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# The pro-polypeptide of von Willebrand Factor is required for the formation of a functional Factor VIII-binding site on mature von Willebrand Factor

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We have established that a recombinant von Willebrand Factor (vWF) mutant (vWFdelpro) that lacks the pro-polypeptide, in contrast with mature wild-type vWF, with which it is identical in terms of primary amino acid sequence, is not able to form a complex with Factor VIII. Wild-type vWF (flvWF) and vWFdelpro were expressed in AtT-20 cells. Under the culture conditions employed, completely processed multimerized flvWF and dimeric vWFdelpro were secreted into the medium. FlvWF and vWFdelpro were compared for their Factor VIII-binding properties in two distinct assay systems. In a direct binding assay, purified human Factor VIII was shown to bind to flvWF that had been immobilized on the surface of microtitre wells by using an anti-vWF monoclonal antibody. In contrast, Factor VIII did not bind to immobilized vWFdelpro. In a competition assay, fluid-phase flvWF appeared to inhibit efficiently the binding of Factor VIII to immobilized vWF isolated from plasma, whereas vWFdelpro did not influence Factor VIII binding. From these observations, it is argued that the pro-polypeptide serves an essential role in the post-translational processes that lead to the expression of a functional Factor VIII-binding site on the mature vWF subunit.

## INTRODUCTION

Factor VIII and von Willebrand Factor (vWF) circulate in plasma as a non-covalently linked protein complex (for review, see Sadler & Davie, 1987). Both proteins serve an essential role in the haemostatic process. Factor VIII participates as a cofactor in the activation of Factor X by Factor IXa in the intrinsic coagulation pathway (Van Dieijen *et al.*, 1981; Mertens *et al.*, 1985; for review see Hoyer, 1987; Kane & Davie, 1988), and vWF mediates the adhesion of platelets to damaged vascular surfaces (Tschopp *et al.*, 1974; Sakariassen *et al.*, 1979).

Factor VIII is synthesized as a single-chain molecule ($M_r$ 260000) (Vehar *et al.*, 1984; Toole *et al.*, 1984), which, owing to limited proteolysis, is present in plasma as a series of heterodimers. These are composed of variable derivatives of the *N*-terminal 'heavy' chain ($M_r$ 50000–180000) and a common *C*-terminal 'light' chain ($M_r$ 80000) (Rotblat *et al.*, 1985; Andersson *et al.*, 1986; Eaton *et al.*, 1986). Regulation of Factor VIII activity involves proteolytic cleavages by thrombin, Factor Xa and activated Protein C (Eaton *et al.*, 1986; Fay *et al.*, 1986).

vWF is a multimeric protein, with $M_r$ ranging between $5 \times 10^5$ and about $20 \times 10^6$ (Van Mourik & Bolhuis, 1978; Hoyer & Shainoff, 1980; Ruggeri & Zimmerman, 1980). It is synthesized as a pre-pro-polypeptide that consists, to a large extent, of repeated domains (Sadler *et al.*, 1985; Verweij *et al.*, 1986; Bonthron *et al.*, 1986). Upon cleavage of the signal peptide, pro-vWF dimerizes through disulphide linkages in its *C*-terminal region (Marti *et al.*, 1987). The dimers serve as protomers for multimerization, which is governed by disulphide linkages between the free *N*-termini (Marti *et al.*, 1987). The assembly into multimers is followed by the proteolytic removal of the pro-polypeptide. This cleavage is not always complete (Lynch *et al.*, 1983; Wagner & Marder, 1984).

The formation of a complex between Factor VIII and vWF is of fundamental importance in the maintenance of normal haemostasis. Several patients have been described to suffer from bleeding disorders associated with an aberrant interaction between Factor VIII and vWF, resulting from a structural defect in either Factor VIII (Higuchi *et al.*, 1989) or vWF (Nishino *et al.*, 1989; Mazurier *et al.*, 1990). The formation of a Factor VIII–vWF complex substantially affects the half-life of Factor VIII in the circulation (Over *et al.*, 1981; Tuddenham *et al.*, 1982; Brinkhous *et al.*, 1985). The molecular basis of the interaction between Factor VIII and vWF is largely unknown.

Previous studies employing purified plasma proteins have demonstrated that residues within the acidic region of amino acids 1649–1689 of the Factor VIII light chain are involved in vWF binding (Foster *et al.*, 1988; Lollar *et al.*, 1988; Leyte *et al.*, 1989*a*). The use of recombinant Factor VIII mutants has further revealed that the sequence 1669–1689, and in particular the tyrosine residue 1680, is essential in the formation of a functional vWF-binding site (Leyte *et al.*, 1991).

On vWF, a major Factor VIII-binding site has been assigned to the *N*-terminal 272 amino acid residues of the mature subunit (Foster *et al.*, 1987). This vWF fragment was shown to compete with immobilized vWF for Factor VIII binding (Foster *et al.*, 1987; Koedam *et al.*, 1989). Furthermore, the sequence 1–272 appeared to contain the binding site of an anti-vWF monoclonal antibody that inhibits the formation of a Factor VIII–vWF complex (Foster *et al.*, 1987). Recently, the epitopes of other inhibitory antibodies have been mapped within the sequence 1–272 to residues 1–106 (Pietu *et al.*, 1989) and 78–96 (Bahou *et al.*, 1989) respectively.

Previous studies have shown that the Factor VIII–vWF complex dissociates under mildly reducing conditions (Counts *et al.*, 1978; Vehar & Davie, 1980). It has also been reported that

Abbreviations used: HSA, human serum albumin; vWF, von Willebrand Factor.

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reduced and alkylated vWF is unable to bind Factor VIII (Foster *et al.*, 1987). These data suggest that disulphide bonds within vWF are critical for complex formation with Factor VIII. The functioning of the Factor VIII-binding site may therefore depend on certain conformational preconditions.

In the present study, we have investigated the role of the pro-polypeptide in the formation of a functional Factor VIII-binding site. Previously, it has been shown that a recombinant vWF mutant that lacks the pro-polypeptide (vWFdelpro) is unable to assemble into multimers (Verweij *et al.*, 1987). vWFdelpro forms dimers only, and as a consequence differs from mature wild-type vWF with respect to the intermolecular disulphide linkages in the region 283–695 (Marti *et al.*, 1987) that are involved in the polymerization of dimers into multimers. It is possible that in vWFdelpro also the region adjacent to residues 283–695, containing the Factor VIII-binding site (1–272) (Foster *et al.*, 1987), does not possess the structural organization of the wild-type molecule. As a result, the mutant protein might interact with Factor VIII in a manner different from wild-type vWF. To examine this possibility, we have expressed recombinant wild-type vWF (flvWF) and vWFdelpro in AtT-20 cells and analysed these proteins with respect to their Factor VIII-binding properties.

## MATERIALS AND METHODS

### Materials

Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). [$^{35}$S]Methionine was from The Radiochemical Centre (Amersham, Bucks., U.K.). Culture media and antibiotics were purchased from GIBCO Europe (Breda, The Netherlands). Protein A–Sepharose and gelatin–Sepharose were obtained from Pharmacia (Uppsala, Sweden). Human serum albumin (HSA) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Immulon microtitre plates were from Dynatech (Plockingen, Germany).

### Plasmid constructions

The plasmid RSVvWF, containing the full-length vWF cDNA under control of the RSV-LTR promoter, was constructed as follows. The plasmid pRSVtPA (MacDonald *et al.*, 1986) was digested with endonucleases *Bgl*II and *Hin*dIII, and the cohesive ends were filled in with DNA polymerase I (Klenow fragment). The vector fragment was isolated. The plasmid pSP8800vWF (Verweij *et al.*, 1986) was digested with endonuclease *Eco*RI, and the cohesive ends were filled in with DNA polymerase I (Klenow fragment). The blunt-ended *Eco*RI fragment, comprising the full-length vWF cDNA, was isolated and ligated to the blunt-ended vector fragment from pRSVtPA. A plasmid containing the vWF cDNA in the desired orientation was isolated. To obtain the plasmid RSVvWFdelpro, containing the cDNA encoding the mutant vWFdelpro (Verweij *et al.*, 1987) under control of the RSV-LTR promoter, the same strategy as outlined above was followed. DNA-sequence determination has established that the mutagenesis procedure employed to obtain the mutant cDNA encoding vWFdelpro has not resulted in additional mutations (Verweij *et al.*, 1987).

### Establishment of stable cell lines expressing wild-type or mutant vWF

Murine AtT-20/D16V cells (a gift from Dr. John Tooze, EMBL, Heidelberg, Germany) were maintained in Iscove's medium, supplemented with 10% (v/v) fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu$g/ml). At 24 h after seeding, the semi-confluent cells were exposed for 4 h to a calcium phosphate co-precipitate of RSVvWF or RSVvWF-delpro and pSV2neo (Southern & Berg, 1982) (20 $\mu$g and 1 $\mu$g per 10 cm$^2$ well respectively), essentially by the procedure of Graham & Van der Eb (1973). No carrier DNA was used. The cells were 'shocked' with 15% (v/v) glycerol in phosphate-buffered saline (150 mM-NaCl/10.5 mM-sodium phosphate buffer, pH 7.4) for 2 min. After a recovery period of 24 h, the cells were treated with trypsin and re-plated. Selection was applied 24 h after trypsin treatment and consisted of 0.25 mg of G418/ml in Iscove's medium supplemented with 10% (v/v) fetal-calf serum and penicillin/streptomycin. After 2–3 weeks, clones were isolated and grown to confluency in the same medium containing 0.125 mg of G418/ml. Positive clones were identified both by a dot-blot assay and by immunofluorescence employing polyclonal rabbit IgG against human vWF (see below). For the production of recombinant vWF for Factor VIII-binding studies, the cells were grown and maintained in roller bottles in serum-free Iscove's medium containing penicillin/streptomycin (100 ml per bottle). Every 48 h, conditioned media were harvested and samples were assayed for the presence of vWF antigen. Expression levels varied between 1 and 4 $\mu$g/ml for flvWF, and between 3 and 7.5 $\mu$g/ml for vWFdelpro.

### Metabolic labelling and immunoprecipitation

Cells were grown and maintained in Iscove's medium containing 10% fetal-calf serum and penicillin/streptomycin. At 30 min before radiolabelling, the medium was replaced by RPMI medium lacking methionine. After this preincubation, the medium was removed and the cells received the same medium, supplemented with [$^{35}$S]methionine (50 $\mu$Ci/ml). Labelling was for 24 h. Immunoprecipitations employing a polyclonal antiserum against human vWF (see below) were performed and analysed as described elsewhere (Leyte *et al.*, 1989*b*).

### Antibodies

The anti-vWF monoclonal antibody CLB-RAg 41 has been described previously (Stel *et al.*, 1984). This antibody is directed against an epitope on the *C*-terminal portion of the vWF subunit (Verweij *et al.*, 1985) and appeared not to influence the binding of Factor VIII to vWF in concentrations up to 50 $\mu$g/ml. Polyclonal rabbit IgG against human vWF and peroxidase-conjugated polyclonal rabbit IgG against human vWF were obtained from Dakopatts a/s (Glostrup, Denmark).

### vWF multimer analysis

Conditioned media were analysed with regard to the multimeric composition of secreted vWF by the method of Ruggeri & Zimmerman (1981).

### Purified human Factor VIII and vWF

Human Factor VIII and multimeric vWF were purified from Factor VIII concentrate (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) as described previously (Leyte *et al.*, 1989*a*, *b*).

### Factor VIII–vWF binding assay

The binding of Factor VIII to vWF purified from human plasma and immobilized directly on to microtitre wells was studied as described in detail elsewhere (Leyte *et al.*, 1989*a*). To study the binding of Factor VIII to flvWF and vWFdelpro, the recombinant proteins were immobilized on to microtitre wells coated with the anti-vWF monoclonal antibody CLB-RAg 41, as follows. The wells were incubated with 5 $\mu$g of the monoclonal antibody/ml in 50 mM-$NaHCO_3$/HCl (pH 9.5) (100 $\mu$l/well) for 16 h at 4 °C and washed twice with 150 mM-NaCl/50 mM-Tris/HCl (pH 7.4). Subsequently, the wells were incubated with

2 % (w/v) HSA in 0.1 % (w/v) Tween 20/150 mM-NaCl/50 mM-Tris/HCl (pH 7.4) (100 μl/well) for 1 h at 37 °C, and washed twice with the same buffer containing 0.2 % HSA. Then conditioned media diluted in 2 % HSA/0.1 % Tween 20/150 mM-NaCl/50 mM-Tris/HCl (pH 7.4) up to vWF concentrations of 100 m-units/ml were incubated in the antibody-coated wells for 16 h at 4 °C (100 μl/well). The wells were washed twice with the same buffer containing 0.2 % HSA. vWF concentrations in the diluted conditioned media before and after incubation in the antibody-coated wells were determined by an e.l.i.s.a. (see below). This provided for approximate measures of the amounts of immobilized flvWF and vWFdelpro: $1.5 \pm 0.4$ and $1.9 \pm 0.7$ m-units/well (mean ± S.E.M., $n = 4$) respectively. When, alternatively, the antibody-bound vWF was detected directly in the wells by using peroxidase-conjugated polyclonal anti-vWF IgG (see below), no difference between wells containing flvWF and vWFdelpro was found. The binding of Factor VIII to immobilized recombinant vWF was measured as described for the binding of Factor VIII to directly immobilized plasma vWF (Leyte *et al.*, 1989*a*). Leakage of vWF from microtitre wells throughout this procedure was assayed in parallel experiments, as follows. After the binding of vWF to the wells coated with CLB-RAg 41 or after the subsequent Factor VIII-binding assay, 1 μg of peroxidase-conjugated polyclonal anti-vWF IgG/ml in 2 % HSA/0.1 % Tween 20/150 mM-NaCl/50 mM-Tris/HCl (pH 7.4) was incubated in the wells (100 μl/well) for 1 h at 37 °C. The wells were then washed twice with the same buffer containing 0.2 % HSA. Subsequently, the peroxidase enzyme reaction was started by addition of 10 % (w/v) 3,3′,5,5′-tetramethylbenzidine and 0.006 % (w/v) $H_2O_2$ in 0.1 M-sodium acetate/0.1 M-citric acid (pH 5.5) (100 μl/well). The enzyme reaction was halted by addition of 100 μl of 1 M-$H_2SO_4$ per well, and the $A_{450}$ was determined as a measure of the amount of CLB-RAg 41-bound vWF present in the wells. This amount did not change during the course of the Factor VIII-binding experiment.

### Quantification of proteins

vWF antigen was quantified by an e.l.i.s.a. as described previously (Reinders *et al.*, 1987). The spectrophotometric determination of Factor VIII activity was performed as has been described (Leyte *et al.*, 1989*b*). Factor VIII and vWF concentrations were expressed in units per ml; 1 unit represents the amount of activity or antigen in 1 ml of pooled normal human plasma [1 unit of Factor VIII and 1 unit of vWF (wild type) are equivalent to approx. 0.1 μg and 10 μg of protein respectively (Sadler & Davie, 1987)].

## RESULTS

### Expression of recombinant vWF in AtT-20 cells

For the production of recombinant wild-type vWF (flvWF) and the mutant vWFdelpro, stable cell lines were established by using murine AtT-20 cells. Metabolic labelling with [$^{35}$S]methionine and immunopurification of radiolabelled vWF from the conditioned medium yielded with both flvWF and vWFdelpro a protein with the apparent $M_r$ of the mature vWF subunit (about 250000) under reducing conditions (Fig. 1). Since no protein with the size of uncleaved pro-vWF was detectable, flvWF secreted by AtT-20 cells is apparently completely processed. This finding was confirmed by immunoblotting of the conditioned media, after SDS/PAGE under reducing conditions. In these experiments, vWF was detected with polyclonal rabbit IgG against human vWF, and solely the presence of vWFdelpro and mature flvWF was apparent (results not shown). As illustrated in Fig. 2 (lane 1), flvWF is present in the condition medium of AtT-20 cells as a multimeric protein. On the other hand, vWFdelpro secreted by these cells is not assembled beyond the dimer stage (Fig. 2, lane 2).

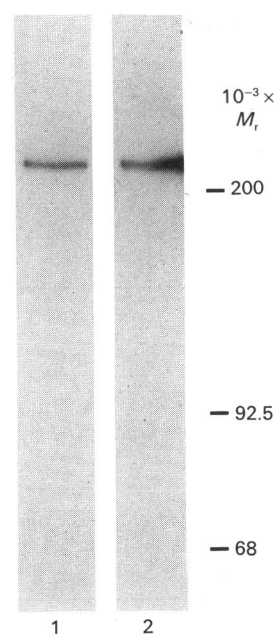


**Fig. 1. Expression of recombinant vWF by AtT-20 cells**

AtT-20 cell lines expressing flvWF or vWFdelpro were metabolically labelled with [$^{35}$S]methionine as described in the Materials and methods section. Radiolabelled vWF was immunopurified from the conditioned media. Immunoprecipitates were analysed by SDS/PAGE (5 % gel) under reducing conditions, followed by autoradiography. The positions of $M_r$ markers are indicated. Lane 1: flvWF. Lane 2: vWFdelpro.

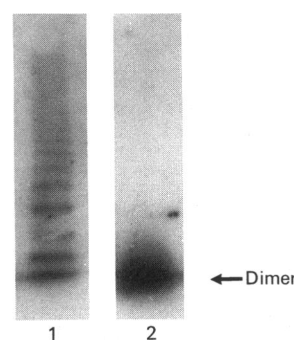


**Fig. 2. Multimeric composition of recombinant vWF secreted by AtT-20 cells**

The composition of vWF in the conditioned media of AtT-20 cell lines expressing flvWF and vWFdelpro was analysed on 1.4 % (w/v)-agarose gels containing 0.1 % SDS as described in the Materials and methods section. Lane 1: flvWF. Lane 2: vWFdelpro. The dimer composed of two mature subunits is indicated by an arrow.

### Direct Factor VIII-binding studies

Fig. 3 shows the results of direct Factor VIII-binding studies employing flvWF and vWFdelpro. For this purpose, recombinant vWF was selectively adsorbed from the conditioned media on to microtitre wells by using the anti-vWF monoclonal antibody CLB-RAg 41. Factor VIII was found to bind to immobilized

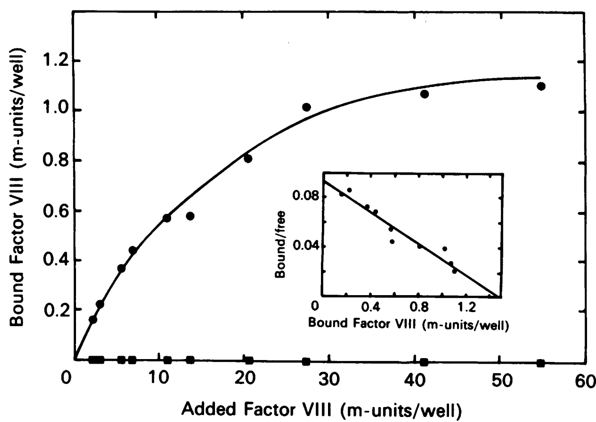


**Fig. 3. Factor VIII binding by immobilized wild-type and mutant recombinant vWF**

FlvWF (●) or vWFdelpro (■) expressed by AtT-20 cell lines was immobilized on to microtitre wells, and Factor VIII binding to the recombinant vWF was assayed as described in the Materials and methods section. The inset shows a Scatchard plot of the data.

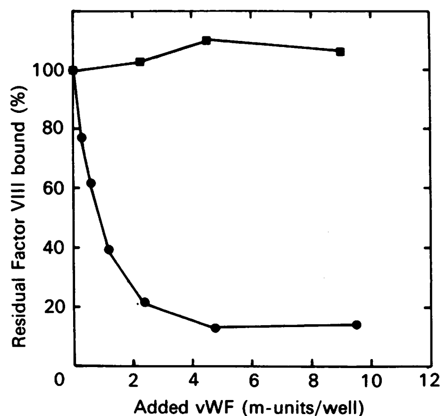


**Fig. 4. Competition between fluid-phase recombinant vWF and immobilized plasma vWF for Factor VIII binding**

Dilutions of serum-free conditioned media of AtT-20 cell lines expressing flvWF (●) or vWFdelpro (■) were mixed with purified Factor VIII (0.5 unit/ml). Subsequently the vWF binding assay was performed as described in the Materials and methods section.

flvWF in a dose-dependent manner. Scatchard analysis of binding data (Fig. 3, inset) demonstrated the presence of a single class of binding sites with a $K_d$ of 1.1 ($\pm$0.17)$\times 10^{-10}$ M (mean$\pm$S.E.M.; $n = 4$). The number of Factor VIII-binding sites was equivalent to 1.5 m-units (or 0.15 ng) per well containing approx. 1.5 m-units (or 15 ng) of immobilized flvWF (see the Materials and methods section). Since Factor VIII and the monomeric mature vWF subunit have approximately the same $M_r$ (260000 and 250000 respectively), this number indicates that the Factor VIII/vWF stoichiometry of the interaction is about 1:100 for completely saturated flvWF. When, instead of flvWF, vWFdelpro had been immobilized on to the microtitre wells, no Factor VIII binding was detectable (Fig. 3).

### Competition studies

As an alternative approach, competition studies were performed between flvWF or vWFdelpro in solution and plasma vWF immobilized directly on to the micro-titre wells. Recombinant flvWF inhibited the binding of Factor VIII to plasma vWF in a concentration-dependent manner, resulting in over 85% inhibition at a flvWF concentration of 5–10 m-units/100 $\mu$l. It is possible that the residual Factor VIII binding relates to non-specific interactions. In contrast with flvWF, vWFdelpro did not inhibit Factor VIII binding to immobilized vWF in concentrations up to 9 m-units/100 $\mu$l (Fig. 4) or 4-fold higher.

## DISCUSSION

The present study demonstrates that of two recombinant proteins with identical amino acid sequences, mature flvWF and vWFdelpro, only one (flvWF) possesses a functional Factor VIII-binding site.

The interaction of flvWF or vWFdelpro with Factor VIII was studied in a system in which the recombinant vWF molecules were immobilized on to microtitre wells by using a monoclonal antibody directed against the *C*-terminal moiety of the vWF subunit, CLB-RAg 41 (see Fig. 3). In this system, the affinity and stoichiometry of the interaction between Factor VIII and flvWF were in the same order of magnitude ($10^{-10}$ M and 1:100 respectively) as has been described for the high-affinity binding of Factor VIII to directly immobilized plasma vWF (Leyte *et al.*, 1989*a*). In the latter study, the existence of additional binding sites on vWF with lower affinity was suggested. The Factor VIII concentrations employed here (less than 1 unit/ml) did not allow the detection of such sites on recombinant vWF.

The apparent lack of Factor VIII binding to vWFdelpro (Fig. 3) cannot be explained by inadequate immobilization of this mutant, since direct immunological detection of CLB-RAg 41-bound vWF did not reveal a difference between flvWF and vWFdelpro (see the Materials and methods section). Furthermore, it is not likely that the interaction with CLB-RAg 41 affects the Factor VIII-binding properties of vWFdelpro. First, the antibody recognizes an epitope remote from the Factor VIII-binding site located on the *N*-terminal region of the vWF subunit (Foster *et al.*, 1987), and does not interfere with the binding of Factor VIII to flvWF (cf. Fig. 3; see also the Materials and methods section). Second, the use of a competitive binding system in which the recombinant proteins in solution were assayed for their inhibition of Factor VIII binding to immobilized plasma vWF (cf. Fig. 4) revealed that, unlike flvWF, vWFdelpro did not possess inhibitory activity. It was therefore concluded that the defective binding of Factor VIII to vWFdelpro is an intrinsic property of this mutant.

Upon expression and secretion by AtT-20 cells, the proteolytic removal of the pro-polypeptide from flvWF was virtually complete. Consequently, both flvWF and vWFdelpro have the entire primary sequence of mature vWF, including the region 1–272 known to contain the Factor VIII-binding site in plasma vWF (Foster *et al.*, 1987). This raises the question why the presence of the primary sequence 1–272 is apparently insufficient to confer Factor VIII-binding properties to vWFdelpro. A major difference between the two vWF species studied here lies in their multimeric composition. In agreement with previous findings (Verweij *et al.*, 1987), flvWF in the conditioned medium of AtT-20 cells had assembled into high-$M_r$ multimers, whereas vWFdelpro had only dimerized. This may suggest a direct relationship between vWF multimerization and Factor VIII binding. However, Foster *et al.* (1987) have shown that the monomeric plasma vWF fragment of amino acid residues 1–272 carries sufficient information to compete with whole vWF for Factor VIII binding. This strongly suggests that multimer assembly is not a prerequisite in this process.

The formation of vWF multimers is dependent on a non-covalent interaction between pro-polypeptides present on different pro-vWF dimers or oligomers (Verweij *et al.*, 1987; Wise *et al.*, 1988; Mayadas & Wagner, 1989). It has been suggested that this process initiates similar interactions between the *N*-terminal

areas of mature vWF. As a result, these areas would be correctly positioned for the formation of intermolecular disulphide bonds between the regions of residues 283–695 (Marti *et al.*, 1987). In agreement with this model, we have demonstrated recently that, within the *N*-terminus of mature vWF, not only the domain involved in these intermolecular covalent bonds (D3), but also the preceding domain (D′), plays an essential role in vWF multimerization (Voorberg *et al.*, 1990). The domains D′ and D3 encompass the Factor VIII-binding region of residues 1–272 (Foster *et al.*, 1987), and one might conceive that an array of intermolecular interactions directed by the pro-polypeptide as outlined above will also have consequences for the intermolecular interactions within this region. As a result, the conformational properties of the vWF sequence involved in Factor VIII binding would be strongly dependent on the presence of the pro-polypeptide during vWF biosynthesis. These properties might include the location of intramolecular disulphide bonds. The sequence 1–272 contains 24 Cys residues (Titani *et al.*, 1986; Verweij *et al.*, 1986), all of which have been shown to be involved in intrachain disulphide bonds (Marti *et al.*, 1987). Since reduction of vWF destroys its ability to bind Factor VIII (Foster *et al.*, 1987), it is likely that such bonds are needed for a functional conformation of the Factor VIII-binding site. We propose that, in addition to its role in vWF multimerization, the pro-polypeptide also directs the folding and disulphide-bond formation within the *N*-terminus of mature vWF required for Factor VIII binding.

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