Platelet factor 4 (PF4) is an abundant platelet α-granule heparin-binding protein. We have previously shown that PF4 accelerates up to 25-fold the proteolytic conversion of protein C to activated protein C by the thrombin-thrombomodulin complex by increasing its affinity for protein C 30-fold. This stimulatory effect requires presence of the γ-carboxyglutamic acid (Gla) domain in protein C and is enhanced by the presence of a chondroitin sulfate glycosaminoglycan (GAG) domain on thrombomodulin. We hypothesized that cationic PF4 binds to both protein C and thrombomodulin through these anionic domains. Qualitative SDS-polyacrylamide gel electrophoresis analysis of avidin extracts of solutions containing biotinylated PF4 and candidate ligands shows that PF4 binds to GAG⁺ but not GAG⁻ forms of thrombomodulin and native but not Gla-domainless protein C. Quantitative analysis using the surface plasmon resonance-based BIACore™ biosensor system confirms the extremely high affinity of PF4 for heparin ($K_d = 4 \text{ nm}$) and shows that PF4 binds to GAG⁺ thrombomodulin with a $K_d$ of 31 nm and to protein C with a $K_d$ of 0.37 μm. In contrast, PF4 had no measurable interaction with GAG⁻ thrombomodulin or Gla-domainless protein C. Western blot analysis of normal human plasma extracted with biotinylated PF4 demonstrates PF4 binding to protein C in a physiologic context. Thus, PF4 binds with relative specificity and high affinity to the GAG⁻ domain of thrombomodulin and the Gla domain of protein C. These interactions may enhance the affinity of the thrombin-thrombomodulin complex for protein C and thereby promote the generation of activated protein C.

Thrombomodulin is a critical mediator of endothelial anticoagulant defenses (1, 2). This 575 amino acid protein, expressed on endothelial cell surfaces, binds to thrombin with high affinity ($K_d = 0.5 \text{ nm}$) and modulates its procoagulant activity by (a) altering thrombin substrate specificity so that proteolytic activation of fibrinogen and factor V and platelet activation by thrombin is abrogated, (b) increasing inactivation of bound thrombin by antithrombin III and, perhaps most importantly, (c) accelerating up to 20,000 times the activation by thrombin of protein C to activated protein C (APC)¹ (1, 2). APC is a potent anticoagulant, which, together with its cofactor protein S, attenuates clotting by proteolytic inactivation of clotting factors Va and VIIIa (1, 2). The importance of the thrombin/thrombomodulin-protein C system is shown by the lethal thrombomobilism that occurs in newborns with severe homozygous protein C deficiency (3), the arterial and venous thrombotic diathesis in individuals heterozygous for protein C deficiency (4), and the elucidation of resistance to the anticoagulant action of APC due to a point mutation of factor V as the single most common cause of thrombophilia in young adults (5, 6).

Thrombomodulin is strongly anionic (pI = 4) (7), primarily due to the post-translational addition of variable amounts of an O-linked, chondroitin sulfate glycosaminoglycan (GAG) (8, 9). Several cationic substances, including polybrene, poly-L-lysine, histidine-rich glycoprotein, and S-protein have been reported to interact with thrombomodulin and interfere with its anticoagulant activity (10–12). We previously found that highly cationic protein (pI > 11) eosinophil-specific granule proteins known to accumulate on endothelial surfaces in various hypereosinophilic conditions (13) also strongly inhibit thrombomodulin anticoagulant function (14). This suggests a mechanism for the prominent thrombomobilism that accompanies hypereosinophilic syndromes (15).

In considering other proteins that might interact with thrombomodulin on a more physiologically relevant basis, we examined platelet factor 4 (PF4). PF4 is a cationic (pI = 7.8) tetramer composed of four identical 70-amino acid 7.8-kDa chains (16–18). PF4, together with β-thromboglobulin and thrombospordin, comprise the major constituents of platelet α-granules that are released from platelets physiologically activated by thrombin (16–17). Perhaps the most salient feature of this abundant protein ($20 \mu g/10^9$ platelets) is its high affinity for heparin, requiring 1.5 mM NaCl to dislodge it from heparin-Sepharose (19, 20). Because of this strong interaction, PF4 prevents complex formation between heparin and antithrombin III, thus diminishing the heparin-dependent acceleration of thrombin inactivation by antithrombin III (21). This, along with the fact that PF4 increases platelet aggregation and degranulation initiated by other agonists (22), suggests PF4 functions as a procoagulant. On the other hand, PF4 inhibits factor XII activation (23, 24) during contact activation of plasmatic coagulation, suggesting an anticoagulant function.

¹The abbreviations used are: APC, activated protein C; GAG, glycosaminoglycan; PF4, platelet factor 4; bPF4, biotinylated PF4; BSA, bovine serum albumin; CHO, Chinese hamster ovary; RU, resonance units; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis.
Platelet Factor 4 Binding to Protein C and Thrombomodulin

We found that PF4, in contrast to other cationic proteins, unexpectedly accelerates up to 25-fold APC generation by the thrombin-thrombomodulin complex (25). This increase in APC formation is attributable to a 30-fold decrease in the $K_m$ for protein C of APC generation by the thrombin-thrombomodulin complex that occurs only in the presence of protein C containing a $\gamma$-carboxyglutamic acid (Gla) domain. PF4 stimulation of APC generation is far more prominent with the glycanated (GAG+) form of thrombomodulin as opposed to the GAG$^-$ form (a 25-fold versus a 4-fold increase, respectively). Moreover, PF4, as does Ca$^{2+}$ (26), induces a change in native protein C but not Gla-domainless protein C autofluorescence emission (25). These data suggest that PF4 induces conformational changes in protein C by interacting with its Gla domain.

To explain these observations, we hypothesize that cationic PF4 forms an electrostatic complex with both the anionic Gla domain of protein C and the anionic GAG domain of thrombomodulin. In combination, these interactions might function to enhance the affinity of protein C for the thrombin-thrombomodulin complex and thereby promote the catalytic generation of APC. To test these hypotheses, we employed qualitative analysis of potential thrombomodulin:PF4 and protein C:PF4 complex formation by analyzing avidin extracts of specimens containing biotinylated PF4 (bPF4) and putative ligands. We also performed quantitative analysis using surface plasmon resonance-based BlAcore™ technology to confirm the presence of any detectable binding interactions and measure their avidity as a gauge of their potential biologic significance.

EXPERIMENTAL PROCEDURES

Materials

Differentially glycanated polypeptide isoforms of a recombinantly produced extracellular domain human thrombomodulin were generated by Chinese hamster ovary (CHO) cells transfected with a pRC/CMV vector containing the sequence for the entire extracellular thrombomodulin domain (amino acids 1–497), as previously described (27, 28). CHO cells express two forms of the thrombomodulin polypeptide separable by anion exchange chromatography. The first has a molecular mass of 116–120 kDa and contains a chondroitinase ABC-cleavable GAG moiety (GAG$^+$ CHO thrombomodulin); the second is unglycanated (GAG$^-$ CHO thrombomodulin) with a mass of 95 kDa. A second glycanated recombinant extracellular form of thrombomodulin was obtained by transfection of the human melanoma cell line CHL with the same thrombomodulin DNA construct as described above. This thrombomodulin form, henceforth referred as GAG$^+$ CHO thrombomodulin, utilizes both the Ser$^\text{Thr}$ and Ser$^\text{Gly}$ O-linked glycanation sites (29) and has therefore an even more heavily glycanated than GAG$^+$ CHO thrombomodulin, with a resulting apparent molecular mass of 200 kDa as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Upon digestion with chondroitinase ABC, both GAG$^+$ CHO and GAG$^+$ CHL thrombomodulin collapse to form a single sharp band with an apparent molecular mass of 95 kDa. Bovine protein C was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Human protein C was isolated from human plasma, and Gla-domainless protein C was prepared by digestion of native protein C with chymotrypsin as described previously (49) and generously provided by N. L. Esmon, Oklahoma Medical Research Foundation (Oklahoma City, OK). Human PF4 was isolated from units of outdated platelet packs as described previously (30, 31) utilizing heparin-agarose affinity chromatography, with the final stage purification achieved by Sephadex G-100 chromatography as described previously by Deuel et al. (18). The purified PF4 migrated as a single band of apparent molecular mass 9,000 Da on overloaded SDS-PAGE gels. bPF4 was kindly provided by C. K. Lai and Ted Maione, Repligen Corporation (Cambridge, MA). PF4 was selectively biotinylated, targeting its three N-terminal glutamate residues (which are not known to be involved in heparin binding) by exposing the native protein with biotin hydrazide in a mildly acidic (pH 5.0–5.4) environment. The resulting bPF4 had 0.92 mol of biotin/1 mol of PF4 monomer and had N-terminal biotinylation confirmed by N-terminal sequencing. Further characterization of this reagent showed preservation of three key native PF4 properties: (a) formation of tetramers at physiologic ionic strength as judged by molecular sieving chromatography mobility, (b) binding to heparin with an affinity similar to that of native PF4 as indicated by its also requiring 1.4–1.5 M NaCl to dislodge it from heparin-Sepharose, and (c) biologic activity with regard to inhibition of endothelial cell proliferation identical to that of native PF4 with an ID$_{50}$ of 1–2 $\mu$g/ml.² Heparin sodium salt grade I-A from porcine intestinal mucosa (average molecular mass, 16,000 Da) was obtained from Sigma. SDS-PAGE analysis on 4–15% acrylamide gels (see Figs. 1–3) was performed using a Protean™ II apparatus from Bio-Rad. Molecular mass standards for SDS-PAGE were obtained from Life Technologies, Inc. Hanks’ balanced salt solution and HEPES were obtained from Life Technologies, Inc. Buffer for bPF4 incubation with thrombomodulin and protein C consisted of Hanks’ balanced salt solution (HBSS) (1 × with HEPES, pH 7.4) containing (unless otherwise indicated) 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$. Avidin immobilized on 6% agarose beads (having a binding capacity of 42 $\mu$g of b-avidin/ml of packed gel), bovine serum albumin, HEPES, EGTA, EDTA, and calcium chloride were obtained from Sigma.

The BlAcore™ instrument, surfactant P20, and sensor chip SA-5 with immobilized streptavidin from Streptomyces avidinii were all obtained from BlAcore, Inc. (Piscataway, NJ). Equilibration buffer used in the kinetic measurements by BlAcore™ consisted of 10 mM HEPES with 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 0.005% (v/v) surfactant P20 adjusted to pH 7.4 with 1 M NaOH at room temperature. Measured buffer osmolality was 280 mosmol. In BlAcore™ experiments measuring the calcium dependence of GAG$^+$ thrombomodulin and protein C binding, the equilibration buffer was supplemented to contain 0.5 mM EGTA and no added CaCl$_2$. All buffers were sterile-filtered through a 0.2-µm CA Costar® bottle-top filter (Corning Costar Corporation, Cambridge, MA) and degassed for 3 min before use.

Methods

Complex Formation Between PF4 and Thrombomodulin, Protein C, and Gla-domainless Protein C: Analysis by bPF4/avidin-agarose Extraction and SDS-PAGE—10 $\mu$M bPF4 in HBSS/HEPES buffer was incubated separately with the following proteins in a final volume of 100 $\mu$L: 2 $\mu$M GAG$^+$ CHO thrombomodulin, 2 $\mu$M GAG$^+$ CHL thrombomodulin, or 2 $\mu$M GAG$^-$ thrombomodulin for 30 min at 37 °C. 30 $\mu$L (4-fold excess binding capacity over bovine thrombomodulin as opposed to the GAG$^+$ CHO thrombomodulin); the second is unglycanated (GAG$^-$ CHO thrombomodulin) with a mass of 95 kDa. A second glycanated recombinant extracellular form of thrombomodulin was obtained by transfection of the human melanoma cell line CHL with the same thrombomodulin DNA construct as described above. This thrombomodulin form, henceforth referred as GAG$^+$ CHO thrombomodulin, utilizes both the Ser$^\text{Thr}$ and Ser$^\text{Gly}$ O-linked glycanation sites (29) and has therefore an even more heavily glycanated than GAG$^+$ CHO thrombomodulin, with a resulting apparent molecular mass of 200 kDa as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Upon digestion with chondroitinase ABC, both GAG$^+$ CHO and GAG$^+$ CHL thrombomodulin collapse to form a single sharp band with an apparent molecular mass of 95 kDa. Bovine protein C was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Human protein C was isolated from human plasma, and Gla-domainless protein C was prepared by digestion of native protein C with chymotrypsin as described previously (49) and generously provided by N. L. Esmon, Oklahoma Medical Research Foundation (Oklahoma City, OK). Human PF4 was isolated from units of outdated platelet packs as described previously (30, 31) utilizing heparin-agarose affinity chromatography, with the final stage purification achieved by Sephadex G-100 chromatography as described previously by Deuel et al. (18). The purified PF4 migrated as a single band of apparent molecular mass 9,000 Da on overloaded SDS-PAGE gels. bPF4 was kindly provided by C. K. Lai and Ted Maione, Repligen Corporation (Cambridge, MA). PF4 was selectively biotinylated, targeting its three N-terminal glutamate residues (which are not known to be involved in heparin binding) by exposing the native protein with biotin hydrazide in a mildly acidic (pH 5.0–5.4) environment. The resulting bPF4 had 0.92 mol of biotin/1 mol of PF4 monomer and had N-terminal biotinylation confirmed by N-terminal sequencing. Further characterization of this reagent showed preservation of three key native PF4 properties: (a) formation of tetramers at physiologic ionic strength as judged by molecular sieving chromatography mobility, (b) binding to heparin with an affinity similar to that of native

² C. K. Lai, personal communication.
immobilized as described above at a flow rate of 20 μl/min for 90 s, following which equilibration buffer alone was perfused at 20 μl/min. Injections were carried out in triplicate for each concentration and with increasing concentrations of heparin. After each analyte injection, the chip surface was regenerated by perfusion with 1 M NaCl for 30 s at a flow rate of 20 μl/min. This regeneration protocol reproducibly brought RU values back to within 10 RU of those seen before analyte perfusion. For the thrombomodulin preps, GAG+ thrombomodulin at five different concentrations ranging from 100 to 350 nM in equilibration buffer and GAG– thrombomodulin at five different concentrations ranging from 400 to 1300 nM was substituted for heparin; the washout and regeneration protocols were the same as those described for heparin. Similar experiments were performed with human protein C at 4 concentrations ranging from 2150 to 6800 nM and Gla-domainless protein C at five concentrations from 1,000 to 3,180 nM. For each analyte used, the interaction with a SA-5 chip without immobilized PF4 was assayed and found to be negligible.

Binding of ligand to the streptavidin-gold-dextran surface is expressed in resonance units (1 RU = 1 pg/mm²/s). Changes in RU over time form a “sensorgram,” and these changes have been shown to be proportional to the changes in mass bound to the streptavidin-gold surface. The dissociation rate constant (k_diss) was obtained by averaging (k_avgdiss) determinations from each sensorgram calculated using the equation,

$$\ln(R_t/R_0) = k_{diss}(t - t_0)$$

where R_t is response at time t and R_0 in RU is response at the beginning of dissociation phase, t_0. These calculations were performed using the nonlinear model provided by the manufacturer’s BIA™ evaluation software (Version 2.1) that fit data from perfusions of ligands at 4–5 different concentrations, each done in triplicate. The association rate constant (k_assoc) was calculated using a linear model that plots k_s values against concentration (C) using the formula, k_s = k_assoc + k_diss. The slope values (k_s) were obtained from analysis of each sensorgram using the equation,

$$R = (R_0/k_s)[1 - e^{(-k_s t)}]$$

where R is the binding rate at t. These calculations were performed using a nonlinear model provided by the manufacturer’s BIA evaluation software. The equilibrium dissociation constant (K_d) for PF4 and ligand interaction was obtained using equation,

$$K_d = k_{diss}/k_{assoc}$$

Detection of Protein C in bPF4 Extracts of Normal Human Plasma by Western Blot Analysis—100 μl of three normal human plasma specimens was incubated with either 20 μl of 700 μg/ml bPF4 or 20 μl of phosphate-buffered saline for 10 min at 37 °C. 20 μl of avidin-agarose beads (42 units of biotin binding/ml) was added to each sample and incubated for 10 min at 37 °C. Samples were vortexed twice during incubation. The samples were centrifuged at 12,000 × g for 5 s, and the bead-protein complex was washed three times with ice-cold phosphate-buffered saline. The pellet was reconstituted in 20 μl of 1 × sample buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0, 2.5% SDS, 5% 2-mercaptoethanol, and 0.01% bromphenol blue). Samples were boiled for 5 min and centrifuged at 12,000 × g for 5 s. 4 μl of each supernatant or 50 μg of authentic human protein C (Enzyme Research Laboratories, IN) were loaded into each lane of a 12.5% Phastgel (Pharmacia Biotech Inc.) SDS-PAGE device, separated, and transferred to a nitrocellulose membrane following the manufacturer’s instructions. The membrane was blocked with blocking buffer (5% nonfat dry milk in TBS (20 mM Tris, 130 mM NaCl, pH 7.5)) overnight at 4 °C, washed, then incubated for 2 h with a 1:1,000 dilution of rabbit anti-human protein C serum (Celsius Labs, OH) in the blocking buffer. The membrane was rinsed three times in distilled water and three times in TBS for 5 min each then incubated with a 1:20,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) in blocking buffer for 1 h. Immobilized antigen was detected with enhanced chemiluminescence (ECL; Amersham Corp.) and autoradiography.

**RESULTS**

To demonstrate potential complex formation between PF4 and either thrombomodulin or protein C, we developed an assay system that exploits the extremely high affinity between biotin and avidin. Native PF4 was biotinylated to a final molar ratio of ~1:1 biotin to PF4 monomer as described above. To assure that this structural modification had not significantly impaired the biologic activity of PF4 with respect to its capacity to accelerate APC generation by the GAG+ form of thrombomodulin (25), we performed preliminary experiments comparing bPF4 to native PF4 in this regard. We found (not shown) that the ED₅₀ for acceleration of APC catalysis was identical for native and bPF4 (i.e. 3.3–10 μg/ml), as was the magnitude of acceleration at saturation (approximately 25-fold over base line).

To assay the ability of PF4 to bind to differentially glycanated forms of thrombomodulin, bPF4 was added to solutions containing GAG– CHO thrombomodulin, GAG+ CHO thrombomodulin, or GAG+ CHL thrombomodulin. These solutions were then extracted with avidin beads and thoroughly washed, and the captured protein was solubilized in sample buffer, run on SDS-PAGE gels, and stained with Coomassie Blue. Lane 1, GAG– CHO thrombomodulin, no bPF4; lane 2, GAG– CHO thrombomodulin, + bPF4; lane 3, GAG+ CHO thrombomodulin, no bPF4; lane 4, GAG+ CHO thrombomodulin, + bPF4.

**FIG. 1.** PF4 binding to differentially glycanyated forms of thrombomodulin. Solutions containing 2 μg thrombomodulin (TM) of the indicated GAG status were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μM bPF4 and extracted with avidin-agarose beads, and the resulting extracts were solubilized in sample buffer, run on 4–15% gradient SDS-PAGE gels, and stained with Coomassie Blue. Lane 1, GAG– CHO thrombomodulin, no bPF4; lane 2, GAG– CHO thrombomodulin, + bPF4; lane 3, GAG+ CHO thrombomodulin, no bPF4; lane 4, GAG+ CHO thrombomodulin, + bPF4.

Our previous work demonstrated that PF4 induces significant changes in protein C autofluorescence emission in both the presence and absence of calcium, suggesting that PF4 induces conformational changes in protein C (25). To investigate the possibility of a physical complex formation between PF4 and protein C as the basis for these conformational changes and to examine the calcium dependence of this phenomenon, we analyzed avidin extracts of solutions containing bPF4 and protein C in the presence of calcium concentrations between 0–1 mM (Fig. 2, lanes 1–3). To establish the specificity of this interaction, we added a concentration of BSA equimolar to that of
protein C to these solutions. As shown in lanes 1–3, large amounts of protein C (bands at 46 and 28 kDa, corresponding to the heavy and light chains, respectively) but only small amounts of BSA (69 kDa band) were bound to bPF4 at all calcium concentrations. Both in the total absence (lane 1) and presence (lane 3) of physiologic concentrations of calcium, similar amounts of protein C are bound by PF4. Lanes 4–6 are avidin bead extracts of protein C at various calcium concentrations in the absence of added bPF4 to control for nonspecific adsorption of protein C to avidin. These lanes are devoid of protein C. As shown in lanes 7–9, there is a considerable nonspecific interaction between BSA and avidin alone. Indeed, the amounts of BSA detected in lanes 1–3 are nearly identical to that in lanes 7–9, suggesting that there is no PF4-specific interaction with BSA. Taken together, these data indicate that PF4 binds protein C in a calcium-independent manner.

Because the N-terminal end of the protein C light chain contains a Gla domain with nine Gla residues and is therefore intensely anionic, we hypothesized that cationic PF4 might bind to native but not Gladomainless protein C. We therefore compared avidin extracts of native and Gladomainless protein C incubated in the presence of bPF4 (Fig. 3). As expected, large amounts of native human protein C bound to bPF4 (lane 2) but not to avidin beads alone (lane 1). The two bands of protein C heavy chain at 44 and 40 kDa (lane 5) represent previously described α and β subforms of human protein C heavy chain (46, 47). The 25-kDa band in lane 2 is the native protein C light chain. In striking contrast, only scanty amounts of Gladomainless protein C heavy chain bound to PF4 (lane 3). The presence or absence of Gladomainless protein C light chain could not be ascertained, because its mobility is the same as that of bPF4 dimer (see lanes 4 and 5). However, because the protein C light and heavy chains are covalently linked by a disulfide bond under physiologic conditions, these results suggest that PF4 binds to protein C through its Gla domain.

Although the experiments depicted in Figs. 1, 2, and 3 qualitatively demonstrate an interaction between PF4 and GAG+ forms of thrombomodulin as well as protein C, they do not quantitate their avidity. Because the avidity of these interactions may give insights into their potential physiologic significance, we employed surface plasmon resonance-based analysis (BIACore™) to quantitate the strength of these interactions. To accomplish this, bPF4 was first immobilized upon a streptavidin sensor chip, candidate ligands were perfused into the chamber surrounding the chip, and potential binding interactions were monitored. To validate the use of our bPF4 reagent in this system, we measured its afffinity for heparin, an interaction well characterized by other methodologies using native PF4 (16, 17, 32–34). Fig. 4 shows a series of sensograms resulting when increasing concentrations (60–3,200 nM) of heparin were perfused over a chip upon which −1500 RU of bPF4 had previously been immobilized. Equilibration buffer alone was perfused to establish a stable base line. Then, at time point A (beginning of association phase), buffer containing various concentrations of heparin was perfused across the chip. The initial steep rise in RUs is attributable to differences in the refractive indices of the sample and equilibration buffer. This artifact is followed by a continuous binding curve at each heparin concentration. At point D (beginning of dissociation phase), perfusion of heparin-containing buffer is discontinued, and perfusion with equilibration buffer alone is resumed. Another buffer artifact can be seen followed by a smooth dissociation phase. As expected, increasing concentrations of heparin yield increasingly steep binding curves as well as increasing amounts of ligand bound as revealed during the dissociation phase. By analyzing separately the association and dissociation phases as described under “Methods,” we determined a $k_a$ of $4.1 \times 10^5$ M$^{-1}$ s$^{-1}$ and a $k_d$ of $1.8 \times 10^{-3}$ s$^{-1}$, yielding an equilibrium dissociation constant ($K_D$) of 4.4 nM (Table I). This value is similar to that of previously published estimates of the PF4-heparin interaction as measured by other methodologies (16, 17, 32–34).

We performed a similar quantitative analysis of the interaction of bPF4 with GAG+ CHO thrombomodulin and compared it with that of its GAG counterpart, GAG− CHO thrombomodulin (Fig. 5). As shown in the left panel (A), perfusion of the chip with increasing concentrations of GAG+ CHO thrombomodulin yields a graded series of pronounced binding and dissociation curves. Binding above base line is proportional to the concentration of the soluble analyte. In marked contrast, when the GAG− isoform of CHO thrombomodulin was substituted for the GAG+ isoform, little if any binding occurs (Fig. 5B). Analysis of the dissociation phase suggests that almost no (≤12 RU) of GAG− thrombomodulin are bound to the chip. The insert in Fig. 5A shows a $k_s$ versus concentration plot of the bPF4/GAG+ CHO thrombomodulin interaction. Analysis of
This curve yields an association constant \( k_a \) of 4.8 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\) (Table I). Analysis of the dissociation phase at the different concentrations yields an average \( k_d \) of 1.5 \( \times 10^{-3} \) s\(^{-1}\), yielding an equilibrium dissociation constant \( K_d \) of 31 nM (Table I). The interaction of GAG–CHO thrombomodulin to bPF4 was too weak to measure using this methodology.

Fig. 6 shows a similar quantitative analysis of the interaction between bPF4 and native or Gla-domainless protein C. As shown in panel A, there is a prominent binding interaction between bPF4 and native protein C. In striking contrast, as shown in the right panel (B), there is no detectable interaction between bPF4 and Gla-domainless protein C. Kinetic analysis of the bPF4-native protein C interaction reveals a \( K_d \), of 37 \( \mu \)M (Table I). Because there was no detectable binding of Gla-domainless protein C to bPF4, no kinetic parameters could be calculated for this interaction.

To ascertain whether the bPF4-protein C interaction described above occurs in a physiologic plasma milieu, avidin-agarose extracts of normal human plasma supplemented with bPF4 were separated by SDS/PAGE, transferred to nitrocellulose filter paper, and probed for protein C with an anti-protein C antibody (Fig. 7). In the absence of PF4, there was no detectable protein C (lane 2). In the presence of PF4, however, two bands whose mobility corresponds to that of the \( \alpha \) and \( \beta \) subforms of authentic human protein C heavy chain (lanes 1 and 6) are evident in extracts of three normal human plasmas (lanes 3–5).

**DISCUSSION**

Nature of the PF4-Thrombomodulin and PF4-Protein C Interactions—Thrombomodulin is strongly anionic at physiologic pH (pI \( \approx 4 \), Ref. 7), largely due to variable and potentially extensive (15–100 kDa) post-translational, O-linked glycanation with a chondroitin sulfate-like GAG (8, 9, 55). O-Linked glycanation occurs predominantly at Ser-472, but it can also occur at Ser-474 in the serine/threonine-rich domain of thrombomodulin (29) located between the hydrophobic membrane-anchoring domain and the thrombin-binding epidermal growth factor-like domain. Glycanation is highly variable in extent, depending upon the cell type and vascular bed origin of endothelial cells in which thrombomodulin is expressed, suggesting that thrombomodulin can be considered a “part-time proteoglycan” (29). The presence or absence of this GAG domain strongly influences several critical functional aspects of thrombomodulin function, including antithrombin III inactivation of thrombin, affinity for binding thrombin, capacity to quench thrombin procoagulant effects, and the catalytic rate and calcium dependence of APC generation (9–12, 52–55).

Given the pronounced anionic charge of thrombomodulin and the important role of the anionic GAG domain in thrombomodulin function, potential interactions between thrombomodulin and various cationic compounds and proteins has been of considerable interest. The vast majority of such substances, including polybrene, poly-L-lysine, vitronectin, histidine-rich glycoprotein, S-protein, and the eosinophil cytotoxic granule proteins (major basic protein, eosinophil peroxidase, and eosinophil cationic protein) have been found (10–13) to have potent inhibitory effects upon the direct anticoagulant and protein C-activating cofactor activity of thrombomodulin. We were therefore surprised to find that cationic PF4, a major platelet \( \alpha \)-granule component released during aggregation, could promote a massive (up to 25-fold) acceleration of thrombomodulin protein C-activating cofactor activity (25). Further highlighting the unusual nature of this interaction is the finding that the other two major heparin-binding platelet \( \alpha \)-granule components, \( \beta \)-thromboglobulin and thrombospondin, failed to influence thrombomodulin function.

Our current study was designed to explore potential mechanisms underlying this novel interaction. We previously demonstrated that PF4 stimulation of thrombomodulin APC generation is mostly attributable to a pronounced increase in the apparent affinity of the thrombin-thrombomodulin complex for protein C shown by a 30-fold decrease in the \( K_m \) for protein C of APC generation by the GAG+ thrombomodulin-thrombin complex (25). In contrast, the affinity of thrombin for thrombomodulin is not significantly affected by PF4. This PF4 effect was seen with native protein C (but not Gla-domainless protein C) and more prominently with GAG+ thrombomodulin than with GAG– thrombomodulin. Moreover, PF4, like calcium, caused changes in native (but not Gla-domainless) protein C autofluorescence emission. Using qualitative and quantitative analytic techniques, we now demonstrate specific, strong, and calcium-independent binding interactions between PF4 and GAG+ thrombomodulin and between PF4 and the Gla domain of protein C.

Relative specificity of PF4 binding interactions is shown by its ability to bind to GAG+ but not GAG– thrombomodulin (Figs. 1 and 5) and its ability to blockade this binding with excess heparin GAG (not shown) to protein C but not bovine serum albumin (Fig. 2), to native but not Gla-domainless protein C (Figs. 3 and 6), and to protein C in the complex mixture of proteins in human plasma (Fig. 7). Though not formally...
proven by generating binding curves at varying salt concentrations and generating a Record plot, these interactions are probably electrostatic because they are reversible either spontaneously (see dissociation phase in Figs. 5 and 6) or immediately after the addition of heparin (25). In the case of thrombomodulin, cationic PF4 binds to the polyanionic chondroitin sulfate GAG domain; in the case of protein C, PF4 binds to the polyanionic Gla domain. PF4 binding of other potential ligands might have relative specificity based upon negative charge density, as is the case for the relative affinities of PF4 for various types of GAGs, which correlates directly with their degree of sulfation (20).

**Proposed Mechanism for PF4 Acceleration of Thrombin-Thrombomodulin Protein C-activating Cofactor Activity**—The well characterized interaction between PF4 and heparin is probably the best model for PF4 binding to the GAG domain of thrombomodulin. PF4 monomer secondary structure consists of a large open loop (residues 24–34), three stranded antiparallel β sheets (residues 39–68), and one α helix at the carboxyl terminus (residues 75–85), which lies diagonally across the β sheets (36). The C-terminal α helix end of PF4 contains a cluster of four lysines (Lys-76, Lys-77, Lys-80, and Lys-81) thought to be important for heparin binding because their guanidination strongly impairs PF4 heparin binding (20). At physiological pH, PF4 forms a tetramer in which, based on x-ray crystallography coordinates, one model predicts a ring of strongly positive charge running perpendicularly across the helices that are comprised of these lysine residues as well as His-38 (37). Another more recent model using NMR and mutational analysis of human PF4 suggests that an internal loop containing Arg-20, Arg-22, His-23, Tyr-25, Lys-46, and Arg-49, not the C-terminal α-helix lysines, mediates heparin binding (33). Presumably the PF4 interactions with thrombomodulin GAG involve these same cationic, externally oriented domains. As is the case for PF4 binding to heparin, PF4 binding to GAG also contains calcium-independent (BIAcore data not shown).

**Table I**

| Kinetic parameters of PF4 interaction with heparin, thrombomodulin, and protein C |
|-------------------------|-----------------------------|-----------------------------|-----------------------------|
|                         | Heparin                    | GAG+ TM                     | GAG- TM                     | Native protein C            | Gla-domainless protein C |
| kₐ (M⁻¹s⁻¹)             | 4.1 × 10⁵                  | 4.8 × 10⁴                   | NM                          | 1.2 × 10⁴                   | NM                        |
| k_d (s⁻¹)               | 1.8 × 10⁻³                 | 1.5 × 10⁻³                  | NM                          | 4.5 × 10⁻³                  | NM                        |
| K_D (nM)                | 4.4 nM                     | 31 nM                       | NM                          | 0.37 μM                     | NM                        |

*Fig. 5.* Sensorgrams of surface plasmon resonance-based analysis of GAG+ and GAG− thrombomodulin binding to PF4. Panel A, GAG+ CHO thrombomodulin (TM). Using protocols described in the legend for Fig. 4, equilibration buffer supplemented with increasing concentrations (1–5: 1, 350 nM; 2, 260 nM; 3, 200 nM; 4, 150 nM; 5, 100 nM) of GAG+ thrombomodulin were perfused across a chip containing immobilized bPF4. One representative curve (of triplicates) at each concentration is shown. Inset, kₐ plot used to determine kₐ. Panel B, GAG− thrombomodulin. The protocol is as for panel A, except GAG− thrombomodulin was substituted for GAG+ thrombomodulin. 1, 1,300 nM; 2, 1,000 nM; 3, 750 nM; 4, 550 nM; 5, 410 nM. Because no binding interaction is evident, no kₐ plot is shown.
negatively charged Gla groups densely clustered in the 41 NH2-terminal amino acid residues of the light chain of protein C that interact with Ca2+ as low affinity binding sites (26). That PF4 binds native but not Gla-domainless protein C (Figs. 3 and 6) strongly implicates the protein C Gla domain, not the high affinity (6 × 10⁻⁷ M, Ref. 26) Ca²⁺-binding site (Glu-80) localized by Rezaie et al. (38) as the locus of this interaction. As is the case with heparin and GAG+ thrombomodulin, PF4 binding interactions with protein C are also calcium-independent (Fig. 2), further supporting a possible electrostatic interaction. We propose that PF4 binding to protein C induces conformational changes that are responsible for the changes in protein C autofluorescence emission that occur (25). Such conformational changes may mimic (or synergize with) those caused by the binding of calcium, which exposes the scissile bond region of protein C (39), thereby rendering protein C more susceptible to proteolytic activation by the thrombin-thrombomodulin complex. This model also explains why in the presence of PF4 the thrombin-thrombomodulin complex rapidly activates protein C in the total absence of calcium (25), whereas in the absence of PF4, this reaction proceeds at a negligible rate.

Based on our demonstration of PF4 binding to GAG+ thrombomodulin and to protein C, a model can be proposed to explain the 30-fold decrement in the $K_m$ for protein C of APC generation by the GAG+ thrombomodulin-thrombin complex seen in the presence of PF4. Two discreet but simultaneous interactions occur: one between a molecule (or molecules) of PF4 and protein C and another between a different molecule (or molecules) of PF4 and the GAG domain of thrombomodulin. The enhanced affinity between protein C and the thrombin-thrombomodulin complex caused by PF4 binding to the protein C Gla domain could occur either at the protein C-thrombomodulin interface in the fourth epidermal growth factor domain of thrombomodulin (40) at the Asp-349 Ca²⁺-binding site (41) or, alternatively, at the thrombin-protein C interface. This latter interaction, interestingly, has been proposed (42) to depend upon the anionic residues Glu-39 and Glu-192 in thrombin and Asp P3 and Asp P3’ in protein C. A separate interaction between the GAG domain of thrombomodulin and PF4 may further enhance the affinity of protein C for the thrombin-thrombomodulin complex by neutralizing the anionic charge of the GAG. Depending upon the orientation and stoichiometry of GAG-bound PF4, this domain might even be rendered cationic.
and thus increase the apparent affinity of protein C for the thrombin-thrombomodulin complex by immobilizing (and thus in effect, concentrating) protein C in the vicinity of its receptor, much as local GAGs have been proposed to facilitate the interaction of heparin-binding growth factors to their specific polypeptide receptors (43).

Implications of Findings—We have shown that at high concentrations, PF4 binds to both GAG+ forms of thrombomodulin and to protein C, the functional consequence of which is a pronounced increase in the protein C-activating cofactor activity of thrombomodulin. The apparent $K_m$ values we measured for the PF4-GAG+ thrombomodulin and PF4-protein C interactions (31 nM and 0.37 µM, respectively) were similar to or lower than those for the interaction of vitamin K-dependent clotting proteins with phospholipid membranes, which are typically in the 200–1,000 nM range (48). Because of the high concentrations of PF4 required to exert this effect (approximately 3–10 µg/ml, the concentration found in human serum (16)), it is difficult to imagine this mechanism as having a physiologic role except perhaps in the immediate vicinity of an actively degranulating platelet. Nonetheless, these findings do provide a mechanistic basis for predicting enhanced pharmacologic generation in vitro of APC or creation of a corresponding anticoagulant state in vivo using a combination of purified PF4, exogenous or endogenous thrombin, and endothelial or recombinant extracellular domain forms of thrombomodulin. We note that these last reagents have already shown promise as a cofactor for more definitive experimentation.

Given the conservation of Gla domains among vitamin K-dependent clotting factors, it is easy to envision PF4 interacting with other Gla domain-containing proteins in addition to protein S. In human plasma (45),3 it is, therefore, not clear whether the possibility that PF4 not only increases generation of APC in a factor X α-initiated clotting of human plasma by 30–40% (45), raising the anticoagulant effect of APC in a factor X α-initiated clotting process, it is easy to envision PF4 interacting with other Gla domain-containing proteins in addition to protein S, but not to clotting factors V and XII as well as protein S, but not to clotting factors V and XII in human plasma (45).3 It is, therefore, not clear whether the net effect of PF4 at such concentrations would be pro- or anti-coagulant. More definitive in vitro and in vivo experimentation will be required to resolve this issue.

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8.管理办法，200–1,000 nM range (48). Because of the high concentrations of PF4 required to exert this effect (approximately 3–10 µg/ml, the concentration found in human serum (16)), it is difficult to imagine this mechanism as having a physiologic role except perhaps in the immediate vicinity of an actively degranulating platelet. Nonetheless, these findings do provide a mechanistic basis for predicting enhanced pharmacologic generation in vitro of APC or creation of a corresponding anticoagulant state in vivo using a combination of purified PF4, exogenous or endogenous thrombin, and endothelial or recombinant extracellular domain forms of thrombomodulin. We note that these last reagents have already shown promise as a clinically effective anticoagulant in animal models (35, 40, 44), and recombinant PF4 has been infused to achieve blood concentrations of 30–50 µg/ml in rats (30), baboons (50), and humans (51) to reverse heparinization without noticeable side effects. Moreover, in experiments not shown, 3–10 µg/ml PF4 increases the anticoagulant effect of APC in a factor Xα-initiated clotting of human plasma by 30–40% (45), raising the possibility that PF4 not only increases generation of APC by thrombomodulin but also potentiates its anticoagulant activity.

Given the conservation of Gla domains among vitamin K-dependent clotting factors, it is easy to envision PF4 interacting with other Gla domain-containing proteins in addition to protein C and exerting influences upon other aspects of the coagulation cascade. Supporting this possibility, we have found that at high concentrations such as those used in our studies with thrombomodulin and protein C, PF4 binds to clotting factors X and II as well as protein S, but not to clotting factors V and XII in human plasma (45).3 It is, therefore, not clear whether the net effect of PF4 at such concentrations would be pro- or anti-coagulant. More definitive in vitro and in vivo experimentation will be required to resolve this issue.

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