# Specific Membrane Binding of Factor VIII Is Mediated by *O*-Phospho-L-serine, a Moiety of Phosphatidylserine†

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ABSTRACT: Phosphatidylserine, a negatively charged lipid, is exposed on the platelet membrane following cell stimulation, correlating with the expression of factor VIII receptors. We have explored the importance of the negative electrostatic potential of phosphatidylserine vs chemical moieties of phosphatidylserine for specific membrane binding of factor VIII. Fluorescein-labeled factor VIII bound to membranes containing 15% phosphatidic acid, a negatively charged phospholipid, with low affinity compared to phosphatidylserine-containing membranes. Binding was not specific as it was inhibited by other proteins in plasma. Factor VIII bound to membranes containing 10% phosphatidylserine in spite of a varying net charge provided by 0–15% stearylamine, a positively charged lipid. The soluble phosphatidylserine moiety, *O*-phospho-L-serine, inhibited factor VIII binding to phosphatidylserine-containing membranes with a $K_i$ of 20 mM, but the stereoisomer, *O*-phospho-D-serine, was 5-fold less effective. Furthermore, binding of factor VIII to membranes containing synthetic phosphatidyl-D-serine was 5-fold less than binding to membranes containing phosphatidyl-L-serine. Membranes containing synthetic phosphatidyl-L-homoserine, differing from phosphatidylserine by a single methylene, supported high-affinity binding, but it was not specific as factor VIII was displaced by other plasma proteins. *O*-Phospho-L-serine also inhibited the binding of factor VIII to platelet-derived microparticles with a $K_i$ of 20 mM, and the stereoisomer was 4-fold less effective. These results indicate that membrane binding of factor VIII is mediated by a stereoselective recognition *O*-phospho-L-serine of phosphatidylserine and that negative electrostatic potential is of lesser importance.

Factor VIII (antihaemophilic factor) is a plasma glycoprotein that prevents hemorrhaging through its function as an enzyme cofactor bound to the platelet membrane [for reviews, see Kane and Davie (1988) and Mann et al. (1990)]. Transfer of factor VIII from plasma to the platelet membrane after vascular injury serves several purposes. First, membrane binding localizes the procoagulant function to the vascular defect where the stimulated platelet has adhered (Nesheim et al., 1988; Gilbert et al., 1991). Second, membrane-bound factor VIII provides a high-affinity binding site for factor IXa, the enzyme for which it serves as a cofactor, on either platelets (Ahmad et al., 1989) or phospholipid vesicles (Duffy et al., 1992), whereas factor VIII in solution does not efficiently bind factor IXa. Third, membrane binding decreases the $K_M$ of factor X, the substrate for assembled factor VIII and factor IXa (Mann et al., 1988). Although membrane binding of factor VIII is critical, the molecular constituents of the factor VIII receptor have not been identified.

Factor VIII, with $M_r$ 280 000, is homologous to another procoagulant protein, factor V, in amino acid sequence (Church et al., 1984; Gitschier et al., 1984; Toole et al., 1984) and in function as a membrane-bound enzyme cofactor (Furie & Furie, 1988; Kane & Davie, 1988; Gilbert et al., 1990; Mann et al., 1990). The proteins share a repeating domain structure of A1–A2–B–A3–C1–C2, in which the A domains are homologous with ceruloplasmin, the B domain is unique to each protein, and the C domains are homologous with discoidin I, a phospholipid-binding lectin (Bartles et al., 1982), and with a murine milk fat globule membrane protein (Stubbs et al., 1990). Both factor VIII and factor V bind with high affinity to phospholipid membranes via the "light chain" composed of the A3–C1–C2 segment (Krishnaswamy & Mann, 1988). However, factor VIII requires more phosphatidylserine per binding site than factor V (Gilbert et al., 1990), and current evidence implicates different domains in membrane binding. While binding of factor V is mediated by the A3 domain (Kalafatis et al., 1990) and the C2 domain (Ortel et al., 1992), binding of factor VIII is apparently mediated by the C2 domain (Arai et al., 1989; Foster et al., 1990). In plasma, factor VIII differs from factor V in that it circulates in a noncovalent complex with von Willebrand factor (Lollar, 1991). von Willebrand factor competitively inhibits factor VIII from binding to phosphatidylserine-containing membranes (Andersson & Brown, 1981; Gilbert et al., 1992) and to activated platelets (Nesheim et al., 1991). Proteolytic activation by thrombin removes a von Willebrand factor binding peptide of factor VIII (Foster et al., 1988; Pittman & Kaufman, 1988), causing dissociation of the two proteins and allowing factor VIII to bind to activated platelets (Nesheim et al., 1991) and to phosphatidylserine-containing membranes (Gilbert et al., 1992).

A physiological role for negatively charged phospholipids in promoting membrane binding of blood coagulation proteins has been hypothesized. These phospholipids are normally sequestrated on the inner surface of blood cell membranes (Zwaal et al., 1977), but are translocated to the outer surface upon activation of platelets (Bevers et al., 1983) and other cells (Bach & Rifkin, 1990; Bevers et al., 1992). The same agonists that lead to translocation of platelet phosphati-

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dylserine lead to the release of membranous vesicles called platelet-derived microparticles (Sandberg et al., 1985; Sims & Wiedmer, 1986), which have a high concentration of factor VIII receptors (Gilbert et al., 1991). Phosphatidylserine is exposed on the surface of platelet-derived microparticles, and the exposure of both factor VIII receptors and phosphatidylserine is stable over a time course of many minutes in comparison to exposure upon the platelet surface, which is transient (Gilbert et al., 1991; Chang et al., 1993). The role of negatively charged phospholipids such as phosphatidylserine has been questioned because the capacity to provide specific binding sites is doubted. The plasma concentration of factor VIII, 0.2 nM, is as much as 20 000-fold lower than those of other plasma proteins that bind to membranes with negative electrostatic potential, e.g., prothrombin, 1.4 $\mu$M, $\beta_2$-glycoprotein I, 4 $\mu$M. It is believed that a binding interaction based upon net charge would not provide sufficient specificity to select trace proteins such as factor VIII for a critical location upon the platelet membrane (Nesheim et al., 1988; Neuenschwander & Jesty, 1988). We have recently reported that membranes containing phosphatidylserine have binding sites with sufficient specificity to select factor VIII from plasma, although the concentration of other phospholipid binding proteins is as much as 20 000-fold higher (Gilbert et al., 1992). Accordingly, we have hypothesized that the binding of factor VIII to the exterior leaflet of the cell membrane is mediated by recognition of specific lipid moieties rather than via electrostatic potential. This would be analogous to the binding of protein kinase C to the inner leaflet of the cell membrane via stereoselective recognition of phospho-L-serine of phosphatidylserine (Lee & Bell, 1989). We have found that factor VIII binds to negatively charged membranes lacking phosphatidylserine with low affinity and without specificity, but that high affinity and specificity are provided by phosphatidylserine. Further, *O*-phospho-L-serine, a soluble moiety of phosphatidylserine, is an inhibitor of membrane binding of factor VIII.

## MATERIALS AND METHODS

Bovine brain phosphatidylserine, phosphatidic acid prepared from egg yolk phosphatidylcholine, egg yolk phosphatidylcholine, and stearylamine were from Avanti Polar Lipids (Pelham, AL). Cholesterol was from Calbiochem (La Jolla, CA). L-Serine, D-serine, L-homoserine, *O*-phospho-L-serine, *O*-phospho-D-serine, *glycero*-phosphoryl-L-serine, phosphoglycerol, phosphoethanolamine, and the calcium ionophore A23187 were from Sigma (St. Louis, MO). Lot characteristics of *O*-phospho-L-serine and *O*-phospho-D-serine included specific optical rotations of 15.9° and −15.1°, respectively, in 2 M HCl. Frozen von Willebrand factor deficient plasma, from a patient with homozygous type III von Willebrand disease, was from George King Biomedical (Overland Park, KS). Fluorescein 5-maleimide and 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate ($DiIC_{16}(3)$)[1] were from Molecular Probes (Eugene, OR). Phospholipase D from cabbage was from Boehringer Mannheim (Indianapolis, IN).

Recombinant human factor VIII, a generous gift of Dr. Randy Kaufman and Debbie Pittman (Genetics Institute, Cambridge, MA), was labeled with fluorescein maleimide as described (Gilbert et al., 1991, 1992). Lipid bilayers supported by glass microspheres (liposphere) were prepared by allowing sonicated phospholipid vesicles (Barenholz et al., 1977) to fuse on clean glass microspheres of 2.0 $\mu$m diameter (Gilbert et al., 1992). Liposphere were stored at 4 °C in the dark and used within 48 h of preparation. Large multilamellar vesicles were prepared by hydration of a lipid film evaporated onto glass (Bangham et al., 1965). The size distribution of multilamellar vesicles was restricted by sedimentation and filtration through laser-etched polycarbonate membranes as previously described (Gilbert et al., 1991). When sonicated vesicles were prepared for the evaluation of electrostatic membrane potential, the fluorescent membrane lipid $DiIC_{16}(3)$ was included at 0.1 mol %, which does not affect the membrane binding of factor VIII (Gilbert et al., 1991). The relative electrostatic potential of vesicles was evaluated by agarose gel electrophoresis (Rosing et al., 1988) with vesicles visualized by fluorescence of $DiIC_{16}(3)$. Binding of fluorescein-labeled factor VIII to lipospheres was evaluated by flow cytometry using a Coulter Epics Profile II (Gilbert et al., 1992).

Citrate-anticoagulated von Willebrand factor deficient plasma, stored at −80 °C until use, was thawed at 37 °C, and $CaCl_2$ and sodium heparin were added to 13 mM and 4 international units/mL final concentrations, respectively. Calcium was included to offset the 13 mM sodium citrate with which the plasma was supplied. The control buffer for binding experiments was prepared with 14 mM $CaCl_2$, 13 mM sodium citrate, and 4 units/mL sodium heparin in buffer A.

*O*-Phospho-L-serine, *O*-phospho-D-serine, phosphoethanolamine, phosphoglycerol, and *glycero*-phospho-L-serine were dissolved in water, the pH was adjusted to 7.4 with 1 M NaOH, and the concentration of the stock solution was determined by elemental phosphorus assay (Chen et al., 1956). Phosphatidyl-D-serine, phosphatidyl-L-serine, and phosphatidyl-L-homoserine were synthesized by transphosphatidylation of phosphatidylcholine (Comfurius & Zwaal, 1977). The phosphatidylserine synthesis products were dissolved in chloroform and separated by HPLC on a 4.6 × 250 mm silica gel column with 5 $\mu$m bead diameter (AllTech Associates, Inc., Deerfield, IL) using an isocratic mobile phase of acetonitrile, methanol, and phosphoric acid (Chen & Kou, 1982). Fractions correlating to the retention time of natural phosphatidylserine were collected into 1 M $Na_2HPO_4$ at a ratio of 4 parts eluent to 1 part sodium phosphate to convert phosphoric acid to a sodium salt. Solvents were evaporated under reduced pressure, and the lipid was extracted from sodium phosphate salts in chloroform. Aliquots of synthesized phosphatidylserine were analyzed for purity and quantitative phosphatidylserine content by HPLC as described above. The phosphatidyl-L-homoserine synthesis products were dissolved in chloroform/methanol (1:1) and subjected to HPLC on a 10 × 250 mm silica gel column with 5 $\mu$m bead diameter (AllTech Associates, Inc., Deerfield, IL) at 1 mL/min using a 20-min linear gradient of 10–37% solvent A followed by a 40-min plateau. Solvents were as follows: A, methanol/acetic acid/water, 74:22:4; B, chloroform. The retention times of phosphatidic acid, phosphatidylserine, and phosphatidylcholine were identified using an evaporative light-scattering detector (Varex Corp., Burtonsville, MD), and fractions corresponding to the retention time of phosphatidylserine were collected and lyophilized. The purity and identity of the product were determined by subjecting a fraction of the product, suspended in chloroform/methanol (1:1), to HPLC. Samples were eluted and quantitated using a modification of an established solvent system at a flow rate of 1 mL/min with a gradient of 2–8% solvent

[1] Abbreviations: buffer A, 0.14 M NaCl, 0.02 M Tris-HCl, 0.5 mM $CaCl_2$, 0.1% bovine albumin, and 0.001% Tween 80, pH 7.4; $DiIC_{16}(3)$, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

B over 15 min (Lutzke & Braughler, 1990). Solvents were as follows: A, isopropyl alcohol/hexane, 1:1; B, 0.5 mM serine/NaOH (pH 7.7) in HPLC-grade water.

Gel-filtered platelets were prepared as previously described (Gilbert et al., 1991). Platelets were stimulated by 2 $\mu$M A23187, diluted into a suspension of platelets at $1 \times 10^8$/mL from a 200 $\mu$M stock solution in dimethyl sulfoxide. Binding of factor VIII to platelet-derived microparticles was measured by flow cytometry as described for lipospheres (Gilbert et al., 1992).

## RESULTS

If the properties of a membrane binding site for factor VIII are determined by net membrane potential, then a negatively charged phospholipid such as phosphatidic acid should provide binding sites with affinity and specificity equivalent to those of membranes containing phosphatidylserine. We have compared the binding of fluorescein-labeled human factor VIII to membranes supported by glass microspheres (lipospheres) (Gilbert et al., 1992) containing 15% phosphatidic acid vs 15% phosphatidylserine (Figure 1A,B). Factor VIII bound saturably to phosphatidylserine-containing membranes ($K_D$ = 5 nM), but the binding to phosphatidic acid containing membranes was of lower affinity. To further investigate the effect of membrane potential, binding experiments were performed at pH 8.5. At this pH phosphatidic acid carries a double negative charge, whereas phosphatidylserine carries a single negative charge. The binding of factor VIII was unaffected even though phosphatidic acid containing membranes carried a negative potential twice as large as membranes with phosphatidylserine (data not shown). In order to be certain that the difference in factor VIII binding observed with these membranes was not an artifact arising from the use of glass-supported membranes, control experiments were performed in which the binding of factor VIII to large, multilamellar vesicles was evaluated. Factor VIII bound saturably to vesicles containing 15% phosphatidylserine with a $K_D$ equivalent to those of experiments with lipospheres (data not shown). In confirmation of experiments with lipospheres, binding of factor VIII to phosphatidic acid containing vesicles was much less than that to phosphatidylserine-containing vesicles with no evidence of saturation at a factor VIII concentration of 40 nM. An increase in the mole fraction of phosphatidic acid to 70% resulted in more bound factor VIII, but at factor VIII concentrations of 1–20 nM, bound factor VIII remained less than with membranes of 15% phosphatidylserine (data not shown).

The specificity of membrane binding sites for factor VIII was evaluated by measuring the quantity of factor VIII that bound in the presence of von Willebrand factor deficient plasma (Figure 1C). Factor VIII was inhibited from binding to phosphatidic acid containing membranes by as little as 1% plasma, whereas binding to phosphatidylserine-containing sites was affected minimally. The quantity of factor VIII binding to phosphatidic acid containing membranes was small before the addition of plasma (Figure 1B), and the residual binding in the presence of plasma was within 2-fold of background with residual fluorescence possibly representing artifacts from increased light scatter or fluorescence of plasma. Thus, phosphatidylserine contributes to both the affinity and specificity of factor VIII binding sites by a property other than negative charge.

The negative membrane electrostatic potential provided by phosphatidylserine, although not sufficient, may still be necessary for specific, high-affinity binding. To clarify this

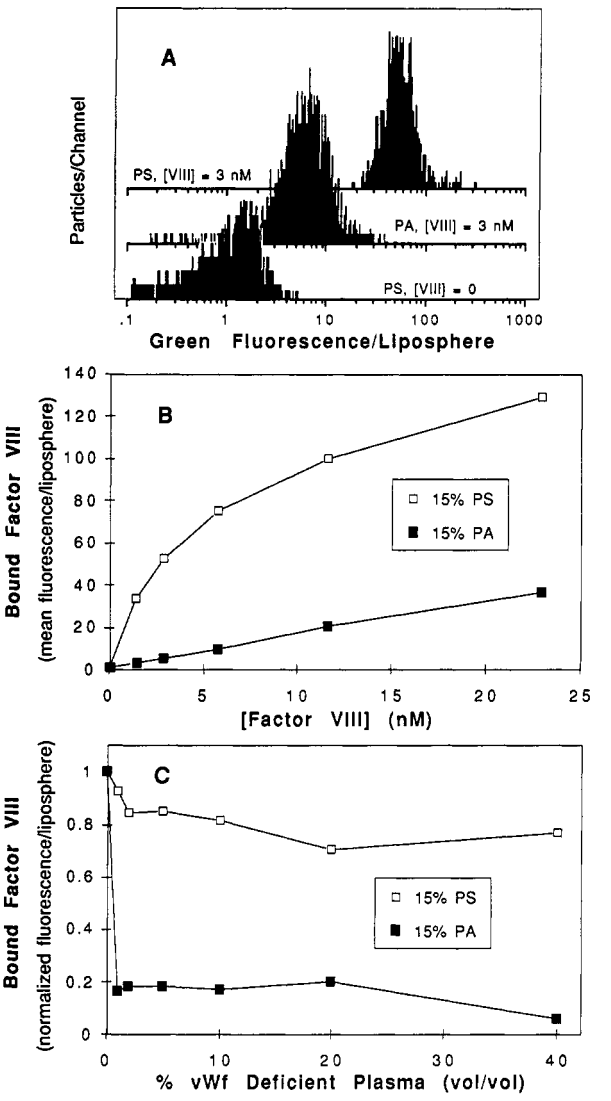


FIGURE 1: Interaction of factor VIII with membrane binding sites containing phosphatidylserine vs phosphatidic acid. (A) Fluorescein-labeled factor VIII was incubated with lipospheres in buffer A, and binding was evaluated by measuring the green fluorescence per liposphere. Histograms of liposphere fluorescence with no added factor VIII were equivalent with both lipid preparations. After incubation with 3 nM fluorescein-labeled factor VIII, lipospheres with phosphatidylserine-containing (PS) membranes had more bound factor VIII than those with phosphatidic acid containing (PA) membranes. (B) Factor VIII bound saturably to lipospheres with membranes containing phosphatidylserine (□). Factor VIII bound with lower affinity to membranes containing phosphatidic acid with no evidence of binding saturation at 23 nM factor VIII (■). (C) In a competition binding experiment, fluorescein-labeled factor VIII (5 nM) was mixed with varying amounts of von Willebrand factor deficient plasma prior to the addition of lipospheres. While plasma proteins had little effect upon the binding of factor VIII to phosphatidylserine-containing membranes, 80% of the factor VIII binding to phosphatidic acid containing lipospheres was displaced by 1% plasma. Binding was evaluated after a 10-min incubation of lipospheres with a mixture of factor VIII and plasma as described (Gilbert et al., 1992). Data were corrected for background fluorescence readings of lipospheres in the presence of plasma and normalized to facilitate comparison of the degree of binding displacement caused by plasma. The lipid compositions were phosphatidylserine/phosphatidylcholine/cholesterol, 15:85:20, and phosphatidic acid/phosphatidylcholine/cholesterol, 15:85:20.

issue, experiments were performed with neutral and positively charged membranes. Stearylamine, a positively charged lipid, was added to phosphatidylserine, phosphatidylcholine, and cholesterol in various quantities, and vesicles were synthesized by sonication. The phosphatidylserine composition was

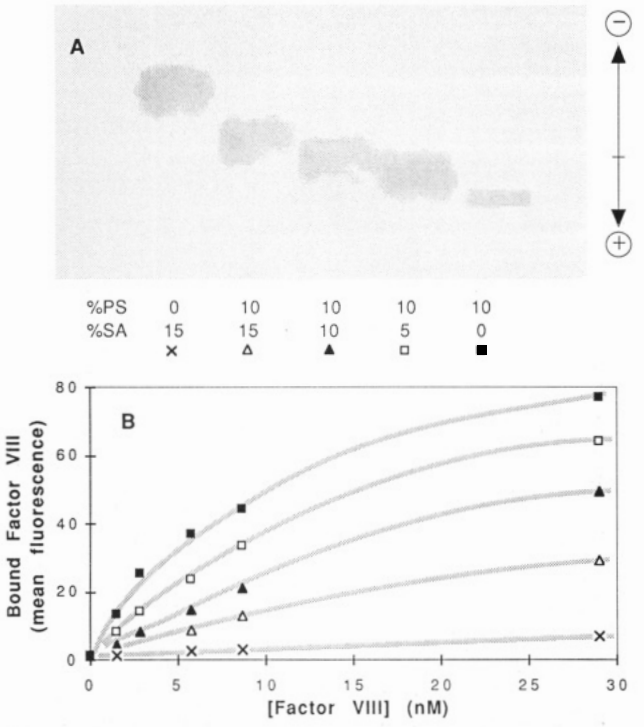


FIGURE 2: Effect of electrostatic membrane potential upon binding of factor VIII. (A) The net potential of phospholipid vesicles was evaluated by agarose gel electrophoresis. The vesicles contained stearylamine, a positively charged lipid, as well as phosphatidylserine and phosphatidylcholine. The final position of vesicles was visualized using the fluorescence of incorporated DiIC16(3) lipid dye. Failure to migrate from the origin (tick on arrow at right) indicated that vesicles with 10% each of stearylamine and phosphatidylserine had no net charge. Other preparations had relative migration rates as predicted on the basis of their composition of positive and negatively charged lipid. (B) Fluorescein-labeled factor VIII was incubated with lipospheres of varying membrane potential, and binding was evaluated. Lipospheres containing stearylamine bound less factor VIII, and the decrease was proportional to the quantity of stearylamine. However, at a factor VIII concentration of 28 nM, more than one-third of the factor VIII that bound in the absence of stearylamine bound to positively charged liposphere membranes containing phosphatidylserine. In contrast, stearylamine-containing membranes that lacked phosphatidylserine did not bind factor VIII. Vesicles and liposphere membranes had phospholipid mole fractions of phosphatidylserine (PS) and stearylamine (SA) as indicated, the balance being phosphatidylcholine, 20 mol % cholesterol, and 0.1 mol % DiIC16(3).

reduced from 15% to 10% because vesicle aggregation was a problem when stearylamine and phosphatidylserine were both present at 15% of total lipid. The net membrane charge of these vesicles was evaluated by observing migration in an electric field using agarose gel electrophoresis (Figure 2A). Vesicles containing more stearylamine than phosphatidylserine migrated toward the anode and those containing less migrated toward the cathode, indicating positive and negative net charge, respectively, as predicted by vesicle composition. Phospholipid from the vesicle preparations was used to prepare lipospheres, and binding of factor VIII was evaluated by flow cytometry (Figure 2B). Bound factor VIII decreased as the electrostatic potential varied from negative to positive. However, at a factor VIII concentration of 28 nM, membranes with neutral net charge bound approximately 60% of the factor VIII that bound to the most negatively charged membranes; positively charged membranes with 15% stearylamine and 10% phosphatidylserine bound 35% of the factor VIII that bound to the most negatively charged membranes. Control lipospheres, containing stearylamine and lacking phosphatidylserine, did not bind factor VIII, indicating that stearylamine did not contribute to factor VIII binding. The specificity of factor VIII binding to these lipospheres was evaluated in the presence of von Willebrand factor deficient plasma as described above. While lipospheres containing stearylamine bound less factor VIII than those without, binding was not decreased more than 15% by 40% plasma (vol/vol). Thus, while stearylamine decreased the net binding of factor VIII to synthetic phosphatidylserine-containing membranes, the remaining binding sites were specific for factor VIII in the presence of plasma. Therefore, a negative membrane potential is not required to support specific binding of factor VIII.

We hypothesized that factor VIII binds specifically to the hydrophilic portion of phosphatidylserine that differs from phosphatidic acid: the L-serine moiety attached to a phosphate oxygen via a phosphoester linkage. Our hypothesis predicted that the stereochemical configuration of the amine and carboxyl moieties about the $\alpha$-carbon of serine may be critical to specific binding (Figure 3). Therefore, we tested the capacity of soluble analogs of phosphatidylserine and phosphatidic acid to displace factor VIII from phosphatidylserine-containing membranes. L-Serine did not inhibit the binding of factor VIII at concentrations up to 100 mM (data not shown). *O*-Phospho-L-serine displaced factor VIII with a $K_i$ of approximately 20 mM (Figure 4). In contrast, *O*-phospho-D-serine had little effect at concentrations up to 40 mM. At higher concentrations, inhibition of binding did occur with 50% inhibition of factor VIII binding at approximately 100 mM. The ionic strength of the buffer increased from 0.17 to 0.40 as the phosphoserine concentration increased from 0 to 100 mM. To determine whether simple ionic effects accounted for binding inhibition by *O*-phospho-D-serine, inhibition experiments were performed with other less complete phosphatidylserine analogs. Phosphoethanolamine, which differs from *O*-phosphoserine in that it lacks a carboxyl at the $\alpha$-carbon, did not inhibit binding of factor VIII at concetrations as high as 70 mM. Phosphoglycerol, which lacks serine altogether but carries the same net charge as *O*-phospho-D-serine, was approximately equivalent to *O*-phospho-D-serine as an inhibitor of factor VIII binding. This suggested that the limited inhibition by *O*-phospho-D-serine may be largely due to phosphate and possibly an ionic effect. *glycero*-Phosphoryl-L-serine was equivalent to *O*-phospho-L-serine in inhibiting membrane binding of factor VIII, suggesting that neither the valence of phosphate nor the interaction of factor VIII with the glycerol moiety of phosphatidylserine contributes significantly to factor VIII binding. These results indicate that factor VIII recognizes *O*-phospho-L-serine as a portion of the specific membrane binding site on phosphatidylserine-containing membranes.

The stereoselective inhibition of factor VIII binding by *O*-phospho-L-serine suggested that the binding of factor VIII to membranes containing phosphatidyl-D-serine would be decreased compared to membranes containing the naturally occurring phosphatidyl-L-serine. To investigate this prediction, we synthesized phosphatidyl-D-serine by trans-phosphatidylation of egg phosphatidylcholine in the presence of excess D-serine using the phospholipase D from cabbage. Phosphatidyl-L-serine was synthesized as a control. Lipid was purified by HPLC and detected by absorbance at 203 nm as described under Materials and Methods. The identity of purified phosphatidylserine as the major product was confirmed by an HPLC retention time equivalent to that of natural phosphatidylserine (Figure 5A). The products were quantitated by integration of the peak area monitored at 203 nm vs a standard curve developed from egg phosphatidylcholine.

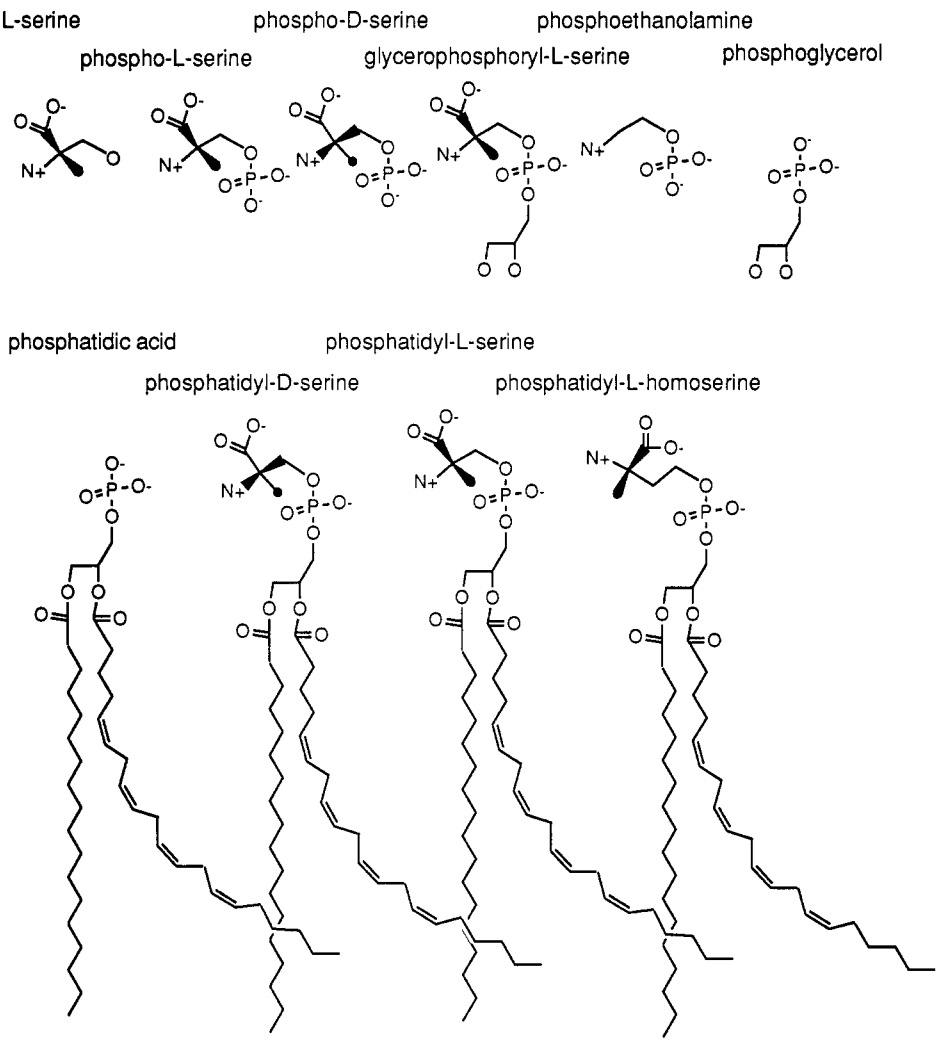


FIGURE 3: Structures of phosphatidylserine and soluble analogs. The structure of a characteristic phosphatidylserine molecule is depicted third from left. The $\alpha$-carbon of serine is chiral, and except in some bacteria, biological molecules exhibit the L conformation. The two fatty acid chains make phosphatidylserine insoluble in aqueous buffers. Except for the hydrogen attached to the serine $\alpha$-carbon, hydrogen atoms are not shown; carbon atoms are not labeled.

Egg phosphatidylcholine was used because the absorbance at 203 nm is related to the particular unsaturated fatty acid chains of a lipid, and those of bovine brain phosphatidylserine may not be the same fatty chains as those of egg phosphatidylcholine. Synthesis of phosphatidyl-D-serine was less efficient than synthesis of phosphatidyl-L-serine, with yields of 64 vs 1070 $\mu$g from a starting material of 10 mg of phosphatidylcholine. These results indicate that base exchange by phospholipase D is partially stereoselective with regard to L-serine and D-serine. Purified lipid was mixed with phosphatidylcholine and cholesterol, organic solvents were evaporated, and phospholipid vesicles were prepared by sonication. The resulting vesicles were incubated with cleaned glass microspheres to prepare lipospheres, and binding of factor VIII was evaluated. Binding of factor VIII to lipospheres containing synthesized phosphatidyl-L-serine was equivalent to binding to vesicles with natural phosphatidylserine (Figure 5B). In contrast, binding to lipospheres with phosphatidyl-D-serine was decreased. When the factor VIII concentration was less than 10 nM, binding to membranes containing phosphatidyl-D-serine was about 15% of binding to membranes containing phosphatidyl-L-serine. These results support the hypothesis that factor VIII binding to membranes is supported by stereoselective recognition of *O*-phospho-L-serine.

We wished to determine whether the dimensions of *O*-phospho-L-serine were critical for recognition by factor VIII. For this purpose we synthesized phosphatidyl-L-homoserine, which differs from phosphatidyl-L-serine by the addition of a single methylene between the serine $\alpha$-carbon and the phosphoester bond (Figure 3). Phosphatidyl-L-homoserine was synthesized as described for phosphatidyl-D-serine, except that trans-phosphatidylation was performed in the presence of excess L-homoserine rather than excess D-serine. The purity of the product was evaluated by HPLC with detection by an evaporative light-scattering detector. The product had a retention time slightly longer than that of phosphatidyl-L-serine (Figure 6A), comparable to the slightly slower migration of phosphatidylhomoserine previously demonstrated on thin layer chromatography in a similar solvent system (Lee & Bell, 1989). The quantity of phosphatidyl-L-homoserine was evaluated by integration of the peak area with reference to a standard curve established with phosphatidyl-L-serine. The final yield was 24 $\mu$g from 10 mg of phosphatidylcholine. Factor VIII bound to liposphere membranes of 15% phosphatidyl-L-homoserine, and the affinity was similar to that of membranes of 15% phosphatidyl-L-serine but with approximately 30% fewer sites (Figure 6B). However, when the binding of factor VIII was evaluated in the presence of other plasma proteins, it was greatly reduced. As little as 3% plasma reduced bound factor VIII by 65% while having little effect upon the binding to membranes with phosphatidyl-L-serine. These results suggest that the distances between the phosphate

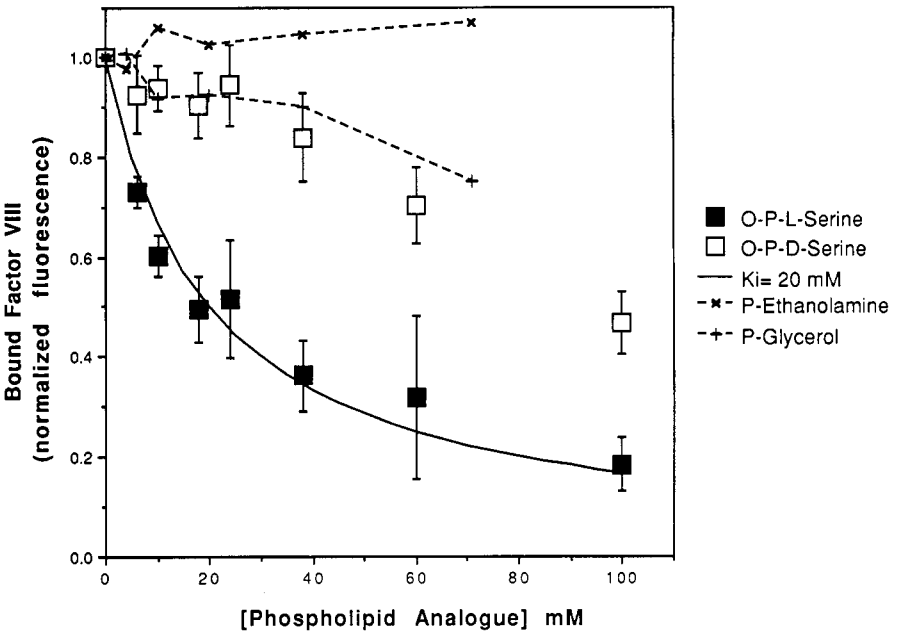


FIGURE 4: Effect of *O*-phospho-L-serine and *O*-phospho-D-serine upon factor VIII binding to phospholipid membranes. Fluorescein-labeled factor VIII was incubated with the indicated concentration of phosphatidylserine analog in buffer A, and lipospheres were added. After 10 min, binding of factor VIII to lipospheres was evaluated by flow cytometry. *O*-Phospho-L-serine (■) displaced factor VIII from lipospheres corresponding to a $K_i$ of approximately 20 mM (—). By comparison, *O*-phospho-D-serine was a less effective inhibitor (□), with 50% inhibition at approximately 100 mM. Phosphoethanolamine did not inhibit binding at concentrations as high as 70 mM (×). Phosphoglycerol was approximately as effective as *O*-phospho-D-serine as a binding inhibitor with 20% inhibition at 70 mM (+). Displayed results with error bars represent mean ±SD for five independent experiments. The liposphere membrane composition was phosphatidylserine/phosphatidylcholine/cholesterol, 10:90:20.

and the amine and carboxyl moieties of phospho-L-serine are critical for specific recognition by factor VIII in the presence of competing plasma proteins.

To determine whether recognition of *O*-phospho-L-serine is involved in the binding of factor VIII to platelet receptors, we examined the effect of soluble phosphatidylserine analogs upon the binding of factor VIII to platelet-derived microparticles (Figure 7). Because the peak expression of platelet receptors for factor VIII is transient at 37 °C, we studied the interaction with platelet-derived microparticles which have longer lived receptor expression (Gilbert et al., 1991). Platelet-derived microparticles were added to a mixture of fluorescein-labeled factor VIII and a phosphatidylserine analog. *O*-Phospho-L-serine inhibited factor VIII binding to microparticles, and inhibition was equivalent to that in experiments with lipospheres (Figure 4). *O*-Phospho-D-serine had a greater inhibitory effect upon factor VIII binding to microparticles than to lipospheres at concentrations less than 40 mM. However, 50% inhibition of binding occurred at approximately 80 mM, equivalent to the results with lipospheres. This result supports the hypothesis that recognition of *O*-phospho-L-serine by factor VIII contributes to receptor binding on stimulated platelets.

## DISCUSSION

We have observed that a negative electrostatic potential is not sufficient to provide specific membrane binding sites for factor VIII. This was investigated by comparing the effects of two different negatively charged lipids, phosphatidic acid and phosphatidylserine. While factor VIII bound to membrane sites containing phosphatidic acid, the binding was of low affinity compared with membrane sites containing phosphatidylserine. Furthermore, membrane sites containing phosphatidylserine were specific for factor VIII in the presence of all plasma constituents, but those containing phosphatidic acid were not. We observed that the affinity of factor VIII for phosphatidylserine-containing membrane sites is reduced when the negative electrostatic potential is decreased by a positively charged lipid but that the binding sites for factor VIII remain specific. Therefore, although a negative electrostatic potential apparently contributes to binding affinity, it is not necessary to provide specific binding sites for factor VIII.

Rosing et al. (1988) reported that positively charged membranes containing phosphatidylserine support activity of the prothrombinase enzyme, while those containing other negatively charged lipids do not. Factor V, which is structurally and functionally homologous to factor VIII, is a critical cofactor in the prothrombinase complex. Therefore, this report implies that factor V, like factor VIII, may recognize a phosphatidylserine-containing membrane site in the absence of negative electrostatic potential. The function of the prothrombinase complex also requires membrane binding of the γ-carboxyglutamic acid containing proteases, factor Xa and prothrombin. Because prothrombinase function was preserved in the face of positive electrostatic potential, it is likely that membrane binding sites of prothrombin and factor Xa are also determined by lipid structures rather than by net membrane potential. In earlier experiments, Bangham (1961) found that high concentrations of phosphatidic acid containing membranes increased the rate of plasma coagulation but that the effect could be negated by the addition of sufficient positively charged lipid to neutralize the negative membrane charge. Our results confirm Bangham's observation that phosphatidic acid is not a structure recognized by procoagulant proteins and that its effect is limited to influence upon membrane electrostatic potential.

The requirement for phosphatidylserine, rather than phosphatidic acid, in the binding site of factor VIII indicates that L-serine, the structural moiety that distinguishes these two polar lipids, is critical to the specific binding of factor VIII.

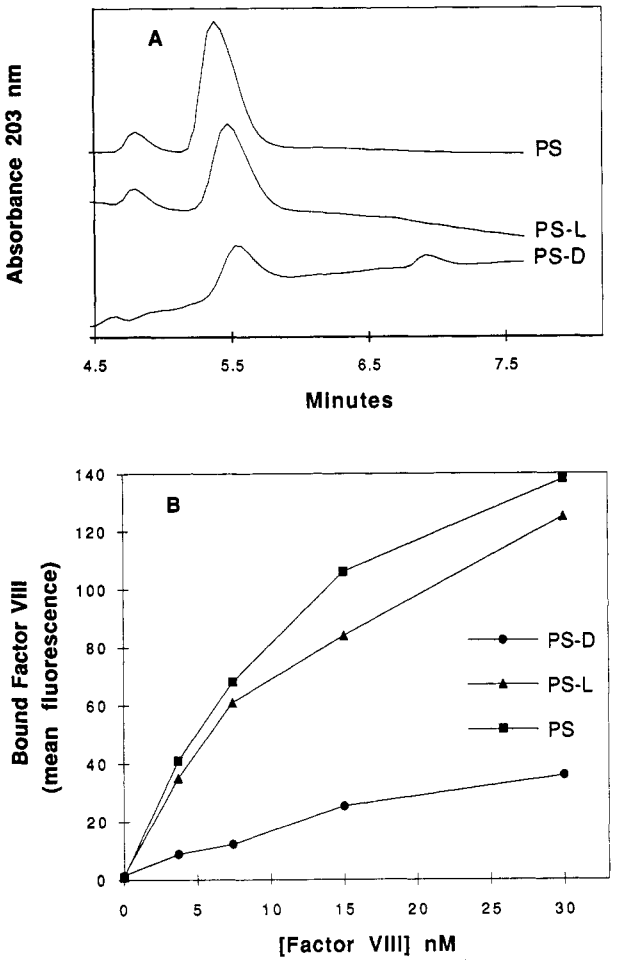


FIGURE 5: Evaluation of synthesized phosphatidylserines and their capacity to support binding of factor VIII. (A) Synthesized, purified phosphatidyl-L-serine (PS-L) and phosphatidyl-D-serine (PS-D) were subjected to HPLC over a silica column. Both synthetic phosphatidylserines yielded a dominant peak with a retention time of 5.6 min. No phosphatidylcholine, phosphatidic acid, or phosphatidylethanol was detected contaminating these products. The natural phosphatidylserine standard retention time was 5.5 min, which is slightly less than the synthetic products. The slightly shorter retention time relates to a slightly shorter column equilibration time rather than a varying product as previously reported (Chen & Kou, 1982). Displayed data have been corrected by subtraction of the background peak due to absorbance by chloroform, and the phosphatidyl-D-serine chromatogram is displayed at 2.5× relative to phosphatidyl-L-serine chromatograms. (B) When phosphatidylserines were incorporated into liposphere membranes, factor VIII bound equivalently to membranes containing synthetic (▲) and natural (■) phosphatidyl-L-serine. By comparison, binding to membranes containing phosphatidyl-D-serine (●) was reduced to less than one-third.

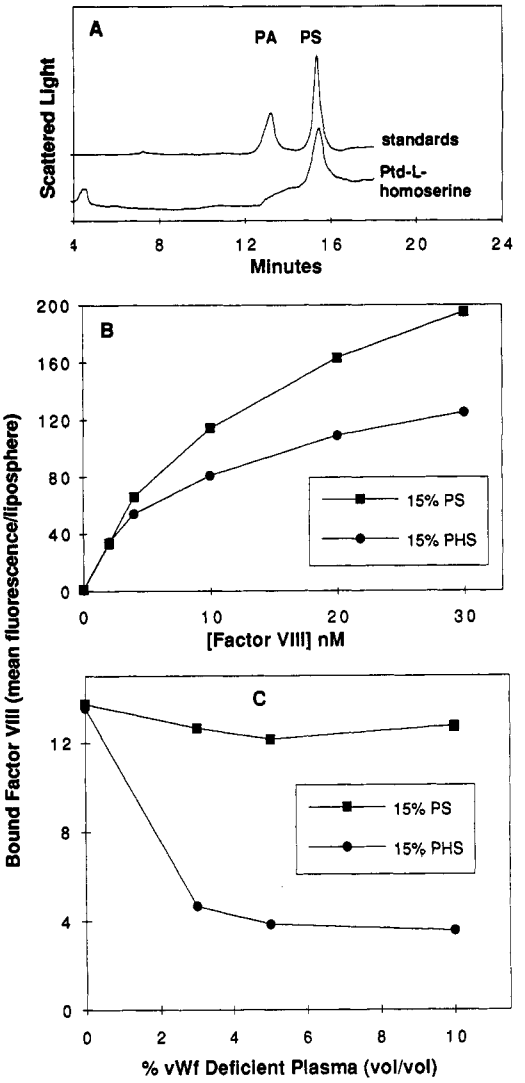


FIGURE 6: Evaluation of synthesized phosphatidylhomoserine and its capacity to support specific binding of factor VIII. (A) Synthesized, purified phosphatidyl-L-homoserine was subjected to HPLC over a silica column (Ptd-L-homoserine). The dominant peak had a retention time a few seconds longer than that of phosphatidylserine (PS, standards). No phosphatidic acid (PA) or residual phosphatidylcholine was detected. The small peak with retention time slightly greater than 4 min is an artifact occassionally seen in tracings with other lipids. The gradual rise prior to the PS peak represents the mobile-phase gradient (see Materials and Methods) and is accentuated in the lower tracing because it is at 3-fold higher magnification than the upper tracing. (B) Phosphatidyl-L-homoserine was incorporated into liposphere membranes and incubated with varying concentrations of fluorescein-labeled factor VIII. Factor VIII bound saturably and with comparable affinity to membranes containing 15% phosphatidyl-L-homoserine (●) and 15% phosphatidylserine (■). Total protein bound to phosphatidylhomoserine-containing membranes was about one-third less. (C) Fluorescein-labeled factor VIII (2 nM) was mixed with varying concentrations of von Willebrand factor deficient plasma prior to the addition of lipospheres. Following a 10-min incubation, bound factor VIII was evaluated. Binding to phosphatidylhomoserine-containing lipospheres was decreased 65% by 3% plasma, with greater decreases at higher concentrations. Plasma had little effect upon the binding of factor VIII to phosphatidylserine-containing liposphere membranes.

This prediction was confirmed by the capacity of O-phospho-L-serine to displace factor VIII from phosphatidylserine-containing membranes and by the decreased affinity of factor VIII for membranes containing synthetic phosphatidyl-D-serine compared to phosphatidyl-L-serine. Because phosphate-containing compounds are low-affinity chelators of calcium ions, it is likely that the calcium ion concentration was decreased below the nominal concentration of 0.5 mM in the experiments depicted in Figures 4 and 7. Calcium ions are not required for the binding interaction between the light chain of factor VIII and phospholipid membrane sites (Gilbert et al., 1991). Therefore, the inhibitory effect of O-phospho-L-serine cannot be explained on the basis of a decreased calcium ion concentration. The displacement was stereoselective as O-phospho-D-serine was at least 4-fold less effective as an inhibitor. The implication is that factor VIII recognizes O-phospho-L-serine via at least three points: the serine amine, the serine carboxyl, and the phosphate of the phosphoester linkage, all in the correct stereochemical configurations. The stereoselective preference for O-phospho-L-serine is relatively small compared to the 20–50-fold selectivity of some amino acid transport proteins (Gazzola et al., 1981; White et al., 1982). However, the degree of preference is comparable to

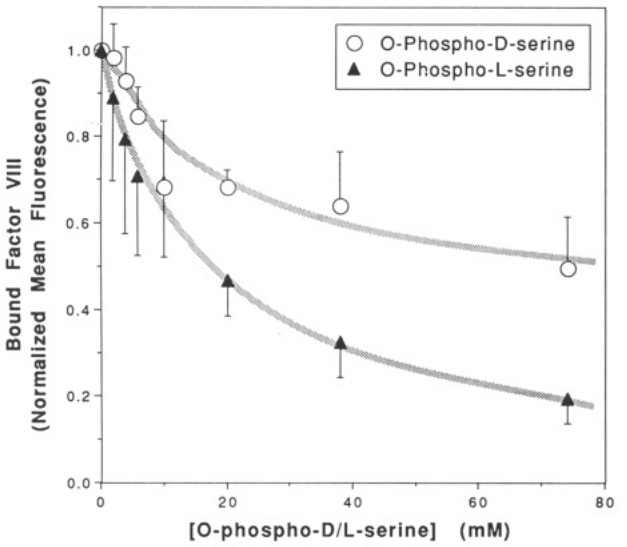


FIGURE 7: Effect of *O*-phospho-L-serine and *O*-phospho-D-serine upon factor VIII binding to platelet-derived microparticles. Gel-filtered platelets, $1 \times 10^8$/mL, were stimulated with 2 $\mu$M A23187 for 5 min at 25 °C. Stimulated platelets and corresponding microparticles were diluted 1:10 into a mixture of fluorescein-labeled factor VIII and *O*-phospho-L-serine or *O*-phospho-D-serine in buffer A and mixed by gently swirling. Binding of factor VIII was evaluated by flow cytometry after 5 min. Displayed results are the mean ± SE for six independent experiments.

transport protein selectivity for D and L isomers of aspartate and an aspartate analogue, cysteinesulfinate (Gazzola et al., 1981). The postulated explanation for limited transport protein selectivity toward these amino acids is the existence of a binding site to which the negatively charged groups of aspartic acid and cysteinesulfinate may bind in either the spatial order $\alpha, \beta$ or $\beta, \alpha$. This explanation implies that both negatively charged groups of the amino acids are sterically accessible for the binding interactions and that there is a limited preference for one aspartate carboxyl over the other or for the carboxyl vs the sulfinate of cysteinesulfinate. The same explanation may apply to the limited stereoselectivity of factor VIII for *O*-phospho-L-serine, i.e., a three-point binding interaction in which steric constraints allow both the carboxyl and the phosphate to interact with the preferred site for the other. The specificity of the interaction between factor VIII and *O*-phospho-L-serine suggests that pharmacological agents incorporating this moiety may be useful in inhibiting the function of factor VIII, factor V, or other procoagulant proteins.

The experiments with phosphatidyl-L-homoserine present an apparent puzzle. How can this lipid support binding of high affinity, but lack specificity? Membrane binding of factor VIII is a multistep process in which recognition of *O*-phospho-L-serine is probably the earliest step (Bardelle et al., 1993). While phosphatidyl-L-homoserine will apparently support an early binding step sufficiently to facilitate some high-affinity binding, detailed kinetic studies will be required to demonstrate whether support for this first step is really equivalent to phosphatidylserine. The fact that other plasma proteins will compete for binding to phosphatidyl-L-homoserine-containing membranes, but not phosphatidylserine-containing membranes, indicates either that factor VIII recognizes phospho-L-homoserine with lower affinity than phospho-L-serine or that other plasma proteins have higher affinity for phospho-L-homoserine. Thus, the precise distances between the carboxyl, the phosphate, and the amine of *O*-phospho-L-serine are important as well as the stereochemical arrangement of these groups.

*O*-Phospho-L-serine inhibited binding of factor VIII to platelet-derived microparticles, suggesting that phosphatidylserine is a critical constituent of a platelet receptor for factor VIII. However, inhibition of binding by *O*-phospho-L-serine occurred at relatively high concentrations and implied a $K_D$ between *O*-phospho-L-serine and factor VIII of 20 mM. This correlates to a binding energy of 2.3 kcal/mol, accounting for only 20% of the 11.3 kcal/mol required to account for a membrane binding $K_D$ of 4 nM. Therefore, unidentified molecular interactions contribute 80% of the binding energy. It is possible that multiple, low-affinity interactions with *O*-phospho-L-serine additively contribute the necessary binding energy. Alternatively, it is possible that factor VIII interacts with other membrane structures such as phosphorylcholine of phosphatidylcholine or phospholipid acyl chains. We have found a lower activation energy for the association of factor VIII to membranes of dioleoylphosphatidylserine and dioleoylphosphatidylcholine compared to the more tightly packed egg yolk phosphatidylcholine and bovine brain phosphatidylserine. This suggests that interaction of factor VIII with phospholipid acyl moieties may be one source of binding energy (Bardelle et al., 1993). We are currently investigating the contribution of acyl chains of phosphatidylserine and moieties of phosphatidylcholine to the membrane binding energy of factor VIII.

We report the first demonstration that a plasma protein may bind to a cell membrane via recognition of a phosphatidylserine moiety. Inside the cell, protein kinase C binds to the inner leaflet of the plasma membrane via stereoselective affinity for phosphatidyl-L-serine (Lee & Bell, 1989). Like factor VIII, protein kinase C binds to phosphatidyl-L-serine but not phosphatidyl-D-serine and has a lower affinity for phosphatidyl-L-homoserine. Gelsolin, an actin binding protein, adheres to the inner leaflet of the plasma membrane with adesion mediated by specific polyphosphoinositides (Janmey & Stossel, 1987; Janmey & Stossel, 1989). Perforin, a lymphocyte pore forming protein, recognizes phosphorylcholine of phosphatidylcholine or sphingomyelin on the membrane of red blood cells (Tschopp et al., 1989). The pentraxins, plasma proteins of unknown function, recognize phosphorylethanolamine or phosphorylcholine and may mediate binding to membranes containing phosphatidylethanolamine or phosphatidylcholine (Schwalbe et al., 1992). The phospholipid-specific binding interactions of this group of proteins suggest that cellular and subcellular localization of diverse phospholipids may function in partitioning proteins to locales on the exterior as well as the interior leaflets of cell membranes.

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## REFERENCES

Ahmad, S., Rawala-Sheikh, R., & Walsh, P. (1989) *J. Biol. Chem. 264*, 3244–3251.

Andersson, L., & Brown, J. (1981) *Biochem. J. 200*, 161–167.

Arai, M., Scandella, D., & Hoyer, L. (1989) *J. Clin. Invest. 83*, 1978–1984.

Bach, R., & Rifkin, D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6995–9.

Bangham, A. (1961) *Nature 192*, 1197–1198.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965) *J. Mol. Biol. 13*, 238–252.

Bardelle, C., Furie, B., Furie, B. C., & Gilbert, G. (1993) *J. Biol. Chem. 268*, 8815–24.
Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806–2810.
Bartles, J., Galvin, N., & Frazier, W. (1982) *Biochim. Biophys. Acta 687*, 129–136.
Bevers, E., Comfurius, P., & Zwaal, R. (1983) *Biochim. Biophys. Acta 736*, 57–66.
Bevers, E., Wiedmer, T., Comfurius, P., Shattil, S., Weiss, H., Zwaal, R., & Sims, P. (1992) *Blood 79*, 380–388.
Chang, C. P., Zhao, J., Wiedmer, T., & Sims, P. J. (1993) *J. Biol. Chem. 261*, 7171–8.
Chen, S., & Kou, A. (1982) *J. Chromatogr. 227*, 25–31.
Chen, P., Toribara, T., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Church, W. R., Jernigan, R. L., Toole, J., Hewick, R. M., Knopf, J., Knutson, G. J., Nesheim, M. E., Mann, K. G., & Fass, D. N. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6934–6937.
Comfurius, P., & Zwaal, R. (1977) *Biochim. Biophys. Acta 488*, 36–42.
Duffy, E., Parker, E., Mutucumarana, V., Johnson, A., & Lollar, P. (1992) *J. Biol. Chem. 267*, 17006–17011.
Foster, P., Fulcher, C., Houghten, R., & Zimmerman, T. (1988) *J. Biol. Chem. 263*, 5230–5234.
Foster, P. A., Fulcher, C. A., Houghten, R. A., & Zimmerman, T. S. (1990) *Blood 75*, 1999–2004.
Furie, B., & Furie, B. C. (1988) *Cell 53*, 505–518.
Gazzola, G., DAll'Asta, V., Bussolati, O., Makowske, M., & Christensen, H. (1981) *J. Biol. Chem. 256*, 6054–6059.
Gilbert, G. E., Furie, B. C., & Furie, B. (1990) *J. Biol. Chem. 265*, 815–822.
Gilbert, G. E., Sims, P. J., Wiedmer, T., Furie, B., Furie, B. C., & Shattil, S. J. (1991) *J. Biol. Chem. 266*, 17261–68.
Gilbert, G. E., Drinkwater, D., Barter, S., & Clouse, S. B. (1992) *J. Biol. Chem. 267*, 15861–15868.
Gitschier, J., Wood, W. I., Goralka, T. M., Wion, K. L., Chen, E. Y., Eaton, D. H., Vehar, G. A., Capon, D. J., & Lawn, R. M. (1984) *Nature 312*, 326.
Janmey, P., & Stossel, T. (1987) *Nature 325*, 362–364.
Janmey, P., & Stossel, T. (1989) *J. Biol. Chem. 264*, 4825–4831.
Kalafatis, M., Jenny, R. J., & Mann, K. G. (1990) *J. Biol. Chem. 265* (35), 21580–9.
Kane, W. H., & Davie, E. W. (1988) *Blood 71*, 539–555.
Krishnaswamy, S., & Mann, K. (1988) *J. Biol. Chem. 263*, 5714–5723.
Lee, M., & Bell, R. (1989) *J. Biol. Chem. 264*, 14797–14805.
Lollar, P. (1991) *Mayo Clin. Proc. 66*, 524–534.
Lutzke, B., & Braughler, J. (1990) *J. Lipid Res. 31*, 2127–2130.
Mann, K. G., Jenny, R. J., & Krishnaswamy, S. (1988) *Annu. Rev. Biochem. 57*, 915.
Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., & Krishnaswamy, S. (1990) *Blood 76*, 1–16.
Nesheim, M. E., Pittman, D. D., Wang, J. H., Slonosky, D., Giles, A. R., & Kaufman, R. J. (1988) *J. Biol. Chem. 263*, 16467–16470.
Nesheim, M., Pittman, D., Giles, A., Fass, D., Wang, J., Slonosky, D., & Kaufman, R. (1991) *J. Biol. Chem. 266*, 17815–17820.
Neuenschwander, P., & Jesty, J. (1988) *Blood 72*, 1761–1770.
Ortel, T., Devore-Carter, D., Quinn-Allen, M., & Kane, W. (1992) *J. Biol. Chem. 267*, 4189–4198.
Pittman, D., & Kaufman, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2429–2433.
Rosing, J., Speijer, H., & Zwaal, R. (1988) *Biochemistry 27*, 8–11.
Sandberg, H., Bode, A., Dombrose, F., Hoechli, M., & Lentz, B. (1985) *Thromb. Res. 39*, 63–79.
Schwalbe, R., Dahlback, B., Coe, J., & Nelsestuen, G. (1992) *Biochemistry 31*, 4907–4915.
Sims, P., & Wiedmer, T. (1986) *Blood 68*, 556–551.
Stubbs, J., Lekutis, C., Singer, K., Bui, A., Yuzuki, D., Srinivasan, U., & Parry, G. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8417–8421.
Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., & Kaufmann, R. J., et al. (1984) *Nature 312*, 342–347.
Tschopp, J., Schafer, S., Masson, D., Peitsch, M., & Heusser, C. (1989) *Nature 337*, 272–274.
White, M. F., Gazzola, G. C., & Christensen, H. N. (1982) *J. Biol. Chem. 257* (8), 4443–9.
Zwaal, R., Comfurius, P., & van Deenen, L. (1977) *Nature 268*, 358–360.