Factor VIII functions in an enzyme complex upon the activated platelet membrane where phosphatidylserine exposure correlates with expression of receptors for factor VIII. To evaluate the specificity of phosphatidylserine-containing membrane binding sites for factor VIII, we have developed a novel membrane model in which phospholipid bilayers are supported by glass microspheres (lipospheres). The binding of fluorescein-labeled factor VIII to lipospheres with membranes of 15% phosphatidylserine was equivalent to binding to phospholipid vesicles (Kd = 4.8 nm). Purified von Willebrand factor (vWF), a carrier protein for factor VIII, decreased membrane binding of factor VIII with a Kd of 10 μg/ml. Likewise, normal plasma decreased bound factor VIII by more than 90% whereas plasma lacking vWF decreased the binding of factor VIII by only 20%. Proteolytic activation of factor VIII by the thrombin, which releases factor VIII from vWF, increased liposphere binding in the presence of vWF and in the presence of normal plasma. Although factor V is homologous to factor VIII and binds to lipospheres with the same affinity, purified factor V was not an efficient competitor for the membrane binding sites of factor VIII. These results indicate that phosphatidylserine-containing membrane sites have sufficient specificity to select thrombin-activated factor VIII from the range of phospholipid-binding proteins in plasma.

Factor VIII functions as a cofactor in an enzyme complex consisting of thrombin-activated factor VIII and the enzyme factor IXa, assembled upon a membrane (for reviews see Refs. 1, 2). The assembled complex, called the factor Xase complex, cleaves the zymogen factor X, to factor Xa which is responsible for prothrombin activation. The importance of the assembled factor Xase complex is illustrated by hemophilia, a disease in which a deficiency of either factor VIII or factor IX leads to life-threatening bleeding. Membrane binding of factor VIII serves several functions. First, procoagulant function is localized to the vascular defect where the activated platelet has adhered (3, 4). Second, membrane-bound factor VIII provides a high affinity binding site for factor IXa, but not factor IX (5), on either platelets (6) or phospholipid vesicles (7), whereas factor VIII in solution does not efficiently bind factor IXa. Third, the KM of factor X for assembled factor VIII and factor IXa is decreased by membrane binding (8). In spite of the critical role of membrane binding in factor Xase function, the molecular details of interaction between factor VIII and its membrane receptor are unknown.

Factor VIII, a plasma glycoprotein of M, 280,000, is homologous to another coagulant protein, factor V, in amino acid sequence (9–11) and in function as a membrane-bound enzyme cofactor (1, 2, 12, 13). 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 1, a phospholipid-binding lectin (14), and with a murine milk fat globule membrane protein (15). The cofactor activity of each protein is increased through limited proteolysis by thrombin, and this results in the removal of the B domain, with the remaining heavy and light chain fragments interacting to form a Ca2+-dependent complex. Both factor VIII and factor V bind with high affinity to phospholipid membranes via the "light chain" composed of the A3-C1-C2 segment (16). However, factor VIII requires more phosphatidylserine/binding site than factor V (13), and current evidence implicates different domains in membrane binding. While binding of factor V is mediated by a peptide in the A3 domain (17), binding of factor VIII is apparently mediated by the C2 domain (18, 19). In contrast to factor V, factor VIII is stabilized through a non-covalent complex with von Willebrand factor (vWF) (20) which prevents binding to receptors on the platelet membrane (21). After proteolytic activation by thrombin, factor VIII is released from its complex with vWF, becoming available to bind to platelet receptors and participate in the factor tenase enzyme complex.

Assembly of the Xase complex on platelets requires cell activation. The development of platelet procoagulant activity correlates with the reorientation of phosphatidyserine from the inner to the outer bilayer of the plasma membrane (22) and the expression of membrane binding sites for factors VIII (3, 4) and V (23). When platelets are activated by agonists that induce procoagulant activity, they release small vesicles derived from the plasma membrane (24–26). These vesicles, also referred to as microparticles, have a high density of membrane receptors for factors VIII (4) and V (27).

* This work was supported by the Medical Research Service of the Department of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of National Institutes of Health Clinical Investigator Award HL02567. To whom correspondence should be addressed: Brockton-West Roxbury VA Medical Center, 1400 VFW Parkway, West Roxbury, MA 02132. Tel: 617-323-3427; Fax: 617-323-8786.

1 The abbreviations used are: vWF, von Willebrand factor; DICa(3), 1,1'dihexadecyl-3,3,3'-tetramethyl indocarbocyanine perchlorate; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; HEPES, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid.
binding characteristics of factors VIII and Va to activated platelets and microparticles is similar to the interaction of these cofactors with synthetic phospholipid vesicles containing phosphatidylserine. The dissociation constants for factor VIII (13) and factor Va (16) binding to platelets and microparticles are similar to dissociation constants for binding to phospholipid vesicles. Phosphatidylserine-containing lipid vesicles compete effectively with both platelets and microparticles for binding of factors VIII and Va (4). In addition, there is some competition for binding between factors VIII and Va on platelets, microparticles, and phospholipid vesicles (4). However, doubt has remained as to whether membrane phosphatidylserine could provide a specific binding site for factor VIII. The plasma concentration of factor VIII, 0.2 nM, is as much as 20,000-fold lower than other plasma proteins that bind to phosphatidylserine-containing membranes, e.g. prothrombin, 1.4 μM; β2-glycoprotein I, 4 μM. Because initiation of membrane binding has been understood as an electrostatic interaction between a positively charged protein region and a negatively charged muscle of uncharged protein, it seemed that a phosphatidylserine-containing membrane could provide sites with sufficient specificity to rapidly bind factor VIII in plasma has seemed small. In this study we have characterized the specificity of phospholipid-binding sites for factor VIII in the presence of plasma proteins that may compete with factor VIII or otherwise modify membrane binding.

Phospholipid vesicles have provided a cell membrane model for evaluating binding properties of proteins such as factor VIII. Phospholipid vesicles, as a model for the platelet membrane, have also supported studies in which binding was mediated by an adhesive membrane protein rather than by membrane lipids. However, unilamellar phospholipid vesicles are not well suited for competition binding studies. Small size and low density make them difficult to physically or analytically separate from unbound protein remaining in solution. In addition, because high concentrations of lipids are required for binding studies with unilamellar vesicles, they are prone to aggregation. Competition membrane binding studies would be facilitated by a model system in which membrane was reconstituted in units of uniform size sufficiently large for study by light microscopy or flow cytometry, not prone to aggregation, and sufficiently dense for separation from unbound molecules by sedimentation. Phospholipid vesicles spontaneously fuse to form a bilayer on a chemically clean glass support (28). This property motivated our characterization of membranes supported by glass microspheres as a model for studying membrane binding interactions. We have found that supported membranes bind factor VIII equivalently to phospholipid vesicles and have used this model system to determine the specificity of phospholipid-binding sites for factor VIII.

**EXPERIMENTAL PROCEDURES**

Polystyrene and glass microspheres were from Duke Scientific, Palo Alto, CA. Bovine brain phosphatidylserine and egg yolk phosphatidylcholine were from Avanti Polar Lipids, Pelham, AL. Recombinant human factor VIII, a gift from Dr. R. Kaufman and D. Pittman of Genetics Institute, has been extensively characterized (29). This material had activity of 4,000 units/mg. Human factor V and anti-factor V antibody, AHB 5102, were purchased from Harwoscreen Technologies, Essex Junction, VT. Factor V had activity of 300 units/mg and ran as a dominant band of approximately 30 kDa on a reduced, silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The monoclonal antibody AC1.2 and pure P-selectin protein were generous gifts from Drs. R. Celi, S. Sj?ger, B. C. Furie, and B. Furie, Tufts University. Purified vWF was a generous gift of Drs. M. Cruz and B. Handin, Brigham and Women’s Hospital. This material had less than 0.01 unit of factor VIII activity/ml at a concentration of 16 μg/ml. vWF-deficient plasma, from a patient with homozygous type III von Willebrand disease, was from George King Biomedical, Overland Park, KA. Fluorescein isothiocyanate, fluorescein-5-maleimide, and 1′,1′-dihexadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiIC8(3)) were from Molecular Probes, Eugene, OR. Polycarbonate membranes were from Nucleopore, Pleasanton, CA. Filters were 60-μm polystyrene and 1.6-μm nominal diameter were suspended in 0.02% filtered Sparkleen detergent and sonicated briefly. Removal of very small particles was accomplished by sedimentation at 80 x g x 3 min in a swinging bucket centrifuge in a solution of 0.02% Sparkleen. Beads in the pellet were resuspended in 0.02% Sparkleen, sonicated briefly to eliminate aggregation, and sedimented as above. This process was repeated three times. Microspheres were passed through a laser-etched polycarbonate membrane with 3-μm diameter pores (Nucleopore) to remove larger spheres. The filter apparatus was submerged in a sonication bath to maintain suspension of the microspheres during filtration. The filtered microspheres were resuspended in 1 ml in a deionized, distilled water while sonicated in a sonication bath. Microspheres were retained in the stream by a 0.22-μm syringe filter and retrieved by reversed flow through the filter.

**Phospholipid-coating of Microspheres—**Cleaned, size-sorted glass microspheres were suspended in 3 ml of Tris-buffered saline (0.02 M Tris, 0.14 M NaCl, 0.05% BSA), and small unilamellar phospholipid vesicles of phosphatidylserine/phosphatidylcholine/DiIC8(3) 15862

**Fluorescence Labeling—**Factor VIII was labeled with fluorescein-5-maleimide as previously described (4) except that unbound fluorescein-5-maleimide was removed by gel filtration over a Sephadex G-25 SF column equilibrated in 0.15 M NaCl, 2 mM CaCl2, 0.1 M betaine, 0.004% Tween 80, 20 mM MES, pH 6.5. Protein concentration of factor VIII was determined using a Micro-BCA Assay (Pierce Chemical Co.) using bovine albumin as a standard. Factor VIII activity was determined using a chromogenic substrate assay with normal plasma as a control (Diagnostics Stago). No loss of factor VIII activity occurred with fluoresce labeling. Monoclonal antibodies were labeled as published previously (31). The labeled antibodies had fluorescein/protein ratio of 1 mol/mol as determined by comparative absorbance at 280 and 490 nm.

**Factor VIII Assay—**Factor VIII-deficient plasma, 75 μl, was incubated for 15 min at 37°C with an equal volume of an activated cephaloplastin reagent (DiIC8(3) Thrombin-activated factor VIII was diluted into 10 mM CaCl2, also at 37°C, and 150 μl was immediately added to the plasma/cephaloplastin mixture. The time from addition of calcium to fibrin strand formation was monitored with a fibrometer.

**Cleaning and Size-restricting Glass Microspheres—**Microspheres, 10 mg, of 1.6-μm nominal diameter were suspended in 0.02% filtered Sparkleen and sonicated briefly. Removal of very small particles was accomplished by sedimentation at 80 x g x 3 min in a swinging bucket centrifuge in a solution of 0.02% Sparkleen. Beads in the pellet were resuspended in 0.02% Sparkleen, sonicated briefly to eliminate aggregation, and sedimented as above. This process was repeated three times. Microspheres were passed through a laser-etched polycarbonate membrane with 3-μm diameter pores (Nucleopore) to remove larger spheres. The filter apparatus was submerged in a sonication bath to maintain suspension of the microspheres during filtration. The filtered microspheres were resuspended in 1 ml in a deionized, distilled water while sonicated in a sonication bath. Microspheres were retained in the stream by a 0.22-μm syringe filter and retrieved by reversed flow through the filter.

**Flow Cytometry—**Flow cytometry was performed on 25-μl aliquots of 100-μl samples with a final microsphere concentration of 1 x 10^7/ml using a Coulter EPICS-Profil II flow cytometer. For evaluation of liposomes binding to U937 cells, the cells were at a final concentration of 1 x 10^6/ml and the liposphere concentration was as specified in the figure legend. Data acquisition was triggered by forward light scatter with all photomultipliers in the log mode. Data acquisition for large multilamellar vesicles was triggered by red fluorescence from the DiIC8(3) probe incorporated into the vesicles. Noise was reduced during analysis by eliminating events with forward and side scatter values different from those characteristic of lipospheres, U937 cells, or vesicles, respectively. Mean log fluorescence was converted to linear fluorescence for values depicted in Figs. 4-8. Only experiments in which the fluorescence histogram indicated a log normal distribution, as judged by inspection, were analyzed quanti-
tatively. Flow cytometry experiments were performed in Tris-buffered saline, 0.5 mM CaCl₂, 0.1% BSA unless otherwise specified.

Lipid Assay—Phospholipid vesicles used to prepare coated microspheres were analyzed for elemental phosphorous content (32) and DiC₁₆(3) fluorescence. Aliquots were diluted into 0.1% Triton X-100, and fluorescence was measured to determine a standard curve (λₑ 547 nm, λₘ 560 nm). Phospholipid-coated microspheres were also diluted into 0.1% Triton X-100 and fluorescence was evaluated in a continuously stirred cuvette. DiC₁₆(3) fluorescence was read against the fluorescence standard curve and phosphorus content determined from the phosphorous to fluorescence ratio of the stock vesicles.

Plasma Preparation—Fresh blood was collected into a polypropylene container containing sodium heparin, 14 international units/ml final concentration. Cells were removed by sedimentation, 2500 x g for 15 min. Plasma was kept at room temperature and used within 2 h of collection. Citrate-anticoagulated von Willebrand factor-deficient plasma, stored at -80 °C until use, was thawed at 37 °C and CaCl₂ and sodium heparin were added to 15 mM and 14 international units/ml, final concentrations, respectively. Calcium was included to offset 13 mM sodium citrate with which the plasma was supplied. The control buffer for binding experiments was prepared with 14 mM CaCl₂, 13 mM sodium citrate, and 14 units/ml sodium heparin.

Tissue Culture—Cell lines HL60 and U937 were maintained in culture in RPMI 1640 medium supplemented with penicillin G sodium (100 units/ml), streptomycin sulfate (100μg/ml), HEPES (10 mM), sodium pyruvate (1 mM), L-glutamine (2 mM), β-mercaptoethanol (0.00004%), and 20 and 10% fetal calf serum, respectively.

RESULTS
Deposition of a Lipid Membrane on Glass Microspheres—We have developed and characterized a technique, in which phospholipid membranes are supported by glass microspheres, for evaluating membrane binding interactions. Microspheres of 1.6-μm nominal diameter were chosen as the smallest size readily evaluable by light microscopy and flow cytometry. Larger glass spheres sedimented more rapidly than desirable for equilibrium binding experiments. The size distribution of microspheres, as obtained from the vendor, was broad and included spheres both smaller and larger than the nominal diameter. By flow cytometry, the smaller microspheres were not completely discriminated from noise using light scatter criteria (Fig. 1A). To obtain a more uniform population, the microspheres were size-restricted by differential sedimentation followed by filtration through a polycarbonate membrane of defined pore size. Cleaning was accomplished by sonication in detergent followed by extensive washing as described under "Experimental Procedures." The cleaned, size-restricted microspheres were incubated with DiC₁₆(3)-labeled phospholipid vesicles, then washed extensively. The resulting population had a narrow size distribution and the very small spheres were absent so that discrimination of the microspheres from background noise was possible (Fig. 1A). After incubation with phospholipid vesicles increased, red fluorescence indicated that all microspheres were coated with fluorescence-labeled lipid (Fig. 1B). Fluorescence microscopy examination confirmed that all microspheres were evenly coated with fluorescent lipid (Fig. 2). Approximately 5% of microspheres were brighter than others (e.g. rightmost sphere, Fig. 2) leading to the hypothesis that occasional spheres receive two bilayer membranes. Glass microspheres which had not been exposed to lipid had no fluorescence under these conditions.

The size distribution of liposomes was evaluated from fluorescence photomicrographs. The mean diameter was 1.99 ± 0.36 μm (n = 57). The amount of lipid/microsphere was determined by counting the number of spheres in a sample and determining the quantity of associated lipid fluorescence from the membrane-incorporated lipid dye, DiC₁₆(3) as described under "Experimental Procedures" (Table I). The quantity of lipid, sufficient for a mean phospholipid area of 0.54 nm², and the even distribution (Fig. 2) are consistent with the hypothesis that glass microspheres, like glass coverslips (28), support a bilayer membrane after incubation with phospholipid vesicles. Membrane-coated microspheres (liposomes) were stored at 4 °C in the dark and used within 96 h of preparation.

Polystyrene microspheres are widely available and prior membrane binding studies have been performed utilizing hydrated phosphatidylserine on a polystyrene support (19, 33). To determine whether polystyrene microspheres support membrane formation polystyrene microspheres of 20-μm di-
ameter were washed as described above and incubated with DiI\textsubscript{340}(3)-labeled small unilamellar vesicles. All treated microspheres had uniform fluorescence as determined by fluorescence microscopy whereas untreated spheres had no fluorescence. After extensive washing of the microspheres in Tris-buffered saline, 0.1% BSA, the phospholipid/microsphere was determined by elemental phosphorous assay. Results indicated an average of 6.6 \times 10^6 phospholipid monomers/microsphere. This correlates to an area of 3.8 nm\textsuperscript{2}/phospholipid molecule assuming a monolayer rather than a bilayer membrane structure. Similar values were obtained when the procedure was modified by sonication the polystyrene microspheres in the presence of vesicles indicating that this low density of phospholipid represents a thermodynamically favorable lipid packing. Because of the low density of lipids upon the polystyrene microspheres, glass microspheres were used in subsequent experiments.

Incorporation of P-selectin into Liposphere Membranes—If phospholipid forms a bilayer upon glass microspheres and if the bilayer structure resembles phospholipid vesicles in lipid packing, then such a bilayer may be able to support the functional reconstitution of an integral membrane protein. To test this hypothesis we evaluated the capacity of membranes supported upon glass microspheres (lipospheres) to support the adhesive function of P-selectin (also called P-GE, GMP-140, CD-62). Phospholipid vesicles containing P-selectin and the fluorescent membrane probe, DiI\textsubscript{110}(3), were synthesized as previously described (34). One ml suspensions of P-selectin-containing vesicles, 2.5 mg of phosphatidylcholine, 25 µg of P-selectin, or control vesicles without P-selectin, were incubated for 40 min with 1 \times 10^6 size-sorted and cleaned glass microspheres prepared as above. After extensive washing, incorporation of P-selectin into the liposphere membrane was demonstrated by flow cytometry with fluorescein isothiocyanate-labeled AC1.2, a monoclonal antibody specific for P-selectin (not shown). Function of the incorporated integral membrane protein was evaluated by determining that P-selectin-containing lipospheres, like activated platelets, adhere to monocytic cells (Fig. 3A). P-selectin-containing lipospheres adhered to HL60 cells with 5–25 lipospheres/cell and frequent aggregates of cells and lipospheres. Likewise, P-selectin-containing lipospheres adhered to U937 cells with 2–15 lipospheres adherent/cell (not shown). Control lipospheres did not adhere to HL60 cells (Fig. 3B) or U937 cells (not shown). Adhesion to U937 cells was also evaluated by flow cytometry. Ninety % of cells demonstrated adherent P-selectin-lipospheres (Fig. 3C) while control lipospheres did not adhere. These results, indicating functional reconstitution of an integral membrane protein, support the hypothesis that liposphere membrane structure resembles the structure of cell membranes and phospholipid vesicles.

**Factor VIII Binding to Lipospheres**—To further investigate the properties of liposphere membranes, the binding of fluorescein-labeled factor VIII was measured (Fig. 4A). Saturable binding was demonstrated, approaching a plateau at 20 nM factor VIII. The dissociation constant obtained from non-linear regression analysis was 4.8 nM, in close agreement with prior measurements of factor VIII binding to phospholipid vesicles (13). In order to demonstrate that liposphere-binding sites were equivalent for fluorescein-labeled factor VIII and non-labeled factor VIII, a competition binding experiment was performed (Fig. 4B). Factor VIII was mixed with 3 nM fluorescein-labeled factor VIII prior to addition of lipospheres. Competition was observed and conformed to that predicted for equivalent ligands.

To determine whether binding sites for factor VIII on lipospheres were equivalent to sites on phospholipid vesicles, competition experiments were performed. Fluorescein-labeled factor VIII was added to a mixture of lipospheres and phospholipid vesicles (Fig. 4C), and displacement was observed with increasing vesicle concentrations. These data agreed with displacement predicted mathematically assuming that lipospheres and vesicles have binding sites of the same affinity for factor VIII. These results indicate that a phospholipid membrane supported on a glass surface exhibits factor VIII-bind-

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lipid/sphere</th>
<th>Area/lipid\textsuperscript{*}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>5.1</td>
<td>0.48</td>
</tr>
<tr>
<td>5</td>
<td>4.3</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean</td>
<td>4.7</td>
<td>0.54</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Area was calculated assuming a uniform bilayer coating of spheres with a 1.99-µm diameter.

**Table I**

**Mean membrane lipid/microsphere**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lipid/sphere</th>
<th>Area/lipid\textsuperscript{*}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6</td>
<td>0.38</td>
</tr>
<tr>
<td>2</td>
<td>3.3</td>
<td>0.75</td>
</tr>
<tr>
<td>3</td>
<td>4.0</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>5.1</td>
<td>0.48</td>
</tr>
<tr>
<td>5</td>
<td>4.3</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean</td>
<td>4.7</td>
<td>0.54</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Area was calculated assuming a uniform bilayer coating of spheres with a 1.99-µm diameter.
brane, and these proteins are present in concentrations that exceed the concentration of factor VIII by as much as 20,000-fold. If a cell membrane containing phosphatidylserine is to promote efficient function of factor VIII in the Xase enzyme complex, then it must provide binding sites of sufficient specificity to select factor VIII over the many alternative membrane binding proteins in plasma. A control experiment indicated that after correction for measured light absorption by plasma, there was no effect of plasma upon the fluorescence emission of fluorescein-labeled factor VIII (data not shown). Because the pathway traversed by excitation and emission light in flow cytometry experiments is short and the calculated correction factor would be less than 5%, no mathematical corrections were introduced into the flow cytometry results. In order to evaluate the specificity of the liposphere sites, fluorescein-labeled factor VIII was mixed with various dilutions of heparinized plasma prior to the addition of lipospheres. Binding of factor VIII was evaluated after 1 min (Fig. 5). Plasma efficiently decreased factor VIII binding to lipospheres with 90% inhibition at 50% plasma (v/v). A control experiment, in which fresh plasma was anticoagulated with hirudin rather than heparin showed equivalent displacement of factor VIII indicating that the results were not affected by heparin anticoagulation.

Factor VIII functions in the Xase complex after proteolytic activation by thrombin, and we postulated that thrombin activation might affect membrane binding. Conditions for optimal factor VIII activation were determined as described in the legend for Fig. 7. Thrombin-activated, fluorescein-labeled factor VIII (factor VIIIa) was immediately cooled to 0°C and used within 30 min of activation. The loss in membrane binding and procoagulant activity of factor VIIIa under these conditions was less than 20%. Factor VIIIa was diluted into plasma samples and lipospheres were added. Membrane binding of factor VIIIa was measured after a 1-min incubation. Although plasma decreased the quantity of factor VIIIa bound to lipospheres 50% of initial binding remained in the presence of 70% plasma. These results suggested that thrombin activation of factor VIII affected phospholipid membrane binding and that 50% of phospholipid sites that will adsorb factor VIIIa in the absence of other proteins are specific for factor VIIIa in the presence of plasma.

To determine the effect of von Willebrand factor upon factor VIII binding to lipospheres, fluorescein-labeled factor VIII was incubated with purified von Willebrand factor prior to the addition of lipospheres (Fig. 6). Increasing concentrations of vWF led to decreased bound factor VIII. The concentration of von Willebrand factor that decreased the factor binding sites that are equivalent to those on phospholipid vesicles.

**Binding of Factor VIII to Lipospheres in the Presence of Plasma**—Many plasma proteins bind to phospholipid membranes, and these proteins are present in concentrations that exceed the concentration of factor VIII by as much as 20,000-fold. If a cell membrane containing phosphatidylserine is to promote efficient function of factor VIII in the Xase enzyme complex, then it must provide binding sites of sufficient specificity to select factor VIII over the many alternative membrane binding proteins in plasma. A control experiment indicated that after correction for measured light absorption by plasma, there was no effect of plasma upon the fluorescence emission of fluorescein-labeled factor VIII (data not shown). Because the pathway traversed by excitation and emission light in flow cytometry experiments is short and the calculated correction factor would be less than 5%, no mathematical corrections were introduced into the flow cytometry results. In order to evaluate the specificity of the liposphere sites, fluorescein-labeled factor VIII was mixed with various dilutions of heparinized plasma prior to the addition of lipospheres. Binding of factor VIII was evaluated after 1 min (Fig. 5). Plasma efficiently decreased factor VIII binding to lipospheres with 90% inhibition at 50% plasma (v/v). A control experiment, in which fresh plasma was anticoagulated with hirudin rather than heparin showed equivalent displacement of factor VIII indicating that the results were not affected by heparin anticoagulation.

Factor VIII functions in the Xase complex after proteolytic activation by thrombin, and we postulated that thrombin activation might affect membrane binding. Conditions for optimal factor VIII activation were determined as described in the legend for Fig. 7. Thrombin-activated, fluorescein-labeled factor VIII (factor VIIIa) was immediately cooled to 0°C and used within 30 min of activation. The loss in membrane binding and procoagulant activity of factor VIIIa under these conditions was less than 20%. Factor VIIIa was diluted into plasma samples and lipospheres were added. Membrane binding of factor VIIIa was measured after a 1-min incubation. Although plasma decreased the quantity of factor VIIIa bound to lipospheres 50% of initial binding remained in the presence of 70% plasma. These results suggested that thrombin activation of factor VIII affected phospholipid membrane binding and that 50% of phospholipid sites that will adsorb factor VIIIa in the absence of other proteins are specific for factor VIIIa in the presence of plasma.

To determine the effect of von Willebrand factor upon factor VIII binding to lipospheres, fluorescein-labeled factor VIII was incubated with purified von Willebrand factor prior to the addition of lipospheres (Fig. 6). Increasing concentrations of vWF led to decreased bound factor VIII. The concentration of von Willebrand factor that decreased the factor binding sites that are equivalent to those on phospholipid vesicles.
VIII binding by 50% was 10 μg/ml. To further evaluate the role of plasma von Willebrand factor in limiting the membrane binding of factor VIII, binding was measured in the presence of plasma that lacked von Willebrand factor. Factor VIII binding was minimally decreased, with 80% of binding remaining in the presence of 70% vWf-deficient plasma. The effect of von Willebrand factor-deficient plasma upon liposphere binding of thrombin-activated factor VIII was equivalent to the effect upon non-activated factor VIII. Because von Willebrand factor-deficient plasma also lacks factor VIII control experiments were performed with factor VIII-deficient plasma containing normal concentrations of von Willebrand factor. The decrease in factor VIII binding to lipospheres caused by factor VIII-deficient plasma was equivalent to normal plasma indicating that the maintained factor VIII binding in the presence of von Willebrand factor-deficient plasma was not a result of decreased competition from native factor VIII in the plasma. In order to verify that the effect of thrombin upon membrane binding of factor VIII was due to altered interaction of factor VIII with vWF rather than altered membrane binding of factor VIII experiments were performed with purified reagents. Factor VIII, in the presence and absence of von Willebrand factor, was incubated with thrombin. At various times, aliquots were removed and evaluated for membrane binding in the presence or absence of vWF (Fig. 7A). Thrombin activation of factor VIII incubated with vWF led to increased membrane binding whereas thrombin activation of factor VIII alone did not increase membrane binding. After 4 min of exposure to thrombin at 37°C, membrane binding decreased whether factor VIII was activated in the presence or absence of vWF. An equivalent increase in binding was observed when factor VIII was activated by thrombin prior to incubation with vWF and exposure to lipospheres. Increased membrane binding of factor VIII in the presence of vWF correlated with increased activity in a coagulation assay with factor VIII deficient plasma (Fig. 7B).

The Effect of Factor V upon Membrane Binding of Factor VIII—A potential competitor for the phospholipid membrane binding sites of factor VIII is the homologous protein, factor V. To investigate the effect of factor V upon factor VIII binding to phospholipid sites, experiments were performed to determine whether purified human factor V would displace factor VIII from lipospheres (Fig. 8). In a preliminary experiment, the affinity of factor V for liposphere membrane sites was equivalent to the affinity of factor VIII with a KD of 4.3 nM (Fig. 8, inset). The presence of factor V led to partial displacement of factor VIII reaching 45% displacement at 60 nM factor V. If both proteins bind with equivalent affinity to the same phospholipid site, the predicted displacement was 92%. Factor V concentrations as high as 250 nM led to only 70% displacement of factor VIII versus a predicted displacement of 98%. In some experiments the presence of 1–10 nM factor V was associated with a 5–20% increase in the amount of factor VIII binding to lipospheres (data not shown).

**DISCUSSION**

Lipospheres—We have developed and characterized a novel technique for evaluating membrane binding interactions in complex systems. Phospholipid vesicles fuse to form a bilayer on a chemically clean glass support (28) where lateral mobility coefficients and phase transition temperatures are comparable to those in phospholipid vesicles suggesting similar membrane structure (35). Membrane deposition upon a glass support is
permits quantitative evaluations, and the capacity to perform competition experiments. Fluorescein-labeled factor VIII, 2 nM, was mixed with varying concentrations of factor V prior to the addition of lipospheres. Factor VIII binding was evaluated by flow cytometry 30 min after addition of lipospheres. Increasing concentrations of factor V led to partial displacement of factor VIII, but the displacement was less than predicted if two equivalent ligands compete for the same receptor (smooth line). To confirm that factor V binds to phospholipid membranes with the same affinity as factor VIII the binding of factor V to lipospheres was studied. Bound factor V was detected using fluorescein-labeled AHB 5102, a monoclonal antibody to human factor V that does not inhibit procoagulant activity (inset). Saturable binding was confirmed and the best fit saturation curve (smooth line) indicated a $K_D$ of 4.3 nM, consistent with prior measurements (13, 16) and equivalent to the affinity of factor VIII for lipospheres.

not hindered by negatively charged lipids and binding of prothrombin to a supported membrane is comparable to binding to phospholipid vesicles (36). Tissue factor, a coagulant integral membrane protein, has been localized with a phospholipid layer on the inner surface of microcapillary tubes where it is functional in protocytic activation of factor X (37). We have extended these findings to show that phospholipid vesicles prepared by sonication or by detergent dialysis spontaneously form a membrane on glass microspheres and that the membrane is sufficiently stable to allow washing and sedimentation at 80 $\times$ g. The quantity of lipid associated with each liposphere is consistent with a single bilayer coating in which the lipid packing is equivalent to packing in hydrated lamellae of phosphatidylcholine (38). Defects such as those observed by Tamm and McConnell (35) upon cooling supported membranes were not observed. Absence of defects after liposphere packing under the force of 80 $\times$ g may indicate either that this is not sufficient pressure to cause membrane defects or it may reflect the tendency of phospholipid membrane to rapidly cover exposed chemically clean glass when small membrane defects occur. High affinity binding sites for factor VIII are equivalent to binding sites on large phospholipid vesicles (13). Further, we have demonstrated that functional P-selectin, an integral membrane adhesive protein can be functionalized upon the microspheres along with the lipid membrane. Together, these data suggest that the membrane on a liposphere is a bilayer, equivalent to model membranes that lack glass support.

The utility of lipospheres for studies of high affinity adhesive interactions has been demonstrated in this work. The strengths of this model system are the rapidity with which an assay can be performed, the uniform size of lipospheres which permits quantitative evaluations, and the capacity to perform competition experiments. The low phospholipid concentration required for a binding experiment, e.g. 1 $\times$ 10$^6$ lipospheres/ml, correlates to a phospholipid concentration of only 80 nM suggesting the utility for studies of very high affinity adhesive processes. It is likely that the high affinity interaction between factor VIII and the phospholipid membrane and between P-selectin and the moncytoid HL60 cells is mediated by multiple lower affinity interactions. In the case of factor VIII, evidence suggests that more than one phosphatidyserine molecule is required per factor VIII-binding site (13). In the case of the P-selectin liposphere-HL60 cell interaction, it is likely that multiple P-selectin molecules interact with multiple carbohydrate moieties on the HL60 cell (34). Lipospheres may also have utility for study of these lower affinity constituent interactions. We are currently characterizing interactions of factor VIII with glass beads prepared in a high performance liquid chromatography column. Under these conditions the contribution of various low affinity constituent interactions can be characterized by their effect on the retention time of the adhesive ligand (39). Alternatively, a low affinity ligand may be identified by increased retention time on a liposphere column when adhesion is difficult to detect by more conventional techniques. Together these uses suggest that lipospheres may have broad application in characterizing adhesion interactions with dissociation constants ranging from millimolar to picomolar.

The Effect of von Willebrand Factor upon Membrane Binding of Factor VIII—In plasma factor VIII circulates in a noncovalent complex with von Willebrand factor. Prior reports indicate that binding of factor VIII to von Willebrand factor and to phospholipid vesicles are mutually exclusive (40–42) and that von Willebrand factor prevents factor VIII from binding to activated platelets (4, 21). In this work we demonstrate that vWF is the major plasma protein that influences the binding of factor VIII to a phosphatidylerine-containing membrane. Although the affinity of factor VIII for von Willebrand factor and the kinetics of association and dissociation are unknown, this report indicates that at plasma concentrations, von Willebrand factor will prevent the majority of membrane binding by factor VIII. The fraction of membrane binding prevented in vivo may be higher as the native plasma concentration of factor VIII is 5-fold lower than concentrations used in these experiments. The purified vWF preparation used in these experiments was enriched in the high molecular weight multimeric forms. It is possible that a preparation enriched for the lower molecular weight vWF multimers would have more factor VIII-binding sites exposed, per unit weight, and would function more efficiently to prevent factor VIII binding to lipospheres. Proteolytic activation of factor VIII by thrombin involves cleavage of a peptide that mediates factor VIII binding to von Willebrand factor (43, 44). In a purified system lacking vWF, cleavage of this vWF-binding peptide is unnecessary to achieve full procoagulant activity of factor VIII (45). Thus, thrombin-activated factor VIII no longer binds to von Willebrand factor with high affinity and is able to bind to receptors upon the activated platelet (21). We have confirmed the observation that thrombin activation of factor VIII, resulting in release from von Willebrand factor, enables phospholipid membrane binding of factor VIII (42). Further, we have shown that thrombin activation does not increase membrane binding of factor VIII in the absence of vWF indicating that the vWF-binding peptide of factor VIII does not influence phospholipid membrane binding.

Not all factor VIII was displaced from lipospheres by increasing vWF concentrations. Whether residual liposphere binding represents an equilibrium between membrane-bound and vWF-bound factor VIII or whether it represents a low affinity interaction between vWF with complexed factor VIII and phospholipid membranes is an area of active investigation. Prior studies apparently exclude a high affinity interaction between vWF and phospholipid membranes (40, 41).
Specificity of the Phospholipid Binding Sites for Factor VIII

Specificity of the Factor VIII-binding Site—This report indicates that the phospholipid membrane sites to which factor VIII binds are specific. These sites preferentially bind factor VIII over all proteins in plasma, including the homologous protein, factor V. The plasma concentration of factor V, 30 nM, exceeds the \( K_D \) for phosphatidylserine-containing membrane sites by 7-fold and is sufficient to saturate 85% of binding sites. The failure factor V, purified and in plasma, to efficiently compete for membrane binding sites of factor VIII indicates that factors VIII and V are not equivalent ligands competing for a single type of binding site. These data are best reconciled by the hypothesis that these two proteins recognize distinct, but overlapping membrane binding sites. The chemical mechanism responsible for formation of distinct binding sites from a two-component lipid mixture is unknown. Prior work indicated that factor VIII recognizes binding sites that differ chemically from those recognized by factor V in having a higher phosphatidylserine content (13). The hypothesis that factor V recognizes specific chemical moieties of membrane lipids, rather than net negative charge, is supported by experiments in which the prothrombinase complex was supported by positively charged membranes that contained phosphatidylserine but not other negatively charged phospholipids (46). Our working model for membrane binding is a multi-step process in which initial adsorption of factor VIII is mediated by interaction with specific lipid moieties. Specificity may involve lateral arrangement of lipids within the membrane as well as the requirement for specific lipids. Adsorption is followed by additional binding steps that contribute to high affinity, such as clustering of specific lipids about factor VIII or insertion of a hydrophobic protein domain into the membrane.

Although 50% of liposphere-binding sites bound factor VIIIIs in the presence of plasma, 50% did not. It is possible that rapid degradation of factor VIIIIs by serine proteases such as protein C may have diminished binding somewhat (42). This supposition is supported by experiments with vitamin K-deficient plasma from which protein C may have diminished binding somewhat (42). The hypothesi is that factor V recognizes specific chemical moieties of membrane lipids, rather than net negative charge, is supported by experiments in which the prothrombinase complex was supported by positively charged membranes that contained phosphatidylserine but not other negatively charged phospholipids (46). Our working model for membrane binding is a multi-step process in which initial adsorption of factor VIII is mediated by interaction with specific lipid moieties. Specificity may involve lateral arrangement of lipids within the membrane as well as the requirement for specific lipids. Adsorption is followed by additional binding steps that contribute to high affinity, such as clustering of specific lipids about factor VIII or insertion of a hydrophobic protein domain into the membrane.

The interaction between factor VIII and lipospheres resembles the interaction between factor VIII and activated platelets in several ways (4, 21). The affinity of factor VIII for platelets and lipospheres is equivalent. In both cases von Willebrand factor prevents membrane binding of factor VIII, but not thrombin-activated factor VIII (21). The homologous membrane binding cofactor, factor V, does not displace bound factor VIII from platelet receptors (3), is a poor competitor in binding to receptors on platelet microparticles (4), and is a poor competitor in binding to phosphatidylserine-containing sites on lipospheres. While the information in this report does not establish whether phosphatidylserine is a critical constituent of the platelet receptor it does indicate that phosphatidylserine-containing membrane binding sites have sufficient specificity to function as specific receptors for factor VIII.

In summary—We have used a novel model, in which synthetic membranes are supported by glass microspheres, to evaluate the specificity of the phospholipid-binding site for factor VIII. Results indicate that phosphatidylserine-containing membrane sites are sufficiently specific to rapidly bind thrombin-activated factor VIII in the presence of plasma. Failure of factor V to efficiently displace factor VIII, in spite of structural homology and equivalent membrane affinity, suggests that these homologous proteins recognize distinct but overlapping membrane sites. The high specificity of phospholipid-binding sites for factor VIII suggests that factor VIII recognizes and adheres to a particular arrangement of lipid moieties rather than the diffuse negative charge of phosphatidylserine-containing membranes.

Acknowledgments—We are grateful to Dr. G. Bush and S. Bennett for extensive use of the Coulter Epics Profile II flow cytometer and to Dr. Kenneth Bauer for helpful discussions of the manuscript.