at the lowest concentrations used. Specific binding is plotted versus the amount of free \(^{125}\)I-APC. Data were analyzed by the nonlinear least squares program, and the curve (-) indicates the best fit line. Inset, Scatchard analysis of the same data. B/F, bound/free; B, bound.

The effective assembly of activated protein C and protein S on the endothelial cell surface with subsequent expression of activated protein C anticoagulant indicates that this mechanism may be an important component of the antithrombotic nature of the vessel wall.
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