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Hemophilia A due to mutations that create new N-glycosylation sites

(factor VIII/denaturing gradient gel electrophoresis/N-Glycanase)

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ABSTRACT **In studying the molecular defects responsible for cross-reacting material-positive hemophilia A, we have identified two patients in whom the nonfunctional factor VIII-like protein has abnormal, slower-moving heavy or light chains on SDS/PAGE. Both patients have severe hemophilia A (<1% of normal factor VIII activity) with a normal plasma level of factor VIII antigen. The molecular defects were identified by denaturing gradient gel electrophoresis screening of PCR-amplified products of the factor VIII-coding DNA sequence followed by nucleotide sequencing of the abnormal PCR products. In patient ARC-21, a methionine-to-threonine substitution at position 1772 in the factor VIII light chain creates a potential new N-glycosylation site at asparagine-1770. In patient ARC-22, an isoleucine-to-threonine substitution at position 566 creates a potential new N-glycosylation site at asparagine-564 in the A2 domain of the factor VIII heavy chain. The mobility of these chains on SDS/PAGE was normal after N-Glycanase digestion and procoagulant activity was generated—to a maximum of 23% and 45% of control normal plasma. Abnormal N-glycosylation, blocking factor VIII procoagulant activity, represents a newly recognized mechanism for the pathogenesis of severe hemophilia A.**

Hemophilia A, an X chromosome-linked recessive disease, is the commonest hereditary coagulation disorder, with a frequency of 1 in 10,000 males. It is caused by a deficiency of factor VIII procoagulant activity (VIII:C). Factor VIII participates in blood coagulation as an essential cofactor in the cleavage of factor X by factor IXa in the presence of Ca^{2+} and phospholipid (1, 2). Factor VIII is produced as a single-chain protein of 2351 amino acids, including a 19-amino acid signal peptide. It is then modified by proteolytic cleavages to generate amino-terminal heavy-chain polypeptides ranging in size from 92 to 200 kDa and an 80-kDa carboxyl-terminal light chain (3–5). Procoagulant activity requires further thrombin cleavage at arginine-372 to yield 54-kDa and 44-kDa heavy-chain fragments and at arginine-1689 to yield a 72-kDa light-chain fragment (6).

A large number of factor VIII gene mutations have been described (see refs. 7 and 8 for reviews). Gross gene rearrangements, such as deletions, insertions, and duplications, account for only 5% of the molecular defects in hemophilia A patients; point mutations are thought to account for the remaining defects. While identification of point mutations in the factor VIII gene is made difficult because of its large size, its many exons, and the high frequency of *de novo* mutations that result in different molecular defects in unrelated affected individuals, the patients whose bleeding disorder is due to a dysfunctional protein are a subgroup that is more accessible to investigation. Nonfunctional factor VIII-like protein is present in 5% of hemophilic plasmas and can be detected by immunoradiometric assays (9). These plasmas are termed cross-reacting material (CRM)-positive (10). The study of CRM-positive plasmas is of special interest, as it has the potential to expand our knowledge of factor VIII structure/function through an understanding of how mutations block procoagulant activity. We have previously characterized CRM-positive hemophilia A point mutations that abolish critical factor VIII heavy- and light-chain thrombin cleavage sites (11, 12). We report here a newly recognized mechanism for the pathogenesis of hemophilia A, abnormal N-glycosylation that blocks VIII:C.

MATERIALS AND METHODS

Patient Samples. We have evaluated a total of 24 plasmas from unrelated CRM-positive hemophilia A patients. Criteria used to establish this diagnosis included a factor VIII antigen (VIII:Ag) concentration of >0.40 unit/ml, a ratio of VIII:C to VIII:Ag of <0.5, and a normal or elevated concentration of von Willebrand factor antigen (vWF:Ag) (>0.5 unit/ml). Approval was obtained from the Institutional Review Boards for these studies. Patients were informed that blood samples were obtained for research purposes and that their privacy would be protected.

Patient ARC-21 (also designated as JH-116 by the Center for Medical Genetics, Johns Hopkins University School of Medicine) has severe hemophilia A with frequent joint hemorrhages. There is no family history of a bleeding disorder. VIII:C was <0.01 unit/ml on repeated assays, VIII:Ag 0.72 unit/ml, and vWF:Ag 1.07 units/ml. The clinical history for patient ARC-22 (JH-117) is similar in that he too has severe hemophilia A with frequent joint hemorrhages. His VIII:C level was <0.01 unit/ml on repeated assays, VIII:Ag 1.54 units/ml, and vWF:Ag 0.76 unit/ml. His maternal grandfather also had hemophilia.

Factor VIII Measurements. VIII:C was measured by a one-stage method using factor VIII-deficient plasma as substrate (13). VIII:Ag was measured by immunoradiometric assay using ^{125}I-labeled Fab′, prepared from the plasma of a patient with an alloantibody to factor VIII (9). vWF:Ag was measured by immunoradiometric assay using a rabbit antibody (14). Citrate-anticoagulated normal plasma, pooled from 10 donors, served as a standard (1 unit/ml) for VIII:C, VIII:Ag, and vWF:Ag measurements and was used as the control for immunopurification studies.

Immunopurification of Factor VIII. The procedure for immunopurifying factor VIII from small volumes of plasma has been published (11). Patient plasmas or pooled normal plasma were incubated with an immunoadsorbent prepared by coupling to agarose the IgG from a high-titer factor VIII inhibitor plasma. The adsorbed protein was analyzed directly

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Abbreviations: CRM, cross-reacting material; DGGE, denaturing gradient gel electrophoresis; VIII:Ag, factor VIII antigen; VIII:C, factor VIII procoagulant activity; vWF:Ag, van Willebrand factor antigen.

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or after incubation with highly purified human thrombin (2 units of thrombin per unit of VIII:Ag). The protein was eluted with SDS, analyzed by SDS/PAGE, and identified by immunoblotting using three monoclonal anti-factor VIII antibodies (11). Molecular weight markers were from Bio-Rad.

Southern Blot Analysis. Analysis was performed as described (15), using *Taq* I as restriction endonuclease and three subfragments of factor VIII cDNA as hybridization probes (15, 16).

Amplification of Genomic DNA. High molecular weight leukocyte DNA was amplified by using *Taq* DNA polymerase (Cetus) (17). PCR amplification (35 cycles) was performed in volumes of 40 μl for denaturing gradient gelelectrophoresis (DGGE) analysis and 100 μl for sequence analysis: the 100-μl reaction mixture contained 200–400 ng of genomic DNA, 400 nM each PCR primer, 200 μM each dNTP, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.02% gelatin, and 2 units of *Taq* DNA polymerase. Each cycle consisted of 94°C for 20 sec, a thermal transition from 94°C to the annealing temperature over 2 min, 52–60°C for 45 seconds, and 72°C for 30–60 sec, followed by extension at 72°C for 10 min. The sequences of the primers used for these amplifications have been published (18).

DGGE Analysis. PCR products (≈80 ng of each) from two patients were combined to form heteroduplexes. After heat denaturation at 95°C for 5 min, the DNA solution was slowly cooled to room temperature (>30 min) and subjected to DGGE under conditions determined empirically for each PCR product (18, 19). DNA was loaded onto a 6.5% polyacrylamide gel (14 cm × 19 cm, 0.75 mm thick) containing a linear gradient of denaturants and was electrophoresed at 2–4 V/cm for 16–23 hr. The gradient difference in denaturants used was 20% [100% denaturants = 7 M urea/40% (vol/vol) formamide]. Gels were then stained in ethidium bromide and photographed with a UV transilluminator. Purified PCR products that showed abnormal migration patterns on DGGE were directly sequenced (17, 18, 20).

Digestion of Factor VIII with N-Glycanase. Two microliters of a 250-unit/ml stock solution of N-Glycanase was added to a 5-μl sample containing 2 units of VIII:Ag for 1 hr at 37°C in the presence of 6 μl of 10 nM phenylmethylsulfonyl fluoride in 2-propanol and 3.5 μl of 10% (vol/vol) Nonidet P-40 (21). The digested factor VIII was then analyzed by SDS/PAGE and immunoblotting as noted above. N-Glycanase is Genzyme's brand name for peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase, EC 3.5.1.52. N-Glycanase hydrolyzes all common asparagine-linked oligosaccharides (high-mannose, hybrid, and complex type) from glycopeptides and glycoproteins to give free oligosaccharides and free peptide or protein. The asparagine residue to which the carbohydrate was linked is converted to aspartic acid. This amidase activity is distinct from the activities of endoglycosidases H and F. Oligosaccharides attached to carboxyl- or amino-terminal asparagines are not substrates for N-Glycanase enzyme.

To determine the effect of digestion on VIII:C, aliquots (100 μl) of plasma were incubated with 1 unit (4 μl) N-Glycanase at 37°C. The residual VIII:C activity was measured by a one-stage clotting assay (13) using as assay standard normal pooled plasma incubated with the same concentration of N-Glycanase for the same period of time.

RESULTS

These CRM-positive plasmas were evaluated by use of an immunopurification method that requires only small volumes of plasma (1–2 ml) in order to detect mutant factor VIII that has an abnormal molecular mass or a missing thrombin

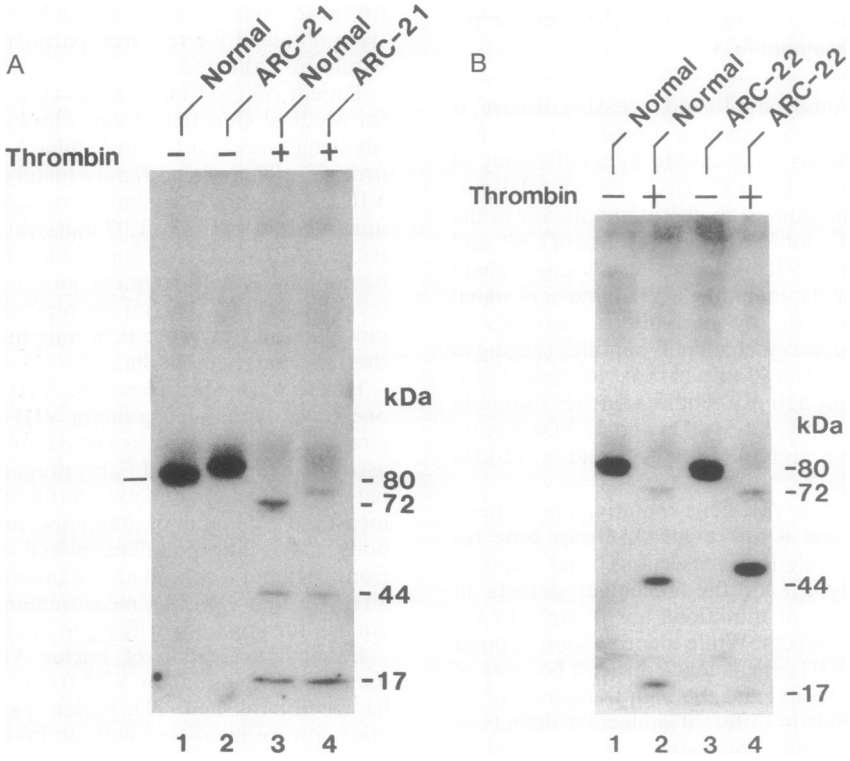

FIG. 1. Immunoblot showing the thrombin cleavage pattern of factor VIII protein. (*A*) Comparison of factor VIII from ARC-21 and normal plasma. Plasmas containing 2 units of VIII:Ag were incubated with immunoadsorbent overnight before washing and treatment with thrombin (4 units) or buffer (Tris-buffered saline) for 30 min at room temperature. The factor VIII fragments were then eluted with 2% SDS and the protein was analyzed by SDS/PAGE and immunoblotting using a mixture of three monoclonal anti-factor VIII antibodies. The factor VIII heavy chain (92–200 kDa) is not detected by this method, but the 44-kDa (A2 domain) heavy-chain fragment is identified after thrombin cleavage. The 17-kDa fragment is a subsequent heavy-chain cleavage product when normal factor VIII is incubated with high concentrations of thrombin (11). (*B*) Similar comparison of factor VIII from ARC-22 and normal plasma. The 17-kDa fragment was not detected for any of the ARC-22 samples.

cleavage site. Of the first 24 CRM-positive samples studied, 2 had unique changes in the molecular mass of a factor VIII chain. The light chain from patient ARC-21 had reduced migration on SDS/PAGE, consistent with a molecular mass of 83 kDa instead of 80 kDa (Fig. 1*A*, lanes 1 and 2). The abnormal migration persisted after thrombin cleavage (lanes 3 and 4), indicating that the abnormality was within the 72-kDa light-chain fragment. ARC-22 plasma had a normal factor VIII light chain, but the A2 domain heavy-chain fragment migrated more slowly than normal (≈47 kDa instead of 44 kDa) and the 17-kDa thrombin-generated fragment (11) was missing (Fig. 1*B*, lanes 2 and 4). The abnormal migration persisted in both cases when the factor VIII was analyzed by SDS/PAGE after reduction.

To characterize the molecular defects in these two patients, leukocyte-derived genomic DNA was first analyzed by Southern blotting using the enzyme *Taq* I. No abnormal restriction endonuclease fragments were detected. As the immunoblot analysis only localized the factor VIII abnormalities to the light chain (ARC-21) or the A2 domain (ARC-22), a systematic characterization of the factor VIII gene was initiated to identify the mutations. The coding regions and almost all of the splice junctions were then amplified by PCR using *Taq* DNA polymerase and 47 oligonucleotide pairs (18), and the PCR-amplified products were analyzed by DGGE. Fragments that had abnormal patterns were then sequenced. A point mutation (ATG → ACG) causing a threonine substitution for methionine-1772 in the light chain was identified in exon 15 for patient ARC-21. This mutation creates a new potential N-linked glycosylation site (Asn-Ile-Thr) at asparagine-1770. In ARC-22, a mutation (ATA → ACA) that causes a threonine substitution for isoleucine-566 was detected in exon 12. This generates a new potential N-linked glycosylation site (Asn-Gln-Thr) at asparagine-564 in the factor VIII heavy chain. No other mutations were found after extensive DGGE analysis of the entire factor VIII coding region, 41 of 50 splice junctions, the promoter region including the TATA box and 150 nucleotides 5′ to the cap site, and 345 nucleotides from the 3′ untranslated and flanking region including the polyadenylylation site. This extensive analysis was performed to establish that no additional mutations were present in the DNA of these patients.

To establish that the abnormal molecular mass of the two mutant factor VIII proteins was solely due to additional carbohydrate, immunopurified factor VIII was digested with N-Glycanase [an enzyme that hydrolyzes all common asparagine-linked oligosaccharides from glycoproteins (21)] and the mixtures were analyzed by SDS/PAGE and immunoblotting. The normal 80-kDa factor VIII light chain migrated more rapidly after digestion with N-Glycanase, indicating that it had an attached oligosaccharide group (Fig. 2*A*, lane 4, and Fig. 2*B*, lane 6). The N-Glycanase-treated ARC-21 light chain (Fig. 2*A*, lane 3) had the same mobility as the treated normal light chain. N-Glycanase treatment did not affect the mobility of the normal 44-kDa (A2 domain) fragment of the factor VIII heavy chain (Fig. 2*B*, lanes 6 and 7), indicating the one potential N-linked glycosylation site in the A2 domain (22) was not glycosylated. The ARC-22 factor VIII heavy-chain fragment had normal mobility after N-Glycanase treatment (Fig. 2*B*, lane 5), demonstrating a normal molecular mass in the absence of carbohydrate.

To determine whether the additional carbohydrate causes the functional defects in these two molecules, we assessed the effect of incubation with N-Glycanase on VIII:C. The enzyme had no consistent effect on VIII:C of normal plasma under the conditions of the experiments; i.e., for as long as 6 hr there was less than a 10% change from that of control samples held at 37°C for the same period of time in the absence of enzyme. Incubation of ARC-21 or ARC-22 plasmas with N-Glycanase led to consistent increases in procoagulant activity, to a maximum at 6 hr of 26% and 45% of normal, respectively (Fig. 3). The generation of VIII:C after digestion with N-Glycanase indicates that the novel post-

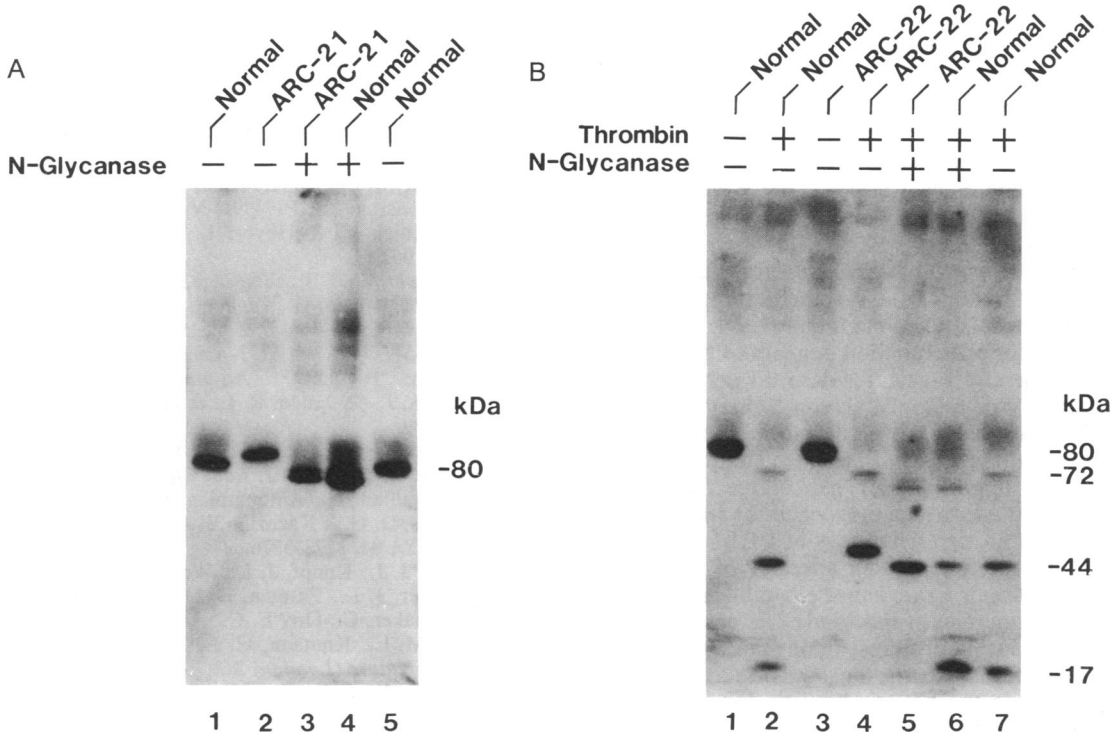

FIG. 2. Effect of N-Glycanase treatment on factor VIII chains. (*A*) Factor VIII from ARC-21 and normal plasma. Only anti-factor VIII light-chain antibodies were used in this study. The normal factor VIII light chain has faster mobility after digestion, and the ARC-21 light-chain mobility is normalized after enzyme treatment. (*B*) Factor VIII from ARC-22 and normal plasma. The light-chain patterns are the same. The mobility of the ARC-22 A2 (44-kDa) fragment is normalized after incubation with N-Glycanase.

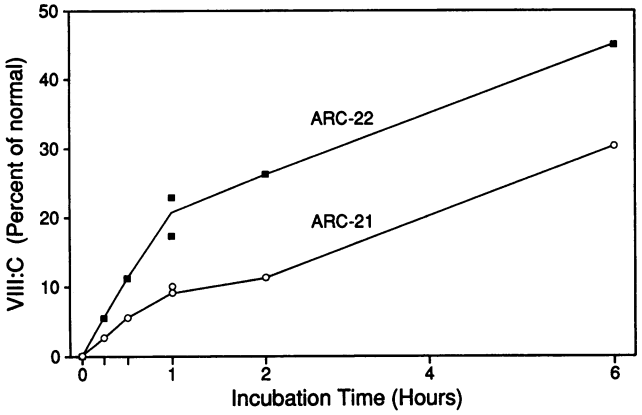

FIG. 3. Effect of N-Glycanase on VIII:C. Aliquots (100 μl) of CRM-positive plasma were incubated with one unit N-Glycanase for periods up to 6 hr. VIII:C of the sample (determined by a one-stage clotting assay) was then compared to that of normal pooled plasma that had been incubated with the enzyme for the same period of time.

translational glycosylation leads to loss of factor VIII function in both of these CRM-positive plasmas.

DISCUSSION

The usefulness of immunopurification and immunoblot characterization of the nonfunctional factor VIII-like protein in CRM-positive hemophilia A is demonstrated by the identification in 2 patients of abnormal, slower-moving factor VIII heavy and light chains. While infrequent, detected in only 2 of 24 samples tested, this type of defect provides new information about the molecular characteristics required for factor VIII procoagulant activity. It supplements data previously reported for hemophilia A mutations that prevent factor VIII activation by thrombin (11, 12, 23–25) and the binding of factor VIII to von Willebrand factor (26). However, the nature of the factor VIII dysfunction for the 2 patients reported here could not be fully established by immunoblot studies. Characterization of the molecular defect was necessary to identify the pathogenesis. While screening methods using Southern blot analysis did not identify an abnormality, the combination of PCR and DGGE was successful in identifying the mutations. This success is consistent with observations for a large number of patients with mild to moderate hemophilia A (18).

The amino acid sequence of factor VIII predicted from DNA sequencing of the factor VIII gene identifies 25 potential N-glycosylation sites, 19 of which are in the connecting (B) domain (16, 27). Although the carbohydrate structure of factor VIII has not been studied in greater detail, the role of carbohydrate in factor VIII function appears to be dispensable. Neuraminidase and β-galactosidase do not affect the procoagulant activity of factor VIII/von Willebrand factor preparations (28), and we found that the cleavage of N-linked chains with N-Glycanase did not inactivate factor VIII. Fay *et al.* (29) removed approximately half of the factor VIII carbohydrate with a mixture of exoglycosidases and endoglycosidases and found no significant loss of VIII:C.

The loss of protein function as a result of abnormal, additional glycosylation is a rare cause of a clinical disorder. To date, two other examples of this kind of defect have been detected in mutant plasma proteins. In one case, the substitution of asparagine for isoleucine-7 establishes a new N-glycosylation sequence in the heparin-binding domain of antithrombin III, and the modified molecule has a decreased affinity for heparin (antithrombin Rouen-III) (30). The other instance, fibrinogen Asahi, has impaired polymerization and delayed cross-linking of the mutant γ chain, associated with a substitution of threonine for methionine-310 and consequent N-glycosylation at asparagine-308 (31). In contrast to our studies (Fig. 3), the functional impact of the additional carbohydrate has not been definitely established by determining the effect of carbohydrate removal on the activity of either antithrombin Rouen-III or fibrinogen Asahi. While neither mutant factor VIII had entirely normal specific activity after the 6-hr incubation with N-Glycanase, they did reach 36% and 21%, respectively, of the VIII:C/VIII:Ag ratio of pooled normal plasma (9). As longer (24-hr) incubation with the enzyme at 37°C reduced both mutant and normal VIII:C levels, it was not possible to determine whether the partial generation of VIII:C was due to incomplete digestion in these experiments (Fig. 3), greater than normal susceptibility to loss of VIII:C during plasma incubation at 37°C, or intrinsic defects in cofactor function that were due to the point mutations *per se*.

The analysis of factor VIII protein dysfunction due to abnormal posttranslational modifications has identified an important mechanism responsible for the pathogenesis of severe hemophilia A. Moreover, the carbohydrate-mediated dysfunction in the two mutant factor VIII-like proteins identifies factor VIII regions that are required for procoagulant function.

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