Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells

(protein processing/Weibel-Palade bodies/extracellular matrix/hemostasis)

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Communicated by Jerrold Meinwald, December 3, 1986

ABSTRACT The intracellular site of cleavage of pro-von Willebrand factor subunit and the subsequent fate of the propolypeptide (von Willebrand antigen II) and of the mature von Willebrand factor (vWf) were investigated. Both the propolypeptide, which was found to be a homodimer of noncovalently linked subunits, and mature vWf were released from Weibel-Palade bodies of endothelial cells following stimulation with secretagogues. The stoichiometry of the two released proteins was essentially equimolar. This indicates that vWf and the propolypeptide were packaged into the Weibel-Palade bodies as one unit, pro-vWf, and that the proteolytic cleavage of pro-vWf is likely to be a post-Golgi event. The association of prosequences into dimers supports their hypothetical role in the multimerization process. After secretion, the two proteins were distributed differently, as based on the following observations. The propolypeptide did not associate with vWf in the culture medium, did not codistribute with vWf in the extracellular "patches of release" on stimulated endothelial cells, and was not detected in the endothelial cell extracellular matrix, which did contain vWf. Additionally, in contrast to vWf, the propolypeptide did not bind to the matrix of human foreskin fibroblasts. Since the propolypeptide does not associate with vWf and does not interact with extracellular matrices *in vitro*, it is highly unlikely that it would promote platelet adhesion to subendothelium *in vivo*.

The bleeding disorder of von Willebrand disease is associated with deficiency or defects in two proteins: von Willebrand factor (vWf) (1) and von Willebrand antigen II (2). A common biosynthetic origin has been found for the two proteins with von Willebrand antigen II being the propolypeptide of vWf (3), separated from the vWf subunit by cleavage during intracellular processing (4, 5). Both vWf and the propolypeptide are secreted constitutively from endothelial cells (6, 7), and they are the only two proteins thus far found in the storage organelles, the Weibel-Palade bodies (7, 8). Only the largest and biologically most active vWf multimers are released from the Weibel-Palade bodies (9) following the action of secretagogues, such as thrombin (10, 11) and the calcium ionophore A23187 (11). It is not known whether the propolypeptide is also released from the organelles or whether it is internally degraded during recycling of the Weibel-Palade bodies. vWf is an adhesive glycoprotein mediating attachment of platelets to subendothelium where it is present (12) bound to a noncollagenous component (13, 14). The biological function of von Willebrand antigen II, the unusually large propolypeptide (100 kDa) is unknown. If the propolypeptide remains associated as a "light chain" with

vWf multimers after cleavage or if it has affinity for extracellular matrices of its own, then it also could be involved in the platelet-basement membrane adhesion process. To answer some of these questions we have investigated the release of the propolypeptide from the Weibel-Palade bodies and examined possible interactions of the propolypeptide with mature vWf, with endothelial cell surface, and with extracellular matrices.

MATERIALS AND METHODS

Cells and Culture Conditions. Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described (8, 15); only primary cultures were used. Cells were cultured in McCoy's 5A medium (Flow Laboratories) containing 20% (vol/vol) fetal bovine serum. Human foreskin fibroblasts in sixth passage were a gift from Sue Schmidt (University of Rochester) and were grown under the same conditions. For metabolic labeling, cells were grown for 3 days in the presence of [³⁵S]methionine (25 μ Ci/ml; 1148 Ci/mmol; 1 Ci = 37 GBq) (New England Nuclear).

Treatment of Cells with A23187 or Thrombin. Endothelial cells were metabolically labeled for 3 days, chased for 6 hr in unlabeled medium (4), and then treated with the secretagogues as described (9).

Antibodies and Immunofluorescent Staining. The preparation of polyclonal antisera against vWf, pro-vWf (which recognizes both the propolypeptide and the mature vWf subunit) and monoclonal antibodies against the propolypeptide were described (8, 16). Monoclonal antibody against vWf was prepared in the same manner as the monoclonal antibodies against the propolypeptide (16). For immunofluorescent staining (8), cells were grown on glass coverslips, fixed in 3.7% (vol/vol) HCHO, and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline. For double-label staining, incubations of cells were performed in the following order: (i) monoclonal anti-propolypeptide antibody, undiluted, for 1 hr; (ii) rhodamine-conjugated goat antibody to mouse IgG, diluted 1:10, for 30 min; (iii) anti-vWf antiserum, diluted 1:30, for 30 min; and (iv) fluorescein-conjugated goat anti-rabbit immunoglobulin, diluted 1:10, for 30 min.

Iodination. Purified vWf (16 μ g) and the vWf propolypeptide (15 μ g) were separately radiolabeled with 0.3 mCi of carrier-free Na¹²⁵I (Amersham) using 0.5 μ g of Iodo-Gen (Pierce). Unbound ¹²⁵I was removed by gel filtration using a PD-10 column (Pharmacia). Similar amounts of vWf (10⁷ cpm, 7.8 μ g) and of the propolypeptide (10⁷ cpm, 9.7 μ g) were incubated with the fibroblasts. Iodination of the endothelial

cell surface was carried out as described by Graham *et al.* (17).

Purification of Propolypeptide, vWf, and Gel Electrophoresis. The proteins were immunopurified from culture medium or cell lysates as described (18). When purification was to be in the absence of detergents, 0.02 M Tris-HCl, pH 8.3, was used for washes. Plasma vWf propolypeptide was purified from therapeutic concentrates (Koate) as a byproduct during the purification of factor VIII (19). Further purification of the propolypeptide was achieved using a Mono Q (HR 5/5, Pharmacia) column with a linear NaCl gradient (0.1–1 M) in 20 mM Hepes, pH 7.2. The propolypeptide eluted as a single peak at 0.35 M NaCl. Homogeneity was confirmed by NaDodSO₄ gel electrophoresis and silver staining (20, 21). NaDodSO₄/polyacrylamide gels were prepared as described by Laemmli (22).

Stoichiometry of Secreted Propolypeptide and Mature vWf Subunit. The [³⁵S]methionine-labeled proteins were copurified with anti-pro-vWf antiserum and electrophoresed, reduced, on 6% polyacrylamide gels. Autoradiographs of these gels were scanned with a densitometer. The stoichiometry was determined based on the number of methionine residues in the proteins: 14 in the propolypeptide and 38 in the mature vWf subunit as determined by Verweij *et al.* (23).

Gel Filtration and Sucrose Density Centrifugation of the Propolypeptide. The vWf propolypeptide was applied to a 0.7 × 50 cm Sephacryl S-300 column (Pharmacia) equilibrated with 20 mM Hepes (pH 7.2), 200 mM NaCl, 10% (wt/vol) glycerol, and 0.02% azide. Thyroglobulin (84 Å), catalase (52 Å), and bovine serum albumin (35 Å) were used as calibration standards (24). Blue dextran (M_r , >10⁶) and orange G (M_r , 452) were used to determine V_0 and V_t , respectively. Fractions (200 µl) were collected at a flow rate of 1 ml/hr. Elution positions were determined by the Bradford protein assay (25). Elution positions of the propolypeptide also were determined by ELISA using the anti-propolypeptide monoclonal antibody. Ultracentrifugation was carried out in a Beckman SW60Ti rotor at 40,000 rpm for 16 hr at 2°C using a 5–20% (wt/vol) sucrose gradient in buffer containing 20 mM Hepes (pH 7.2), 200 mM NaCl, and 0.02% azide. Aliquots of 100 µl of the standards, catalase (11.3 S), fibrinogen (7.9 S), and bovine serum albumin (4.3 S) (24), each at 1 mg/ml, were layered onto parallel gradients and centrifuged simultaneously. Following centrifugation, the gradient tubes were punctured at the bottom, and 29 fractions (150 µl) were collected. Positions of the propolypeptide and the protein standards were determined.

Calculation of Native Molecular Weight of the Propolypeptide. Native molecular weight was calculated from the Stokes radius and sedimentation coefficient by the method of Siegel and Monty (24). A partial specific volume of 0.714 g/cm³ was calculated for the propolypeptide by the method of Cohn and Edsall (26) using the amino acid sequence composition derived from the cDNA sequence of the cloned protein (23).

RESULTS

vWf and its large propolypeptide are secreted constitutively from endothelial cells in culture. To examine if the propolypeptide is also released with vWf by the regulated pathway, human umbilical vein endothelial cells were treated with secretagogues. Cells were metabolically labeled for 3 days with [³⁵S]methionine, chased for 6 hr in unlabeled medium, and then treated for 10 min with the ionophore A23187 or thrombin. vWf and its propolypeptide were purified with polyclonal anti-pro-vWf antiserum and analyzed, reduced, on a 6% polyacrylamide gel (Fig. 1). The nonphysiologic (A23187) and physiologic (thrombin) secretagogues caused release of both vWf and the propolypeptide into the culture medium (Fig. 1, lanes c and d). By scanning of autoradi-

FIG. 1. Constitutive secretion and release of vWf and its propolypeptide by endothelial cells. [³⁵S]Methionine-metabolically labeled vWf and the propolypeptide were purified with anti-pro-vWf antiserum from culture medium from endothelial cells labeled for 3 days (lane a), 10 min (lane b), 10 min in the presence of 10 µM A23187 (lane c), and 10 min in the presence of 1 unit/ml thrombin (lane d). Autoradiograph of a reduced 6% polyacrylamide gel is shown.

graphs of gels from four separate experiments, we have determined that the stoichiometry of the released propolypeptide to the released vWf subunit was 1.1:1, essentially equimolar. The amount of propolypeptide relative to vWf was somewhat lower in the constitutively secreted material (0.7:1). This was probably due to preferential extracellular degradation of the propolypeptide over the 3-day labeling period, since incubation of the material released by A23187 stimulation for prolonged periods with human foreskin fibroblasts changed the ratio of propolypeptide to vWf to that seen in constitutively secreted material (data not shown).

To determine whether extracellular patches of vWf that appear after release also contain the propolypeptide, endothelial cells were grown on glass coverslips and fixed before (Fig. 2 a and d) and after (Fig. 2 b and e) stimulation with A23187. Double-label immunofluorescent staining of the permeabilized cells with polyclonal anti-vWf antiserum and monoclonal anti-propolypeptide antibodies showed that the propolypeptide did not codistribute with vWf in the release patches (Fig. 2 b and e). Several other monoclonals against the propolypeptide also bound to the intracellular propolypeptide but failed to show surface staining. In addition, the extracellular matrix contains vWf (refs. 13 and 14 and Fig. 2 a and c) but not propolypeptide (Fig. 2 d and f).

Since proteins in the extracellular matrix interact very closely and their structure might not be flexible, it is conceivable that the propolypeptide epitopes were not accessible to the different monoclonals. We, therefore, studied the binding of the propolypeptide to foreign matrices with which vWf is known to interact (13, 14). Human foreskin fibroblasts grown on coverslips were covered with culture medium containing vWf at 10 µg/ml or propolypeptide at 10 µg/ml purified from plasma. The coverslips were fixed and stained with polyclonal anti-pro-vWf antiserum at 24 and 72 hr. At both time points, vWf but not the propolypeptide was found to be associated with the matrix, shown for the 72-hr incubation in Fig. 3. In addition, approximately equal amounts of iodinated plasma-derived propolypeptide and vWf were added separately to human foreskin fibroblast cultures, and, after a 2-day incubation, samples from the culture medium and from the lysed cells, which included extracellular matrix, were examined by gel electrophoresis (Fig. 4). While vWf clearly bound to the cells and was seen after a 2-hr autoradiographic exposure, days of exposure were needed to detect a trace of propolypeptide in the cell lysate (data not shown). Similar results were obtained with

FIG. 2. Distribution of vWf and its propolypeptide in control endothelial cultures and cultures treated with ionophore A23187. Double-label immunofluorescent staining of permeabilized cells with anti-vWf antiserum (*a-c*) and monoclonal antibody against the propolypeptide (*d-f*). In control cells (*a* and *d*), vWf and the propolypeptide codistribute in the Weibel-Palade bodies (arrowheads) but only vWf is seen in the strands of extracellular matrix (*m*). (*b* and *e*) Cells treated for 10 min with A23187. The majority of Weibel-Palade bodies have disappeared and instead bright patches of fluorescence containing vWf (arrowhead), but not propolypeptide, are present on the cell surface. (*c* and *f*) Field of dense extracellular matrix containing vWf and lacking the propolypeptide. (*a*, *b*, *d*, *e*, bar = 10 μ m; *c*, *f*, bar = 20 μ m.)

native unpurified vWf and propolypeptide secreted constitutively by endothelial cells. Culture medium from metabolically labeled umbilical vein endothelial cells was added to human foreskin fibroblasts. After a 1-day incubation, vWf and the propolypeptide were purified from the medium and from the washed, lysed fibroblasts using anti-pro-vWf antiserum and analyzed, reduced, on polyacrylamide gels. Only vWf and not the propolypeptide associated with the cells (data not shown). It appears, therefore, that after secretion or stimulated release, vWf and its propolypeptide follow separate routes, with some of the vWf associating with the surface of the activated cells and the extracellular matrix and with all of the propolypeptide remaining in soluble form.

To examine if the secreted vWf forms a noncovalent complex with the 100-kDa propolypeptide, culture medium from metabolically labeled endothelial cells was purified using polyclonal or monoclonal anti-vWf antibodies in the absence of denaturing agents. The purified material was analyzed by gel electrophoresis (data not shown). No propolypeptide was found in the purified samples arguing that the two proteins do not form a noncovalent complex.

To determine if the secreted propolypeptide is monomeric or forms oligomers by noncovalent associations with itself, we have estimated the native molecular weight of the

propolypeptide purified from plasma. Gel filtration analysis of the propolypeptide by a plot of its elution position relative to standards (Fig. 5A) yielded a Stokes radius of 66 Å. Sedimentation of the purified protein in a 5–20% (wt/vol) sucrose gradient produced a single peak with a sedimentation coefficient of 6.7 S (Fig. 5B). Sucrose gradient centrifugation of citrated, pooled normal plasma followed by ELISA of gradient fractions using anti-pro-vWf antiserum to detect the propolypeptide yielded an identical sedimentation coefficient. From these data an estimated molecular size of 176 kDa

FIG. 3. Incorporation of vWf and of the propolypeptide into a foreign extracellular matrix. Human foreskin fibroblasts were incubated for 72 hr in the presence of plasma vWf at 10 μ g/ml (*a*) or the propolypeptide at 10 μ g/ml (*b*). The cells were then fixed and stained by fluorescence with polyclonal anti-pro-vWf antiserum that recognizes both proteins. (Bar = 20 μ m.)

FIG. 4. Binding of iodinated plasma vWf and plasma propolypeptide to fibroblasts. An identical number of counts in radiolabeled propolypeptide (*a*) and vWf (*b*) were added to media to be overlaid onto human foreskin fibroblast cultures. Examination of this media on reduced gels before (data not shown) and after (Medium) a 48-hr incubation with the cells showed that the propolypeptide was labeled more efficiently and was not degraded by the cells. After the incubation period cells were lysed and identical volumes of lysates were reduced and submitted to electrophoresis (Cells). The figure is a composite of autoradiographs of a 6% polyacrylamide gel. Exposures were 4 hr for medium and 1 day for cells.

FIG. 5. (A) Determination of Stokes radius of plasma propolypeptide. Purified propolypeptide (100 μ l, 23 μ g) was submitted to gel filtration in a Sephacryl S-300 column (●). Standards shown were bovine serum albumin (Δ), catalase (□), and thyroglobulin (○). (B) Determination of the sedimentation coefficient of the propolypeptide. Purified propolypeptide (100 μ l, 67 μ g) was sedimented through a 5–20% sucrose gradient (●). Standards included bovine serum albumin (Δ), fibrinogen (□), and catalase (○). Identical elution position was obtained with 50 times less propolypeptide (data not shown), indicating that native size of the propolypeptide was independent of protein concentration.

and a frictional coefficient of 1.79 were calculated. Furthermore, when the same column was equilibrated in 6 M guanidine hydrochloride, the denatured propolypeptide eluted in a lower molecular size region between β -galactosidase (130 kDa) and bovine serum albumin (68 kDa). Thus, the native molecular size value obtained was consistent with the propolypeptide circulating as a dimer composed of noncovalently associated identical subunits of 90–100 kDa as determined by NaDodSO₄/PAGE (2, 3) and examination of cDNA clones covering the prosequence region of pro-vWf, which predicts an unglycosylated protein of 81 kDa (3, 23).

DISCUSSION

vWf and its propolypeptide share the same intracellular path. The near 1:1 stoichiometry of vWf and its propolypeptide released from the Weibel–Palade bodies indicates that they were packaged as intact pro-vWf molecules and that the cleavage occurred only after the protein was sorted into the Weibel–Palade body. A strong noncovalent association between the propolypeptide and the mature vWf is an unlikely explanation for this stoichiometry as it was not observed after secretion. Equimolar stoichiometry of proteolytic fragments of processed hormones has been observed in secretory vesicles (27), and the site of proinsulin cleavage has been localized by sophisticated immunoelectron microscopic methods to the coated secretory granules (28). Since the multimerization of vWf appears to begin in the trans-Golgi apparatus (29) and with cleavage being probably a post-Golgi processing step, it is most likely that multimerization precedes the cleavage of the prosequence. An earlier study demonstrating a high proportion of pro-vWf subunits in newly synthesized vWf multimers (18) supports the same conclusion. The targeting signal for the regulated pathway (30) could be located anywhere on the pro-vWf subunit.

In contrast to their common intracellular path, vWf and its propolypeptide separated after secretion from endothelial cells. This conclusion came from the following observations: both proteins were released from endothelial cells after treatment with secretagogues (Fig. 1), but only vWf associated with the stimulated cells (Fig. 2 *b* and *e*). Some of the secreted vWf participates in the endothelial cells extracellular matrix (refs. 8, 13, and 14 and Fig. 2*c*) but the propolypeptide was not detected in the matrix by immunofluorescence staining (Fig. 2*f*). The propolypeptide also did not bind to extracellular matrices of foreign cells, as seen both by fluorescence staining (Fig. 3*b*) and examination of bound protein by gel electrophoresis (Fig. 4, lane *c*). Even in the soluble fraction the two proteins appeared to be independent

of each other, since the propolypeptide did not copurify with the vWf in the absence of detergents (data not shown).

The Arg-Gly-Asp sequence was found in the C-terminal region of the propolypeptide (23), indicating that it could interact with cellular receptors for adhesive glycoproteins (31). However, while vWf appears to associate with stimulated endothelial cells (ref. 9 and Fig. 2*b*), immunofluorescent staining with monoclonal antibodies against the propolypeptide did not demonstrate the presence of the propolypeptide in the extracellular patches of vWf (Fig. 2*e*). We were unable to detect the propolypeptide or vWf on the stimulated endothelial cells by iodination (data not shown), indicating that the fraction of released vWf associating with the cell surface is very small. After stimulation of release of platelet α granules containing both the propolypeptide and vWf (32–34), the large majority of propolypeptide is found in the supernatant (32), while 65% of platelet vWf remains associated with the platelets (33). Some of this released vWf is clearly exposed on the activated platelet surface (35).

We have estimated the apparent molecular size of the circulating propolypeptide to be around 176 kDa; a number significantly higher than previously thought (2). In contrast, our results were obtained with highly purified protein and included in the calculations both the sedimentation coefficient and the Stokes radius of the molecule. From this native size, we conclude that the propolypeptide is a homodimer. The intracellular multimerization of vWf occurs by disulfide bonding of dimeric protomers in the N-terminal region of the vWf subunit close to the prosequence (16). We have proposed that the prosequence might be involved in the alignment of the protomers, facilitating the cross-linking process (18), and it is possible that the noncovalent propolypeptide dimers are formed at that time.

It seems unlikely that the sole function of the propolypeptide would be its hypothetical role in vWf processing. A domain of much smaller size than 100 kDa could assure binding of the protomers. Since both the propolypeptide and vWf have the same genetic (3) and secretory controls (Fig. 1), a similar function for them in hemostasis is possible. We have, therefore, examined the interaction of the propolypeptide with the extracellular matrix—the *in vitro* model of basement membrane. Unlike vWf (13, 14), the propolypeptide did not bind to the extracellular matrices (Figs. 2, 3, and 4) and, therefore, it could not support the adhesion of platelets to subendothelium. Because of the separate fate of vWf and its propolypeptide after secretion, if a role in hemostasis for the propolypeptide were to be found, it is likely to be independent of vWf.

The authors acknowledge Dr. Duane Schroeder and Cutter Laboratories for supplying the therapeutic concentrates; Susan Malerk, Christina Nicot, and Theresa Smudzin for technical assistance; and Carol Weed for preparation of the manuscript. This work was supported by Grants HL-30616 and HL-34050 from the National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, with additional support to P.J.F. from a Grant-in-Aid from the American Heart Association. This work was done during the tenure of an Established Investigatorship from the American Heart Association (D.D.W.) and with funds contributed in part by the New York Affiliate, Inc.

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