The Binding of $^{35}$S-Labeled Recombinant Factor VIII to Activated and Unactivated Human Platelets*

(Received for publication, June 8, 1988)

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Recombinant-derived human Factor VIII was labeled intrinsically with $[^{35}]$methionine, and its binding to washed human platelets was studied. Binding measurements were performed by incubating Factor VIII and platelets for 5 min at room temperature in Tyrode’s solution supplemented with Ca$^{2+}$ (5.0 mm), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (5.0 mm), 0.50% bovine serum albumin, and the Factor Xa and thrombin inhibitors 5-dimethylaminonaphthalene-1-sulfonylglutamylglyclysarcinyl chloromethyl ketone and 5-dimethylaminonaphthalene-1-sulfonilarginine-N-(3-ethyl-1,5-pentanediyldiamide). Separation of free from bound Factor VIII was accomplished by centrifugation through oil, and nonspecific binding was determined with excess unlabeled Factor VIII. Binding was saturable, reversible, and stimulated 20-fold after platelet activation with thrombin. Furthermore, binding was specific in that bound labeled Factor VIII could be displaced by excess unlabeled Factor VIII, but not by Factor V. Scatchard analysis indicated a single class of binding sites with $K_d = 2.9$ nM and 450 sites/activated platelet. The time course of displacement indicated a $t_1/2$ of bound Factor VIII of approximately 5 min. When platelets were incubated in Ca$^{2+}$, both the heavy and light chains of Factor VIII were bound, whereas exposure to EDTA resulted in the binding of the light chain only. These results demonstrate the specific reversible binding of Factor VIII to human platelets, likely mediated through the light chain.

Factor VIII is a plasma protein that functions as an essential cofactor in the intrinsic pathway of blood coagulation. A deficiency of Factor VIII, either qualitative or quantitative, causes the bleeding tendency characteristic of classic hemophilia A (1). The cofactor circulates at an approximate concentration of 0.2 μg/ml (0.7 nM) and is in large part associated with its carrier protein, von Willebrand factor (1-3).

Until recently, efforts to study Factor VIII were hampered by its relative unavailability consequent to its low plasma concentration and apparent lability. The work of several groups, however, resulted in the isolation of sufficient material in cultured mammalian cell lines (4-6). These accomplishments have provided new and valuable sources of the material for both basic studies and therapeutic uses.

Factor VIII expresses its functional properties by participating as a cofactor in the surface-dependent proteolytic activation of Factor X by the serine protease Factor IXa. Although Factor IXa alone will slowly catalyze the activation of Factor X, the rate of the reaction is enhanced by 4-5 orders of magnitude by the combination of Factor VIII (VIIa) and the catalytic surface (7). In model systems, the surface can be provided by negatively charged phospholipid vesicles, whereas in vivo the surface is presumed to be provided by the platelet. By analogy with the prothrombinase complex (2, 9, 10), which consists of protease Factor Xa, cofactor Factor Va, the platelet surface, and Ca$^{2+}$, interactions of Factor VIII with the platelet can be hypothesized as essential in the activation of Factor X (2). Thus, the following studies were undertaken to investigate the nature of the presumed interaction of Factor VIII with both activated and unactivated human platelets. These studies utilized Factor VIII prepared by recombinant techniques in the presence of $[^{35}]$methionine, which provided a specimen both functionally active and intrinsically labeled.

EXPERIMENTAL PROCEDURES

Recombinant-derived Factor VIII—Intrinsically labeled Factor VIII was prepared from a genetically engineered Chinese hamster ovary cell line that has been previously described (8). The cells were grown to 2 x 10$^8$ cells/20-cm plate, rinsed, and incubated at 37 °C for 15 min in 5 ml of methionine-free medium containing 10% dialyzed fetal calf serum. The medium was then supplemented with 0.5 μCi/ml $[^{35}]$methionine (7800 Ci/mmol, 1 Ci = 37 GBq; Du Pont-New England Nuclear) and 0.1% aprotinin (Sigma). The label was removed at 4 h, and complete medium containing 10% fetal calf serum and 0.1% aprotinin was added. Twenty-four hours later, the conditioned medium was collected and centrifuged to remove cellular debris, and soybean trypsin inhibitor and phenylmethanesulfonyl fluoride were added to final concentrations of 1 mg/ml and 1 mM, respectively. The sample was diluted with 0.5 M NaCl, 20 mM CaCl$_2$ and applied to a monoclonal antibody column specific for the heavy chain of Factor VIII (kindly supplied by Barry Foster, Genetics Institute). The column was washed, and Factor VIII was eluted and subjected to fast protein liquid chromatography on a cation exchange Mono S gel and then an anion exchanger Mono Q gel (both from Pharmacia LKB Biotechnology Inc.). The latter column was eluted in 50 mM Tris-HCl, pH 7.2, 0.1% Tween 80, 5 mM CaCl$_2$ with 1 M NaCl and Factor VIII stored at -80 °C. Purity was determined by analysis on SDS-polycrylamide gels before and after digestion with thrombin. Activity was determined by a chromogenic assay method (Kabi Coatest). The activity of the purified Factor VIII was 5.6 units/ml and $1 x 10^4$ units/ml.

* This work was supported by Grant D6-309 (to M. E. N.) from the Medical Research Council of Canada and by a National Institutes of Health Small Business Innovation Research Grant 2R44-HL35946-02 (to R. J. K.). 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.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; DdSO$_4$, dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; vWF, von Willebrand factor; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
Unlabeled Factor VIII was isolated from conditioned medium from cells as described above by monoclonal antibody affinity fractionation and subsequent chromatography on Mono S and Mono Q fast protein liquid chromatography columns. The Factor VIII obtained was apparently homogeneous by SDS-polyacrylamide gel electrophoresis. 

Binding Experiments—All experiments were performed in 0.4-ml series of tubes prepared by diluting an initial stock solution containing Factor VIII (0.7 nM) and activated platelets were incubated for 30 min in the presence of either 2.5 mM Ca\(^2+\) or 10 mM (total) EDTA, followed by centrifugation and recovery of pellets by digestion in 1% DoDSO\(_4\). Electrophoresis in DoDSO\(_4\) and polyacrylamide according to Laemmli (17) and autoradiography utilizing ENUHANCE (Du Pont-New England Nuclear) were performed in order to characterize the material bound under the two sets of conditions. 

RESULTS

The binding of Factor VIII to human platelets both before and after platelet activation by thrombin is depicted in Fig. 1. Binding to activated platelets, indicated by curve A, was characterized by a simple binding isotherm that yielded a linear Scatchard plot (inset). This plot indicates the existence under these conditions of a single class of 450 binding sites/cell with a dissociation constant of 2.9 nM. A similar experiment with a separate isolate of platelets yielded essentially indistinguishable results (K\(_d\) = 2.0 nM; n = 435 sites/platelet). Binding to unactivated platelets was substantially diminished, although detectable as indicated by the isotherm (curve U) in Fig. 1. Saturation was achieved at about 20 molecules of Factor VIII/platelet. These data suggest that the expression of binding sites for Factor VIII under these conditions is a platelet activation-dependent event.

Reversibility and Specificity of Binding—Fig. 2 represents the time of displacement of previously bound \(^{35}S\)methionine-labeled Factor VIII following the addition of an overwhelming excess of either unlabeled Factor VIII or Factor V. Two preparations of unlabeled Factor VIII (Fig. 3, lanes 1 and 2), one of which is deficient in the B-domain of the heavy chain.

![Fig. 1. Binding of \(^{35}S\)-labeled Factor VIII to activated and unactivated platelets. The units of the vertical axis represent molecules of Factor VIII bound per platelet (PLT), while those of the horizontal axis represent the concentrations of unbound free (f) Factor VIII. The data indicated by curve A were obtained with thrombin-activated platelets, whereas those indicated by curve U were obtained with unactivated platelets. A Scatchard plot of the data obtained with the activated set is shown (inset). A single class of sites is indicated, with K\(_d\) = 2.9 nM and n = 450 Factor VIII sites/platelet. Nonspecific binding was determined with excess unlabeled Factor VIII and was less than 10% of the specific binding.](image-url)
Factor VIII Binding to Platelets

The off-rate constant is approximately $1.1 \times 10^{-3}$ s$^{-1}$, which implies an on-rate constant of $3.8 \times 10^8$ M$^{-1}$ s$^{-1}$. In addition, since Factor V provided no detectable displacement, the binding measured is highly specific for Factor VIII.

**Localization of Platelet-binding Domain of Factor VIII**

The heavy and light chains of Factor VIII associate through a divalent metal ion-dependent interaction which can be dissociated with loss of cofactor function by treatment with a chelator such as EDTA (18-20). In order to determine whether the platelet interaction is specifically expressed through the heavy or light chain, platelet-bound material was collected in the presence of either Ca$^{2+}$ or EDTA and analyzed by electrophoresis in DodSO$_4$ plus autoradiography. Results are shown in Fig. 3. Lane 3 represents the $^{35}$S-labeled Factor VIII preparation used in these studies. The major components are the 200-kDa heavy chain and the 80-kDa light chain doublet. The diffuse material marked with an arrow likely represents an extended form of the light chain with the amino terminus represented by residue 1313 of the mature Factor VIII polypeptide. Lane 4 represents material bound to platelets in the presence of Ca$^{2+}$, and lane 5 represents that bound in the presence of EDTA. In the presence of Ca$^{2+}$, the components at 200, 180, and ~90 kDa are taken as heavy chain and heavy chain fragments, whereas the materials at the arrow and the 80-kDa doublet plus a faint band of 70 kDa represent materials of the light chain domain. In the presence of EDTA, the vast majority of bound material is material of the light chain domain, with only a trace of the heavy chain observed. Laser densitometry was utilized in order to quantify the densities at each position. In the starting material (lane 3), 54 and 46% of the total density was contributed by components of heavy and light chain domains, respectively. In the presence of Ca$^{2+}$ (lane 4), 48 and 52% of the total density was associated with components from these domains, whereas in the presence of EDTA, the corresponding densities were 3 and 97% of the total. Thus, the quantitative analysis plus visual inspection of the autoradiographs indicate that the platelet-binding domain of Factor VIII resides on the light chain. In addition, the results obtained in the presence of Ca$^{2+}$ suggest proteolytic processing of platelet-associated Factor VIII. Both the processing of bound material and exclusive localization of the binding determinant in the light chain are analogous to the properties of the Factor Va-platelet interactions, as shown by Tracy et al. (21, 22).

**DISCUSSION**

Factor VIII, when activated, functions as a cofactor in the surface-dependent, Factor IXa-catalyzed activation of Factor X, an essential reaction of the coagulation cascade (1-3). Since the surface and cofactor together provide 4-5 orders of magnitude rate enhancement (7), control of the expression of the appropriate surface may well provide control of the expression of intrinsic Factor X activating activity. Thus, the interaction of Factor VIII with the platelet likely represents a potential element in the interplay of interactions by which the process of coagulation is regulated.

The data presented here indicate that blood coagulation Factor VIII interacts reversibly and specifically with a limited number of binding sites on human platelets. In addition, the expression of these sites is dependent upon activation of the platelet. The linearity of the Scatchard plot of binding data is consistent with a single class of identical binding sites. The data obtained by electrophoretic and autoradiographic analysis of material bound in the absence and presence of Ca$^{2+}$ suggest that the platelet-binding domain of Factor VIII is specifically associated with the light subunit. Tracy et al. (21)
found that the interaction of platelets with Factor Va, a cofactor structurally and functionally analogous to Factor VIII (VIIIa), is also specifically mediated through the light subunit. This subunit, like the light subunit of Factor VIII, represents the COOH-terminal domain of the parent molecule (29, 23).

Our data alone do not identify the platelet receptor for Factor VIII. Since, however, bound Factor VIII is not displaced by the analogous cofactor protein Factor V, the binding sites for Factor VIII appear clearly distinct from those which exist for Factor V. This specificity for Factor VIII indicates the existence of a receptor distinct from negatively charged phospholipid, as the latter would likely bind both proteins indiscriminately and competitively. von Willebrand factor, the carrier for Factor VIII in plasma, was not included in the incubation media of these studies. Since, however, platelets release von Willebrand factor upon stimulation (24, 25), a role for this protein in the Factor VIII-platelet interaction cannot be precluded by our data. The platelet count utilized in the final incubations of our experiments (1 \times 10^6\mu l) would be expected to release enough vWF to nearly saturate the binding sites for this protein (25, 26), thereby providing on the order of 50,000 vWF subunits/cell. This figure exceeds by a factor of 200 the presently observed capacity, thus suggesting that platelet vWF may not comprise the observed binding receptor. Furthermore, vWF interactions with the platelet are divalent cation-dependent (27), whereas our work indicates that the light chain of Factor VIII interacts in the presence of EDTA.

Others have demonstrated qualitatively the interaction of coagulant Factor VIII with platelets. Moake et al. (28) found that both von Willebrand factor and Factor VIII coagulant could be removed from citrated platelet-poor plasma by formalinization of platelets in the presence of ristocetin. Sewerin and Andersson (29) found by discontinuous albumin density centrifugation that Factor VIII was found associated with platelets. This association was observed when platelets were stimulated with collagen, but not with unstimulated platelets. In addition, activation of Factor VIII with thrombin promoted a further increase in binding. The qualitative results observed previously are consistent with our results in that these studies together both indicate the existence of the Factor VIII-platelet interaction and demonstrate a requirement for platelet stimulation to elicit binding. In another approach, which utilized studies of the kinetics of Factor X activation as stimulated by Factor IXa, Factor VIIIa, and platelets, Rosing et al. (30) inferred the presence of about 1000 functional sites per Factor VIIIa-Factor Xa and 3000 sites for Factor Va-Factor Xa on thrombin-stimulated platelets. A similar number of functional studies of the kinetics of Factor X activation as stimulated by Factor VIIIa-Factor IXa and 26,000 for Factor Va-Factor Xa. These latter sites were interpreted as negatively charged phospholipids. Although the latter numbers greatly exceed the Factor VIII binding capacity observed here, the lower number inferred with thrombin only is similar to the number found here. The smaller numbers measured here (450 Factor VIII sites/cell after thrombin stimulation) are clearly specific for Factor VIII, as indicated by the absence of displacement by Factor V, and might well reflect the low capacity class inferred by Rosing et al. (30) upon stimulation by thrombin only. The current data, together with those of Rosing et al., suggest the possible existence of two classes of sites for the interaction of Factor VIII (and by analogy Factor V) with platelets. One relatively low capacity and high specificity class may comprise specific receptor-like binding entities expressed upon exposure to a stimulus like thrombin, and another might reflect an indiscriminate surface presented by the platelet upon further stimulation. Clearly, further efforts will be required to elucidate fully the nature of the Factor VIII-platelet interaction(s) and the functional consequences of the interaction(s).

Acknowledgments—We wish to acknowledge the valuable technical assistance of Mary Ann Krane and Jack Pettinato. We also wish to express our gratitude to Marilyn McCallum for typing the manuscript.

REFERENCES