Glucosylceramide, a Neutral Glycosphingolipid Anticoagulant Cofactor, Enhances the Interaction of Human- and Bovine-activated Protein C with Negatively Charged Phospholipid Vesicles

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The effect of glucosylceramide (GlcCer) on activated protein C (APC)-phospholipid interactions was examined using fluorescence resonance energy transfer. Human APC, labeled with either fluorescein (Fl-APC) or dansyl (DEGR-APC) donor, bound to phosphatidylcholine/phosphatidylserine (PC/PS, 9:1 w/w) vesicles containing octadecylrhodamine (OR) acceptor with a $K_d$ of 460 nM. The presence of C8-GlcCer selectively enhanced the binding of C16,6-NBD-phosphatidylserine but not C16,6-7-nitrobenz-2-oxa-1,3-diazole (NBD)-phosphatidylcholine to coumarin-labeled APC. These data suggest that APC binds to GlcCer, that PC/PS/GlcCer vesicles like PC/PS vesicles bind to the N-terminal γ-carboxyglutamic acid domain of APC, and that one mechanism by which GlcCer enhances the activity of APC is by increasing its affinity for membrane surfaces containing negatively charged phospholipids.

Blood coagulation has to be carefully regulated by balancing the procoagulant reactions with potent anticoagulant processes. Both procoagulant and anticoagulant reactions occur at a physiologically significant rate only when the respective enzymes form multicomponent complexes on lipid membrane surfaces (1). Thus, membranes play a pivotal role in regulating blood coagulation reactions.

The molecular details of mechanism(s) by which various lipids affect coagulation are not fully understood. Many groups have observed that lipid vesicles containing negatively charged phospholipids, especially phosphatidylserine (PS), bind and markedly enhance the rate of activation of procoagulant enzymes (2–9). It has been proposed that the high rate of activation of procoagulant enzymes in the presence of vesicles containing PS is due in large part to enhancement of substrate binding to the negatively charged surface of the phospholipid bilayer. The resulting high local concentration of substrate on the membrane surface can enhance diffusion of substrate to the activation complex in two dimensions rather than in three dimensions (10). Although the significance of this proposed mechanism has been controversial and alternative models have been proposed to explain many kinetic data (11, 12), current paradigms most often assume that membranes only provide a surface template for the assembly and function of the various procoagulant enzyme-containing multicomponent complexes and that the procoagulant phospholipids do not play a more active role in the blood coagulation reaction. However, it has been recently reported that submicellar concentrations of a short chain variant of phosphatidylserine, namely dicaproyl phosphatidylserine, enhances the rate of activation of both prothrombin by the prothrombinase complex and factor X by the Xase complex (13, 14). These data suggest the existence of a functionally significant binding site(s) for C6PS on one or more procoagulant plasma proteins and that the occupancy of these sites by the phospholipid molecules activates the clotting factors. If there are functionally important PS-binding sites on some procoagulant proteins, do such functionally important lipid-binding sites also exist in anticoagulant proteins? Although both procoagulant and anticoagulant reactions are markedly enhanced by the presence of negatively charged surfaces in vitro, certain lipids and lipoproteins selectively enhance anticoagulant reactions in plasma (15–17). Recently,

The abbreviations used are: PS, phosphatidylserine; GlcCer, glucosylceramide; APC, activated protein C; FRET, fluorescence resonance energy transfer; [14C]FPC, 1-3-phosphatidylcholine-1,2-dial[1-14C]oleoyl; PC, phosphatidylcholine; GalCer, galactosylceramide; GlbCer, globo-tetraosylceramide; BSA, bovine serum albumin; DJ, factor Xa; CVa, factor Va; DEGR-CK, (5-dimethylaminonaphthalene-1-sulfonyl-glutamylglycylglycylarginyl chloromethylketone); DEGR-APC, APC labeled in the active site with 7-diethylamino-3-((4-prolylarginyl chloromethylketone inhibitor; OR, octadecylrhodamine; C8-GlcCer, C8-glucosylceramide; BSA, bovine serum albumin; DJ, factor Xa; CVa, factor Va; DEGR-CK, (5-dimethylaminonaphthalene-1-sulfonyl-glutamylglycylglycylarginyl chloromethylketone); DEGR-APC, APC labeled in the active site with 7-diethylamino-3-(4'-iodoacetyl)-aminophenyl-4-methylcoumarin in a Na+-facetylmethacrylate (Na+-ACT) inhibitor; OR, octadecylrhodamine; C8-GlcCer, C8-glucosyl-β1-1-N-octanoyl-γ-erythro-sphingosine; C8-ceramide, N-octanoyl-N-erythro-sphingosine; OR, octadecylrhodamine; C8-GlcCer, C8-glucosyl-β1-1-N-octanoyl-γ-erythro-sphingosine; C8-ceramide, N-octanoyl-N-erythro-sphingosine; DPH, diphenylhexatriene; NBD, 7-nitrobenz-2-oxa-1,3-diazole; C16,6-NBD-PS, 1-palmitoyl-2-[6-[7-nitro-2-1,3-benzoxadiazol-4-yl]amino]caproyl]-sn-glycero-3-phosphoserine; CMC, critical micellar concentration; Gla, γ-carboxyglutamic acid; D, donor; DA, donor and acceptor; B, blank.

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Deguchi and co-workers reported that plasma glucosylceramide (GlcCer) deficiency is a potential risk factor for venous thrombosis and that depletion or augmentation of GlcCer in normal plasma either reduces or enhances, respectively, the anticoagulant response to activated protein C (APC) (18). Subsequently, they reported that certain glycolipids such as GlcCer, lactosylceramide, and globotriaosylceramide, enhance the anticoagulant response of APC (19). In this study, we examined the mechanism by which such glycolipids may enhance the anticoagulant activity of APC. To test the hypothesis that GlcCer increases the anticoagulant activity of APC by increasing its affinity for lipid surfaces where anticoagulant reactions can occur, the binding of APC to GlcCer-containing phospholipid vesicles was examined using fluorescence resonance energy transfer (FRET). Additionally, we have also examined whether there is a unique GlcCer binding site(s) on APC, the occupancy of which augments APC activity.

**EXPERIMENTAL PROCEDURES**

**Reagents—**5-Dimethylaminonaphthalene-1-sulfonyl-glutamylglycyl arginyl chloromethylketone (DEGR-CR) was purchased from Calbiochem (La Jolla, CA). N-fluorescein-p benzyol phenylalanyl-lysyl (N'-benzoyl-L-amino (LW) amide (LWB) was prepared as described before (20). 7-Di-ethylamino-3-(4'-iodoacetamido)amino-4-methylcoumarin and octadecylrhodamine (OR) were obtained from Molecular Probes (Eugene, OR). Bovine brain phosphatidylcholine (PC), bovine brain PS, and human spleen GlcCer were obtained from Sigma. N-(Glycyl-L-1'-N-octanoyl)-N-erythro-sphingosine (C8-Glc-ceramide), N-octanoyl-N-erythro-sphingosine (C8-Ceramide), 1-palmitoyl-2-6-[7-nitro-2,1,3-benzoazoxadiazol-4-ylamino]caproyl-sn-glycerol-3-phosphocholine (C16,6 NBD-PC), 1-palmitoyl-2-6-[7-nitro-2,1,3-benzoazoxadiazol-4-ylamino]caproyl-sn-glycerol-3-phosphoserine (C16,6 NBD-PS) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). 1,3-Dihexadecyl-1,2-di-(L-lysyl) cephalin (C16,16 PC) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The chromogenic substrate Spectrozyme PCAs (American Diagnostica, Greenwich, CT), normal human plasma, factor V-deficient plasma (George King Bio-Medical Inc., Overland Park, KS), and Innovin® (Dade, Miami, FL) diluted 1:64 in Tris-buffered saline containing 0.1% BSA and 30 mM CaCl2.

**Proteins—**Human APC was obtained from Enzyme Research Laboratories (South Bend, IN). Bovine APC was obtained as a gift from Dr. Mary J. Heeh, The Scripps Research Institute (La Jolla, CA). Factor Va (fVa) and factor Xa (fXa) were purchased from Hematologic Technologies Inc. (Essex Junction, VT).

**Active Site-specific Labeling of APC—**Bovine and human APC were active site-specific-labeled with either fluorescein (20), dansyl (21), or active site-specific-labeled with either fluorescein (20), dansyl (21), or coumarin dye and purified from the excess reagents according to procedures published earlier (22). For the fluorescein and coumarin labeling, N-fluorescein-p benzyol phenylalanyl-lysyl (N'-benzoyl-L-amino amide and 7-diethylamino-3-(4'-iodoacetamido)amino-4-methylcoumarin were used, respectively, instead of the 5-iodoacetamido fluorescein.

**Phospholipid Vesicles—**Multicomponent vesicles of PC/PS (9:1 w/w ratio), PC/PS/GlCer (8:1:1), PC/GlCer (9:1), PC/GalCer (8:1:1), and 100% PC were prepared according to procedures published earlier (23). Briefly, pure lipids in chloroform were mixed in the appropriate ratios, and then the chloroform was evaporated under nitrogen gas to begin the procedure to facilitate determination of postextrusion phospholipid recovery. The concentration of glycolipids recovered was determined by measuring sugar head groups of the glycolipids using an orcinol-based colorimetric assay (18).

For the plane-to-plane FRET experiments, vesicles containing the acceptor dye NBD-rhodamine OR were also prepared as above except that the desired amount of a solution of OR in ethyl acetate was added to the lipid mixture prior to drying with nitrogen. The concentration of OR was determined as before as was σ, the OR acceptor density at the vesicle surface (23).

Clotting Assays—The procoagulant and anticoagulant properties of vesicles containing GlcCer were determined using Fxa-initiated clotting assays with exogenously added human or bovine APC. For these assays, GlcCer-containing vesicles at varying doses (50 μl) were mixed with normal plasma (25 μl), human or bovine APC (34.5 nM final), or buffer (TBS containing 0.1% BSA, 30 μl) and incubated for 3 min at 37 °C. Then, fXa (50 μl, 0.3 nM final) buffer containing 30 mM CaCl2 was added to initiate clotting, and clotting times were recorded using an Amelung KC4 microcoagulometer (Sigma). The response to APC was expressed as a ratio of clotting times that was calculated by dividing the clotting time in the presence of APC by the baseline clotting time in the absence of APC. Modified dilute prothrombin time-based assays were also performed as previously described (17, 18). Briefly, 7.5 μl of plasma was mixed with varying concentrations of C8-GlCer or C8-Cer and incubated for 3 min at 37 °C with fibrinogen (0.6 mg/ml final) and APC (8.7 nM final) plus protein S (28 nM final) or buffer (100 μl total). Clotting times were measured after addition of 50 μl of recombinant tissue factor (Innovin® from DADE, Miami, FL) diluted 1:64 in Tris-buffered saline containing 0.1% BSA and 30 mM CaCl2.

**Factor Va Inactivation Assays—**To study the effect of GlcCer on APC-dependent inactivation of factor Va, APC (0.94 nm) in 50 mM Tris (pH 7.4), 150 mM NaCl, and 5 mM CaCl2 was incubated for 5 min at 37 °C with factor Va (1 nM) in the presence or absence of various concentrations of PC/PS, PC/PS/GlcCer, PC/GlcCer, or PC vesicles, respectively. Then the reaction was quenched by the addition of a small molar excess of EDTA over CaCl2, an aliquot of the reaction mix was withdrawn, and the residual factor Va activity was determined using a prothrombin time-clotting assay using a Va-deficient plasma.

**Spectral Measurements—**All spectral measurements were made using a SLM A2 (or a SLM 8100 spectrofluorometer (SLM-Aminco, Rochester, NY) as described earlier (22, 23). Fluorescein, dansyl, and coumarin dye emissions were detected at excitation and emission maxima of 490, 340, and 390 nm and 525, 462, and 462 nm, respectively. All experiments were performed with 5 × 5-mm quartz cuvettes. Sample was mixed, and adsorption of proteins to cuvette walls was minimized as described (24, 25).

**Plane-to-plane FRET Experiments—**The interaction of APC with various preparations of multicomponent lipid vesicles was monitored using plane-to-plane FRET between donor dyes in the active site of APC and acceptors on the membrane surface. Either fluorescein-labeled APC (FI-APC), or dansyl-labeled APC (DEGR-APC) served as donators and rhodamine imbedded on the membrane surface served as acceptors. FRET experiments were performed as before (26) except that the D (donor-containing)- and DA (donor and acceptor)-containing cuvettes initially received 100 nM of the donor DEGR-APC (or FI-APC), whereas cuvettes A (containing acceptor) and B (blank) received 100 nM unlabeled EGR-APC (or FPR-APC) (22). Spectral parameters including quantum yields (Φ), spectral overlap integrals (I(DA)), and distance of closest approach (R0) for these experiments are described as before (22, 23, 26–31).

The distance of closest approach (R0) was calculated using the relation

$$Q_0/Q_{DA} = 1 + \left(\frac{4\pi R_0^2}{R_{DA}^2}\right)^4$$

where $Q_0/Q_{DA}$ is the ratio of donor quantum yields in the presence of acceptor and σ is the density of acceptor chromophores (OR) at the membrane surface.

**Point-to-plane FRET—**Four samples were prepared in parallel for each energy transfer experiment: cuvette D (containing donor) and cuvette DA (containing donor and acceptor) each received 100 nM of coumarin-labeled APC (donor), whereas cuvettes A (containing acceptor) and B received 100 nM unlabeled FPR-APC. The net initial emission intensity (F0) was obtained by the subtraction of the signal of B from D. For the acceptor, Samples D and B were titrated with short chain lipids lacking the acceptor dye NBD, whereas samples DA and A were titrated with short chain lipids conjugated to the NBD acceptor. The net intensity of D, DA, or A (F0, FDA, and F0, respectively) was obtained by subtracting the signal from the B cuvette and correcting for dilution. To correct for any signal in DA sample due to direct excitation of the acceptor, the net dilution-corrected emission intensity from A was subtracted from the DA sample signal. Making the reasonable assumption that the absorption of coumarin in the active site is unaffected by the presence of NBD-labeled lipids, the ratio of donor quantum yields in D and DA samples is given by Equation 2.

$$Q_0/Q_{DA} = \left[\frac{F_{0,D}}{F_{DA}}\right]/\left[\frac{F_{DA} - F_{D}}{F_{DA} - F_{A}}\right]$$

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where $F$ is the net dilution-corrected emission intensity of a sample at some point in the titration and the subscript "o" is used to denote the initial intensity of the sample.

For titrations in the presence of C8-GlcCer, 100 mM coumarin-APC was first incubated with 15 mM C8-GlcCer before point-to-point FRET experiments were performed with NBD-labeled phospholipids as described above. The distance $R$ between coumarin donor in the active site groove of APC and NBD acceptor conjugated to the phospholipid was determined using the relation in Equation 3,

$$E = R^o_o(R^o_o + R^o)$$  
(3)  

where $R_o$ is the distance at which FRET is 50% efficient and $E$ is the efficiency of FRET given by Equation 4.

$$E = 1 - (Q_{0}/Q_o)$$  
(4)  

Critical Micellar Concentration (CMC) Determination—The critical micellar concentration (CMC) of C8-GlcCer in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 5 mM CaCl$_2$ was determined using diphenyl hexatriene (DPH) fluorescence and 90° light scattering. DPH is an aggregation-sensitive dye and has been used to determine the CMC of lipids (13). Two samples were prepared in parallel for each experiment. The first sample (S) contained DPH (15 μM final) in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 5 mM CaCl$_2$, whereas the second sample contained a dye-free B. DPH fluorescence was monitored at 365 nm excitation and 460 nm emission, respectively. The net initial emission intensity termed $F_o$ was obtained by subtracting the initial intensity of B from the initial intensity S. The samples S and B were then titrated with increasing concentrations of lipids. Relative fluorescence intensity ($F/F_o$), calculated using Equation 5, was plotted against lipid concentration.

$$F/F_o = (F - F_o)/(F_o - F_0)$$  
(5)  

$F$ is the net dilution-corrected emission intensity of a sample at some point in the titration and the subscript $o$ is used to denote the initial intensity of the sample. A plot of $F/F_o$ versus lipid concentration gave two linear slopes, and the intercept of the two linear slopes was taken as the CMC of C8-GlcCer. The CMC of C8-GlcCer in the presence of 100 mM FPR-APC was similarly determined.

The CMC of C8-GlcCer was also determined by 90° light scattering using a SLM AB2 spectrophotometer (SLM-Aminco, Rochester, NY) with emission and excitation wavelengths of 320 nm. Experiments were performed with a band pass of 2 nm on both excitation and emission light paths. All buffers for light scattering were prepared dust-free by filtration using a 0.22-μm Acrodisc syringe filter units (Pall Gelman Laboratory, Ann Arbor, MI) and by centrifuging dust particles using a Microfuge before light-scattering experiments. The scatter intensity ($I$) of monomeric C8-GlcCer is much lower than multimeric aggregates or micelles of C8-GlcCer, and the concentration at which the scatter intensity increased sharply was taken as the CMC of GlcCer.

The CMC of C16,6-NBD phosphatidyserine and C16,6-NBD phosphatidylcholine were determined by monitoring the steady-state fluorescence anisotropy of the NBD reporter for increasing concentrations of lipid. NBD anisotropy decreases sharply upon lipid aggregation due to homo-FRET (27). NBD anisotropy was measured using Glan-Thompson prism polarizers placed on both the excitation and emission beam paths at 466 and 536 nm excitation and emission wavelengths, respectively. The emission intensity measured when the sample was excited by vertically plane-polarized light and the emission detected through a horizontal polarized light was termed $I_{VV}$, $I_{VH}$, $I_{HV}$, and $I_{HH}$ were defined analogously. Anisotropy ($r$) was determined using the relation in Equation 6,

$$r = (I_{VV} - I_{VH})(I_{VH} + 2I_{HH})$$  
(6)  

where the grating factor G equals $I_{HH}/I_{VH}$.

RESULTS

Effect of GlcCer on the Anticoagulant Response of APC—The effect of GlcCer on the anticoagulant response of plasma to human and bovine APC was tested using FXa-induced clotting assays (Fig. 1). Both PC/PS and PC/PS/GlcCer vesicles enhanced the anticoagulant response of both species of APC. The steeper slope for the GlcCer-containing vesicles compared with the vesicles lacking GlcCer (Fig. 1) indicates that incorporation of GlcCer into PC/PS increased the anticoagulant response of both species of APC. A GlcCer-dependent enhancement of APC activity was also observed in a tissue factor-induced dilute prothrombin-clotting assays (data not shown) showing that GlcCer serves as a lipid cofactor for both human and bovine APC.

Effect of GlcCer on APC-dependent Inactivation of Factor Va—The anticoagulant activity of APC observed in a FXa-induced clotting assay, which is due to the inactivation of fVa by APC, was studied using four different vesicle surfaces, 100% PC, PC/PS (9:1 w/w), PC/GlcCer (9:1), and PC/PS/GlcCer (8:1:1) (Fig. 2). Under the experimental conditions used, APC inactivated very little fVa on 100% PC vesicles and PC/GlcCer vesicles, whereas PC/PS and PC/PS/GlcCer vesicles supported APC-dependent inactivation of fVa. The APC-dependent inactivation of fVa was most efficient using 400 μM PC/PS/GlcCer vesicles where ~70% of fVa activity was lost in 5 min (Fig. 2). For PC/PS vesicles, 40% of fVa activity was lost under similar experimental conditions. Therefore, these data show that GlcCer enhanced APC-dependent inactivation of fVa in the presence of 10% PS.

Effect of GlcCer on APC-Phospholipid Binding—To test the hypothesis that GlcCer alters interaction of human APC with membranes, the binding of human APC to lipid vesicles containing GlcCer was compared with binding to vesicles lacking GlcCer. For these experiments, FRET was used to monitor APC binding to lipid vesicles (22, 23). APC labeled in the active site with either a fluorescein (FI-APC) or a dansyl (DEGR-APC) reporter served as a donor, whereas rhodamine dyes in OR at the aqueous-lipid interface served as the acceptor.

When human FI-APC was titrated with PC/PS vesicles, only a very small (3%) decrease in fluorescein emission was observed (data not shown). Likewise, a very small change in fluorescein emission was also detected when FI-APC was titrated with PC/PS/GlcCer, PC, or PC/GlcCer vesicles (data not shown). However, when FI-APC was titrated with PC/PS (or PC/PS/GlcCer, PC, or PC/GlcCer) vesicles containing OR acceptor, the fluorescein intensity decreased until sufficient phospholipids were added to bind all of the FI-APC (22). This OR-dependent decrease in fluorescein intensity results largely from FRET from the fluorescein dye in the active site of APC to the OR at the membrane surface (22). To facilitate data analysis, the data were normalized and expressed as a ratio of...
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GlcCer incorporation into PC/PS vesicles enhances APC-dependent inactivation of factor Va. Human APC (0.94 nM final) in 50 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM CaCl₂, plus 0.1% BSA was incubated with factor Va (1 nM final) and PC (○), PC/GlcCer (□), PC/PS (▲), or PC/PS/GlcCer (●) vesicles, respectively, at 37 °C for 5 min, and the residual factor Va activity was measured using a prothrombin time clotting assay.

Donor quantum yields in the presence (Q_D) or absence (Q_D0) of the acceptor using Equation 1. When no acceptor-containing vesicles were present, the ratio of Q_D/Q_D0 was 1, whereas in the presence of these lipids, the value of Q_D/Q_D0 was less than 1 due to FRET.

Fig. 3A presents data for titrations of human Fl-APC using four different vesicles containing similar OR acceptor density. The ratio of Q_D/Q_D0 showed a similar dependence on lipid concentration for the PC/PS, PC, and PC/GlcCer vesicles titrations, decreasing and reaching a plateau at ~30 μg/ml vesicle concentration. However, the same plateau was reached at a much lower lipid concentration in the PC/PS/GlcCer vesicles. These data suggest that PC/PS, PC, and PC/GlcCer vesicles have approximately the same affinity for human APC, whereas PC/PS/GlcCer vesicles have a greater affinity for FI-APC. Apparent dissociation constant (K_app) calculations based on curve fitting showed that the affinity for FI-APC was 5-fold greater for the PC/PS/GlcCer vesicles (3 μg/ml) compared with PC/PS vesicles (16 μg/ml). Thus, the incorporation of GlcCer into PC/PS vesicles significantly increased the affinity of vesicles for APC.

Although vesicles composed of only PC are inactive in our functional assays (see Fig. 2), these vesicles bound to FI-APC with almost the same affinity as the functionally active PC/PS vesicles as reported in an earlier study (27). This discrepancy between the functional and the binding data is presumably due to the poor binding affinity of IVa, the substrate of APC, for PC vesicles leading to the functional inactivity of PC vesicles (28, 29). Incorporation of GlcCer into PC vesicles did not alter the affinity of these vesicles for APC. Thus, incorporation of GlcCer into phospholipid vesicles-detectable altered the affinity of vesicles for APC only when PS was present.

FRET between the fluorescein donor on human APC and the rhodamine acceptor on the membranes is most efficient when the APC molecule is completely membrane-bound. Although this situation can never be reached experimentally due to the dynamic equilibrium between the bound and the unbound forms, it can be approached in the presence of a large amount of lipid. At high concentrations of lipids, the maximum extents of FRET for all four titrations in Fig. 3A are approximately the same. Since the acceptor densities were similar in all four titrations, the efficiency of FRET is a direct reflection of the distance between the donor-acceptor pair, suggesting that the four vesicles bind similarly to the APC molecule, presumably to the membrane-binding Gla domain of APC.

APC Species Independence of GlcCer-dependent Effect on APC-Phospholipid Binding—Because Fig. 1 showed that GlcCer augmented the anticoagulant activities of both human and bovine APC, the hypothesis that GlcCer similarly alters interaction of bovine APC with PS-containing vesicles was tested. When bovine DEGR-APC was titrated with PC/PS/GlcCer vesicles, the ratio of emission intensities (I_F/F) of the dansyl moiety in APC decreased and reached a plateau value of 0.79 ± 0.02 at ~12 μg/ml of lipid much like its human counterpart (data not shown). Therefore, the emission intensity of bovine DEGR-APC was sensitive to its interaction with PC/PS/GlcCer vesicles. However, the dansyl emission intensity was not altered significantly with control PC/PS vesicles. When the binding curve obtained for the interaction of bovine DEGR-APC with PC/PS/GlcCer was fit to a hyperbolic profile, a K_app of ~6 μg/ml was obtained, a value very similar to the K_app value for the interaction of human DEGR-APC with PC/PS/GlcCer. The binding of bovine DEGR-APC to PC/PS/GlcCer vesicles was also monitored by FRET between the dansyl donor on APC and rhodamine acceptors on PC/PS/GlcCer (OR) vesicles (Fig. 3B). At >12 μg/ml PC/PS/GlcCer (OR) vesicles, the decrease in dansyl emission of bovine DEGR-APC, like that of human DEGR-APC, reached a plateau. When bovine DEGR-APC was titrated with PC/PS (OR) vesicles, the plateau was reached at >35 μg/ml lipids. Therefore, these data suggest that PC/PS/GlcCer (OR) vesicles bind bovine APC and human APC with greater affinity than PC/PS vesicles indicating that the GlcCer-dependent increase in affinity of APC for PS-containing vesicles is not species-specific.

GalCer- and Gb₄Cer-containing vesicles did not significantly alter the ability of APC to inactivate FVa (19). When bovine DEGR-APC was titrated with PC/PS/GalCer or PC/PS/Gb₄Cer vesicles containing OR, the dansyl fluorescence decreased and reached a plateau at lipid concentrations >35 μg/ml much like the PC/PS vesicles. Thus, GalCer and Gb₄Cer did not increase the affinity of APC for PS-containing vesicles (Fig. 3B).

These data obtained with bovine and human DEGR-APC as donor are consistent with those obtained with fluorescein-acceptor donor (i.e. human FI-APC), indicating that the effect of GlcCer on FRET between the APC active site locus and rhodamine on the membrane is independent of the donor dye.

Specificity of GlcCer-dependent Increased Affinity of PS-containing Vesicles for APC—To test if GlcCer-dependent increase in the affinity of negatively charged vesicles for APC is specific for APC, we compared the binding of PC/PS vesicles that contained or lacked GlcCer to DEGR-IXa. When human DEGR-IXa was titrated with PC/PS or PC/PS/GlcCer vesicles, the anisotropy of the dansyl dye in the active site of IXa increased due to IXa binding to the vesicles and reached a plateau value (Fig. 4). Approximately 50 μg/ml of PC/PS and 70 μg/ml of PC/PS/GlcCer were required to bind all the free DEGR-IXa, showing that the incorporation of GlcCer in PC/PS vesicles does not enhance the affinity of PC/PS for IXa and that the GlcCer-dependent enhancement of affinity of PC/PS for APC is specific for APC.

Membrane-bound Topography of APC Is Independent of Membrane Composition—The magnitude of FRET observed for a given donor-acceptor pair depends upon the distance of closest approach (R) and the acceptor density (σ) among other things. Q_D/Q_D0 values were obtained over a wide range of acceptor densities for the different vesicle compositions from different FRET (Fig. 5). R,, the distance at which FRET is 50% efficient, is a constant for the given donor-acceptor pair and has been determined to be 48.6 Å for the dansyl-rhodamine pair,
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assum ing a random orientation of the transition dipoles during the donor lifetime (31). The ratio of quantum yields \( \frac{Q_{DA}}{Q_D} \) was directly proportional to the acceptor density (\( \sigma \)) indicating that increased energy transfer was observed when more acceptor was present on the lipid surface (Fig. 5).

The average values of \( R \) from all experiments for DEGR-APC of either species bound to PC/PS (90:10 w/w), PC/GlCer (80:10:10), PC, and PC/GlCer (90:10) vesicles were calculated to be 64, 63, 63, and 62 Å, respectively. When data for human and bovine molecules were analyzed separately, the \( R \) values were 64, 63, 62, and 61 Å for human APC bound to PC/PS, PC/PS/GlCer, PC, and PC/GlCer, respectively, and 64, 65, 62, and 68 Å for bovine DEGR-APC bound to PC/PS, PC/PS/GlCer, PC, and PC/GlCer, respectively (Table 1). Therefore, there was no significant difference in the average \( R \) value for experiments using PC/PS, PC/PS/GlCer, PC/PS/GalCer, PC, and PC/GlCer vesicles. Thus, we conclude that the higher affinity of PC/PS/GlCer for APC was not associated with a change in the overall topography of membrane-bound APC and that the topography of membrane-bound DEGR-APC is independent of the composition of the bound membrane under the experimental conditions used.

Binding of Short Chain Lipids to APC—To address the question of whether APC has a unique binding site(s) for GlcCer, we used a short-chained analog of GlcCer, namely C8-GlcCer, which does not form micelles under the experimental condi-

Fig. 3. A, GlcCer incorporation into PC/PS vesicles enhances the affinity of human Fl-APC for PC/PS vesicles. Samples containing Fl-APC (100 nM initially) in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 5 mM CaCl2, were titrated with PC (○), PC/PS (□), GlCer (▲), or PC/PS/GlCer (●) in the presence or absence of OR and the ratio of quantum yields in the presence and absence of acceptor (\( \frac{Q_{DA}}{Q_D} \)) was calculated using Equation 2. At the end of the titration 200 mM dithiothreitol was added to the sample cuvette to release the Fl-labeled APC heavy chain from the membrane and reverse the FRET (data not shown). In this experiment, \( \frac{Q_{DA}}{Q_D} \) of the four titrations are plotted against the lipid concentration. The OR acceptor density for the PC, PC/GlCer, and PC/PS/GlCer titrations was 5.2 × 10−14 dyes/Å² and that of PC/PS titration was 5.25 × 10−14 dyes/Å². B, GlcCer-dependent enhancement of bovine APC binding to PC/PS vesicles. Samples containing bovine DEGR-APC (100 nM initially) in 50 mM Hepes (pH 7.4), 150 mM NaCl, and 5 mM CaCl2 were titrated with PC/PS/GlCer (○), PC/PS/Gb4Cer (●), PC/PS/GalCer (▲), or PC/PS (□) vesicles containing or in the absence of OR acceptor, and the binding monitored by FRET as described under “Experimental Procedures.” The OR acceptor density in the PC/PS/GlCer, PC/PS, PC/PS/Gb4Cer, and PC/PS/GalCer titrations was 1.78, 1.34, 1.69, and 1.2 × 10−14 dyes/Å², respectively. Half maximal binding occurred at 4.5, 16, 10, and 16 µg/ml lipid for the PC/PS/GlCer, PC/PS, PC/PS/GalCer, and PC/PS/Gb4Cer vesicles, respectively.

Fig. 4. Binding of PC/PS/GlCer vesicles to DEGR-fxa. DEGR-fxa (initially 200 nM) was titrated with PC/PS (○) or PC/PS/GlCer (●) vesicles, and the binding monitored by measuring the anisotropy (\( r \)) at 340 nm excitation and 540 nm emission. The initial anisotropy of DEGR-fxa before the addition of vesicles is designated the term \( r_0 \), whereas \( r \) is the anisotropy at any point in the titration.
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The concentrations above which NBD labeled PC (C16,6-NBD-PC) and PS (C16,6-NBD-PS) aggregate were determined using homo-FRET measurements. Fluorophores such as NBD, which exhibit a small Stokes’ shift, can efficiently undergo fluorescence self-transfer that is readily detected by the resulting depolarization of emission monitored as a decrease in anisotropy at different lipid concentrations (32). Below the CMC of the short-chained lipid, NBD anisotropy remained constant, whereas when the lipids started to aggregate NBD anisotropy decreased due to homo-FRET. At high concentrations of lipid, the anisotropy reached a constant low value, presumably due to complete energy transfer between the NBD moieties. For example, for C16,6-NBD-PS the anisotropy of the NBD moiety decreased and reached a plateau value of 0.076 \pm 0.005) below 960 nM phospholipid but decreased sharply at higher levels due to homo-FRET, and thus its CMC was 960 nM (data not shown). The CMC values for C16,6-NBD-PS (Fig. 8, inset) and C16,6-NBD-PC (data not shown) were also determined in the presence of 15 \mu M C8-GlcCer. Both C16,6-NBD-PS (190 \mu M) and C16,6-NBD-PC (320 \mu M) aggregated at a lower concentration in the presence of C8-GlcCer compared with the absence of C8-GlcCer.

### Affinity of Coumarin-APC for Phospholipids in the Presence or Absence of C8-GlcCer

To assess if short-chained C8-GlcCer altered the binding affinities of APC for PS and PC, point-to-point FRET was performed between coumarin-APC and NBD-labeled short-chained PC or PS in the presence or absence of an
excess of C8-GlcCer, where coumarin served as the FRET don-
or and NBD as the acceptor. Upon addition of the acceptor-labeled phospholipids, the ratio of quantum yields ($Q_{DA}/Q_{D}$) of coumarin-APC decreased due to APC binding to the lipids (Fig. 8). Both C16,6-NBD-PC and C16,6-NBD-PS bound to APC, and C16,6-NBD-PS bound to coumarin-APC with a 2-fold greater affinity compared with C16,6-NBD-PC. Addition of C8-GlcCer to coumarin-APC prior to NBD-labeled phospholipids altered the titration with NBD-labeled PS but not NBD-labeled PC (Fig. 8). In the presence of C8-GlcCer, the C16,6-NBD-PS transition from soluble to aggregated lipid occurred above 190 nM phospholipid. Because the effect of C8-GlcCer on FRET between C16,6-NBD-PS and cou- marin-APC FRET occurred well below this CMC of C16,6-
NBD-PS, i.e. half-maximal FRET at 50 nM NBD-PS, it is likely that the coumarin to NBD FRET monitored the binding of APC to soluble phospholipids. Because C8-GlcCer itself binds to APC (Fig. 7), we conclude that C8-GlcCer bound to APC increased the affinity of APC for C16,6-NBD-PS.

FXa, an APC homolog, reportedly binds short chain PS at three different loci, namely the Gla domain, and the two epidermal growth factor domains (34). In our experiments, the effect of C8-GlcCer on FRET between the coumarin donor tethered to the active site of APC and the NBD acceptors located on the short-chained phospholipids was very similar at high lipid concentra-
tions for all four titrations (Fig. 8), suggesting that both short-chained phospholipids bind to approximately the same region on APC. A $R_0$ of 39.6 Å has been reported for the coumarin-NBD donor-acceptor pair (33). Assuming that the $R_0$ of this donor-acceptor pair does not change upon covalent at-
tachment of donor and acceptor to protein and lipid, respect-
ively, a distance of closest approach of ~50 Å was calculated using Equation 3 in the FRET experiments placing the phospholipid binding site(s) near the Gla domain of APC. Therefore, these FRET data suggest that C16,6 NBD-PS and C16,6-
NBD-PC bind far away from the active site of APC and that the presence of C8-GlcCer prior to the titration, does not alter the location of phospholipid binding, although its presence does increase the affinity of C16,6-NBD-PS for APC.

DISCUSSION

The present study reveals that GlcCer, a neutral glycosphin-
golipid, enhances the anticoagulant activity of APC by increas-
ing the affinity of APC for negatively charged phospholipid vesicles containing PS. Therefore, these direct binding studies provide a mechanistic rationale for the recently observed aug-
mentation of APC anticoagulant activity by GlcCer and for the venous thrombosis associated with deficiency of GlcCer (18, 19). Furthermore, our data show that C8-GlcCer, a short chain fatty acid-containing analog of GlcCer, can directly bind to a site(s) on APC below the CMC and increase the affinity of APC for soluble PS but not for soluble PC. These results therefore suggest the existence of a functionally significant GlcCer-bind-
ing site(s) on APC and provide a reasonable explanation for the PS dependence of the GlcCer anticoagulant cofactor effects.

Our results are in accordance with the recently reported observations that only certain, but not all, glycolipids enhance the APC-dependent inactivation of fVa (19). For example, whereas GlcCer enhances the anticoagulant response of APC, GalCer, a diastereomer of GlcCer, does not. Thus, the GlcCer effect is stereospecific. Making the reasonable assumption that GlcCer and GalCer produce equivalent effects on membrane fluidity, these data would suggest that a direct interaction of GlcCer with APC and/or fVa and not membrane fluidity changes caused by the introduction of neutral glycolipids in a phospholipid milieu, is responsible for the enhanced lipid bind-
ing by APC.

From the FRET data for membrane-bound topography of APC (Fig. 5) we conclude that the GlcCer-containing phospho-
lipid vesicles bind to the same end of the APC molecule as that of the vesicles lacking GlcCer. Based on the average $R$ of 64 Å, we suggest that the Gla domain of APC harbors the specific GlcCer binding site(s) that is responsible for the enhanced phosphatidylserine binding in APC. Since GlcCer does not af-
fect Xa-induced clotting in a Xa-1 stage assay (19) and tissue factor-induced clotting in a dilute prothrombin time assay in the absence of APC, a comparison of the protein C Gla domain amino acid sequence with those of IX, factor VII, and prothrom-
bin was made, which revealed several interesting differences in amino acids. For example, the amino acid at position 23 in protein C, which is thought to play an important role in mem-
brane-binding properties of protein C, is occupied by a nega-
tively charged Asp or Gla residue, respectively, in human and bovine protein C, whereas this position is substituted by un-
charged Ser residues in human and bovine VII, IX, and pro-
thrombin. Furthermore, we speculate that two other potential candidates for APC interactions with GlcCer are Gln and Asp at position 32 and 36 in protein C, respectively, which are markedly different from those in IX, prothrombin, and VII. Residues 23, 32, and 36 in protein C may potentially interact specifically with the polar head group of the GlcCer molecule by forming suitable hydrogen bonds. The ether oxygen that con-
nects the sugar to the ceramide moiety and the hydroxyl group on the fourth carbon of the glucopyranose have been shown to be critical for the anticoagulant activity of GlcCer since glucose, ceramide, and GalCer are not functionally active. The three aforementioned amino acid residues of protein C are in close pro-
ximity to each other in a three-dimensional computer model of the Gla domain and could very well be collectively involved in dock- ing the GlcCer molecule in the Gla domain of APC.

GlcCer is present in plasma, mainly in lipoproteins, at a concentration of ~10 μM (18). GlcCer in cells is predominantly located on the external leaflet of cellular membrane bilayers, where it can cluster in detergent-insoluble microdomains en-
riched in glycosphingolipids and where these microdomains or so-called rafts can form either in caveolae or at other cell membrane loci (35–38). Based on recently reported studies (18,
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19) and our results here, we hypothesize that microdomains enriched in neutral glycosphingolipids could serve as “anticoagulant microdomains” because GlcCer would promote APC binding. Moreover, such glycosphingolipid-enriched domains could also mediate other APC-dependent functions such as anti-inflammatory or anti-apoptotic activities of APC (39–42).

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