Factor VIII/von Willebrand Factor Protein

GALACTOSE, A CRYPTIC DETERMINANT OF VON WILLEBRAND FACTOR ACTIVITY

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ABSTRACT The normal Factor VIII/von Willebrand factor protein has the ability to agglutinate or aggregate normal platelets in the presence of ristocetin (von Willebrand factor activity). Removal of >95% of the sialic acid from this protein by neuraminidase did not affect the von Willebrand factor or procoagulant activity. However, oxidation of the penultimate galactose of the asialo Factor VIII/von Willebrand factor protein with galactose oxidase resulted in a progressive loss of von Willebrand factor activity with no effect on procoagulant activity. Reduction of the 6-aldehydo intermediate by potassium borohydride caused full regeneration of von Willebrand factor activity. These studies confirm the identification of the intact penultimate galactose moiety as a critical determinant of von Willebrand factor activity.

INTRODUCTION

The normal Factor VIII/von Willebrand factor (FVIII/vWf)[1] protein purified from normal plasma has been characterized as a macromolecular glycoprotein with two major biologic activities (1–3). One activity, procoagulant activity, is related to the intrinsic phase of blood coagulation and is assayed by the specific ability to correct the blood coagulation defect in patients with hemophilia A. The second activity, von Willebrand factor activity, is assayed by the ability to induce agglutination or aggregation of von Willebrand's disease or normal platelets in the presence of ristocetin.

In hemophilia A (FVIII deficiency), procoagulant activity is deficient, whereas vWf activity is normal. In "classic" von Willebrand's disease both the procoagulant and vWf activity are decreased; however, recent studies have indicated that there are forms of von Willebrand's disease in which the vWf activity is the only activity that is decreased or it is more severely reduced than the procoagulant activity. Investigation of these patients has suggested that the reduced vWf activity is related to a deficiency of the carbohydrate moiety of this glycoprotein (4–6). To characterize the carbohydrate(s) involved in vWf activity, we modified the carbohydrate content of the normal FVIII/vWf protein and studied the effect on the interaction between this protein and platelets. The present paper demonstrates the dependence of vWf activity on the intact galactose residues terminated by sialic acid.

METHODS

FVIII/vWf protein was purified from concentrates of intermediate purity obtained from the American National Red Cross, Bethesda, Md. (kindly supplied by Dr. Milan Wickerhauser). Sepharose 4B was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., (Piscataway, N. J.). The materials for polyacrylamide gel electrophoresis were those previously described (7). Neuraminidase from *Clostridium perfringens* and galactose oxidase were obtained from Worthington Biochemical Corp. (Freehold, N. J.). Potassium borohydride was supplied by Matheson, Coleman & Bell (East Rutherford, N. J.).

FVIII/vWf protein was purified by precipitation by polyethylene glycol followed by gel chromatography on Sepharose 4B (8). The column effluent was monitored for protein and procoagulant and vWf activity. The protein in the void volume was collected and concentrated by ammonium sulfate precipitation (8).

The neuraminidase was purified by affinity chromatography using sialic acid as an immobolized ligand (9). The final preparation of neuraminidase in 0.05 M sodium acetate, pH 5.5 contained 2.0–3.35 U of neuraminidase and 0.25 mg of bovine serum albumin per milliliter. 1 U of activity hydrolyzes 1 μmol of sialic acid per minute with human α_1 acid glycoprotein at 37°C. Galactose oxidase was purified by chromatography on DEAE cellulose (10). The purified enzyme contained 270 U/ml. Proteolytic activity was assayed using a radiolabeled hemoglobin substrate (11). Neither enzyme had measurable proteolytic activity. Sialic acid measurements were performed by the thiobarbiturate assay after hydrolysis in 0.1 N sulfuric acid at 80°C for 60 min (12). Free sialic acid was measured in the same assay, however, the acid hydrolysis step was omitted.

Enzymatic modification of the FVIII/vWf protein. The

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[1] *Abbreviations used in this paper*: FVIII, Factor VIII; vWf, von Willebrand factor.

FVIII/vWf protein in concentrations of 0.5–0.75 mg/ml was incubated with neuraminidase, final concentration 0.02–0.03 U/ml, pH 7.0 at 37°C for various periods of time. Over the time-course, aliquots were removed for the measurement of free sialic acid and procoagulant and vWf activities. All assays were performed on fresh material. In some experiments, the FVIII/vWf protein-neuraminidase mixture was chromatographed on a 1 × 8-cm column of Sepharose 4B and the FVIII/vWf protein collected and assayed for vWf activity. The activity per milligram of protein of the original material was compared to that of the protein of the column fractions. The control for the neuraminidase treatment of the FVIII/vWf protein was the FVIII/vWf protein incubated with buffer and carried through the same steps over the identical time period.

Treatment with neuraminidase and galactose oxidase. In some of these experiments, the neuraminidase concentration was increased to 0.05 U/ml final concentration. After >90% of the sialic acid was removed from the FVIII/vWf protein (at 2 h), galactose oxidase, 7.0 U/ml, and horseradish peroxidase, 20 U/ml, final concentrations, were added to the asialo FVIII/vWf protein. All incubations were performed at 37°C. The controls run during this set of experiments included an initial mixture of the FVIII/vWf protein and buffer and FVIII/vWf protein and neuraminidase. At the end of 2 h (at time of >90% sialic acid removal), each control was divided into two aliquots. To one aliquot of the FVIII/vWf protein buffer mixture was added galactose oxidase and buffer was added to the other. To one aliquot of the neuraminidase-treated FVIII/vWf protein was added the galactose oxidase and to the other, buffer. All four of these mixtures (i.e., [*a*] FVIII/vWf protein-buffer-buffer; [*b*] FVIII/vWf protein-buffer-galactose oxidase; [*c*] FVIII/vWf protein-neuraminidase-buffer; and [*d*] FVIII/vWf protein-neuraminidase-galactose oxidase) were simultaneously carried through identical experimental procedures. At specified time intervals, aliquots were removed and vWf and procoagulant activities were measured. A portion of each of the four incubation mixtures was chromatographed on Sepharose 4B. The fractions were collected and the protein and vWf activity were measured. The fractions with peak protein and vWf activity were pooled and the pH was adjusted to 7.4 with 0.5 N NaOH (added slowly with constant stirring). Then potassium borohydride was added at 100 μg per milligram of protein, and the vWf and procoagulant activities were assayed. To insure that there was no nonspecific effect of potassium borohydride, all four incubation mixtures were again chromatographed over a Sepharose 4B column, and the FVIII/vWf protein was collected and assayed again. Protein concentration was estimated by the method of Lowry et al. (13).

Assay procedures. FVIII procoagulant activity was measured as previously described (14). vWf activity was assayed utilizing the washed formalin-fixed platelet method (15). In all experiments, the modified FVIII/vWf protein was tested alone with appropriate controls at the same time intervals; in all assays, the test material was always diluted so that it fit in the middle of the standard curve.

RESULTS

The FVIII/vWf protein from the Sepharose 4B column revealed one band which did not enter a 5% polyacrylamide gel in the presence of sodium dodecyl sulfate and a single subunit of 230,000 mol wt. The mean specific vWf was 39.8 U/mg and procoagulant activity was 27.6 U/mg (pooled normal plasma equals 1 U/ml).

The sialic acid content of the FVIII/vWf protein revealed a mean value of 107.9±8.7 nM/mg (mean±1 SD, n = 12) or 25 mol per 230,000-mol wt subunit. Determination of sialic acid in nine of these preparations by determining the free sialic acid after neuraminidase treatment revealed a value of 112.6±10.3 nM/mg (mean±1 SD, n = 9) or 26 mol per 230,000-mol wt subunit. Despite removal of >95% (mean 96%, range 91–103%, n = 6) of sialic acid from the FVIII/vWf protein, no loss of vWf activity (98±4% of the control, n = 6) was observed and there was no alteration in procoagulant activity (101±5% of the control, n = 6) (Fig. 1). When the FVIII/vWf protein was separated from the neuraminidase by rapid gel chromatography, the intact protein had identical vWf activity (units per milligram) to the control material or the material before separation of neuraminidase from the FVIII/vWf protein.

After desialyation of FVIII/vWf protein, the addition of galactose oxidase resulted in the rapid progressive loss of vWf activity. After a 3-h incubation, the vWf

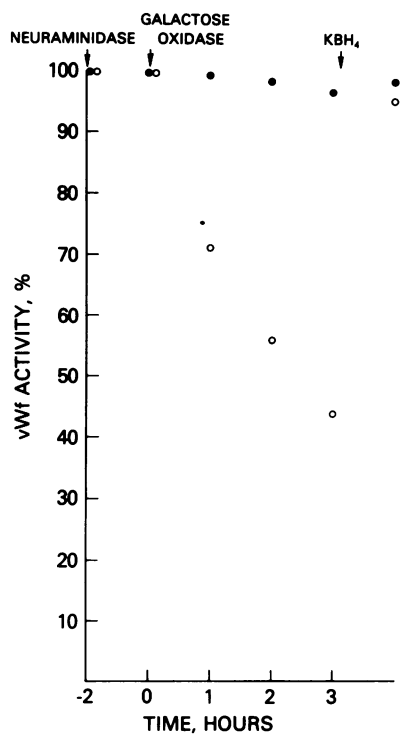

FIGURE 1 In three separate experiments, after a 3-h incubation, the FVIII/vWf protein treated with neuraminidase and galactose oxidase, and the controls (Table I and paper) were treated with potassium borohydride. This resulted in regeneration of the vWf activity of the FVIII/vWf protein in the incubation mixture which had been treated with neuraminidase and galactose oxidase, and did not affect the controls. (○), The neuraminidase-galactose incubation mixture, and (●), the mean values of the three controls used in the experiment. The arrows indicate the time of addition. The neuraminidase was added 2 h before the galactose oxidase or buffer (−2 h).

activity was decreased 50–63% (Fig. 1) and the procoagulant activity remained intact (99±4% of the control, $n = 4$). After a 20-h incubation, 90% of the vWf activity was destroyed compared to the controls (Table I). During the experiment, the controls ([*a*] FVIII/vWf protein-neuraminidase-buffer; [*b*] FVIII/vWf protein-buffer-galactose oxidase; [*c*] FVIII/vWf protein-buffer-buffer) did not vary more than ±10% vWf activity throughout the course of the experiment. When the FVIII/vWf protein was separated from the enzyme (neuraminidase and galactose oxidase), the loss of activity in units per milligram was identical to that found when the entire incubation mixture was assayed.

In four separate experiments, the FVIII/vWf protein activity had decreased to a mean of 44±5% of the control after 3 h (Table I). In three of these experiments, we attempted to regenerate vWf activity by reduction with KBH_4. In each experiment, vWf activity was regenerated in the neuraminidase galactose oxidase-treated FVIII/vWf protein to 87–104% of the control (mean 95%). Similar treatment of the control mixtures revealed no alteration in vWf or procoagulant activity. To insure no nonspecific effect of potassium borohydride, the FVIII/vWf protein was separated from the excess potassium borohydride by rapid gel chromatography. The void-volume FVIII/vWf material had activity comparable to that seen in the control mixtures.

The reduced and nonreduced protein did not reveal any alteration of migration of the major protein band or any new bands in sodium dodecyl sulfate polyacrylamide gel electrophoresis after treatment with neuraminidase or galactose oxidase.

TABLE I
Effect of Neuraminidase and Galactose Oxidase on vWf-Mediated Agglutination of Platelets

Experiments	Incubation I	Incubation II	Activity After 3 h	Activity After 20 h
1	Buffer	Buffer	102	96
2	Buffer	Galactose oxidase	95	90
3	Neuraminidase	Buffer	98	90
4	Neuraminidase	Galactose oxidase	44	10

Experiments 1, 2, 3, and 4 each contained 0.75 mg/ml of purified human FVIII/vWf protein placed in the first incubation mixture with either buffer or neuraminidase (1/100 vol, final concentration of neuraminidase 0.05 U/ml) for 2 h. Free sialic acid was determined in all four mixtures. No free sialic acid was detected in the FVIII/vWf buffer mixtures whereas 96±4% of sialic acid was removed in the neuraminidase mixtures. In the second incubation, galactose oxidase, 7 U/ml and horseradish peroxidase, 20 U/ml final concentration were added to mixtures 2 and 4 with an identical volume of buffer added to mixtures 1 and 3. The second incubation was performed for 20 h with samples removed at various times for assay of vWf activity. In all four experiments, the procoagulant FVIII activity remained constant throughout the incubation period.

DISCUSSION

Studies on the removal of sialic acid and/or the modification of penultimate galactose indicate that neither has an effect on the procoagulant activity of the FVIII/vWf protein. Similarly, vWf activity was unaffected by removal of >95% of the sialic acid; however, modification of the galactose residues of the asialo FVIII/vWf protein resulted in a time-dependent loss of vWf activity. Over the course of 3 h, 50–63% of the vWf activity was lost, and after 20 h, 90% of the vWf activity was destroyed. An advantage of using galactose oxidase to modify the galactose residues is apparent in that, under appropriate conditions, the enzymatically produced 6-aldehydo reaction product can be reconverted to galactose by borohydride.

The sialic acid content in FVIII/vWf protein reported here is different from those previously reported by us and others. Marchesi et al. (7) reported ≅1% sialic acid, Legaz et al. (16) reported 1.2%, and we reported 1.5% (5). Recently, Sodetz and co-workers (8) have reported ≅5% sialic acid content in the FVIII/vWf protein, and our data in this paper indicated approximately a 3.3% sialic acid content. The reasons for these discrepancies are not clear, but certainly different starting material, different purification procedures, and possible preselection of different subsets of FVIII/vWf molecules (i.e. subpopulations) may play a significant role in the reported variances of the sialic acid content. The results of Sodetz and co-workers (8) and ours cannot be explained on this basis, since we both used similar starting material and purification procedures. The possibility that our value is lower because we prepared a partially desialated FVIII/vWf protein would be one explanation. However, our final material had similar activities per milligrams of protein and it is clear from our studies that treatment of our FVIII/vWf protein with galactose oxidase has absolutely no effect on vWf activity unless that material is preincubated with neuraminidase to remove the sialic acid before exposure to galactose oxidase.

Several different groups have tested the effects of neuraminidase and(or) galactose oxidase treatment of FVIII/vWf protein on vWf activity (17). In general, many of these studies have not demonstrated the relative purity of their FVIII/vWf protein preparation or of their enzyme preparations; and in some studies bovine FVIII, which does not require ristocetin, was tested rather than human material. In two studies in which vWf activity was decreased by neuraminidase treatment, although >80% sialic acid was removed, only

50% of activity was lost (8, 18). In our studies, removal of >95% of the sialic acid did not affect vWf activity.

Our results indicate that the intact penultimate galactose residues are necessary for vWf activity. After oxidation by galactose oxidase, >90% of activity is destroyed; and the addition of potassium borohydride results in the concommitant reduction of the aldehydo group and regeneration of vWf activity. These data strongly suggest the loss of vWf activity to be related to specific modification of the galactose residues. Conceivably, borohydride may affect other portions of the protein other than the oxidized galactose residues; however, intact FVIII/vWf protein did not lose activity when treated with galactose oxidase nor was it affected by subsequent incubation with borohydride. These studies confirm the identification of the intact galactose moiety as a critical determinant of vWf activity.

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