It has been suggested that an abnormality in the carbohydrate structure of the blood clotting glycoprotein, human factor VIII/von Willebrand factor (FVIII/vWF), may give rise to the hemorrhagic disorder known as von Willebrand's disease. More recently, we have reported that the sequential removal of sialic acid and galactose from FVIII/vWF causes a progressive diminution in the ability of human FVIII/vWF to support ristocetin-induced platelet aggregation. We now report experiments aimed at defining how modifications of carbohydrate side chains of FVIII/vWF protein cause a loss of platelet-aggregating activity. The ristocetin cofactor activity of human FVIII/vWF was reduced to 39% after removal of 74% of the sialic acid by protease-free neuraminidase. Ristocetin cofactor activity was reduced further to 19% after oxidation of 39% of the galactose residues of asialo-FVIII/vWF by galactose oxidase treatment and was restored to 33% after potassium borohydride reduction of galactose-oxidized asialo-FVIII/vWF. The receptor-binding potency and affinity of each form of FVIII/vWF derivative was determined by employing a FVIII/vWF receptor-binding assay. The effective concentration to inhibit 50% binding of 0.2 µg/ml of 125I-FVIII/vWF to 5 × 10^6 platelets and the binding dissociation constant for each form of FVIII/vWF are: 2.0 µg/ml, 1.1 nM for native FVIII/vWF; 14.5 µg/ml, 12.5 nM for asialo-FVIII/vWF; 66 µg/ml, 53.8 nM for galactose-oxidized asialo-FVIII/vWF; and 30 µg/ml, 18.9 nM, for KBH-reduced galactose-oxidized asialo-FVIII/vWF. Furthermore, a linear correlation between the log of receptor-binding affinity and the log of ristocetin cofactor activity was observed. We conclude that the diminished ristocetin cofactor activity of FVIII/vWF having modified carbohydrate side chains results from reduced binding affinity for platelet FVIII/vWF receptors. Our results also indicate that the binding of FVIII/vWF to platelet receptors is functionally relevant with respect to ristocetin cofactor activity.

Factor VIII/von Willebrand factor (FVIII/vWF) is a plasma glycoprotein or glycoprotein complex (~10^6 daltons) with two distinct biological activities, aspects of which were reviewed recently (1). One function is procoagulant activity which corrects the blood coagulation defect in classical hemophilic patients. The other is platelet-aggregating activity which is essential for the formation of hemostatic platelet plugs at the transected end of small vessels. The molecular basis for the two biological activities of this macromolecule remains unresolved. The two biological activities can be dissociated from each other by treatment of FVIII/vWF with thrombin (2) or high ionic strength buffers (3-5). Some believe that dissociated FVIII procoagulant activity is on a smaller molecule which accounts for 99% of the protein in the FVIII/vWF isolate (3-5). Other investigators have suggested that both FVIII and vWF activities are on the same molecule which, after cleavage by thrombin, gives rise to a derivative with full FVIII procoagulant activity (2, 7). Several of the biochemical and immunological characteristics of the FVIII/vWF macromolecule have been described (8, 9).

Defects in platelet plug formation and blood coagulation have been observed in patients with a hereditary deficiency of FVIII/vWF protein known as von Willebrand's disease (vWD) (10). With refined laboratory techniques, variant forms of von Willebrand's disease have been described in some patients who have normal levels of plasma FVIII/vWF antigen and FVIII procoagulant activity (11-15). Following a proposal by McKee et al. (16), one group of investigators published findings that suggested that modification of carbohydrate side chains on the FVIII/vWF protein accounts for its reduced function in some patients with von Willebrand's disease (14). Sodetz et al. reported that successive removal of sialic acid and galactose residues from FVIII/vWF progressively impaired its ristocetin cofactor activity without affecting FVIII procoagulant activity (17, 18); similar results were then observed by Granick (19) who removed sialic acid and oxidized the galactose residues of the FVIII/vWF protein. Recently, we have identified and established the existence of FVIII/vWF receptors on platelets which relate directly to their function in the ristocetin-induced platelet aggregation assay (20, 21). Now, by employing a FVIII/vWF receptor-binding assay, we have further explored and defined the mechanism by which modifications of carbohydrate side chains on normal human FVIII/vWF causes reduced platelet-aggregating activity in the presence of ristocetin.

METHODS

Portions of this paper (including "Methods," a small part of "Results," and Figs. 3, 4, and 5) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 80M-927, cite author(s), and include a check or money order for $1.20 per set of photocopies. Full-sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Modification of Carbohydrate on FVIII/vWF—As shown in Table 1, 74% of the total sialic acid was removed from native FVIII/vWF by neuraminidase treatment. The same quantity of sialic acid was also removed from native FVIII/vWF during the preparation of galactose-oxidized asialo-FVIII/vWF. After desialylation, the exposed terminal galactose residues were oxidized to galactose aldehyde by treatment with galactose oxidase. Then the residual amount of galactose that had not been oxidized was measured by enzymatic assay for \( \beta \)-galactose. In order to determine whether the enzymatic assay for \( \beta \)-galactose can be used to quantify only galactose in the presence of galactose aldehyde, the substrate specificity of galactose dehydrogenase for galactose aldehyde was studied first. Despite the presence of galactose aldehyde, we found that no more than 3% galactose was detected after galactose (300 nmol/ml) had been incubated for 2 h with galactose oxidase (5 units/ml) and catalase (1 \( \mu \)g/ml) at 87°C. Therefore, the use of the enzymatic assay is valid for quantitating any galactose remaining in the carbohydrate side chains of galactose-oxidized asialo-FVIII/vWF or KBH4-reduced galactose-oxidized asialo-FVIII/vWF.

Platelet Aggregation using Native and Carbohydrate-modified FVIII/vWF—The platelet-aggregating activities of native FVIII/vWF and each of its carbohydrate-modified forms were calculated from the reciprocals of their slopes shown in Fig. 1 and are listed in Table I. After removal of sialic acid, the aggregating activity of FVIII/vWF was reduced to 39% of native FVIII/vWF. This activity was reduced further to 10% of native FVIII/vWF after galactose oxidation and was restored to 33.3% of native FVIII/vWF after borohydride reduction. To confirm that the loss of aggregating activity to the same concentration of potassium borohydride actually decreased its platelet-aggregating activity to \( \sim 75\% \) of normal. Therefore, the restoration of platelet aggregation activity, in spite of direct adverse effects of potassium borohydride on FVIII/vWF, must indicate that the loss of aggregating activity to the exact preoxidation level was due to the direct effects of KBH\(_4\) on the protein (25). It should be noted that all the platelet aggregation activities of native or carbohydrate-modified FVIII/vWF were determined in the same experiment. Moreover, the platelet responsiveness to a given concentration of any FVIII/vWF species tested did not change during the time required for the entire set of analyses.

Receptor-Binding Potency of Carbohydrate-modified forms of FVIII/vWF—The receptor-binding potencies of native or carbohydrate-modified forms of FVIII/vWF were determined from the ability of selected quantities of each FVIII/vWF species to compete with 300 pg/ml of \( ^{125}\)I-FVIII/vWF for the same specific binding sites on the platelet membrane.
Binding of Carbohydrate-modified FVIII/vWF to Platelets

### Table I

| Carbohydrate content, receptor-binding affinity and ristocetin cofactor activity of carbohydrate-modified FVIII/vWF |
|--------------------------------------------------|
| Sialic acid | Galactose | $K_i$ | $K_a$ | Ristocetin cofactor activity |
| nmol/mg protein | | nM | %T/min/µg/ml |
| Native FVIII/vWF | 115.4 ± 1.4 | 165.3 ± 14.8 | 1.1 | 1.8 | 11.3 |
| Asialo-FVIII/vWF | 29.3 ± 1.4 | 169.9 ± 21.5 | 12.5 | 13.4 | 4.4 |
| Galactose-oxidized asialo-FVIII/vWF | 30.8 ± 3.3 | 102.3 ± 3.9 | 53.8 | 59.8 | 2.1 |
| KBH$_4$-reduced galactose-oxidized asialo-FVIII/vWF | 35.7 ± 1.6 | 175.1 ± 7.4 | 18.9 | 27.1 | 3.7 |

$K_i$: Inhibition constant determined from Dixon plot; $1.1 \times 10^6$ used for the molecular weight of FVIII/vWF (8). $K_a$: Inhibition constant determined from effective concentration which inhibits 50% specific binding (EC$_{50}$) of $^{125}$I-FVIII/vWF (0.2 µg/ml) to 5 x 10$^6$ platelets by using the equation: $K_i = EC_{50}/L + (L/K_d)$ where $L$ is the concentration of free $^{125}$I-FVIII/vWF and $K_d$ is the dissociation constant of $^{125}$I-FVIII/vWF binding to platelet receptors.

Rate of platelet aggregation (% transmittance (2') per min for a given concentration of FVIII/vWF or its derivatives). These numbers were determined from the reciprocals of the slopes shown in Fig. 1.

Correlation of Receptor-Binding Affinity and Platelet-aggregating Activity—We have reported recently that FVIII/vWF receptor occupancy is proportional to platelet-aggregating activity (21). Thus, the affinity of each form of FVIII/vWF for the receptors on platelets should be proportional to its platelet-aggregating activity. The platelet-aggregating activity of each form of FVIII/vWF was calculated from the reciprocal of the slope shown in Fig. 1. Then the log of platelet-aggregating velocity is plotted against the reciprocal of each concentration of FVIII/vWF.
Binding of Carbohydrate-modified FVIII/vWF to Platelets

FIG. 6. Correlation of ristocetin cofactor activity and receptor-binding affinity for the different forms of FVIII/vWF. The ristocetin cofactor activities of the carbohydrate-modified forms of FVIII/vWF were determined from the reciprocals of the slopes shown in Fig. 1 and then plotted against the receptor-binding affinity of each respective species. The two data points indicated by (x₁ and x₂) were determined from experiments in which FVIII/vWF (x₁) and its asialo derivative (x₂) were prepared from a different lot of starting FVIII/vWF concentrate than the data points indicated by ○.

aggregating activity was plotted against the log of receptor binding affinity (Kᵢ) for each form of FVIII/vWF (Fig. 6). The linear correlation observed between these two parameters indicates that platelet-aggregating activity of FVIII/vWF is a function of receptor-binding affinity. Therefore, the diminished platelet-aggregating activity of carbohydrate-modified FVIII/vWF is related directly to a decreased binding affinity for the receptors on platelets.

DISCUSSION

We recently established that FVIII/vWF receptors are present on human platelets and that the extent of FVIII/vWF binding to platelets is directly proportional to the extent of ristocetin-induced platelet-aggregating activity (20, 21). Our present results demonstrate that carbohydrate-modified forms of FVIII/vWF compete with ¹²⁵I-FVIII/vWF for the same binding sites, although at a markedly reduced binding affinity. Significantly, the receptor-binding affinity of each form of FVIII/vWF shows excellent correlation with its platelet-aggregating activity (Fig. 6). Hence, the potency of carbohydrate-modified forms of FVIII/vWF to aggregate platelets is directly proportional to their ability to compete for binding sites on platelets. This observation fulfills an important criterion for establishing that binding measurements truly reflect the interaction of ligand with physiologically relevant receptors (29). Thus, diminished receptor-binding affinity must be responsible for the loss of ristocetin cofactor activity observed after modification of the carbohydrate on FVIII/vWF. In Fig. 1, the reciprocal plot of platelet-aggregating activity versus the concentrations of the different carbohydrate-modified forms of FVIII/vWF suggests that in the presence of ristocetin, the different modified forms of FVIII/vWF induce platelet aggregation by the same mechanism. Hence, the loss of platelet-aggregating activity observed with the carbohydrate-modified FVIII/vWF results from reduced receptor-binding affinity. Whether the reduced receptor-binding affinity of carbohydrate-modified FVIII/vWF is due to conformational change(s) of the molecule, or due to the removal of carbohydrate residues directly involved in receptor binding, remains to be determined. Recently, much attention has been focused on the biological significance of the constituent oligosaccharide units of different glycoproteins (30-34); it has been suggested by some that the carbohydrate side chains may play a role in maintaining the conformational stability of glycoproteins (31-33). However, it has also been found that removal of sialic acid groups from the (Fab')₂ fragments of immunoglobulin G abolishes antigen-binding ability, presumably without major conformational change (34). Thus, there are precedents
with other glycoproteins which would support either a con-
formational change or a direct alteration in binding affinity
when the carbohydrate side chains are modified.

The importance of FVIII/vWF carbohydrate side chains
for the full expression of ristocetin-induced platelet-aggregat-
ing activity has been recognized by biochemical studies of the
FVIII/vWF glycoprotein from von Willebrand’s disease pa-
tients (14) as well as by functional studies of enzymatically
modified normal FVIII/vWF (17–19). In the present report,
we have confirmed our previous observations (17, 18) that
both sialic acid and galactose residues of FVIII/vWF protein
are indeed important determinants of ristocetin cofactor ac-
tivity. When 74% of the sialic acid residues were removed
from native FVIII/vWF, we observed a 62% loss of ristocetin
cofactor activity (Table I). These values are in good agreement
with our earlier report. Recently, we also showed that only
62% of the galactose residues can be cleaved enzymatically
from asialo-FVIII/vWF (>95% desialylated) and that this is
accompanied by a reduction in ristocetin cofactor activity to
~12% of normal (18). In the present experiments, we purpose-
fully performed our desialylation at pH 6.8 since procoagulant
activity is not destroyed at this pH, believing this to be a
desirable indication that little or no degradation occurred;
however, only 74% of the sialic acid could be removed. Hence,
26% of the expected galactose remained unexposed; another
38% could not be exposed despite tryptic digestion of >95%
desialylated FVIII/vWF (18). Therefore, it is to be expected
that a total of 64% of the galactose residues will not be
accessible to galactose oxidase; this predicted value is es-
sential that which we observed (61%). Importantly, reduction of
the oxidized galactose groups restored platelet-aggregating
activity almost to that seen for the asialo-FVIII/vWF. These
observations clearly extend our earlier results by showing that
the reduced ristocetin cofactor activity seen with either asialo-
FVIII/vWF or galactose-oxidized asialo-FVIII/vWF is due to
proportional reductions in binding affinity for the platelet
receptors.

Gralnick (19) disagrees that removal of sialic acid from
FVIII/vWF causes a loss of ristocetin cofactor activity. There
are several possible reasons for the discord between his results
and ours. For example, paraformaldehyde-fixed platelets
were used by Gralnick (19) whereas we have always used freshly
washed human platelets. Moreover, our data indicate that
reduced ristocetin cofactor activity observed with the carbo-
hydrate-modified forms of FVIII/vWF becomes most obvious
when the assay is performed over a range of low-concentra-
tions of the particular FVIII/vWF species (Fig. 1). Therefore,
unless one is aware that the assay sensitivity is amplified for
detecting differences in ristocetin cofactor activity over the
lower concentration range, the decreased ability of asialo-
FVIII/vWF to support ristocetin-induced platelet-aggregating
activity might be missed. Lastly, but of equal importance,
whether the decrement in ristocetin cofactor activity is de-
tected when sialic acid is removed from native FVIII depends
in part on the initial ristocetin cofactor activity of the purified,
native FVIII/vWF used as starting material. We find that
normal human FVIII/vWF preparations purified from different
lots of starting concentrates have different amounts of ristocetin cofactor activity. However, when the sialic acid is
removed from any of these purified FVIII/vWF preparations,
all have about the same level of ristocetin cofactor activity,
albeit reduced in varying proportion from that observed for
the starting material. An example of this phenomenon is given
in Fig. 3. For this reason, purified native FVIII/vWF with a
low starting ristocetin cofactor activity may make it difficult
to detect significant reductions in ristocetin cofactor activity
after desialylation.

Our results continue to support plausible models for the
two most frequently encountered forms of von Willebrand’s
disease. In the instance of asialo-FVIII/vWF, low plasma
levels of the antigen would be expected because of rapid
clearance by the hepatic receptor which recognizes asialo-
glycoproteins; this we established in the past (17). Although
containing to possess full FVIII procoagulant activity, the
decreased sialic acid on the asialo-FVIII/vWF molecule also
contributes to decreased ristocetin cofactor activity by virtue
of its reduced affinity for its receptor on the platelet. Our
findings with agalacto-FVIII/vWF suggest analogies to those
features of the less frequently encountered form of von Wil-
lebrand’s disease in which normal plasma levels of FVIII/
vWF antigen and FVIII procoagulant activity are observed
(11–15). For example, the agalacto-FVIII/vWF has normal
FVIII procoagulant activity, no affinity for the hepatic recep-
tor (suggesting a normal circulatory survival and therefore
a normal antigen level), but a markedly reduced ristocetin co-
factor activity (18). Our present study expands the latter
finding by showing that galactose-oxidized FVIII/vWF has
only about 20% of the expected affinity for the FVIII/vWF
platelet receptor and this correlates well with its decreased
function in the ristocetin cofactor activity assay. Albeit neither
of these models have been proven, we do suggest that our
findings may in part explain or provide leads to the eventual
definition of the molecular defects responsible for the spec-
tural of von Willebrand’s disease.

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