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Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor

(von Willebrand disease/proteolytic degradation/bleeding disorder)

JUDITH A. DENT*, SCOTT D. BERKOWITZ*, JERRY WARE*, CAROL K. KASPER†, AND ZAVERIO M. RUGGERI*‡

*Roon Research Center for Arteriosclerosis and Thrombosis, Division of Experimental Hemostasis and Thrombosis, Department of Molecular and Experimental Medicine and Committee on Vascular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037; and †The Orthopaedic Hospital, Los Angeles, CA 90007

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ABSTRACT **Proteolytic cleavage of the von Willebrand factor subunit may be important for processing and/or function of the molecule and is altered in certain subtypes of von Willebrand disease. It results in the generation of two main fragments with apparent molecular masses of 140 kDa and 176 kDa from the 225-kDa subunit. We have now obtained chemical evidence to locate the protease-sensitive bond between residues Tyr-842 and Met-843, a site that appears to reflect the specificity of calcium-dependent neutral proteases (calpains). Antibodies were raised against four synthetic peptides that represented sequences immediately preceding or following or including the cleavage site. One antibody (against the fragment from Ala-837 through Asp-851) reacted only with the intact subunit, and its epitope included the cleavage site. All others reacted specifically with either the 140-kDa or the 176-kDa fragment, demonstrating their origin from a single cleavage. In samples of purified von Willebrand factor from four of five patients with type IIA von Willebrand disease, the anti-peptide antibodies showed markedly decreased reactivity with either the 140-kDa or the 176-kDa fragment, suggesting the existence of distinct molecular abnormalities clustered around the cleavage site. Thus, in the majority of type IIA patients, a common pathogenetic mechanism may lead to the disappearance of the larger multimers as a consequence of structural changes that may expose a sensitive bond to the action of specific proteases. These studies demonstrate the use of anti-peptide antibodies directed at a relevant structural domain for the immunochemical differentiation of normal and mutant molecules.**

von Willebrand factor (vWF) is an adhesive glycoprotein that mediates adhesion and aggregation of platelets at sites of vascular injury (1), a process essential in hemostasis and thrombogenesis. It is synthesized by endothelial cells (2, 3) and megakaryocytes (4) and, in its mature structure, is represented by an array of multimeric forms with molecular masses estimated to range from 500 kDa to >10,000 kDa (1). Genetic abnormalities of this molecule lead to the most common bleeding disorder in humans, von Willebrand disease (1). Both quantitative and qualitative defects of vWF have been identified in von Willebrand disease (1, 5). Type IIA is probably the most common of the qualitative defects and is characterized by selective loss of large and intermediate-sized multimers (6) with heterogeneous phenotypic manifestations (7). In these patients there is increased concentration of two fragments of the vWF subunit with apparent molecular masses of 140 kDa and 176 kDa. They are present only in trace amounts in normal individuals (8), and it has been proposed that their increase may be caused by heightened susceptibility of vWF to the action of a proteolytic enzyme or enzymes, yet to be identified with certainty (9).

Studies on the molecular genetics of type IIA von Willebrand disease have revealed the presence of point mutations resulting in amino acid substitutions within a discrete region of the molecule (10, 11) that corresponds to the previously identified tryptic fragment of 55 kDa, extending between residues 730 and 1114 (12, 13). The presence of distinct abnormalities in the few kindreds examined to date leaves open the question of what common pathogenetic mechanism may be responsible for the structural and functional abnormality of vWF in these patients. To address this issue, we have used microsequencing techniques to define the location of the cleavage site in the normal vWF subunit responsible for the appearance of the two major proteolytic fragments of the molecule. We have then generated peptide-specific antibodies directed at epitopes adjacent to or including this cleavage site and have demonstrated by immunoblotting studies that vWF molecules in four of five type IIA patients had a markedly decreased reactivity with distinct antibodies. These results provide evidence for the presence of structural alterations in a region of the vWF subunit that may result in increased susceptibility to protease action. The identified site of cleavage defines an important domain of vWF that may be involved (*i*) in the normal processing of the molecule as it is secreted from cellular sites into the circulation and (*ii*) in the regulation of its multimeric size.

MATERIALS AND METHODS

Patient Characterization. Five patients representing four different kindreds, P_1 and S_1 (mother and son), P_2, P_3 and P_4, were studied. They all fulfilled the accepted criteria for the diagnosis of type IIA von Willebrand disease (1, 5). Their bleeding time measurements and plasma levels of factor VIII procoagulant activity, vWF antigen, and ristocetin cofactor activity (in % of normal) were as follows: P_1, <20 min, 35%, 46%, 22%; S_1, <30 min, 30%, 50%, 22%; P_2, <40 min, 27%, 3.4%, 0%; P_3, <30 min, 65%, 33%, 6%; and P_4, <15 min, 43%, 45%, 10%. Multimeric analysis of nonreduced plasma vWF was performed by agarose gel electrophoresis (1.5% HGT-P agarose; FMC) in the presence of sodium dodecyl sulfate (SDS) as described (14).

Affinity Purification of vWF. Collection of blood in the presence of protease inhibitors (EDTA, leupeptin, and *N*-ethylmaleimide) and affinity purification of plasma vWF was carried out essentially as reported (8). In brief, a monoclonal anti-vWF antibody (2.2.9), prepared and characterized as described (15, 16), was coupled to cyanogen bromide-

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Abbreviation: vWF, von Willebrand factor.

‡To whom reprint requests should be addressed.

activated Sepharose CL-4B (Pharmacia) at a ratio of 25 mg of antibody per ml of beads. One ml of beads had the ability to bind ≈15 μg of vWF.

Microsequencing. The vWF to be used for this purpose was obtained from 200 ml of Sepharose–antibody beads incubated with 300 ml of plasma, followed by elution with 2% SDS/1 mM dithiothreitol (8). After concentration (Centricon 30 microconcentrators; Amicon) and dialysis against elution buffer (8) containing 0.05% SDS and 0.5 mM dithiothreitol, the reduced vWF (≈40 μl at about 6 μg/μl) was electrophoresed in a 5% polyacrylamide gel containing SDS according to a modification of the original method of Laemmli (8, 17). Electrophoretic transfer was carried out essentially as described by Matsudaira (18) by using polyvinylidene difluoride (PVDF) membranes with a pore size of 0.45 μm (Immobilon-P transfer membrane; Millipore). After transfer, the membrane was stained with Coomassie Blue R-250 in 50% methanol, destained with 50% methanol/10% acetic acid, and rinsed in deionized H_2O for 10 min. The relevant bands were excised from the air-dried membrane and transferred to the cartridge block of the sequenator (Applied Biosystems model 470), which was equipped with on-line HPLC for analysis of the phenylthiohydantoin derivatives. Separation was obtained with a Brownlee C_{18} column (220 mm × 2.1 mm; Alltech Associates).

Peptide Synthesis. Peptides were synthesized by the method of simultaneous multiple-peptide synthesis (19). They were analyzed and purified by reversed-phase HPLC as described (20).

Antibody Production and Characterization. Polyclonal anti-peptide antibodies were generated as described (21) by coupling synthetic peptides to keyhole limpet hemocyanin with glutaraldehyde and injecting them into New Zealand White rabbits. Murine monoclonal antibodies against human vWF were produced as reported (15, 16), and their epitope specificity was determined as described (22).

SDS/Polyacrylamide Gel Electrophoresis and Immunoblotting. SDS/polyacrylamide gels were prepared as described by Laemmli (17). Affinity-purified vWF from normal individuals and patients with type IIA von Willebrand disease was reduced with 65 mM dithiothreitol and electrophoresed in SDS/5% polyacrylamide gels (8). Transfer to nitrocellulose membranes and labeling with polyclonal or monoclonal antibodies by using a double-antibody technique has been described in detail (8, 23). The second antibody, rabbit anti-mouse IgG or goat anti-rabbit IgG, was labeled with ^{125}I. Bands were visualized by autoradiography; intensity of the bands was monitored by soft-laser-scanning densitometry (Biomed Instruments, Fullerton, CA).

Genetic Characterization of the Patients. Genomic DNA of P_1, S_1, P_2, and P_3 was isolated from whole blood as described (24). A cloned fragment, pSVIIa, containing an amino acid substitution at codon 1613 (residue 850 of the mature subunit), was provided by Dennis Lynch (Dana–Farber Cancer Institute, Boston). This mutation corresponds to the one described in P_4 (11). Based on the published cDNA sequence of vWF (25), oligonucleotides for the polymerase chain reaction (26) were synthesized as follows: vWF802, 5′-*ATCTC*GAGATCCGCTACCAGGGCGCA-3′; vWF905-2,5′-*ACTGAAAGCTT*AGCCTCTCGGGGGAGCGTCTCAAA-3′; vWF822, 5′-TCTCTGACCACAGCTTCTTGG-3′; and vWF855, 5′-CCTGGATGTCTCCAGGCAGGCTCTT-3′. The 5′ ends of these oligonucleotides correspond to codons 1567, 1661, 1585, and 1625, respectively, of prepro-vWF cDNA (25). Additional nucleotides were added to vWF802 and vWF905-2 (indicated by italics) to incorporate restriction sites at the 5′ ends; a base change (A → G) (indicated by boldface G) was introduced in vWF855. PCR amplification of the genomic DNA or cloned cDNA was performed by using a DNA thermal cycler and *Thermus aquaticus Taq* polymerase (both from Perkin–Elmer/Cetus) in a 100-μl reaction volume containing ≈2 μg of genomic DNA, 1 μg of each primer, and buffer consisting of 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, and 200 mM each dNTP. Reaction conditions were 30 cycles of 30 sec at 94°C, 30 sec at 52°C, and 30 sec at 72°C. The fragments obtained with oligonucleotides vWF802 and vWF905-2 were gel-fractionated on 2% agarose, and the DNA was purified by using Gene Clean II (Bio 101, La Jolla, CA). The fragments obtained with oligonucleotides vWF822 and vWF855 were phenol-extracted and ethanol-precipitated prior to restriction enzyme digestion. *Bst*EII and *Mnl* I digestions were performed according to the manufacturer's suggested conditions (Stratagene).

RESULTS

Methionine was identified as the amino-terminal residue of the 176-kDa proteolytic fragment of normal plasma vWF, corresponding to position 843 of the mature vWF subunit; this methionine and the following 11 residues represented a perfect match with residues 843–854 of the mature vWF subunit (Table 1). In contrast, amino-terminal sequence analysis of the 140-kDa fragment gave a lower yield (<2 pmol) and multiple peaks. Nevertheless, the main sequence identified started at residue 1 of the mature vWF subunit, followed by four additional residues that were identical to those in positions 2–5 in mature vWF.

On the basis of the identified site of cleavage occurring in the vWF subunit, four 15-residue peptides were synthesized to represent sequences immediately preceding, following, or including this site (Fig. 1). Thus, one peptide corresponded to the carboxyl terminus of the 140-kDa fragment; one to the amino terminus of the 176-kDa fragment; and two overlapped both fragments (Fig. 1). Antibodies were raised against these peptides, and all of them reacted with the intact vWF subunit in immunoblotting (see below). These antibodies were used to test the reactivity of the vWF subunit and its naturally occurring proteolytic fragments in normal controls and in five patients with typical autosomal dominant type IIA von Willebrand disease belonging to four distinct families. All patients showed variable decrease of the intermediate and/or larger multimers of vWF when analyzed by SDS/agarose gel electrophoresis, with a broad heterogeneity typical of type IIA von Willebrand disease (Fig. 2).

The antibody raised against the peptide from Ala-837 through Asp-851 reacted only with the intact subunit of vWF but not with any of the proteolytic fragments, thus demonstrating that the corresponding epitope is destroyed by the cleavage occurring between residues 842 and 843 (Fig. 3). This antibody reacted in an identical manner with all normal and type IIA vWF samples tested. Scanning laser densitometry failed to reveal proteolytic fragments in any of the patients.

Table 1. Amino-terminal sequence analysis of the 176-kDa fragment

	Amino acid residues											
	M	V	T	G	N	P	A	S	D	E	I	K
Relative quantity, pmol	4.0	4.3	3.8	5.8	3.2	7.5	6.0	4.4	6.4	4.4	3.6	5.3

Amino acid residues are indicated with the standard one-letter abbreviation (27).

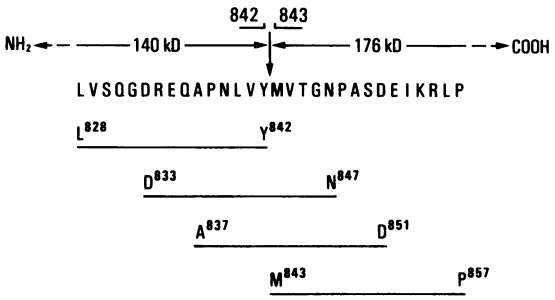

FIG. 1. Amino acid sequences recognized by the anti-peptide antibodies used. The sequence of a segment of the mature vWF subunit (residues 828–857), which includes the cleavage site between residues Tyr-842 and Met-843 (vertical arrow) giving origin to the 140-kDa and 176-kDa fragments, is shown. Synthetic peptides utilized to generate the polyclonal antibodies used in these studies represent sequences adjacent to and overlapping the cleavage site. Each peptide was composed of 15 amino acid residues, as follows: Leu-828 through Tyr-842, Asp-833 through Asn-847, Ala-837 through Asp-851, and Met-843 through Pro-857. The position of each peptide in the sequence of the vWF subunit is schematically represented by a continuous line below the sequence. Amino acid residues are identified by the standard one-letter abbreviation (27).

The two antibodies raised against the peptides from Leu-828 through Tyr-842 and Asp-833 through Asn-847 displayed the same specificity, and only the results obtained with the former are shown. It reacted poorly with the intact subunit of vWF and failed to react with the 176-kDa fragment but showed good reactivity with the 140-kDa fragment (Fig. 4). This suggests, in agreement with the location of the identified cleavage site, that the corresponding epitope (or epitopes) is located at the carboxyl terminus of the 140-kDa fragment, immediately preceding the cleaved bond, and is more accessible to the immunologic probe in the fragment than in the intact subunit. In samples from four of the five IIA patients tested (P_1, S_1, P_3, and P_4), the 140-kDa fragment displayed a strong reactivity with the antibody, and the intensity of the corresponding band was greater than in normal samples. This is justified by the increased concentration of this fragment in type IIA von Willebrand disease, as demonstrated by reaction with the pool of monoclonal antibodies against many distinct epitopes of vWF (Fig. 4). Only in samples of patient P_2 was the reactivity of the 140-kDa fragment relatively decreased as compared with normal samples, assuming that the intensity of the band seen after reaction with the pool of monoclonal antibodies is representative of the amount of protein present. Thus, by laser densitometry, the 140-kDa band in samples of patient P_2 was 1.64 times more intense than the corresponding normal band when probed with the monoclonal pool, whereas it was only 0.75 times the intensity of the normal band when probed with the antibody against the peptide from Leu-828 through Tyr-842 (Fig. 4).

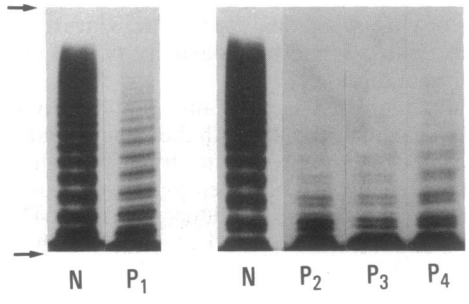

FIG. 2. Multimeric analysis of normal vWF (N) and vWF from patients (P) with type IIA von Willebrand disease. Electrophoresis of denatured unreduced samples was performed in a 1.5% HGT-P agarose gel containing 0.1% SDS. Multimers were visualized with ^{125}I-labeled affinity-purified rabbit anti-human vWF antibody. (*Left*) Samples from a normal control (N) and patient P_1 run in the same gel. (*Right*) Samples from a normal control (N) and patients P_2, P_3, and P_4 run in another similar gel. The upper arrow indicates the stacking gel/running gel interface; the lower arrow indicates the dye front.

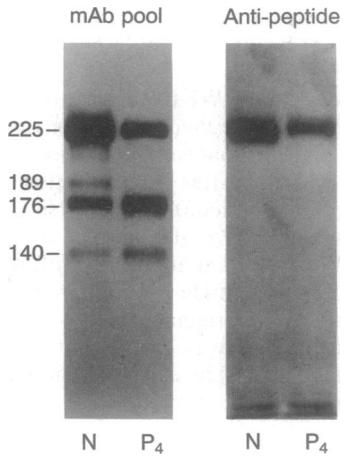

FIG. 3. Immunoblot analysis of reduced vWF using the antiserum against the peptide from Ala-837 through Asp-851. Reduced immunopurified vWF was analyzed in a 5% polyacrylamide gel containing 0.1% SDS. Protein was transferred to nitrocellulose by Western blotting and treated with the anti-peptide antibody followed by ^{125}I-labeled goat anti-rabbit IgG (*Right*). After exposure to obtain a suitable autoradiograph, the same blot was then treated with the pool of murine monoclonal antibodies (mAb) and ^{125}I-labeled rabbit anti-mouse IgG (*Left*). The apparent molecular mass of each major band is indicated at the left in kDa. One normal (N) and one patient (P_4) sample are shown.

The antibody against the peptide from Met-843 through Pro-857 reacted with the intact subunit and the 176-kDa fragment of normal vWF but not with the 140-kDa fragment (Fig. 5). Thus, in agreement with the sequence data, the corresponding epitope is in the amino-terminal region of the 176-kDa fragment. The band corresponding to the 176-kDa fragment was more intensely visualized by reactivity with this antibody in samples from two of the five type IIA patients tested (P_2 and P_3) than in normal samples, in agreement with the increased concentration of the proteolytic fragment shown by reactivity with the pool of monoclonal antibodies. In contrast, the 176-kDa band was poorly reactive with this anti-peptide antiserum in samples from the remaining three patients tested (P_1 and S_1, mother and son, respectively, and P_4), even though reactivity with the monoclonal antibody pool demonstrated that the vWF fragment was present in

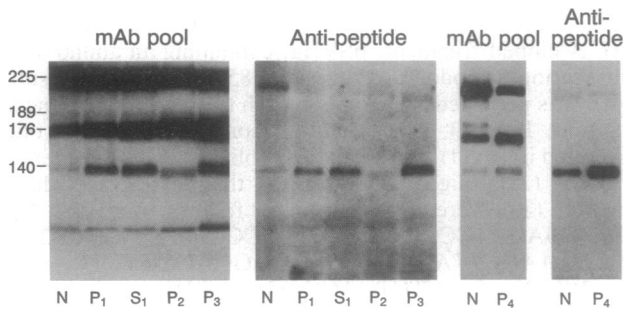

FIG. 4. Immunoblot analysis of reduced vWF using the antiserum against the peptide from Leu-828 through Tyr-842. The methodology used is identical to that described in the legend to Fig. 3 except that a different anti-peptide antiserum was used. The two panels labeled mAb pool represent the corresponding nitrocellulose filter subsequently treated with the monoclonal antibody pool. Apparent molecular masses are indicated at the left in kDa. Lanes containing normal (N) and patient (P) samples are identified at the bottom.

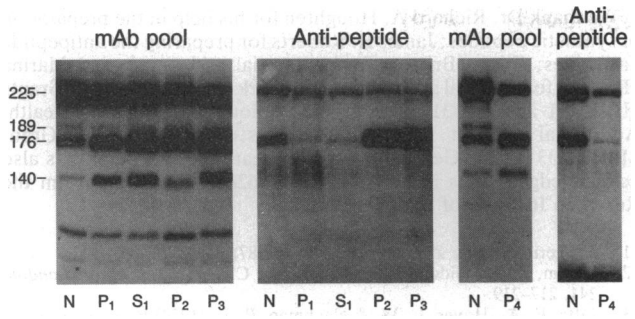

FIG. 5. Immunoblot analysis of reduced vWF using the antiserum against the peptide from Met-843 through Pro-857. The methodology used is identical to that described in the legends to Figs. 3 and 4 except that a different anti-peptide antiserum was used. The two panels labeled mAb pool represent the corresponding nitrocellulose filter subsequently treated with the monoclonal antibody pool. Apparent molecular masses are indicated at the left in kDa. Lanes containing normal (N) and patient (P) samples are identified at the bottom.

concentrations greater than normal also in these patients (Fig. 4).

To test the hypothesis that some of the patients described here might have genetic mutations similar to those reported previously (10, 11), restriction fragment analysis of amplified genomic DNA or cDNA was performed. *Mnl* I digestion of the amplified fragment containing the mutation at base 1613 described in vWF from P_4 (11) yielded the expected uncleaved band of 120 bp, while bands of 83 bp and 37 bp (not shown) were generated by digestion of the amplified fragments from P_1, who had the same immunochemical reactivity as P_4, and P_3, whose vWF had immunochemical reactivity with the anti-peptide antibodies different from that of P_1 and P_4. *Bst*EII digestion of the amplified fragments from P_1, P_2, and P_3 gave bands of 175 bp, 95 bp, and 31 bp (not shown), different from those expected if one of the other two mutations already described (10) were present in vWF. Thus, all of the three new kindreds described here (P_1 and her son S_1, P_2, and P_3) must have mutations different from one another and from those already reported.

DISCUSSION

The present studies provide definitive chemical evidence that cleavage of the peptide bond between Tyr-842 and Met-843 results in proteolytic fragmentation of human vWF *in vivo*. The binding specificities of anti-peptide antibodies directed at epitopes surrounding this bond support the concept that the 140-kDa and 176-kDa fragments of the vWF subunit originate from a single cleavage. The antibody generated against the peptide comprising residues 837–851 reacted only with the intact subunit and not with any of its proteolytic fragments, demonstrating that the corresponding epitope is sensitive to the occurrence of cleavage and is likely to overlap with the site recognized by the protease(s) responsible for it. Consequently, binding of this antibody to vWF may shield the susceptible bond from the action of the enzyme(s). Such a property, particularly if reproduced in smaller molecules with similar binding specificity, may prove useful in the preparation of undegraded purified vWF. The anti-peptide antibodies failed to react with the normal 189-kDa fragment seen with the monoclonal antibody pool, even though this fragment is thought to extend on the amino-terminal side of the 176-kDa fragment and should contain all of the sequences recognized by the anti-peptide antibodies (22). The finding may be explained by the low concentration of this fragment in normal plasma, so that it may only be detected when several distinct IgG molecules bind to different epitopes, as with the monoclonal pool. Alternatively, it is possible that the additional sequence on the amino-terminal side of the cleavage site presents a conformation after blotting onto nitrocellulose paper that masks the epitopes recognized by the anti-peptide antibodies.

The concentration of proteolytic fragments of the vWF subunit is either increased or decreased in different subtypes of von Willebrand disease (8), suggesting that congenital abnormalities of vWF resulting in a dysfunctional molecule with an absence of larger multimers may occur within the structural domain containing the sequence identified here. Moreover, an increase in the 140-kDa and 176-kDa fragments has been reported in myeloproliferative disorders characterized by an acquired loss of the larger vWF multimers (28), a situation that has been attributed to proteolytic degradation due to a protease, or proteases, possibly derived from platelets and/or leukocytes. The exact identification of the cleavage site and the availability of anti-peptide antibodies directed at relevant epitopes surrounding or including the susceptible peptide bond should permit evaluation of whether the degradation of vWF in the different conditions described is due to similar mechanisms.

The nature of the protease, or proteases, responsible for the generation of the vWF fragments is not known. Plasmin (29, 30), calpains (31), also known as calcium-activated neutral proteases, and protease(s) derived from polymorphonuclear cells (32) have all been implicated in the degradation of vWF. The cleavage site identified with the present studies, a tyrosine-methionine bond preceded by a valine residue, is compatible with the preferential specificity of the calpains (33), and no other proteolytic enzyme appears a better candidate for this site specificity. Indeed, the present results, along with the demonstration that inhibitors of calcium-dependent proteases can partially prevent the degradation of vWF *in vitro* (9), strongly favor the concept that calpains are the enzymes responsible for generating the main naturally occurring proteolytic fragments of vWF. This conclusion contrasts with that from previous *in vitro* studies (34) in which the use of calpain I, purified from porcine erythrocytes, and calpain II, purified from porcine kidney, failed to produce the 176-kDa and 140-kDa fragments. However, this discrepancy may reflect species-dependent or tissue-dependent substrate specificities or the fact that the calpains, which are intracellular and membrane-bound enzymes, may have different behavior when used in solution after purification (34) than in the intact organism. Nevertheless, vWF may be susceptible to calpains and/or other as-yet-unidentified proteases yielding fragments similar in size to those described here.

The distinct amino acid substitutions documented to date in vWF from three type IIA patients—namely, Arg-834 → Trp (10), Val-844 → Asp (10), and Ser-850 → Pro (11)—all cluster within 10 residues of the cleavage site identified here. The Ser-850 → Pro mutation has been found in patient vWF of P_4, whose vWF has been studied also with the immunochemical approach described here. Accordingly, his 176-kDa fragment exhibited decreased reactivity with the anti-peptide antibody binding within residues 843–857. Of the new patients studied here, P_1 and S_1 (belonging to the same family) appear to have a mutation in this same region of vWF but distinct from that of P_4, since their 176-kDa fragment exhibited the same decreased reactivity with the appropriate anti-peptide antibody, but their DNA gave a different pattern of restriction fragments. Similarly, the mutation in vWF of patient P_2 appears to be near the carboxyl terminus of the 140-kDa fragment but different from that reported previously in the same region of the molecule at residue 834 (10). Only vWF of patient P_3 was immunochemically indistinguishable from that of normal controls, a finding suggesting that the mutation in this case might be more remote from the cleavage site. Alternatively, this patient might still have a mutation in

the proximity of the cleavage site but not such as to alter reactivity of the fragments with any of the antibodies presently available. Altogether, the majority of molecular abnormalities identified to date in type IIA von Willebrand disease appears to occur within 10 residues on either side of the protease-sensitive bond between Tyr-842 and Met-843.

The present results suggest that the pathogenesis of type IIA von Willebrand disease may be explained, at least in some patients, by amino acid substitutions that change the conformation of the molecule around the peptide bond between Tyr-842 and Met-843 and thereby increase its susceptibility to proteolytic cleavage. These mutations may be in close proximity to the protease-sensitive bond, as found in the majority of cases analyzed to date, but might also be at a distance in the linear sequence of the subunit. Cleavage between residues 842 and 843 may result in disappearance of the larger multimers only when there are no intra- or interchain disulfide bonds linking the 140-kDa and 176-kDa fragments. Indeed, limited proteolysis of native vWF with trypsin yields a single-chain fragment extending between residues 730 and 1114 (12, 13), proving that there are no interchain disulfide bonds in this domain of the subunit. Moreover, vWF has no cysteine between residues 730 and 842 (35), so that there cannot be any intrachain disulfide bond linking the two fragments originated by cleavage of the 842–843 bond. Thus, cleavage of a single bond can indeed cause splitting of a vWF multimer. Consequently, it is clear why the phenotypic expression of type IIA von Willebrand disease is manifest in heterozygotes, since in these individuals abnormal subunits highly susceptible to proteolysis should be randomly intermixed with normal subunits. Clearly, different mutations could result in variable degrees of increased proteolysis, thus accounting for the well-known heterogeneity of type IIA patients with respect to the loss of larger vWF multimers (7, 9).

While the kind of pathogenetic mechanism hypothesized here may be operative in a majority of patients, it is possible in other cases either that mutations in more distant regions of the molecule affect exposure of this or other cleavage sites or that entirely different structural abnormalities result in disappearance or lack of synthesis of larger vWF multimers. Confirmation of the amino-terminal sequence of the 176-kDa fragment from type IIA vWF has not been possible because of the difficulty in obtaining sufficient quantities of plasma for purification. However, if proteolytic fragmentation of vWF occurred in regions of the subunit different from the one identified here, then a distinct pattern of reactivity should become apparent with the panel of antibodies used for the present experiments. Indeed, any putative fragments different from the 140-kDa and 176-kDa species should contain in various possible combinations the epitopes recognized by the anti-peptide antibodies depending on whether or not they include the region between residues 828 and 857. Thus, use of the immunochemical approach described here strongly suggests that in the type IIA patients studied to date, cleavage of the vWF subunit occurs at the bond between Tyr-842 and Met-843. When applied to larger groups of patients, this method may lead to a more refined molecular classification of type IIA von Willebrand disease.

In conclusion, the chemical and immunological data presented here identify an important domain of the vWF molecule that appears to be relevant in the pathogenesis of type IIA von Willebrand disease. Proteolytic cleavage at this site may have important effects on the structure and function of vWF, particularly if, as suggested by others (10, 36), this region of the molecule is involved in regulating the binding to the platelet glycoprotein Ib site.

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