FEBS Letters 351 (1994) 345–348

FEBS 14474

Structural analysis of recombinant von Willebrand factor: identification of hetero- and homo-dimers

Bernhard Fischer, Artur Mitterer, Uwe Schlokat, Renate DenBouwmeester, Friedrich Dorner*

IMMUNO AG, Biomedical Research Center, Uferstraße 15, A-2304 Orth a.d. Donau, Austria

Received 6 June 1994; revised version received 21 July 1994

Abstract Wild-type full-length cDNA of von Willebrand factor (vWF) was expressed in CHO cells. Recombinant vWF (rvWF) was obtained and its molecular composition investigated by two-dimensional electrophoresis. Results of first dimension electrophoresis under non-reducing conditions showed that rvWF-dimer represents a mixture of three different species. Second dimension electrophoresis under reducing conditions revealed, that these protein species represent (i) homo-dimers of either two unprocessed or two fully processed, mature vWF polypeptides, and (ii) the hetero-dimer of unprocessed and mature rvWF monomers. Compared with vWF-dimers from human plasma, which contained predominantly proteolytically degraded polypeptides, recombinant vWF-dimers were shown to consist of non-proteolyzed subunits only.

Key words: Von Willebrand factor; Recombinant protein; Dimer; Structure analysis

1. Introduction

The adhesive protein von Willebrand factor exists in human plasma as a series of heterogeneous multimers ranging in size from about 450 kDa to more than 10,000 kDa [1–4]. The precursor polypeptide produced in endothelial cells, pre-pro-vWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma 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 primary dimer, containing two pro-vWF subunits, through disulphide bond linkage. Then protomeric units of the multimeric series are assembled into higher order multimers by disulphide bonding of dimers. vWF pro-peptide is cleaved from multimeric vWF before release from intracellular storage sites into circulation [1,3,5,6].

Electrophoretic analysis of plasma vWF dimer demonstrated a more complex pattern of repeating multimers, characterized by the presence of a more prominent band (primary dimer) surrounded by two (triplet structure) or more satellite bands [2,4,7]. Recently, two faster and two slower migrating satellite bands have been identified, running in a complex with the prominent band of the primary dimer of plasma vWF [8,9]. More detailed investigations showed, that only the minority of plasma vWF primary dimer molecules consisted of two intact vWF-monomer polypeptide chains, whereas other protein species represented more or less proteolytically degraded vWF fragments [9]. A cleavage site between Tyr^{842} and Met^{843} in vWF-monomer polypeptide chain has been identified [10].

In this study, wild-type vWF full-length cDNA was expressed in CHO cells. Recombinant vWF was obtained and its molecular structure investigated for the first time by two-dimensional electrophoresis to detect the polypeptide composition of recombinant von Willebrand factor dimers.

*Corresponding author. Fax: (43) (2212) 2716.

Abbreviations: Ag, antigen; CHO cells, Chinese hamster ovary cells; ELISA, enzyme-linked immunoabsorbent assay; PBS, phosphate-buffered saline; SDS, sodium dodecylsulfate; vWF, von Willebrand factor; rvWF, recombinant von Willebrand factor.

2. Materials and methods

2.1. Materials

Seakem Agarose HGT(P) and Gel Bond Film were purchased from FMC BioProducts. Acrylamide, *N,N'*-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), glycine, ammonium persulfate and alkaline phosphatase coloration chemicals were from Bio-Rad Laboratories. Normal plasma was from Immuno AG, Austria. Rabbit anti-human vWF polyclonal antibody and alkaline phosphatase conjugated anti-rabbit polyclonal antibody were from Dakopatts. Enzyme-linked immunoadsorbent assay for vWF was from Boehringer Mannheim. All other reagents were purchased from Sigma.

2.2. Construction of the expression vector phAct-vWF

The expression vector phAct-vWF originally represented a derivative of pSVβ [11]. Essentially, the SV40 promoter and SV40 16/19s intron of pSVβ were substituted by the human β-actin promoter and intron, and the bacterial β-galactosidase gene was replaced by the human vWF cDNA [12]. Human vWF cDNA was kindly provided by Dr. J.A. van Mourik (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).

2.3. Transfection and cell culture

20 μg phAct-vWF and 1 μg pSV-dhfr were transfected into dihydrofolate reductase deficient CHO-DXB11 cells [13] according the calcium phosphate cotransfection technique by Graham and Van der Eb [14]. Six hours after transfection, the medium was aspirated and the cells were exposed to 1 ml 15% glycerol in phosphate-buffered saline (PBS) for 1 min. Cells were washed with PBS and culture medium (DMEM/F12 1:1, containing 2 mmol/l glutamine, 0.075% bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, 10 μg/ml deoxyadenosine, 10 μg/ml adenosine and 10 μg/ml thymidine) was added. 48 hours later, cells were split into selection medium and single colonies were isolated after 2 weeks. Positive clones were identified by testing the supernatants for rvWF with anti-vWF ELISA. For scale up, rvWF producing CHO clone #808-68 was grown in selection medium in roller bottles.

2.4. SDS-agarose gel electrophoresis

SDS-agarose gel electrophoresis was performed in a vertical electrophoresis system using the buffer system and running conditions of Ruggeri and Zimmermann [2]. The separating gels (1.5% to 2% agarose concentration) and the stacking gel (0.8% agarose), both 1.5 mm thick, were prepared as described by previously [2]. Prior electrophoresis protein samples were solubilized as described earlier [2].

2.5. Two-dimensional SDS electrophoresis

Two-dimensional SDS electrophoresis was performed by a combination of a high-resolution SDS-polyacrylamide/agarose gel for the first

SSDI 0014-5793(94)00861-2

dimension and a SDS-polyacrylamide gel for the second dimension. SDS-polyacrylamide/agarose gels, containing 0.5% agarose and 3% acrylamide with 5% crosslinking, were prepared by melting 0.15 g agarose in 19 ml of distilled water. 3 ml acrylamide solution (30% acrylamide/1.6% bis-acrylamide) and 7.5 ml of 1.5 mol/l Tris-HCl buffer pH 8.8, containing 0.4% SDS were mixed and heated to 60°C. Agarose solution and acrylamide solution were mixed, and 15 μl TEMED and 0.5 ml ammonium persulfate (10 mg/ml) were added. The separation gel was poured between two prewarmed, inverted glass plates to form a 1.5 mm thick gel. Acrylamide polymerization was performed at 50°C for 30 min. Then agarose was solidified at room temperature. A 2.5% acrylamide stacking gel was prepared as described by Laemmli [15]. Protein samples were denatured in 60 mmol/l Tris-HCl buffer pH 6.8, containing 2% SDS. Electrophoresis was performed at constant voltage of 30 V for 16 h. For resolution in the second dimension, a 5% acrylamide SDS-polyacrylamide gel was prepared as described by Laemmli [15]. Individual gel lanes from the first dimension were excised and soaked for 30 min in 50 mmol/l Tris-HCl buffer pH 8.8, containing 1% SDS and 3% 2-mercaptoethanol at 65°C. Gel strips were washed three times with 50 mmol/l Tris-HCl buffer pH 6.8, containing 0.1% SDS, and were then immobilized at the top of the second dimension gel. Electrophoresis was performed at constant voltage of 200 V for 5 h. As reference proteins, prestained proteins with molecular weights of 205,000, 116,500, and 80,000 were used.

2.6. Immunoblotting

Polypeptides resolved by electrophoresis were transferred onto nitrocellulose membranes in 25 mmol/l Tris, 192 mmol/l glycine, 20% (vol/vol) methanol buffer [16]. Nitrocellulose membranes were blocked with 20 mmol/l Tris-HCl, pH 8.3, containing 0.9% NaCl and reacted with 1:1000 diluted rabbit anti-human vWF antibody for 16 h, followed by alkaline phosphatase conjugated goat anti-rabbit antibody. Coloration was done by incubation of the membranes in 100 ml substrate solution of 0.1 mol/l sodium bicarbonate buffer pH 9.8, containing 1 mmol/l $MgCl_2$, 1 ml *p*-nitro blue tetrazolium chloride (30 g/l in dimethylformamide), 1 ml 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine (15 g/l in dimethylformamide). The reaction was stopped by rinsing the membranes in water.

2.7. vWF antigen determination

The amount of vWF antigen in conditioned media of rvWF producing permanent cell lines was determined using an enzyme-linked immunoadsorbent assay.

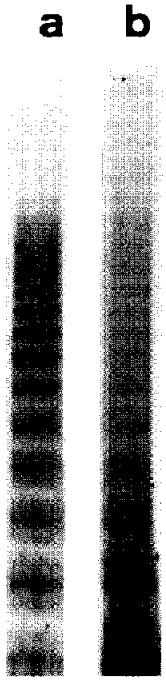

Fig. 1. Analysis of (a) plasma derived and (b) recombinant vWF by non-reducing 1.5% SDS-agarose gel electrophoresis.

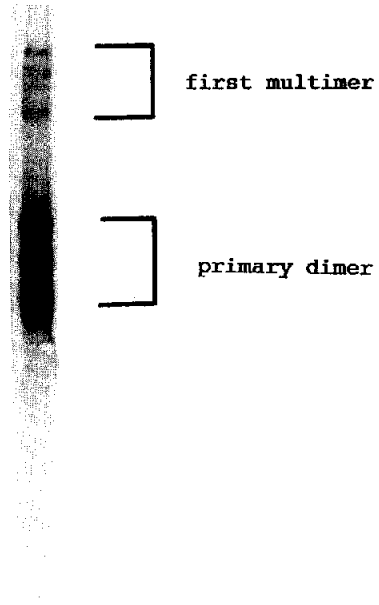

Fig. 2. Analysis of recombinant vWF by non-reducing 3% SDS-acrylamide/0.5% agarose gel electrophoresis.

3. Results

The wild-type full-length cDNA of von Willebrand factor, cloned in the expression vector phAct-vWF, was expressed under the control of the human β-actin promotor in CHO cells. Expression levels of secreted rvWF reached about 5 mg/liter in the conditioned media.

Multimer analysis was performed by SDS-agarose gel electrophoresis and SDS-polyacrylamide/agarose gel electrophoresis. Using 1.5% agarose, rvWF exhibited a multimeric pattern comparable with plasma vWF (Fig. 1). However, no separation into triplet structure of plasma vWF was obtained for rvWF. To get a high resolution picture of rvWF heterogeneity, SDS-3% acrylamide/0.5% agarose gel electrophoresis was carried out (Fig. 2). Results showed, that the primary dimer of rvWF can be separated into three protein bands containing approximately equal amounts. The first multimer (consisting of two dimers) was separated into even more bands. To analyse the molecular structure of the primary dimer of vWF, two-dimensional electrophoresis was carried out with non-reducing SDS-polyacrylamide/agarose gel electrophoresis in the first dimension and reducing SDS-polyacrylamide gel electrophoresis in the second dimension. On both sides, additional sample wells were prepared for prestained molecular weight marker proteins and denatured-reduced vWF. Plasma vWF and rvWF were analysed simultaneously using identical conditions. Fig. 3 shows the two-dimensional electrophoresis of plasma vWF. The results showed, that in the first dimension, the primary dimer of plasma vWF was separated into several protein bands. These individual protein bands were numbered using a nomenclature similar to the terminology introduced by Dent et al. [9]. In agreement with results obtained by Dent et al. [9], the protein band identified as 1U was comprised exclusively of intact monomers of vWF with a molecular weight of about 225 kDa. From the apparent molecular weight of about 450 kDa in the first dimension, protein IU contained two subunits linked by

disulphide bonds. All the other bands migrated faster than 1U and contained proteolytically cleaved vWF fragments. However, due to an apparent higher resolution of vWF in the SDS-polyacrylamide/agarose gel system used in this study, the broad protein band labeled as $1P_1$ by Dent et al. [9] was shown to consist of at least four different species, labeled $1P_{1a}$, $1P_{1b}$, $1P_{1c}$ and $1P_{1d}$ in the present investigation. In the second dimension gel, vWF fragment compositions of the protein species $1P_{1b}$, $1P_2$ and $1P_3$ were similar to those reported previously [9].

In contrast, two-dimensional electrophoresis of rvWF exhibited a quite different pattern (Fig. 4). High-resolution SDS-polyacrylamide/agarose gel electrophoresis showed three protein bands, labeled $1U_a$, $1U_b$ and $1U_c$. Second dimension electrophoresis showed, that species $1U_a$ contained exclusively mature vWF-monomer peptide chains. From the apparent molecular weight of 450 kDa in the first dimension, $1U_a$ consisted of two identical vWF subunits, each of about 225 kDa. The position of protein $1U_a$ in the first and second dimension gel corresponded to the position of the protein species 1U of plasma vWF (Fig. 3). Protein $1U_b$ apparently contained one mature vWF subunit and one pro-vWF subunit, whereas protein $1U_c$ apparently contained two pro-vWF subunits linked by disulphide bonds.

4. Discussion

Full-length cDNA of vWF has been cloned and rvWF has been expressed in heterologous cells for several years [6,17–25]. Expression of rvWF in heterologous cells, such as monkey kidney COS-1 [17–19,21,22] and CV-1 cells [23], baby hamster kidney cells [20], Chinese hamster ovary cells [20,22], human fibroblasts and mouse fibroblasts [20] resulted in the secretion of rvWF dimers and multimers, in a pattern apparently similar

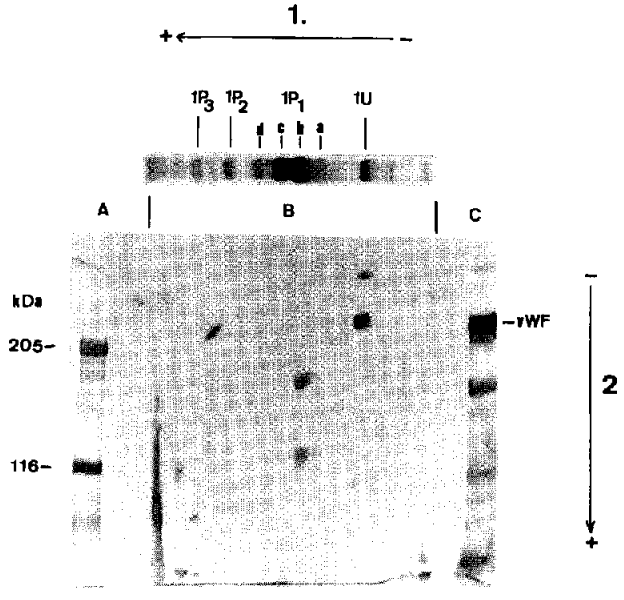

Fig. 3. Two-dimensional analysis of plasma vWF. Plasma vWF was separated in the first dimension by non-reducing high resolution SDS-3% acrylamide/0.5% agarose gel electrophoresis. Individual gel lanes containing the primary dimer vWF-related proteins were cut out, incubated in reducing buffer, mounted on top of a 5% acrylamide gel, and electrophoresed in the second dimension (B). Simultaneously, denatured-reduced vWF (C) and prestained reference proteins (A) were analysed.

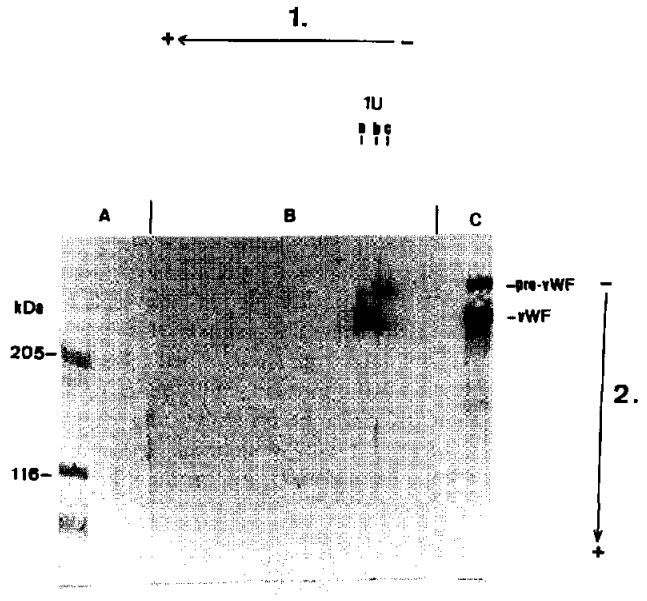

Fig. 4. Two-dimensional analysis of rvWF. rvWF was separated in the first dimension by non-reducing high resolution SDS-3% acrylamide/0.5% agarose gel electrophoresis. Individual gel lanes containing the primary dimer vWF-related proteins were cut out, incubated in reducing buffer, mounted on top of a 5% acrylamide gel, and electrophoresed in the second dimension (B). Simultaneously, denatured-reduced rvWF (C) and prestained reference proteins (A) were analysed.

to plasma vWF, when analysed in non-reducing low-resolution SDS-agarose gel electrophoresis systems. In contrast, SDS-PAGE at reducing conditions revealed that large proportions of secreted rvWF still contained the pro-polypeptide [6,17–23]. However, since this structural property is not reflected by conventional low-resolution SDS-agarose gel electrophoresis it has been ignored in interpretation of conventional agarose gel electrophoresis of recombinant vWF. More detailed structural analyses of rvWF molecules have not been reported.

In this study, for the first time two-dimensional electrophoresis has been used to analyse structural properties of rvWF and were compared to the plasma vWF structure. The investigation showed that rvWF differs significantly from plasma vWF. Plasma vWF at non-reducing conditions resulted at least seven polypeptides related to the primary dimer of vWF. In contrast, structural analysis of rvWF under non-reducing conditions resulted in only three separate protein species related to the primary dimer. Second dimension electrophoresis of rvWF under reducing conditions separated the polypeptide compositions of each protein band related to the primary dimer: species $1U_a$ consisted exclusively of two mature vWF polypeptide chains linked by disulphide bonds, $1U_b$ represented a hybrid molecule of one mature vWF polypeptide chain and one pro-vWF polypeptide chain linked by disulphide bonds, and $1U_c$ was the pro-vWF-dimer. In contrast, the primary dimer of plasma vWF consisted of predominantly proteolytically degraded polypeptides.

From previous experiments it has been established, that (a) vWF pro-polypeptide is not directly involved in dimerization of pro-vWF-monomers, and (b) the pro-peptide is an obligatory structural component for multimerizing vWF-dimers [6,18]. However, before release of vWF from endothelial

cells or intracellular storage sites, the pro-peptide is nearly completely cleaved from multimeric vWF [1]. Thus, the concentration of pro-vWF in the circulation is very low. In contrast, this study showed that all three possible combinations of pro-vWF polypeptide and mature vWF polypeptide, to form the primary dimer, were found in significant amounts. This phenomenon shows that, using current expression systems, the rates of vWF expression, multimerisation, secretion and propeptide processing may not be sufficiently balanced.

The biotechnological production of recombinant proteins, such as vWF, represents an important step in large scale manufacturing of protein-based drugs. The results of this investigation show, that rvWF obtained from transformed cells, in contrast to plasma derived vWF, represents fully intact proteins rather then a mixture of different proteolytically degraded products. Thus, recombinant von Willebrand factor could prove to be a much better defined and purer product than vWF isolated from human plasma.

References

[1] Ruggeri, Z.M. and Ware, J. (1992) Thromb. Haemost. 67, 594–599.
[2] Ruggeri, Z.M. and Zimmerman, T.S. (1981) Blood 57, 1140-1143.
[3] Ruggeri, Z.M. and Ware, J. (1993) FASEB J. 7, 308–316.
[4] Mannucci, P.M., Abildgaard, C.F., Gralnick, H.R., Hill, F.G.H., Hoyer, L.W., Lombardi, R., Nilsson, I.M., Tuddenham, E. and Meyer, D. (1985) Thromb. Haemost. 54, 873–877.
[5] Lynch, D.C. (1993) Ann. N.Y. Acad. Sci. 138, 138–152.
[6] Pannekoek, H. and Voorberg, J. (1989) Bailliere's Clin. Haematol. 2, 879–896.
[7] Baillod, P., Affolter, B., Kurt, G.H. and Pflugshaupt, R. (1992) Thromb. Res. 66, 745–755.
[8] Ciavarella, G., Ciavarella, N., Antonecchi, S., De Mattia, D., Ranieri, P., Dent, J., Zimmerman, T.S. and Ruggeri, Z. (1985) Blood 66, 1423–1429.
[9] Dent, J.A., Galbusera, M. and Ruggeri, Z.M. (1991) J. Clin. Invest. 88, 774–782.
[10] Dent, J.A., Berkowitz, S.D., Ware, J., Kasper, C. and Ruggeri, Z.M. (1990) Proc. Natl. Acad. Sci. USA 87, 6306–6310.
[11] MacGregor, G.R. and Caskey, C.T. (1989) Nucleic Acid Res. 17, 2365–2368.
[12] Verweij, C.L., Diergaarde, P.J., Hart, M. and Pannekoek, H. (1986) EMBO J. 5, 1839–1847.
[13] Urlaub, G. and Chasin, L. (1980) Proc. Natl. Acad. Sci. USA 77, 4216–4220.
[14] Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456–467.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Ribba, A.S., Voorberg, J., Meyer, D., Pannekoek, H. and Pieto, G. (1992) J. Biol. Chem. 267, 23209–23215.
[18] Wise, R.J., Pittman, D.D., Handin, R.I., Kaufman, R.J. and Orkin, S.H. (1988) Cell 52, 229–236.
[19] Verweij, C.L., Hart, M. and Pannekoek, H. (1987) EMBO J. 6, 2885–2890.
[20] Meulien, P., Nishino, M., Mazurier, C., Dott, K., Pietu, G., Jorieux, S., Pavirani, A., Girma, J.P., Oufkir, D., Courtney, M. and Meyer, D. (1992) Thromb. Haemost. 67, 154–160.
[21] Mayadas, T.N. and Wagner, D.D. (1992) Proc. Natl. Acad. Sci. USA 89, 3531–3535.
[22] Kaufman, R.J., Wasley, L.C., Davies, M.V., Wise, R.J., Israel, D.I. and Dorner, A.J. (1989) Mol. Cell. Biol. 9, 1233–1242.
[23] Voorberg, J., Frontijn, R., Calafat, J., Janssen, H., Mourik, van J.A. and Pannekoek, H. (1993) EMBO J. 12, 749–758.
[24] Journet, A.M., Saffaripour, S., Cramer, E.M., Tenza, D. and Wagner, D.D. (1993) Eur. J. Cell Biol. j0, 31–41.
[25] Lynch, D.C., Zimmerman, T.S. and Ruggeri, Z.M. (1986) Br. J. Haematol. 64, 15–20.