Control of von Willebrand factor multimer size by a fibronectin-related substance

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Fraction (F) II and FIII obtained by heparin-Sepharose after digestion of partially purified fibronectin (FN) with cathepsin D and F3, obtained like FIII but from untreated FN, exerted activity (arFN) on unfolded purified von Willebrand factor (vWF) that controls vWF multimer size. Our aim was to evaluate the arFN of F from commercial FN, commercial 30 kDa (with heparin affinity), 45 kDa (gelatin affinity) and 70 kDa FN fragments (gelatin and heparin affinity) and whole FN. The arFN was detected in FII, FIII, F2, F3, 30 kDa, 45 kDa and 70 kDa fragments. The least contaminated sample was the 30 kDa commercial fragment. Characterization studies of this sample revealed two bands: a blurred band of approximately 60 kDa and a sharp major band of 32 ± 6 kDa. The 32 ± 6 kDa band fragment failed to produce arFN because it was stronger than in F2 and FIII band fragments at the same position and with the same arFN. Our data suggest that a fragment of approximately 60 kDa that co-purified with FN, with affinity to heparin and gelatin, has the arFN that controls vWF multimer size. ***Blood Coagul Fibrinolysis*** **14:441–448**

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Introduction

von Willebrand Factor (vWF) is a multimer glycoprotein required for platelet adhesion to exposed subendothelium [1], released from endothelial cells as unusually large multimers, while in normal plasma unfolded vWF undergoes proteolysis by a specific plasma protease (vWFCP) [2,3]. Recently, vWFCP has been partially purified, characterized [2,3] and incompletely amino acid sequenced [4,5]. The apparent molecular weight (M_r) of the enzyme is approximately 300 kDa as estimated by gel filtration and requires divalent cations for catalytic activity. Serine protease or thioprotease inhibitors have no effect on the enzyme, but ethylenediamine tetraacetic acid (EDTA) inhibits protease activity [2,3]. vWFCP has been identified as a new member of the metalloproteinase family [4,5].

Recently, it has been found that the incubation of purified thrombospondin 1 (TSP-1) with vWF resulted in formation of thiol-dependent TSP-1 and vWF complexes, generation of new thiols in vWF and reduction in average multimer size [6,7].

Our previous reports have demonstrated that a polyclonal antibody against fibronectin (FN) causes the immunoprecipitation or blockade of a substance other than whole FN that controls vWF multimer size [8]. As an adhesive glycoprotein of blood and extracellular matrix, FN contains two distinct sites with affinity for heparin [9,10]. The Hep 1 site [9,10] is coincident with the 29 kDa amino-terminal domain, active in binding to fibrin [11,12], TSP [13] and other substances. In turn, the Hep 2 site, near the carboxy-terminal end of the protein, comprises one or more disulfide-lacking type III FN modules [14,15].

Digestion of FN by cathepsin D and further separation of generated fragments by different chromatographic methods allowed the isolation of FN-fibronectinase, FN-laminase, FN-gelatinase and FN-type-IV collagenases [16–19]. The separation of digest on heparin-Sepharose 4B and elution with 0.1 mol/l NaCl [Fraction (F) I] contains mainly reduced 95 and 45 kDa polypeptides. Elution with 0.25 mol/l NaCl (FII) contains predominantly a major single-chain 70 kDa fragment (Hep 1) together with a 190 kDa fragment. Elution with 0.5 mol/l NaCl (FIII) contains a 140 kDa fragment (Hep 2), which yields two 75 and 65 kDa polypeptides after reduction [16,20]. Our preliminary data indicated that FII and FIII obtained from partially purified FN (homemade) digested with cathepsin D and a FIII-like

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fragment from undigested FN, lead to a loss of large vWF multimers induced by high shear rate [21].

In the present study, we analyzed the effect on vWF multimer size of the same fractions from commercial FN, and specifically that of the commercial 70 kDa amino-terminal FN fragment (with gelatin and heparin affinity) and its trypsin digestion fragments: 30 kDa (amino-terminal fragment responsible for heparin/fibrin binding) and 45 kDa (with gelatin affinity).

Materials and methods

Materials

FN (F-0895) from human plasma, FN fragments of 30 kDa (F9911), 45 kDa (F0162) and 70 kDa (F0287), cathepsin D, type I agarose, type III collagen (from calf skin), 4-chloro-1-naphthol, *N*-ethylmaleimide (NEM), Tween 20, pre-stained M_r standard, polyethylene glycol (PEG) 10000, PEG 20000 and 10 mm flat width dialysis tubing (D9277) were from Sigma (St Louis, Missouri, USA). Rabbit antibodies to human: vWF (A082), FN (A-245), biotinylated antiserum to rabbit immunoglobulin (E353) and avidin–biotin–peroxidase complex (ABComplex/HRP) (K377) were from Dako Corp (Carpinteria, California, USA). Sephadex G-100, Gelatin-Sepharose 4B, Heparin-Sepharose 4B, CNBr-Sepharose 4B and Sephacryl S-1000 were from Pharmacia (Uppsala, Sweden). Acrylamide and bis-acrylamide were from Fluka (Buchs, Switzerland). Polystyrene beads with 1.531 μm diameter size were from Polysciences, Inc. (Warrington, Pennsylvania, USA). Pefabloc was from Boehringer Mannheim (Mannheim, Germany). Nitrocellulose membranes were from BIO-RAD Laboratories (Hercules, California, USA). All other reagents were of analytical grade.

Blood collection

Plasma was collected from whole human blood anticoagulated with 12.9 mmol/l sodium citrate using two-step centrifugation ($2500 \times g$, 60 min; $10\,000 \times g$, 30 min) at 4°C. A pool ($n = 30$) of normal plasma (PNP) was used as control.

Purified vWF

vWF was isolated from fresh frozen plasma of normal volunteers following the procedures described by Thorell and Blombäck [22]. The cryoprecipitate obtained was dissolved in 55 mmol/l sodium citrate buffer, 5 mmol/l EDTA (pH 7.4) at room temperature and purified by gel filtration with Sephacryl S-1000. Elution was performed with 55 mmol/l sodium citrate buffer, 5 mmol/l EDTA (pH 7.4). Fractions were collected at a 3 ml/cm^2 per h flow rate. Eluted fractions containing vWF were pooled and concentrated using 20% PEG 10 000 in 0.15 mol/l NaCl (pH 8.5). We discarded fractions containing FN detected by immunoelectrophoresis with a-FN [23].

FN fractions

A commercial FN (F-0895, 15 mg) solution was dialyzed against 0.1 mol/l sodium acetate (pH 3.5) and was digested with cathepsin D (0.05 mg) at 25°C. After 4 h, digestion was stopped by adjusting to pH 7.4. The soluble sample was then dialyzed against 50 mmol/l Tris–HCl (pH 7.4) buffer, which contained 0.1 mol/l NaCl and 1 mmol/l EDTA. After dialysis, any precipitate was discarded after centrifugation at $12\,000 \times g$ for 15 min. The supernatant was applied to a 1.6×2.5 cm heparin-Sepharose 4B column equilibrated with 50 mmol/l Tris–HCl, 1 mmol/l EDTA, 0.1 mol/l NaCl (pH 7.4). The same buffer containing 0.25 and 0.5 mol/l NaCl, respectively, was used for stepwise elution. FI was eluted with 0.1 mol/l NaCl, FII with 0.25 mol/l NaCl and strongly bound FIII with 0.5 mol/l NaCl [20]. Fractions were concentrated and dialyzed against 30% PEG 10 000 in 5 mmol/l Tris and 0.15 mol/l NaCl (pH 8.5) and stored at −20°C. F1, F2 and F3 were obtained by affinity chromatography separation of non-treated FN. Commercial fragments of 30, 45 and 70 kDa FN were reconstituted with 5 mmol/l Tris–HCl, 0.15 mol/l NaCl (pH 8.5). In another set of experiments, the 30 kDa fragment was incubated with 10 mmol/ml EDTA or 12 mmol/ml NEM or 1 mmol/ml Pefabloc.

Commercial FN plus active and inactive (pH 7.4) cathepsin D was also tested.

A plasma F of $M_r < 90$ kDa was obtained on a column of Sephadex G-100 equilibrated in 0.05 mol/l Tris–HCl (pH 7.5) containing 0.05 mol/l EACA, 0.02 mol/l sodium citrate and 0.02% sodium azide. It was applied to a column of gelatin-Sepharose 4B equilibrated with the same buffer. F retained was eluted with 3 mol/l urea [24]. Eluted F was dialyzed against 50 mmol/l Tris–HCl (pH 7.4) buffer, which contained 0.1 mol/l NaCl and 1 mmol/l EDTA, and applied to a column of heparin-Sepharose 4B as already described [20]. The 0.25 mol/l and 0.5 mol/l NaCl-eluted F, F2a and F3a, respectively, were dialyzed against 5 mmol/l Tris–HCl, 0.15 mol/l NaCl (pH 8.5). Besides, F2a and F3a were applied to a column of CNBr-Sepharose 4B column coupled with a-FN (according to the manufacturer's instructions) equilibrated with 5 mmol/l Tris–HCl, 0.15 mol/l NaCl (pH 8.5). The retained F was eluted with glycine 0.1 mol/l (pH 2.5), neutralized, concentrated and dialyzed against PEG 20 000 in 5 mmol/l Tris–HCl, 0.15 mol/l NaCl (pH 8.5). The unretained F were called F2b and F3b.

Control of multimer size on unfolded vWF assay

All above samples were diluted with 5 mmol/l Tris-HCl, 0.15 mol/l NaCl (pH 8.5) and full protease activation of 120-μl aliquots achieved by preincubation for 30 min at 37°C with 18 mmol/l $BaCl_2$. PNP aliquots

of 120 μl diluted from 1/10 to 1/320 in 5 mmol/l Tris-HCl, 0.15 mol/l NaCl (pH 8.5) buffer (pH 8.0) (also incubated with $BaCl_2$) were used for the calibration curve. Purified vWF (0.5 U/ml) containing 18 mmol/l $BaCl_2$ was added to each sample, and then incubated overnight at 37°C [25] in dialysis tubing against 1.5 mol/l urea, in 5 mmol/l Tris (pH 8.0). After dialysis for 120 min against 0.15 mol/l NaCl, digestion was stopped by addition of 20 mmol/l EDTA.

The activity of proteases to control multimer size was measured in PNP. The arFN was the activity able to control multimer size in FN fractions and fragments. Some samples were incubated as controls without urea in polyethylene tubes overnight at 37°C, and 20 mmol/l EDTA were added. vWF degradation was examined by analysis of vWF multimer pattern and vWF collagen binding capacity (vWF : CB).

vWF multimer pattern analysis

The technique of sodium dodecyl sulfate (SDS)–1% agarose gel electrophoresis was performed as previously described [26]. Stained gels were analyzed by densitometric scanning with a SharpScanner (JX 330; Hamburg, Germany), using ImageMaster Software (Pharmacia, Newcastle, UK) to yield the retardation factor (Rf) function, intended for use in deriving a linear relative measurement of band position. A default Rf value is given to each band in the lane for its measurement, assuming that Rf equals zero at the start and 1 at the end of the lane. In each assay, purified vWF was considered Rf = 0. A curve of Rf versus PNP dilutions with unfolded purified vWF was plotted to interpolate the sample Rf values and extrapolate the percentage of arFN. We established that 1/10 PNP dilution corresponded to 100% protease activity.

vWF collagen binding capacity

The arFN of FN fractions and fragments of 30, 45 and 70 kDa commercial FN on large vWF multimers was determined according to our previously described method [27].

A calibration curve was set up using PNP (1 : 20–1 : 640). A curve with the median fluorescence value (vWF : CB) versus PNP dilutions (1/10 = 100% protease activity) with unfolded purified vWF was plotted to interpolate sample median fluorescence (WF : CB) values and extrapolate the percentage of arFN.

Assay for vWF antigen

This assay is based on our previously described method [27].

Protein quantification

Protein quantification was performed by Bradford's method [28].

Electrophoresis and immunoblotting

Fractions were resolved on 7, 8 or 10% SDS-polyacrylamide gel electrophoresis (PAGE) [29]. Samples were prepared for electrophoresis by solubilizing in an equal volume of 0.125 mol/l Tris–HCl (pH 6.8) containing 1.6 mol/l urea, 4% SDS, 20% glycerol and 0.04% bromophenol blue with or without 5% 2-mercaptoethanol for 5 min at 100°C. Unreduced and reduced samples were electrophoresed at 40 mA. Gels were stained with Coomassie brilliant blue. For western blotting, proteins were transferred to a nitrocellulose membrane by electroblotting in 25 mmol/l Tris, 192 mmol/l glycine and 20% methanol buffer (pH 8.3). Transfer conditions were 100 V for 1 h using a Mini Trans-Blot cell (BIO-RAD Laboratories, Hércules, California, USA). After blotting, the membrane was blocked with a 5% solution of milk in phosphate-buffered saline (PBS) (10 mmol/l sodium phosphate, 150 mmol/l NaCl; pH 7.4) for 30 min, then incubated for 19 h with 50 μg/ml polyclonal rabbit anti-human fibronectin antibodies (A-245) in the same solution. After thorough washing with PBS containing 0.05% Tween 20, the blot was incubated for 240 min with biotin-conjugated swine anti-rabbit antibodies, diluted 1/100 in the blocking solution. After further thorough washing, the blot was incubated with ABComplex for 240 min. Following washing, the blot was stained with a substrate solution consisting of 60 mg 4-chloro-1-naphthol per 20 ml methanol and 80 ml PBS, to which was added 5 μl of 30% hydrogen peroxide.

Statistical analysis

Means ± standard deviations (SD) were calculated for experiments based on more than three determinations. Statistical significance was analyzed using an unpaired Student's *t* test.

Results

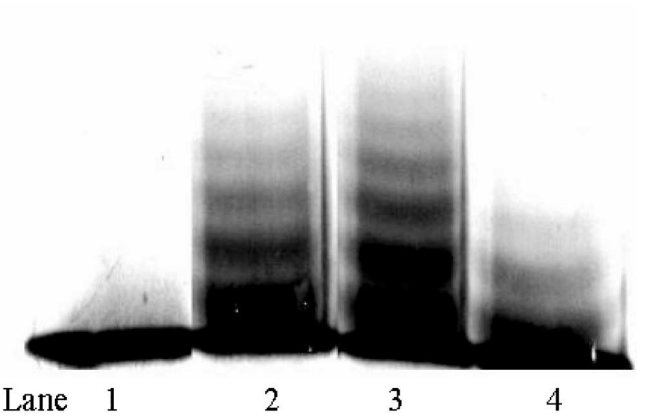
Effect of the commercial 30 kDa fragment on average vWF multimer size

Rf values of unfolded purified vWF plus PNP dilutions were as follows: 0.70 (100% protease activity), 0.48 (50% protease activity), 0.24 (25% protease activity) and 0 (0% protease activity) (data not shown) ($n = 5$).

Incubation of vWF (0.5 U/ml) with commercial 30 kDa fragment (0.5 mg/ml) (Fig. 1, lane 1) resulted in a decrease in average vWF multimer size, Rf = 0.68; according to the Rf values already described, corresponds to 91% arFN. Unfolded vWF (0.5 U/ml) showed no changes in vWF multimer size distribution (Fig. 1, lane 3), with Rf = 0.02 and 0% arFN.

As regards inhibitors, the addition of 10 mmol/l EDTA to vWF (0.5 U/ml) with commercial 30 kDa fragment (0.5 mg/ml) induced a slight reduction in average multimer size (Fig. 1, lane 2,) with Rf = 0.11, 8% arFN.

Fig. 1



Sodium dodecyl sulfate–1% agarose gel analysis of unfolded von Willebrand factor (vWF) multimer distribution from the following samples: lane 3, purified vWF (0.5 U/ml); lane 1, purified vWF (0.5 U/ml) plus commercial 30 kDa fibronectin (FN) fragment (0.5 mg/ml); lane 2, purified vWF (0.5 U/ml) plus commercial 30 kDa FN fragment (0.5 mg/ml) plus 10 mmol/l ehtylenediamine tetraacetic acid; and lane 4 purified vWF (0.5 U/ml) plus commercial 30 kDa FN fragment (0.5 mg/ml) plus 12 mmol/l *N*-ethylmaleimide

Addition of NEM (12 mmol/l) resulted in partially decreased vWF (Fig. 1, lane 4) with Rf = 0.36, arFN = 44%. With the addition of 1 mmol/l Pefabloc, no inhibition was found (data not shown).

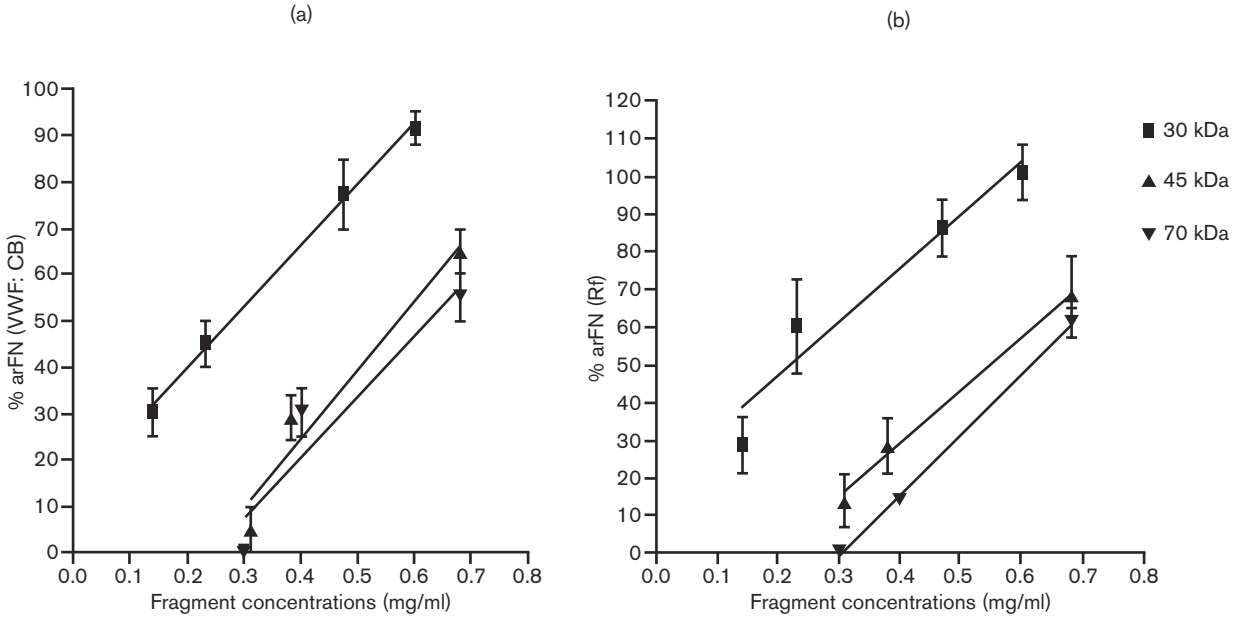
Incubation of vWF (0.5 U/ml) with commercial 30 kDa fragment (0.5 mg/ml) without urea showed no changes in vWF multimer size distribution (Rf = 0.05) (data not shown).

Effects of commercial 30, 45 and 70 kDa fragments on average vWF multimer size

In the following assays, vWF multimer size was evaluated by vWF : CB and vWF multimer pattern assay.

The curve of the means ± SD of arFN ($n = 3$) measured by vWF : CB (Fig. 2a) and Rf (Fig. 2b) versus concentration of commercial FN fragments of M_r 30, 45 and 70 kDa showed the following results: 40% arFN was reached with 0.21 mg/ml of 30 kDa fragment, 0.51 mg/ml of 45 kDa and 0.58 mg/ml of 70 kDa. The 30 kDa fragment had more than two-fold arFN versus

Fig. 2



(a) Relationship between the mean values of percentage activity controlling von Willebrand factor (vWF) multimer (% arFN) of commercial 30, 45 and 70 kDa FN fragments and their concentrations (mg/ml). Samples were obtained by dialyzing purified vWF plus FN fragments activated with $BaCl_2$ overnight with 1.5 mol/l urea. Residual vWF was detected and quantified by vWF collagen binding capacity (vWF : CB) assay as described in Materials and methods. The percentage of arFN was calculated against activated and dialyzed with urea dilutions of a pool of normal plasma (PNP) added to purified vWF in each assay. (b) Relationship between the mean values of arFN percentage in commercial 30 kDa, 45 kDa and 70 kDa kDa FN fragments and their concentrations (mg/ml). Samples were obtained as described in (a). Residual vWF was detected and quantified by sodium dodecyl sulfate–1% agarose gel electrophoresis and immunoenzymatic vWF stain as described in Materials and methods. Rf, retardation factor

the 45 kDa fragment and three-fold arFN versus the 70 kDa fragment.

Effect of fractions from commercial FN on the average vWF multimer size

The curve of the means ± SD of arFN ($n = 3$) measured by vWF : CB (Fig. 3a) and Rf (Fig. 3b) versus FI, II, III, F1, F2 and F3 concentration disclosed that 40% arFN was reached with 0.05 mg/ml F3, 0.14 mg/ml FIII, 0.10 mg/ml F2, 0.18 mg/ml FII or 0.30 mg/ml F1. FI was insufficient to obtain 40% arFN.

The 30 kDa fragment had a similar arFN as FII and less than four-fold arFN versus F3 and two-fold arFN versus F2 and FIII.

Effect of commercial FN on the average vWF multimer size

Commercial FN plus active 0.60 mg/ml (pH 3.5) and inactive 0.50 mg/ml (pH 7.4) cathepsin D showed 29 and 27% arFN, respectively (data not shown).

Effect of fractions from plasmatic aliquots with $M_r < 90$ kDa on the average vWF multimer size

F2a (0.034 mg/ml) and F3a (0.023 mg/ml) exhibited 72 and 87% arFN, respectively. F2b (0.028 mg/ml) and F3b (0.039 mg/ml) exhibited 90 and 68% arFN, respectively (data not shown).

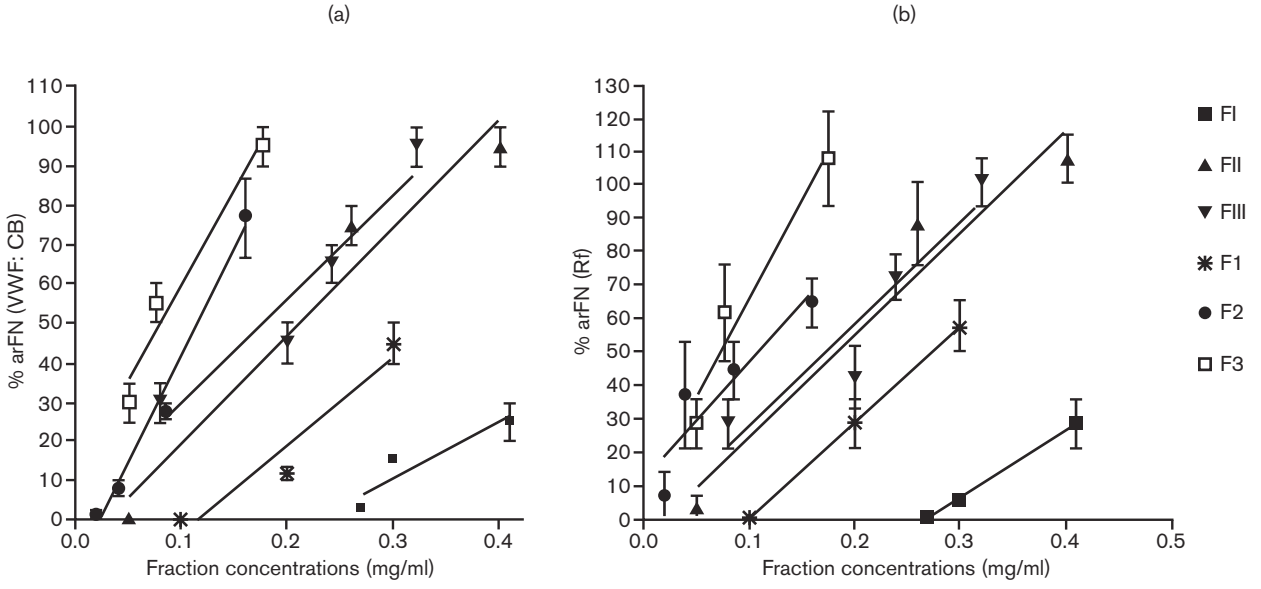
Characterization studies

Several bands more than expected were found in 7% SDS-PAGE under non-reducing conditions of FI, FII, FIII, F1, F2, F3, and FN treated with cathepsin D (pH 3.5 and 7.4) (data not shown). The commercial 45 kDa fragment disclosed a set of bands with very high M_r and bands of 61 ± 5, 56 ± 6 and 43 ± 4 ($n = 3$), but lacked bands with low M_r. The commercial 70 kDa fragment revealed a set of bands with very high and low M_r, and the following bands: 71 ± 3, 61 ± 7, 58 ± 3 and 50 ± 4 ($n = 3$) (data not shown).

Figure 4 shows 10% SDS-PAGE under non-reducing conditions and immunoblotting with a-FN from the 30 kDa fragment (50 μg/ml), F2 (20 μg/ml) and FIII (28 μg/ml).

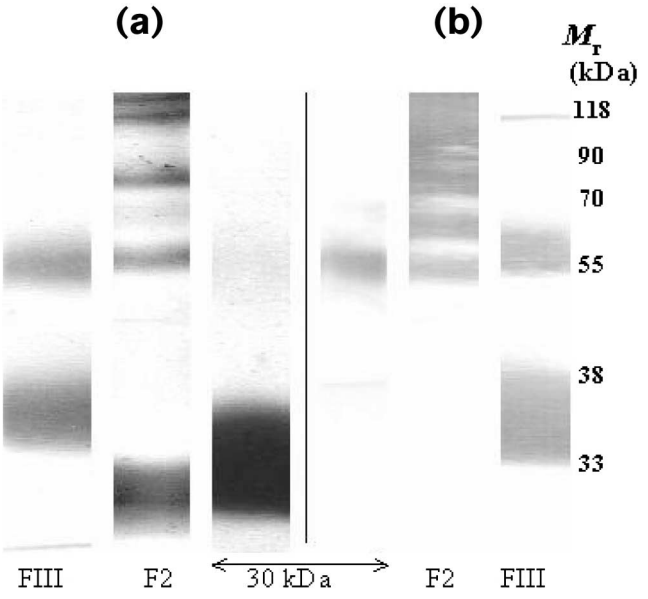
The 30 kDa commercial fragment revealed the following bands: a blurred M_r band of approximately 60 kDa and a well-defined band of 32 ± 6 kDa ($n = 3$) (Fig. 4a). Immunoblotting with a-FN revealed at least two bands of 70 ± 10 and 55 ± 12 kDa within the blurred 60 kDa band ($n = 3$) (Fig. 4b). The 30 kDa fragment

Fig. 3



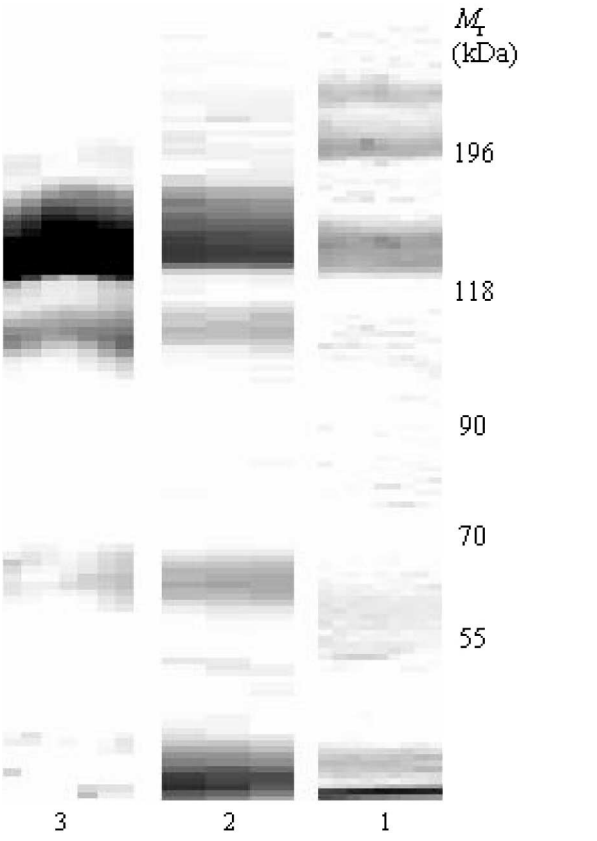
(a) Relationship between the mean values of percentage activity controlling von Willebrand factor (vWF) multimer size (% arFN) of fraction (F) I, FII, FIII obtained by elution with 0.1 mol/l NaCl, 0.25 mol/l NaCl and 0.5 mol/l NaCl heparin-Sepharose chromatography from partially purified FN degraded with cathepsin D, and F1, F2 and F3 obtained by the same method but from untreated FN, versus their concentrations (mg/ml). Samples were obtained by dialyzing purified vWF plus FN activated with $BaCl_2$ overnight with 1.5 mol/l urea. Residual vWF was detected and quantified by vWF collagen binding capacity (vWF : CB) assay as described in Materials and methods. (b) Relationship between the mean values of percentage arFN activity in fraction (F) I, FII, FIII obtained by elution with 0.1 mol/l NaCl, 0.25 mol/l NaCl and 0.5 mol/l NaCl of heparin-Sepharose chromatography from partially purified FN degraded with cathepsin D, and F1, F2 and F3 obtained by the same method but from untreated FN, versus their concentrations (mg/ml). Samples were obtained as described in (a). Residual vWF was detected and quantified by sodium dodecyl sulfate–1% agarose gel electrophoresis and immunoenzymatic stain of vWF as described in Materials and methods. Rf, retardation factor

Fig. 4



(a) 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions of 30 kDa commercial fragment (50 μg/ml), Fraction (F) 2 (20 μg/ml) obtained by elution with 0.25 mol/l NaCl of heparin-Sepharose from commercial FN, and FIII (28 μg/ml) with 0.5 mol/l NaCl of heparin-Sepharose from commercial FN degraded by cathepsin D. F2 and FIII yielded M_r bands of approximately 60 kDa; FIII showed the same band only expected under reducing conditions. The 30 kDa fragment revealed the following bands: a blurred M_r band of approximately 60 kDa and a well-defined band of 32 kDa. The latter band proved significantly stronger than those in the same position of F2 and FIII. (b) Immunoblotting with a-FN of 10% SDS-PAGE in (a). Commercial 30 kDa fragment revealed two bands of 70 kDa and 55 kDa. The latter was also observed in F2 and FIII. M_r indicates the positions of the bands with respect to known marker proteins run in the same gel

Fig. 5



Eight percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions of fibronectin (FN) plus inactive (41 μg/ml) (lane 1) and active (63 μg/ml) (lane 2) cathepsin D. Both reveal a 65 kDa M_r band. Immunoblotting with a-FN from 8% SDS-PAGE. FN plus active cathepsin D (lane 3) sample shows the 65 kDa band. M_r indicates the positions of the bands of a known marker protein run in the same gel

band was not revealed by a-FN. In addition, the 30 kDa commercial fragment was analyzed by 7% SDS-PAGE and no further bands were detected (data not shown).

F2 and FIII yielded M_r bands of approximately 60 kDa. This FIII band was expected only under reducing conditions (Fig. 4a).

The 32 ± 6 kDa ($n = 3$) band from the 30 kDa commercial fragment was stronger than those in the same position of F2 and FIII (Fig. 4a), diluted to obtain similar arFN.

FN plus cathepsin D (active and inactive) under non-reducing conditions revealed a set of bands with very high and low M_r, as well as a band of approximately 60 kDa (data not shown). In Figure 5, 8% SDS-PAGE under reducing conditions of FN plus inactive (41 μg/ml) (lane 1) and active (63 μg/ml) (lane 2) cathepsin D disclosed several bands including one of 64 ± 5 kDa ($n = 5$). Immunoblotting with a-FN of the FN plus active cathepsin D sample (lane 3) showed the same 64 ± 5 kDa band.

Discussion

All experiments for evaluating the effects of FN F and fragments on vWF multimer size were developed by vWF : CB and vWF multimer pattern assay for mutual confirmation. Both assays gave similar results in spite of probable interference between collagen-coated beads (enzyme-linked immunosorbent assay) and the collagen domain of certain FN F and fragments.

To investigate the FN domain involved with arFN, we searched for arFN in FI, FII, FIII and commercial fragments from FN. Since arFN was present in FIII, FII and 30 kDa commercial fragment, the arFN fragment had high affinity to heparin. The arFN found in 45 and 70 kDa commercial fragments suggested that the arFN fragment had affinity to gelatin as well.

On comparing bands, concentrations and activities between the least contaminated commercial 30 kDa fragment versus all commercial fractions and fragments tested, results strongly suggested that the arFN is generated from a fragment in the 60 kDa band.

Commercial FN (with active and inactive cathepsin D) had a fragment with M_r close to 60 kDa but with very low arFN, even with the high concentrations used, indicating that the increase in arFN was obtained by separation of FN and F was individually concentrated.

The arFN found in FII and FIII would indicate that arFN fragment belongs to the FN N-terminal domain and, as it was found in F2 and F3 from untreated FN, this would be evidence that it is circulating in blood, like the free N-terminal FN 30 kDa domain [30].

The arFN fragment did not derive from a contaminant of the commercial initial material because arFN was found in homemade FN [21] and in F from plasmatic aliquots with $M_r < 90$ kDa, as starting material. The arFN products from this F with gelatin and heparin affinity were not retained in a CNBr-activated Sepharose 4B coupled with a-FN column, suggesting that the arFN fragment was not derived from FN. However, the free N-terminal FN 30 kDa domain of the 30 kDa commercial product was not revealed by a-FN.

The arFN was inhibited by 10 mmol/l EDTA and partially by 12 mmol/l NEM but not by Pefabloc, suggesting that the arFN fragment is divalent ion dependent and not a granulocyte protease in nature.

Under non-reducing conditions, the vWFCP shows M_r bands of 150, 140, 130 and 110 kDa by SDS-PAGE derived from the same polypeptide chain (300 kDa) [4]. The enzyme specifically cleaves the Y (842)-M (843) bond of the unfolded vWF A2 domain [31]. The protease was inhibited by 10 mmol/l EDTA and partially by 10 mmol/l NEM [3]. Furlan *et al.* observed that it is difficult to judge whether this inhibition is caused by a slowly reacting SH group in the protease or by a side reaction of another amino acid. Binding occurred to a heparin-Sepharose column [5] provided it was not equilibrated with 0.1 mol/l NaCl [4]. Therefore, the arFN fragment can hardly be associated with vWFCP due to its different M_r and to its binding to heparin-Sepharose column equilibrated with 0.1 mol/l NaCl.

TSP-1 showed a M_r value of 500 kDa, its type 1 properdin domains and the vWF A3 domain resulting in formation of complexes mediated the interaction of TSP-1 with folded vWF. Complex formation was increased by NEM and inhibited by EDTA [6,7]. The arFN fragment is unlikely to be associated with TSP-1 because of its different M_r, and there was also the finding that fragment activity is partially inhibited by 10 mmol/l NEM and that interaction between TSP-1 and vWF requires no unfolded vWF.

Matrix metalloproteinases are responsible for the degradation of extracellular macromolecules [32]. In particular, matrix metalloproteinase 2 (MMP-2, a 72 kDa gelatinase), a zinc-dependent proteinase active at extracellular environment pH, interacts with TSP-1 via its FN-like gelatin binding domain or a closely mapping site [33]. In turn, fibronectin induces MMP-2 secretion and activation [34]. MMP-2 co-purified with FN in chromatography on Sepharoses conjugated with gelatin, arginine or heparin. FN may only be resolved from MMP-2 by resorting to immobilized metal affinity chromatography (Co^{2+}, Ni^{2+}, Zn^{2+}) or a method employing a Fe^{3+} gel pH gradient [35,36]. However, it has not yet been demonstrated that MMP-2 controls vWF multimer size.

To the best of our knowledge, our data provide a demonstration of an FN-related substance responsible for the control of vWF multimer size. Further work will elucidate its role in clinical pathology.

References

1 Sakariassen KS, Bolhuis PM, Sixma JJ. Human blood platelets adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to subendothelium. *Nature* 1979; **279**:636–638.
2 Tsai H. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood* 1996; **87**:4235–4244.
3 Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood* 1996; **87**:4223–4234.
4 Gerritsen HE, Robles R, Lämmle B, Furlan M. Partial amino acid sequence of purified von Willebrand factor-cleaving protease. *Blood* 2001; **98**:1654–1661.
5 Fujikawa K, Suzuki H, Mc Mullen B, Chung D. Purification of human von Willebrand factor-cleaving protease and its identification as a new member of the metalloproteinase family. *Blood* 2001; **98**:1662–1666.
6 Xie L, Chesterman CN, Hogg PJ. Control of von Willebrand factor multimer size by thrombospondin-1. *J Exp Med* 2001; **193**:1341–1349.
7 Xie L, Chesterman CN, Hogg PJ. Reduction of von Willebrand factor by endothelial cells. *Thromb Haemost* 2000; **84**:506–513.
8 Kempfer AC, Farias CE, Carballo GA, Silaf MR, Lazzari MA. The effect of polyclonal antibody against fibronectin on von Willebrand factor multimeric size. *Thromb Res* 1999; **96**:161–167.
9 Mosesson M, Amrani DL. The structure and biologic activities of plasma fibronectin. *Blood* 1980; **56**:145–158.
10 Hynes R, Yamada K. Fibronectins: multifunctional modular glycoproteins. *J Cell Biol* 1982; **95**:369–377.
11 McDonagh RP, McDonagh J, Peterson TE, Thogersen HC, Skorstengaard K, Sottrup-Jensen L, *et al.* Amino acid sequence of the factor XIIIa acceptor site in bovine plasma fibronectin. *FEBS Lett* 1981; **127**: 174–178.
12 Rostagno A, Williams MJ, Baron M, Campbell ID, Gold LI. Further characterization of the NH2-terminal fibrin-binding site on fibronectin. *J Biol Chem* 1994; **269**:31938–31945.
13 Homandberg GA, Kramer-Bjerke J. Thrombospondin binds to amino-terminal fragments of plasma fibronectin. *Thromb Res* 1987; **48**: 329–335.
14 Barkalow FJB, Schwarzbauer JE. Localization of the major heparin-binding site in fibronectin. *J Biol Chem* 1991; **266**:7812–7818.
15 Ingham KC, Brew SA, Migliorini MM, Busby TF. Binding of heparin by type III domains and peptides from the carboxy terminal hep-2 region of fibronectin. *Biochemistry* 1993; **32**:12548–12553.

16 Lambert Vidmar S, Lottspeich F, Emod I, Planchenault T, Keil-Dlouha V. Latent fibronectin-degrading serine proteinase activity in N-terminal heparin-binding domain of human plasma fibronectin. *Eur J Biochem* 1991; **201**:71–77.
17 Lambert Vidmar S, Imhoff JM, Blondeau X, Emod I, Lottspeich F, Keil-Dlouha V. Potential proteolytic activity of human plasma fibronectin: fibronectin gelatinase. *Biol Chem Hoppe Seyler* 1990; **371**:117–128.
18 Emod I, Lafaye P, Planchenault T, Lambert Vidmar S, Imhoff JM, Keil Dlouha V. Potential proteolytic activity of fibronectin: fibronectin laminase and its substrate specificity. *Biol Chem Hoppe Seyler* 1990; **371**: 129–135.
19 Lambert Vidmar S, Lottspeich F, Emod I, Imhoff JM, Keil-Dlouha V. Collagen-binding domain of human fibronectin contains a latent type-IV collagenase. *Eur J Biochem* 1991; **201**:79–84.
20 Keil-Dlouha V, Planchenault T, Potential proteolytic activity of human plasma fibronectin. *Proc Natl Acad Sci USA* 1986; **83**:5377–5381.
21 Kempfer AC, Farías C, Silaf MR, Carballo GA, Lazzari MA. Degradation products of partially purified fibronectin induced the loss of large von Willebrand factor multimers at high shear rate [abstract]. *Thromb Haemost* 1999; **suppl 82**:100.
22 Thorell L, Blombäck B. Purification of the FVIII complex. *Thromb Res* 1984; **35**:431–450.
23 Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal Biochem* 1966; **15**:45–52.
24 Mekka S, Ingham K, Menache D. Rapid methods for isolation of human fibronectin. *Thromb Res* 1982; **27**:1–14.
25 Furlan M, Robles R, Galbusera M, Remuzzi G, Kyrle PA, Brenner B, *et al*. Von Willebrand factor-cleaving protease in thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome. *N Engl J Med* 1998; **339**:1578–1584.
26 Aihara M, Sawada Y, Ueno K, Morimoto S, Yoshida Y, de Serres M, *et al*. Visualization of von Willebrand factor multimers by immunoenzymatic stain using avidin-biotin peroxidase complex. *Thromb Haemost* 1986; **55**:263–267.
27 Kempfer AC, Silaf MR, Farías CE, Carballo GA, Woods AI, Lazzari MA. Binding of von Willebrand factor to collagen by flow cytometry. *Am J Clin Pathol* 1999; **111**:418–423.
28 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248–254.
29 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**:680–685.
30 Skrha J, Richter H, Hormann H. Evidence for the presence of a free N-terminal fibronectin 30-kDa domain in human plasma by quantitative determination with an indirect immunosorbent assay. *Anal Biochem* 1988; **173**:228–234.
31 Dent JA, Berkowitz SD, Ware J, Kasper CK, Ruggeri ZM. Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proc Natl Acad Sci USA* 1990; **87**:6306–6310.
32 Saito T, Oda Y, Sakamoto A, Tamiya S, Iwamoto Y, Tsuneyoshi M. Matrix metalloproteinase-2 expression correlates with morphological and immunohistochemical epithelial characteristics in synovial sarcoma. *Histopathology* 2002; **40**:279–285.
33 Bein K, Simons M. Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. *J Biol Chem* 2000; **275**:32167–32173.
34 Martin J, Eynstone L, Davies M, Steadman R. Induction of metalloproteinases by glomerular mesangial cells stimulated by proteins of the extracellular matrix. *J Am Soc Nephrol* 2001; **12**:88–96.
35 Smilenov L, Forsberg E, Zeligman I, Sparrman M, Johansson S. Separation of fibronectin from a plasma gelatinase using immobilized metal affinity chromatography. *FEBS Lett* 1992; **302**:227–230.
36 Johansson S, Smedsrod B. Identification of a plasma gelatinase in preparations of fibronectin. *J Biol Chem* 1986; **261**:4363–4366.