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Human Annexin V Binds to Sulfatide: Contribution to Regulation of Blood Coagulation

Michiru Ida*, **Ayano Satoh, Isamu Matsumoto and Kyoko Kojima-Aikawa**†

The Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610

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Annexin V is a calcium-dependent phospholipid-binding protein that exhibits anticoagulant activity on binding to phosphatidylserine exposed on the activated surfaces of endothelial cells and platelets, inhibiting activation of factor X and prothrombin in the blood coagulation cascade. Sulfatide (galactosylceramide I^3-sulfate), one of the glycosphingolipids of the platelet cell membrane, is thought to be involved in blood coagulation systems *via* activation of factor XII. In this study, we examined whether or not annexin V binds to sulfatide and affects the coagulant activity of sulfatide. Solid phase assaying of annexin V revealed that it binds specifically to sulfatide, *i.e.* not to galactosylceramide or gangliosides, in the presence of calcium ions. Affinity analysis by means of surface plasmon resonance showed that the K_D of the interaction between annexin V and sulfatide is 1.2 µM. Kinetic turbidometric assaying of plasma coagulation initiated by $CaCl_2$ revealed that the coagulation rate in the presence of sulfatide or phosphatidylserine was decreased by annexin V. These results suggest that annexin V regulates coagulability in the blood stream by binding not only to phosphatidylserine but also to sulfatide.

Key words: annexin, anticoagulant, lectin, plasma coagulation, sulfatide.

Abbreviations: GAG, glycosaminoglycan; GST, glutathione S-transferase; NHS, *N*-hydroxysuccinimide; PC, phosphatidylcholine; PS, phosphatidylserine; RU, response units; TBS, Tris-buffered saline.

Annexins comprise a structurally related protein family composed of four (or eight in the case of annexin VI) repeating domains of 70 amino acids and an N-terminal domain specific for each member. They have the common property of binding to calcium and phospholipids. They are found in a variety of cell types in higher and lower eukaryotes but not yeast. Their functions *in vivo* are unclear, but their close association with cell membranes suggests their involvement in various processes, which include membrane trafficking, cytoskeletal-membrane interactions, signal transduction, cell adhesion, and regulation of the activities of ion channels and other membrane-bound proteins (*1*, *2*). Although annexins were originally considered to be intracellular proteins, many extracellular events are now thought to be annexin-mediated: blood coagulation (*2*), cell adhesion (*3*, *4*), neurotrophy (*5*), and interaction with extracellular matrix molecules such as collagen (*6*) and tenascin-C (*7*).

Annexin V was formerly called vascular anticoagulant α (*8*), and is known to inhibit the binding of coagulant factors Xa and Va to phosphatidylserine (PS) on the exposed surfaces of activated platelets due to its phospholipid-binding property. It is also known to inhibit the activation of factor X (*9*) and the production of thrombin (*8*). PS is usually localized on the inner plasma membrane, but it is exposed on the outer membrane when cells are injured and platelets are activated. When PS is exposed on the surfaces of endothelial cells and platelets, annexin V is thought to prevent concentration of coagulant factors in PS-rich domains on cell surfaces and amplification of the coagulation response in the blood coagulation cascade (*10*).

Sulfatide (galactosylceramide I^3-sulfate), a sulfated glycosphingolipid, exists on the surface of oligodendrocytes (*11*), erythrocytes (*12*), and platelets (*13*). Sulfatide is thought to have complicated functions in coagulation systems. Formerly, it was thought that sulfatide activates blood coagulation factor XII *in vitro* (*14*). Recently, it was shown that sulfatide also has strong anticoagulant activity that prolongs the anticoagulation time and bleeding time *in vivo*, and that it interacts with fibrinogen (*15*). On the other hand, it has been reported that sulfatide promotes thrombus formation due to vein ligation in the rat deep vein thrombosis model (*16*, *17*). Moreover, the interaction between sulfatide and P-selectin, both of which are expressed on platelets, is important in platelet adhesion and aggregation, and causes the formation of fibrin clots (*18*).

We previously reported that annexin V binds to glycosaminoglycans (GAGs) in the presence of calcium ions and also suggested that annexin V recognizes mainly sulfate groups on heparin (*19*). These findings raised the possibility that annexin V binds to sulfatide, as another ligand, besides PS in the regulation of blood coagulability. In this study, we examined the binding of annexin V

*Present address: Department of Perinatology, Institute for Developmental Research, Aichi Human Service Center, 713-8 Kagiya-cho, Kasugai, Aichi 480-0392.

†To whom correspondence should be addressed. Tel: +81-3-5978-5345, Fax: +81-3-5978-5345, E-mail: kyoko@cc.ocha.ac.jp

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to sulfatide, and showed that *in vitro* acceleration of coagulation by sulfatide is inhibited by annexin V.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Human Annexin V—The open-reading frame of human annexin V was cloned by RT-PCR and sequenced. We extracted total RNA from human colon adenocarcinoma cell line HT29 by the standard guanidium thiocyanate procedure, and used PCR primers based on EMBL/GenBank/DDBJ accession number J03745 with *Bam*HI and *Eco*RI sites. Human annexin V cDNA was ligated into plasmid pGEX-3X (Amersham Pharmacia Biotech, Piscataway, NJ) for expression in *E. coli* as a glutathione S-transferase (GST)–annexin V fusion protein. For binding assays on microtiter plates and surface plasmon resonance assays, GST-free annexin V was used. To remove GST from the fusion protein, we used restriction protease factor Xa (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentrations were determined using a Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Biotinylated GST-annexin V was prepared as follows: 1 ml of 2 mg/ml GST-annexin V dissolved in 50 mM $NaHCO_3$ (pH 8.5) was incubated with 74 μl of 1 mg/ml *N*-hydroxylsulfosuccinimide ester of biotin (EZ-link™ sulfo-NHS-biotin: Pierce, Rockford, IL) for 1 h on ice, followed by blocking with 20 μl 1 M Tris-HCl (pH 7.5) to remove unreacted biotin.

Glycolipid-Binding Assay on Microtiter Plates—Sulfatide (bovine brain), ganglioside GM1 (bovine brain), GM3 (canine blood), galactosylceramide (bovine brain), and ceramide (bovine brain) were purchased from Sigma (St. Louis, MO). A glycolipid (2.5 μg) dissolved in methanol was added to each microtiter well (Immulon 1B; Dynex Technologies, Chantilly, VA) and adsorbed on the polystyrene surface by evaporating off the solvent at 37°C. The plate was blocked with 2% skim milk in Tris-buffered saline (TBS) for 2 h. Annexin V at various concentrations was added to the wells, followed by incubation for 2 h in the presence of 5 mM $CaCl_2$. The wells were then washed with TBS containing 1 mM $CaCl_2$ three times, and blocked with 2% skim milk for 1 h. Anti-bovine annexin V polyclonal antibodies, which were prepared in our laboratory (*19*) and cross-react with human annexin V, were added to the wells, followed by incubation for 1 h. The wells were then washed and blocked with 2% skim milk for 15 min. Peroxidase-labeled affinity-purified antibodies to rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to the wells, and then the plate incubated for 30 min. The wells were then washed, and developed by the addition of 200 μl 0.04% (w/v) *o*-phenylenediamine and 0.01% (v/v) H_2O_2 in citrate-phosphate buffer (pH 5.0); color development was stopped by the addition of 50 μl 4 N H_2SO_4. The absorbance of each well was then read at 490 nm. For the dose-dependent assays, the lipids used comprised 2.5 μg per well of sulfatide and dimyristoylphosphatidylcholine (PC, Sigma) in various ratios, and the wells were examined by the same method. In the dose- and calcium-dependent assays, the amount of annexin V added was 50 μl per well, the concentration being 50 μg/ml.

Glycolipid-Binding Assay on TLC Plates—Glycolipids (2.5 μg) were developed on silica thin-layer plastic plates (Polygram SIL G; Macherey-Nägel, Duren, Germany) with chloroform/methanol/0.2% aqueous $CaCl_2$ (60:30:6). Glycolipid visualization was performed by the orcinol-surfuric acid method (*20*). After separation, each plate was blocked with 1% polyvinylpyrrolidone-1% BSA-TBS and then incubated with biotinylated GST-annexin V (10 μg/ml) in 5 mM $CaCl_2$-TBS overnight at 4°C. The plate was then washed with 0.1% Tween 20-1 mM $CaCl_2$-TBS three times, and blocked with the blocking solution for 15 min. The plate was then incubated with streptavidin-peroxidase (ICN Pharmaceuticals, Costa Mesa, CA) for 1 h. The plate was washed and then stained with 4-chloro-1-naphthol.

Surface Plasmon Resonance Assay—We examined the interaction between annexin V and sulfatide by means of surface plasmon resonance assays using a BIAcore 2000™ (BIAcoreAB, Uppsala, Sweden). Liposomes composed of PC alone or 10% sulfatide in PC were prepared by sonication and extrusion through the membrane (pore diameter, 100 nm) of a Liposofast™ apparatus (Avestin, Ottawa, ON). The hydrophobic sensor chip, HPA, was initially washed with 40 mM β-octylglucoside for 5 min at a flow rate of 5 μl/min. Liposomes (2 mg/ml) in TBS were then injected for 30 min at a flow rate of 1 μl/min. To remove unbound liposomes, 10 mM NaOH was injected for 5 min at a flow rate of 5 μl/min. Lipid surfaces were blocked with 5 μM BSA. Forty microliters of annexin V in TBS was injected in the presence of 1 mM $CaCl_2$ at a flow rate of 20 μl/min. The surface was regenerated after each cycle of measurement by injecting 10 μl of 0.5 M EDTA. The binding parameters were calculated with the evaluation program BIAevaluation 3.1 (BIAcoreAB).

Plasma Coagulation Assay—Sulfatide or PS (bovine brain, Sigma, St. Louis, MO) (2.5 μg) was added to each microtiter well and then adsorbed by evaporating off the solvent at 37°C. The plate was blocked with 3% BSA in TBS for 30 min. GST-annexin V at various concentrations was added to the wells, followed by incubation for 1 h in the presence of 5 mM $CaCl_2$. The wells were then washed twice with TBS containing 1 mM $CaCl_2$, and 100 μl of 50% human control plasma (Dade Behring Marburg GmbH, Marburg, Germany) in PBS was added to them. After incubation for 1 min at 37°C, 25 μl of 0.08 M (final concentration, 16 mM) $CaCl_2$ was added to each well in order to start coagulation. This calcium concentration allowed efficient performance of experiments with normal serum coagulation starting at 20 min and finishing at 30 min. The absorbance of each well was read at 405 nm and recorded every 15 s with a Wellreader SK603 (Seikagaku, Tokyo). The onset time of fibrin formation in the analysis was defined as the time when the absorbance value in each well was elevated by more than 0.1 above the baseline.

RESULTS AND DISCUSSION

Annexin V Bound to Sulfatide—In this study, we examined the interaction between annexin V and sulfatide using several assay systems: glycolipids were adsorbed on a plastic or thin-layer silica plate surface, and then solid-phase binding to annexin V was exam-

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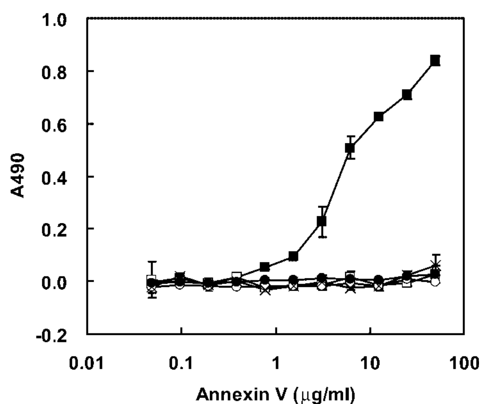

Fig. 1. **Binding of annexin V to glycolipids on microtiter plates.** 2.5 µg of sulfatide (closed squares), GM1 (open circles), GM3 (closed circles), galactosylceramide (open squares), and ceramide (crosses) immobilized on plastic plates were allowed to interact with annexin V in the presence of 5 mM $CaCl_2$, followed by detection with anti-annexin V polyclonal antibodies. Each point is the mean ± SD of two determinations.

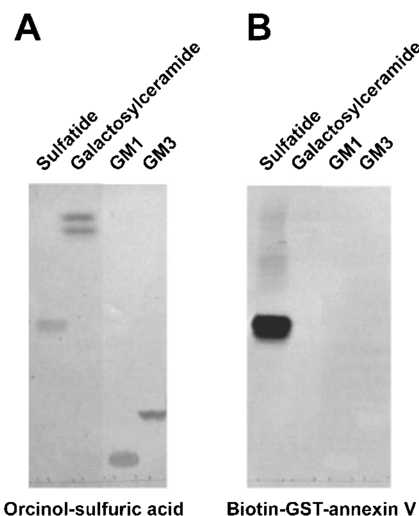

Fig. 3. **Binding of annexin V to glycolipids on TLC plates.** 2.5 µg aliquots of lipids were chromatographed on TLC plates. Glycolipids were visualized with orcinol-sulfuric acid (A). The binding of annexin V to various lipids was examined on TLC plates (B). TLC plates were incubated with biotinylated-GST-annexin V in the presence of 5 mM $CaCl_2$, followed by detection with streptavidin-peroxidase.

ined. First, annexin V was incubated with sulfatide in the presence of calcium ions on a plastic plate, and then annexin V bound to the sulfatide was detected with anti-annexin V antibodies. On a microtiter plate, annexin V bound only to sulfatide among the glycolipids tested in the presence of calcium ions (Fig. 1). Annexin V failed to bind to galactosylceramide or ceramide, which are constituents of sulfatide. Further, annexin V did not bind to gangliosides GM1 and GM3, even though they have a negatively charged sialic acid. In the absence of calcium ions, annexin V did not bind to sulfatide (data not shown). These results suggested that annexin V does not recognize the sulfate groups of sulfatide through a simple electrostatic interaction.

Moreover, to determine the binding specificity to sulfatide, we performed a dose-dependent assay. Sulfatide mixed with PC, to which annexin V does not bind, in various ratios was immobilized on a microtiter plate, and then binding of annexin V was assayed in the same way. Annexin V bound to sulfatide in a dose-dependent manner in the presence of calcium ions (Fig. 2). Next, we examined the binding of annexin V to sulfatide directly on the TLC silica plate surface. Annexin V was labeled with biotin in order to increase the level of detection and to reduce the number of steps in the experiment. We used GST-annexin V for biotinylation to prevent artificial inactivation of annexin V through modification of the amino groups important for the interaction with sulfatide. On the TLC plate, annexin V also bound only to sulfatide, *i.e.* not to gangliosides or galactosylceramide (Fig. 3), in agreement with the results of the microtiter plate assay (Fig. 1). Biotinylated GST did not bind to sulfatide or the other glycolipids (data not shown). To determine the concentration of calcium ions required for the interaction between annexin V and sulfatide, we next carried out binding experiments with varying concentrations of $CaCl_2$. As shown in Fig. 4, annexin V exhibited binding activity toward sulfatide in the presence of mM levels of calcium ions. Since the concentrations of calcium ions in the extracellular spaces and blood are of mM order, the interaction of annexin V with sulfatide in the blood circulation is feasible as a physiological phenomenon.

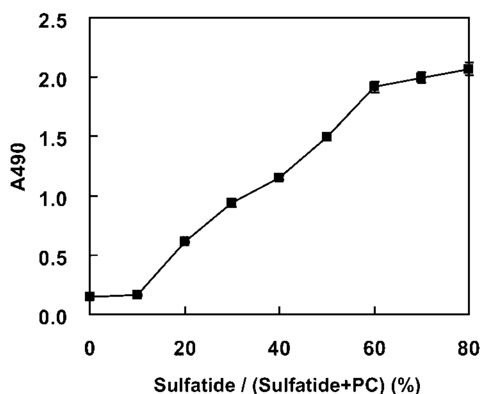

Fig. 2. **Dose-dependent binding of annexin V to sulfatide.** Microtiter wells coated with 2.5 µg of mixtures of sulfatide/PC in various ratios were incubated with annexin V in the presence of 5 mM $CaCl_2$, followed by detection with anti-annexin V polyclonal antibodies. Each point is the mean ± SD of two determinations.

Kinetics of Interaction between Annexin V and Sulfatide—To determine the binding parameters of the interaction between annexin V and sulfatide, we performed surface plasmon resonance assays using the BIAcore system. Liposomes were prepared with sulfatide and PC, which was used as a control, in a ratio of 1:9 and then immobilized on a HPA chip. Various concentrations of annexin V were allowed to flow over the chip in the presence of 1 mM calcium ions. The amount of lipid immobilized at 10% sulfatide–90% PC was 1,700 response units (RU); for PC alone, it was 2,000 RU. The sensorgram of sulfatide was subtracted from that of PC alone as a control, as shown in Fig. 5A. Under the same conditions, BSA did not bind to sulfatide or PC (data not

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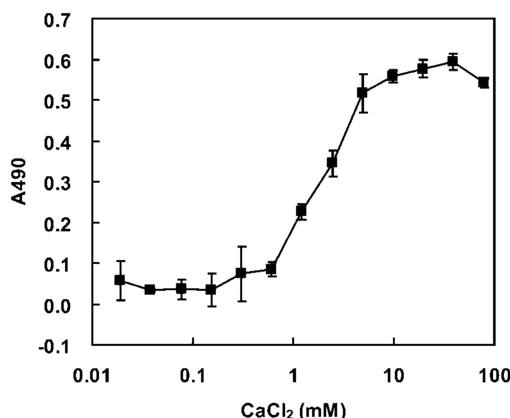

Fig. 4. **Effects of calcium ions on the binding of annexin V to sulfatide.** Annexin V binding to sulfatide was examined with various concentrations of $CaCl_2$. 2.5 μg of sulfatide immobilized on a plastic plate was allowed to interact with annexin V, followed by detection with anti-annexin V polyclonal antibodies. Each point is the mean ± SD of two determinations.

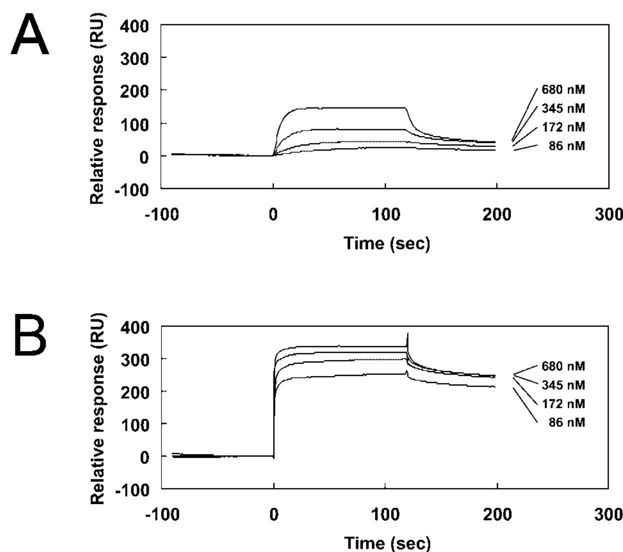

Fig. 5. **Sensorgrams of annexin V binding to immobilized lipids on surface plasmon resonance assaying.** Liposomes comprising 10% sulfatide–90% PC (A) or 10% PS–90% PC (B) were immobilized on a HPA chip. Various concentrations of annexin V (690, 345, 172 and 86 nM, in order of the highest response) were allowed to flow over the chip at a rate of 20 μl/min in the presence of 1 mM $CaCl_2$ at 25°C. The sensorgram data were deducted from sensorgram data with immobilized PC alone as a control.

shown). The K_D of the interaction between annexin V and sulfatide was 1.2 μM. As shown in Fig. 5B, annexin V bound to PS, an annexin V–binding phospholipid, the K_D being 180 nM (the amount of lipid immobilized at 10% PS–90% PC was 1,700 RU; for PC alone, it was 1,800 RU). These results showed that annexin V bound to sulfatide, but that the interaction with sulfatide was weaker than that to PS. Kinetic analyses of the interaction between annexin V and heparin have already reported (*21*, *22*). As compared with the K_D of the interaction between annexin V and heparin, 20 nM, the binding of sulfatide was weak in this study. However, sulfatide may comprise a condensation domain, such as a raft, on the membrane surface and may have a multivalent effect; therefore, the true K_D might be greater than the value obtained in this study.

Effect of Annexin V on Plasma Coagulation Activity of Sulfatide—It has been reported that sulfatide exhibits coagulation activity that accelerates the coagulation of plasma, and that the activity is factor XII–dependent (*17*). Annexin V is known as an anticoagulant factor because it prevents the concentration of coagulant factors in PS-rich domains and amplification of the coagulation response in the blood coagulation cascade (*10*); however, the effect of annexin V on the activity of sulfatide in blood coagulation has not been studied. To determine whether or not annexin V affects the coagulant activity of sulfatide, we next performed kinetic turbidometric assaying of plasma coagulation initiated by $CaCl_2$. In this experiment, we used GST-annexin V because GST-free annexin V is contaminated by factor Xa, which is used as a protease for cleavage of the linker region between GST and annexin V. Factor Xa is a blood coagulation factor and contamination by it in the assay is inconvenient. As shown in Fig. 6A, sulfatide started to coagulate plasma at 4.3 min and remarkably increased the coagulation rate in comparison to in a non-lipid control well, where coagulation started at 20 min. These results agree with previous reports (*16*, *17*). Sulfatide is thought to activate factor XII, the factor that starts the coagulation cascade, suggesting that sulfatide strikingly advances the onset time of coagulation. On the other hand, PS alone did not promote plasma coagulation (Fig. 6C). This may be because PS binds to coagulant factors Xa and Va, which are involved in steps that are rather late in the coagulation cascade. As shown in Fig. 6B, when GST-annexin V was added to plasma in a sulfatide-coated well, the onset of coagulation was delayed; a concentration of 20 μg/ml of GST-annexin V delayed the onset time of coagulation to 7.35 min. These results demonstrated that annexin V inhibits the plasma coagulation activity of sulfatide. This activity of annexin V is probably due to interruption of the binding between sulfatide and factor XII. When GST-annexin V was added to plasma in a PS-coated well, the onset time of coagulation was also delayed. For both lipids, the effects of GST-annexin V were dose-dependent, but the rate of coagulation was remarkably different in a PS-coated well as compared with in a sulfatide-coated well. Because the K_D value of annexin V binding to PS is smaller than that to sulfatide (Fig. 5), GST-annexin V might exhibit different effects on the coagulation rates with the two lipids. In a control experiment, GST did not delay the onset time of coagulation with either lipid.

Mechanism Underlying Anticoagulant Activity of Annexin V—Coagulability, the balance between blood coagulation and fibrinolysis in the blood circulation, is regulated through complex mechanisms in which many steps and a variety of molecules are involved. Annexin V has been considered one of the modulatory molecules for coagulation in the blood circulation. The amount of annexin V in the blood is 1.7 ng/ml (*23*), and there have been several reports of anticoagulant activity of annexin V *in vivo*. Fibrin accretion on injured vessel walls decreased when annexin V was administered to rabbit

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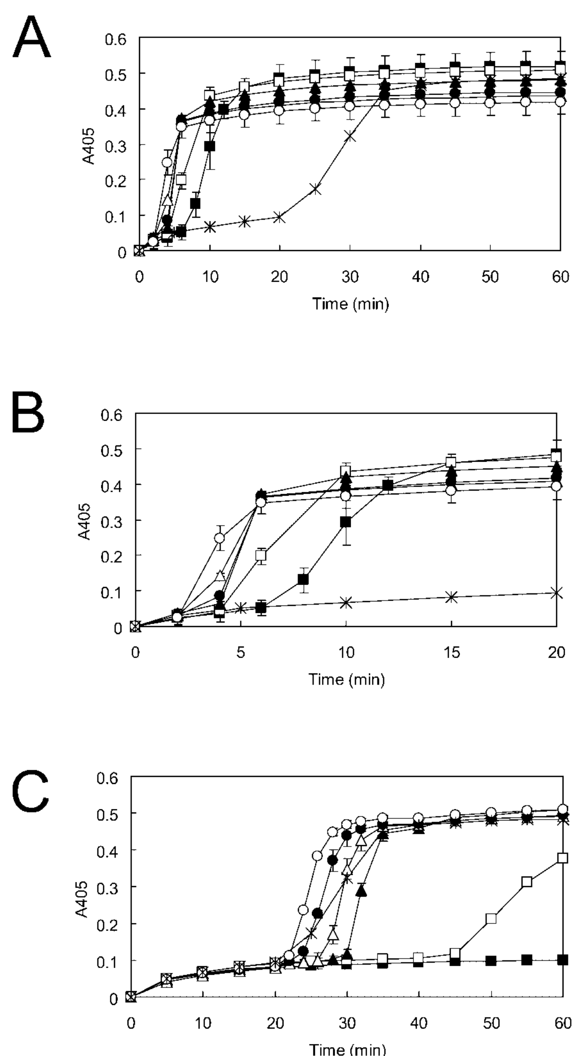

Fig. 6. **Effect of GST-annexin V on the plasma coagulation time.** The effect of GST-annexin V on the plasma coagulation time with sulfatide (A, B) or PS (C) was examined. Panel B is an enlargement of the x-axis in panel A. GST-annexin V [20 μg/ml (closed squares), 10 μg/ml (open squares), 5 μg/ml (closed triangles), and 2.5 μg/ml (open triangles)], or 20 μg/ml of GST (closed circles) or nothing (open circles) was incubated in lipid-coated microtiter wells in the presence of 5 mM $CaCl_2$ for 1 h. Control wells were not coated with any lipid (crosses). Aliquots of 100 μl of plasma in PBS were added to the wells. Plasma coagulation was initiated by adding $CaCl_2$, and the absorbance at 405 nm was monitored. Each point is the mean ± SD of two determinations.

jugular veins injured by air perfusion (*24*), and annexin V exhibited significant antithrombotic activity when it was administered to veins in a laser-induced thrombosis formation model rat (*25*). Recently, the hypothesis that annexin V has a thrombomodulatory function on the surfaces that line the placental and systemic vasculature has been supported by epidemiological studies. It has been reported that annexin V plays roles in the physiological control of blood coagulation reactions and also that abnormalities of annexin V expression are associated with clinical diseases: annexin V is decreased on placental trophoblasts and on endothelial cells in the antiphospholipid syndrome, an antibody-mediated effect that is associated with pregnancy loss and with thrombosis (*26*). Overall, undoubtably annexin V plays significant roles in the regulation of coagulability, though the entire regulatory mechanism remains unclear. It might be important that annexin V exists in the blood stream at a constant level to control coagulation reactions. Overall, the interactions of annexin V with plural ligands in the blood circulation, including PS and sulfatide, may contribute to the regulation of anticoagulant activity.

Sulfatide exists in the blood circulation as a cell surface molecule on erythrocytes (*12*) and platelets (*13*). Factor XII is activated in the presence of prekallikrein and high molecular weight kininogen with negatively charged inorganic surfaces (glass and kaolin), and macromolecules with biological properties (collagen, GAG, lipopolysaccharide, and amyloid β). Sulfatide is thought to exhibit the same activity toward negatively charged surfaces and to initiate the blood coagulation cascade; however, it is not clear how much sulfatide contributes to the regulation of blood coagulability *in vivo.* Cerebroside sulfotransferase-null mice, which do not express sulfated glycolipids such as sulfatide and seminolipid, have already been produced. These mice exhibit abnormalities in myelin function and spermatogenesis (*27*), but no abnormality in blood coagulation systems was found under the optimized conditions.

PS is normally located in the inner leaflet of the lipid bilayer, but is exposed on the extracellular surface when cells are damaged and stressed chemically and/or physically. It is thought that complexes of coagulation factors, Xa-prothrombin-Va and IXa-X-VIIIa, are concentrated on PS-rich cell surfaces and that the blood coagulation reactions proceed there. A mechanism by which annexin V conceals PS and inhibits the binding of coagulation factors to PS-rich cell surfaces to prevent coagulation has been proposed. However, there is no obvious evidence that annexin V inhibits the coagulant activity of PS. Also, in our experiments, GST-annexin V was clearly observed to strongly repress plasma coagulation in the presence of PS, while it is still unclear if GST-annexin V actually inhibits the coagulant activity of PS. It is possible that these results mean that annexin V not only inhibits PS activity but also exhibits an intrinsic anticoagulant activity that is expressed on binding to PS.

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