Triplet structure of human von Willebrand factor

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Human von Willebrand factor (hp-vWF) is a high-molecular-mass protein found in plasma as a series of multimers. It consists of subunits comprising 2050 amino acids linked by disulphide bonds into multimers of various size ranging in molecular mass up to greater than 10000 kDa. Partial proteolysis at position Tyr^{842}–Mer^{843} of the subunit [Dent et al. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 6306–6310] by a vWF-specific protease [Furlan et al. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7503–7507] results in the generation of an N-terminal and a C-terminal fragment and the appearance of hp-vWF triplet bands. It has been suggested [Furlan et al. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7503–7507] that (i) the intermediate triplet band of the primary dimer represents a dimer of two C-terminal fragments, (ii) the slower migrating satellite band of the primary dimer represents an asymmetric structure composed of a mature subunit to which one N-terminal and one C-terminal fragment are linked by disulphide bonds, and (iii) the faster migrating satellite band of the primary dimer contains two N-terminal fragments. Here we used recombinant vWF (r-vWF) for structural analysis of hp-vWF multimers. r-vWF exhibited no proteolytic degradation and all multimers contained mature subunits. High-resolution agarose-gel electrophoresis and two-dimensional electrophoresis demonstrated that (i) r-vWF multimers and hp-vWF intermediate triplet bands exhibited identical molecular mass and electrophoretic mobilities, (ii) the faster and slower migrating satellite bands of hp-vWF differ by less than the molecular mass of one subunit from the corresponding intermediate triplet band, and (iii) the triplet bands of hp-vWF are composed of mature and degraded subunits. The results support a structural model of hp-vWF triplet bands according to which the intermediate triplet bands represent multiple numbers of symmetric and/or asymmetric dimers, the slower migrating satellite bands have one extra N-terminal fragment, and the faster migrating satellite band lacks one N-terminal fragment respectively in comparison with the corresponding intermediate triplet band.

INTRODUCTION

Human von Willebrand factor (hp-vWF) is the largest protein found in plasma. It is an adhesive plasma glycoprotein synthesized in vascular endothelial cells and megakaryocytes. It circulates in human plasma as a series of multimers ranging in molecular mass from about 450 kDa to more than 10000 kDa [1–4]. The precursor polypeptide produced in endothelial cells, prepro-vWF, consists of a 22-residue signal peptide, a 741-residue propeptide and a 2050-residue polypeptide found in mature hp-vWF. After removal of the signal peptide, the resulting pro-vWF subunits are engaged in a complex biosynthetic process thought to begin with the formation of a dimer, containing two pro-vWF subunits linked through disulphide bonds. The protomeric units of the multimeric series are then assembled into higher-order multimers through disulphide bonding of dimers. The hp-vWF propeptide is cleaved from the multimeric hp-vWF before it is released into the circulation [1,3,5,6].

Once released, hp-vWF serves a dual purpose in haemostasis. It is an adhesive protein that acts as a bridge between platelet glycoproteins and vascular subendothelium. In addition, it binds coagulation factor VIII and circulates with it as a non-covalently linked complex. In this manner, hp-vWF plays a key role in haemostasis by initiating platelet adhesion at sites of vascular injury and by localizing coagulation factor VIII to sites where it can participate in generation of thrombin and fibrin clot [7].

The key structural element of multimeric hp-vWF is a dimer formed of two identical subunits connected by intermolecular disulphide bonds at their C-terminal regions [1–3]. Dimers are linked through disulphide bonds at their N-termini and assembled into large multimers [1–3]. The multimeric pattern of hp-vWF is seen on SDS/agarose-gel electrophoresis by regularly spaced bands. However, some of the subunits of circulating hp-vWF appear to be modified by specific proteolytic cleavage *in vivo* and *in vitro* [8]. Dent et al. [9,10] identified the cleavage site at position Tyr^{842}–Met^{843} of the mature subunit. Furlan et al. [11,12] purified and characterized a vWF-specific protease, which generates the N-terminal and C-terminal fragment of the subunit by a single cleavage at this position. The intact subunit (2050 amino acids), the C-terminal fragment (1208 amino acids) and the N-terminal fragment (842 amino acids) have been identified by SDS/PAGE of completely reduced hp-vWF [8–13] as a pattern of protein bands in order of their molecular mass.

If this cleavage occurs in multimeric hp-vWF after secretion, molecular species of hp-vWF can derive which have unequal numbers of N-terminal and C-terminal fragments [1,3], because there is no intrachain linkage across the 842–843 peptide bond and no interchain linkage between the N- and C-termini of the two subunits [1,3]. This situation is reflected by SDS/agarose-gel electrophoresis of hp-vWF under non-reducing conditions. The multimeric pattern of hp-vWF consists of regularly spaced bands on low-resolution SDS/agarose-gel electrophoresis. However, high-resolution SDS/agarose-gel electrophoresis demonstrated that each hp-vWF multimer exhibits a triplet structure composed of an intermediate triplet band, a faster migrating satellite band and a slower migrating satellite band [1,3], apparently resulting from variation in the number of N-terminal and C-terminal fragments.

On the basis of the binding pattern of monoclonal antibodies to hp-vWF satellite bands, Furlan et al. [11] proposed a structural model of the triplet structure of hp-vWF, in which (i) the intermediate triplet band of the primary dimer represents a dimer

Abbreviations used: hp-vWF, human plasma-derived von Willebrand factor; r-vWF, recombinant von Willebrand factor.

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of two C-terminal fragments, (ii) the slower migrating satellite band of the primary dimer represents an asymmetric structure composed of a mature subunit to which one N-terminal and one C-terminal fragment are linked by disulphide bonds, and (iii) the faster migrating satellite band of the primary dimer contains two N-terminal fragments. However, in their study Furlan et al. acknowledged that the immunological reactivity of the monoclonal antibodies decreased or was completely abolished during experiments [11].

vWF deficiency or abnormality leads to von Willebrand disease, which is the most common inherited human bleeding disorder. Treatment and prevention of this disease and development of therapeutic substances essentially depend on molecular and structural characterization of hp-vWF.

In this study we used r-vWF as a reference molecule for structural characterization of hp-vWF. Our results demonstrate that r-vWF multimers are composed exclusively of mature and intact subunits, while whereas hp-vWF contain mature subunits, N-terminal and C-terminal fragments. Structural analysis by high-resolution SDS/agarose-gel electrophoresis demonstrated that the multimeric bands of r-vWF correspond to the intermediate triplet bands of hp-vWF. Slower migrating satellite bands and faster migrating satellite bands of hp-vWF exhibited slightly increased and reduced molecular mass respectively relative to r-vWF multimeric bands. Apparently, slower migrating and faster migrating satellite bands of hp-vWF either contain additional or lack structural elements respectively in comparison with mature multimers.

MATERIAL AND METHODS

Materials

Seaken Agarose HGT(P) was purchased from FMC BioProducts, Rockland, ME, U.S.A. Acrylamide, *N*,*N*′-methylenebisacrylamide, SDS, glycine, prestained calibrated proteins for electrophoresis, ammonium persulphate, alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG (H+L) and alkaline phosphatase coloration chemicals were from Bio-Rad Laboratories, Hercules, CA, U.S.A. Normal human plasma was from Immuno (Vienna, Austria). Rabbit anti-human vWF polyclonal antibody and alkaline phosphatase-conjugated goat anti-rabbit polyclonal antibody were from Dako (Glostrup, Denmark). vWF antigen was quantified by ELISA (Asserachrom vWF; Boehringer-Mannheim, Mannheim, Germany). Fractogel EMD-TMAE and Fractogel EMD-Heparin were from Merck (Darmstadt, Germany). N-Glycosidase F, O-glycosidase and neuraminidase were from Boehringer-Mannheim. All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.). Preparation of r-vWF produced by fermentation of recombinant CHO cells was as described previously [14].

Purification of vWF

Purification of r-vWF and hp-vWF was achieved by anion-exchange chromatography and heparin affinity chromatography as described in detail previously [15].

Electrophoretic characterization of vWF

Multimer analysis of vWF was performed by SDS/agarose-gel electrophoresis [16], followed by blotting on to nitrocellulose membrane as described in detail previously [14,15]. Before SDS/agarose-gel electrophoresis under reducing conditions, proteins were incubated at 90 °C for 2 min in sample buffer containing 20 mM dithiothreitol. Two-dimensional electrophoresis was performed by SDS/2 % agarose gel electrophoresis in the first dimension under non-reducing conditions, and SDS/2 % agarose gel electrophoresis under reducing conditions in the second dimension as describe previously [14]. Prestained calibrated proteins were used as molecular-mass markers in each run. Proteins resolved by electrophoresis were transferred on to nitrocellulose membranes [17]. Detection of vWF multimers was carried out by immunoenzymic staining, modified as described by Aihara et al. [18]. SDS/PAGE was performed in 80 g/l acrylamide gels under reducing conditions as described by Laemmli [19].

Deglycosylation of r-vWF and h-vWF

Deglycosylation of r-vWF and h-vWf was carried out with a combination of N-glycosidase F, O-glycosidase and neuraminidase, using standard protocols suggested by the manufacturer. Deglycosylation was verified by complete loss of binding to *N*- and *O*-glycan-specific lectins.

RESULTS

r-vWF and hp-vWF were analysed by SDS/2 % agarose-gel electrophoresis (Figure 1). Non-reduced r-vWF as well as hp-vWF exhibited a multimeric protein pattern. However, only hp-vWF multimers exhibited triplet bands, composed of an intermediate band, a faster migrating and a slower migrating satellite band. Complete reduction of disulphide bonds in r-vWF demonstrated that this molecule is composed exclusively of mature subunits (Figure 1, lane c). By contrast, reduction of hp-vWF produced three peptide bands (Figure 1, lane b). The pattern of these protein bands confirms that hp-vWF multimers contain intact subunits, C-terminal fragment and N-terminal fragments, in order of decreasing molecular mass. The mature subunits of reduced r-vWF and reduced hp-vWF apparently, exhibit identical molecular mass.

To exclude the possibility that the differences in glycosylation between hp-vWF and r-vWF affect the molecular mass and electrophoretic mobility, N- and O-linked glycans were removed from r-vWF and hp-vWF. Deglycosylated hp-vWF and r-vWF

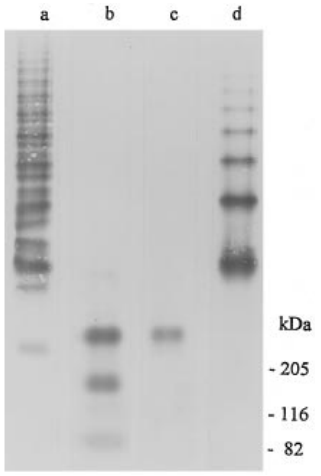

Figure 1 Multimer analysis of r-vWF and hp-vWF by SDS/2% agarose-gel electrophoresis under non-reducing and reducing conditions

Lane a, hp-vWF; lane b, reduced hp-vWF; lane c, reduced r-vWF; lane d, r-vWF. Positions of molecular-mass markers are indicated on the right.

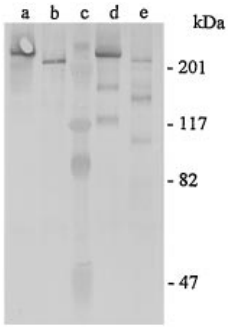

Figure 2 SDS/PAGE under reducing conditions of r-vWF and hp-vWF

Lane a, r-vWF; lane b, deglycosylated r-vWF; lane c, molecular-mass marker; lane d, hp-vWF; lane e, deglycosylated hp-vWF. Positions of molecular-mass markers are indicated on the right.

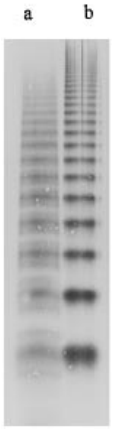

Figure 3 Multimer analysis of hp-vWF (lane a) and r-vWF (lane b) by SDS/2% agarose-gel electrophoresis

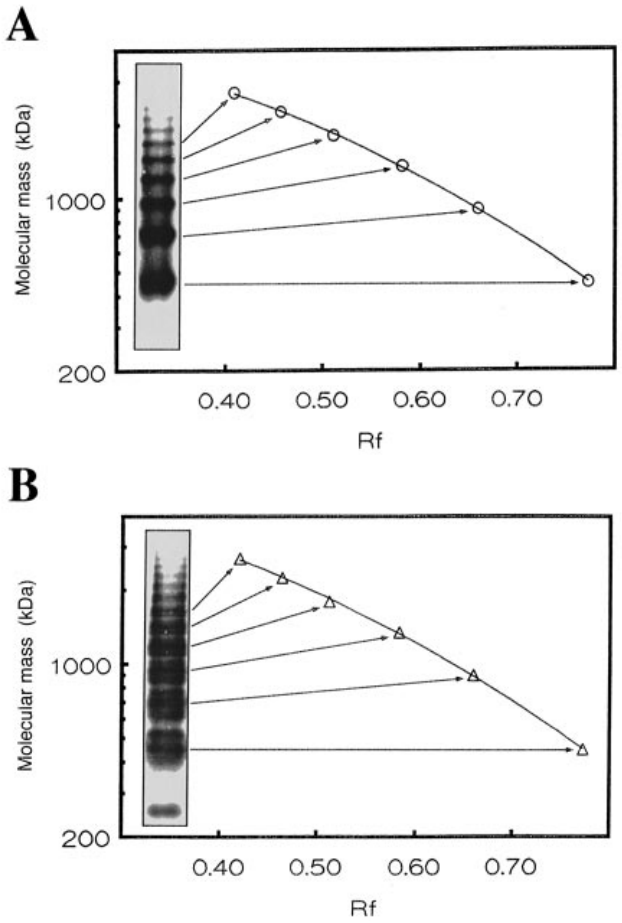

Figure 4 Plot of $\log_{10}$ (molecular mass) against R_F of r-vWF multimers (A) and hp-vWF intermediate triplet bands (B)

The inserts represent the multimer analysis of r-vWF and hp-vWF by SDS/2% agarose-gel electrophoresis. Multimer bands used for plot calculations are indicated.

were analysed by SDS/PAGE under reducing conditions (Figure 2). Mature subunits of r-vWF and hp-vWF and deglycosylated subunits of r-vWF and hp-vWF respectively exhibited identical molecular mass. Deglycosylation of r-vWF and hp-vWF resulted in equivalent reductions in apparent molecular mass of the mature subunit of hp-vWF and r-vWF and in the molecular mass of the N-terminal and C-terminal fragment of hp-vWF.

To identify the hp-vWF triplet bands that correspond to r-vWF multimers, the two proteins were analysed side by side by high-resolution SDS/agarose-gel electrophoresis (Figure 3). It can be seen that intermediate triplet bands of hp-vWF correspond to the multimeric bands of r-vWF.

From a biosynthesis point of view, it has been supposed that multimerization of hp-vWF is a linear process in which multimeric hp-vWF is formed by covalent association of dimers via disulphide bonds [1,3]. Because each dimer contains two mature subunits (molecular mass 225 kDa), the molecular-mass difference between two neighbouring multimeric bands in SDS/agarose-gel electrophoresis should be 450 kDa. However, it could be possible that two dimers form a tetrameric molecule, that two tetramers build an octameric hp-vWF molecule, and so forth. To revise these assumptions and to verify the correlation between r-vWF multimers and hp-vWF intermediate triplet bands, plots of $\log_{10}$ (molecular mass) against R_F of individual multimers of r-vWF and individual intermediate triplet bands of hp-vWF were constructed (Figure 4). The insets show the multimer analysis and protein bands used for the plot construction. For plot constructions a molecular mass of 450 kDa was assumed for the primary vWF dimer and a gradual increase in molecular mass of 450 kDa for each following protein band. It can be seen that the mobility of the multimer bands used is proportional to the logarithm of the molecular mass, in a similar way to protein separation by SDS/PAGE [20,21]. r-vWF and hp-vWF gave identical plots. Using the plot derived for hp-vWF intermediate triplet bands as an intermolecular calibration curve, the faster and slower migrating satellite bands differed by less than 100 kDa from corresponding intermediate triplet bands.

r-vWF and hp-vWF were analysed by two-dimensional electrophoresis (Figure 5). r-vWF and hp-vWF were first separated by high-resolution SDS/agarose-gel electrophoresis under non-reducing conditions. Agarose-gel lanes were incubated for 30 min at 60 °C in 5% 2-mercaptoethanol to reduce disulphide bonds, and were then mounted on top of a second high-resolution SDS/agarose gel. During second-dimension SDS/agarose-gel electrophoresis, the individual components of hp-vWF triplet bands and r-vWF multimer bands were separated according to their molecular mass. Mature subunits and the C- and N-

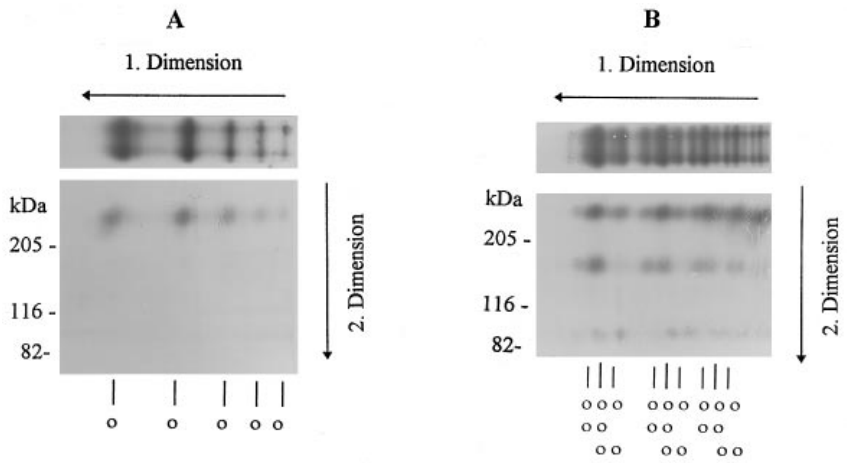

Figure 5 Two-dimensional SDS/2% agarose-gel electrophoresis of r-vWF (A) and hp-vWF (B)

Bottom: schematic representation of r-vWF multimers or hp-vWF triplets (|) resolved in the first dimension (non-reducing conditions) and corresponding spots (○) obtained in the second dimension (reducing conditions). Positions of molecular-mass markers are indicated on the left.

terminal fragments were identified as peptide spots in horizontal lines. In agreement with previous results (Figures 1 and 2), r-vWF multimers contained only mature subunits identified as a single line of peptide spots (Figure 5A). In contrast, mature subunits and C- and N-terminal fragments made up the hp-vWF triplet bands (Figure 5B). The peptide spots are arranged in three horizontal lines corresponding to mature subunits, C-terminal fragments and N-terminal fragments, in order of decreasing molecular mass. The intermediate triplet bands of hp-vWF contained all three components. The faster migrating satellite bands contained mature subunits and C-terminal fragments, and the slower migrating satellite bands contained mature subunits and N-terminal fragments.

DISCUSSION

The key structural element of hp-vWF is a dimer composed of two identical subunits of 2050 amino acids each. Two or more dimers are linked through disulphide bonds at their N-termini and are thus assembled into multimeric molecules [1,3]. hp-vWF molecules with different degrees of multimerization are apparently secreted from endothelial cells into the circulation by the constitutive pathway *in vivo* [1–3]. The smallest secreted hp-vWF molecule is a dimer (primary dimer) with an apparent molecular mass of 450 kDa, and the largest molecules contain more than 20 dimers and have a molecular mass up to 10000 kDa. The multimeric composition of hp-vWF can be visualized by SDS/agarose-gel electrophoresis, showing a characteristic pattern [4].

Limited proteolysis at position Tyr^{842}–Met^{843} of the mature subunit [9] by a specific plasma protease [11,12] produces an N-terminal fragment and a C-terminal fragment, which are contained in hp-vWF multimers in addition to the intact subunit. Mature subunits and N- and C-terminal fragments were detected by electrophoresis after complete reduction of hp-vWF, whereas r-vWF contained only mature subunits because of its recombinant preparation (Figures 1 and 2). The presence of a plasma vWF-specific protease apparently results in the degradation of large hp-vWF multimers *in vivo* and *in vitro* [8,11,12]. A single cleavage at position Tyr^{842}–Met^{843} in just one subunit of multimeric hp-vWF generates two smaller hp-vWF molecules, one with an additional N-terminal fragment and the other with this N-terminal fragment missing, because there are no intra- and inter-chain linkages across the Tyr^{842}–Met^{843} peptide bond [1–3]. Thus, the appearance of satellite bands on high-resolution SDS/agarose-gel electrophoresis of hp-vWF reflects the variation in structural composition and size of hp-vWF multimers (Figure 3). In contrast, r-vWF exhibited no satellite bands (Figure 3) because of the lack of proteolysis.

The nature of the architecture of hp-vWF satellite bands has been the subject of a previous study. Furlan et al. [11] suggested that (i) the intermediate band of the first triplet represents a dimer of two C-terminal fragments, (ii) the slower migrating satellite band of the first triplet represents an asymmetric structure composed of an intact subunit to which one N-terminal and one C-terminal fragment are linked by disulphide bonds, and (iii) the faster migrating satellite band of the first triplet is a dimer of two N-terminal fragments (Figure 6A). In contrast, the present study demonstrates that the intermediate triplet bands of hp-vWF correspond to r-vWF multimer bands (Figure 3). Because r-vWF multimers are composed exclusively of intact dimers (each composed of two mature subunits), intermediate triplet bands of hp-vWF must contain equal numbers of N-terminal and C-terminal fragments which in total represent multiple numbers of dimers. Consequently, identical plots of $\log_{10}$ (molecular mass) against R_F of r-vWF multimers and of intermediate triplet bands of hp-vWF were obtained (Figure 4).

Figure 1 shows that the faster migrating satellite band of the primary hp-vWF dimer exhibited a molecular mass significantly above that of the mature subunit. According to Furlan et al. [11], the faster migrating satellite band of the primary hp-vWF dimer is composed of two N-terminal fragments, which in total contain 1684 amino acids (Figure 6A). However, the mature subunit contains 2050 amino acids. Thus it is most unlikely that the faster migrating satellite band represents an N-terminal dimer. Owing to a lack of molecular-mass markers in the molecular-size range of hp-vWF multimers, intermediate triplet bands of hp-vWF were used to construct a reference plot to estimate the molecular-mass differences between the intermediate triplet bands and the faster and slower migrating satellite bands. Satellite bands differed by less than 100 kDa from the corresponding intermediate triplet bands of hp-vWF multimers.

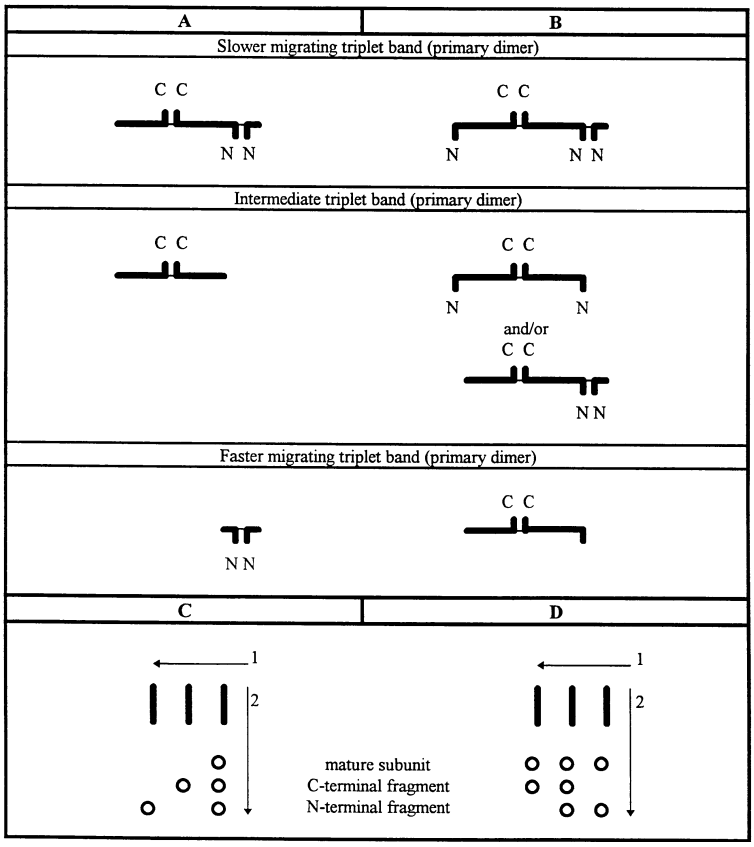

Figure 6 Schematic model of hp-vWF structure

(**A**) Molecular structures of primary triplet bands according to Furlan et al. [11]. (**B**) Molecular structures of primary triplet bands according to the present analysis. (**C**) Schematic drawing of two-dimensional electrophoresis of the primary triplet bands that would result according to the structural model suggested by Furlan et al. [11]. (**D**) Schematic drawing of two-dimensional electrophoresis of the primary triplet bands that would result according to the structural model suggested in the present study.

Complete reduction of hp-vWF produces a pattern of three protein bands corresponding to the mature subunit and the C- and N-terminal fragments in order of decreasing molecular mass. In contrast with r-vWF multimers, which generated a single horizontal line of peptide spots corresponding to mature subunits (Figure 5A), two-dimensional SDS/agarose-gel electrophoresis of hp-vWF produced three horizontal lines of peptide spots (Figure 5B) corresponding to the peptide pattern produced by reduced hp-vWF (Figure 1b). The present results suggest that the intermediate triplet bands of hp-vWF contain mature subunits and C- and N-terminal fragments. Because each intermediate triplet band of hp-vWF corresponded to a multimeric r-vWF band, hp-vWF intermediate triplet bands may exhibit symmetric as well as asymmetric multimeric structures. Symmetric intermediate triplet bands of hp-vWF are structurally identical with r-vWF multimers, i.e. composed of intact dimers. Asymmetric molecules contain intact subunits, and an N-terminal fragment as one and a C-terminal fragment at the other side of the multimer chain. The structural composition of symmetric and asymmetric dimers is shown in a schematic drawing (Figure 6B). Asymmetric molecules are apparently removed from large hp-vWF multimers by two proteolytic cleavages in two dimers. Slower migrating satellite bands of hp-vWF gave peptide spots corresponding to mature subunits and N-terminal fragments (Figure 5B). Thus slower migrating satellite bands represent multiple intact dimers with one N-terminal fragment attached. The proposed structure of the slower migrating satellite band of the first triplet (primary dimer) is shown in Figure 6(B). Such a molecule would contain 4942 amino acids corresponding to a molecular mass of about 545 kDa. The faster migrating triplet bands apparently contain mature subunits and C-terminal fragments (Figure 5B). Thus the faster migrating satellite band of the first triplet (primary dimer) contains one mature subunit and one C-terminal fragment (Figure 6B) comprising 3258 amino acids and having a molecular mass of about 360 kDa. The molecular-mass estimates of the faster and slower migrating satellite band of the primary hp-vWF dimer correspond to experimental results (Figure 4). Faster and slower migrating satellite bands may result from a single proteolytic cleavage of hp-vWF multimers.

Figures 6(C) and 6(D) compare in a schematic drawing peptide bands (first dimension) and peptide spots (second dimension) that would result from two-dimensional electrophoresis of the primary triplet bands of hp-vWF according to the structural model proposed by Furlan et al. [11] and in this paper respectively. It is obvious that Figure 6(C) does not correspond to two-dimensional electrophoresis of hp-vWF (Figure 5B), but that Figure 6(D) does.

In summary, our results support a structural model according to which (i) intermediate triplet bands of hp-vWF represent symmetric and/or asymmetric multimers, (ii) slower migrating satellite bands of hp-vWF are composed of variable numbers of dimers having one N-terminal fragment attached, and (iii) faster

migrating triplet bands of hp-vWF are composed of variable numbers of dimers with one subunit lacking one N-terminal fragment.

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