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Research Article

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Endothelial Cell Synthesis of von Willebrand Antigen II, von Willebrand Factor, and von Willebrand Factor/von Willebrand Antigen II Complex

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Abstract

von Willebrand antigen II (vW AgII) and von Willebrand factor (vWf) are immunochemically distinct proteins that are deficient in the plasma and platelets of patients with severe von Willebrand's disease. Normal human umbilical vein endothelial cells were cultured in the presence of [³⁵S]methionine. Crossed immunoelectrophoresis of endothelial cell supernates and detergent-solubilized endothelial cells demonstrated specific incorporation of the [³⁵S]methionine into vW AgII. Furthermore, when endothelial cells were lysed in the presence of proteolytic inhibitors, a second, less anodal peak was identified on crossed immunoelectrophoresis. This peak represented a complex of vW AgII and vWf and demonstrated a reaction of complete identity with the vW AgII immunoprecipitate. When plasma, serum, or platelets were evaluated by crossed immunoelectrophoresis, this "complex" peak was not present. When antibodies to vWf, fibronectin, or fibrinogen were present in the first dimension of crossed immunoelectrophoresis, only the antibodies to vWf removed the complex. Radioiodinated polyclonal and monoclonal antibodies to vWf also localized vWf to this complex. Under reducing conditions, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled immunoprecipitates indicated that the molecular weight of vW AgII is 98,000 and that vWf was present as two species of 220,000 and 260,000 mol wt, respectively. Immunofluorescent microscopy of endothelial cells demonstrated colocalization of vW AgII and vWf in endothelial cells with intense immunostaining of the same subcellular granules.

Introduction

von Willebrand's disease is characterized by a deficiency or structural abnormality in von Willebrand factor (vWf)¹ (1-3). A second protein, von Willebrand antigen II (vW AgII), is also deficient or absent in von Willebrand's disease (4). While both vWf and vW AgII have not been shown to share antigenic

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1. *Abbreviations used in this paper:* DDAVP, 1-desamino-8-D-arginine-vasopressin; PMSF, phenylmethylsulfonylfluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vW AgII, von Willebrand antigen II; vWf, von Willebrand factor.

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determinants (4, 5), their levels in plasma are linearly associated (6). vWf and vW AgII are both synthesized by cultured human endothelial cells and released concomitantly after *in vivo* stimulation with 1-desamino-8-D-arginine-vasopressin (DDAVP) (7). Both proteins are also found in platelets and are released together after platelet activation (8).

vWf is a glycoprotein that circulates in plasma as a series of high molecular weight multimers (1-3) and mediates the adhesion of platelets to exposed subendothelium (9). These multimers are comprised of 220,000-mol-wt subunits that are held together by disulfide bonds and range in molecular weights up to 20×10^6 . vWf is measured antigenically as von Willebrand antigen or Factor-VIII-related antigen (10) and functionally as the cofactor that mediates platelet aggregation in the presence of the antibiotic ristocetin (11). vWf is synthesized in endothelial cells (12-14) and megakaryocytes (15). Studies of vWf synthesized in cultured human umbilical vein endothelium indicate processing that includes not only glycosylation but also cleavage of a 260,000-mol-wt precursor molecule into the 220,000-mol-wt vWf subunit (14). Intracellularly, vWf appears to be present as a series of multimers with molecular weights equivalent to those of normal plasma, reaching at least 10×10^6 mol wt (16). Most of the protein, however, appears to be present in the dimeric (14) or tetrameric (16) form. vWf has demonstrated in Weibel-Palade bodies (17), although the significance of this subcellular localization is not well understood. vWf secreted into the culture fluid of endothelial cells possesses the multimeric conformations typical of plasma vWf (14, 16).

vW AgII is a second protein described as deficient in von Willebrand's disease (4). vW AgII is found in plasma and platelets, with approximately two-thirds circulating in platelets (8). It is released from platelets during activation by thrombin, ADP, or collagen and is present at increased levels in the plasma of individuals with syndromes associated with platelet activation, such as disseminated intravascular coagulation and hemolytic uremic syndrome (8). vW AgII shows an immunochemical reaction of nonidentity with vWf (4) by crossed immunoelectrophoresis (5). Thus an enigma is presented: two distinct proteins are both absent in a single genetic disorder, and yet they are found in amounts that are linearly correlated in the circulation of normal individuals and of individuals with classic von Willebrand's disease. Their potential relationship, therefore, may be pertinent to the pathophysiology of von Willebrand's disease. This study was undertaken to characterize vW AgII more fully and to determine the association between vW AgII and vWf within the endothelial cell.

Methods

Materials. Unless otherwise noted, supplies and reagents used in endothelial cell culture were purchased from Gibco Laboratories,

Grand Island, NY. Other materials included Type I collagenase (Worthington Biochemicals Corp., Freehold, NJ), fetal calf serum (Biocell, Carson, CA), bovine pituitaries (Pel-Freeze Biologicals, Rogers, AR), [³⁵S]methionine (800 mCi/mM sp act; Amersham Corp., Arlington Heights, IL), fluorescein- and rhodamine-conjugated, preabsorbed antibodies to mouse and rabbit IgG (Tago Inc., Burlingame, CA), Hemofil (Hyland Laboratories, Costa Mesa, CA), protein A Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ), Biogel A15 (Bio-Rad Laboratories, Richmond, CA), SeaKem Agarose ME (FMC Corporation, Marine Colloids Division, Springfield, NJ), and nonionic detergent NP40 (Particle Data, Inc., Elmhurst, IL). All other reagents were purchased from commercial sources and were of the best available grade.

Endothelial cell cultures. Normal human umbilical vein endothelium was cultured by methods previously described (18). Complete medium consisted of RPMI 1640 with 20% fetal calf serum augmented with 5.9 g/liter Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Umbilical cords < 24 h old were cleaned, and the veins rinsed free of blood with lactated Ringer's solution. Cords were clamped and filled with 25 U/ml Type I collagenase. Enzymatic digestion was allowed to proceed at 37°C for 15 min. Cells were removed from the collagenase by centrifugation at 1,000 g for 5 min and then resuspended in complete medium. Cells from several cords were pooled and distributed at $1-5 \times 10^4$ cells/cm² onto 75 cm² flasks precoated with gelatin. Cells were grown at 37°C in 5% CO₂ and reached confluence within 5 d.

Secondary cultures were derived by splitting primary cultures 1:3. Complete medium was removed from confluent cultures and the cells "trypsinized" with 10 ml phosphate-buffered saline (PBS), pH 7.4, containing 500 mg trypsin and 20 mg EDTA. Cells were harvested by centrifugation and subcultured as described for primary cultures. Secondary cultures contained 100 µg/ml endothelial cell growth factor prepared as described elsewhere (19) from bovine pituitaries.

Metabolic labeling of cellular proteins was achieved by incubation of confluent cultures at 37°C for 4 h in serum-free RPMI 1640 that lacked methionine. Methionine was supplied as 200 µCi [³⁵S]methionine/ml. All radiolabeled cells were washed three times in RPMI 1640 supplemented with a 100-fold excess of unlabeled methionine (150 mg/liter) and twice in lactated Ringer's solution immediately before harvesting. Labeled cells were harvested by lysing, in situ, in 0.1 M Tris HCl, pH 8.6, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) in 5 mM EDTA, 1% NP40, 0.15 M NaCl, and 1 mM iodoacetic acid. Endothelial cell lysate or culture supernate was centrifuged at 12,000 g and stored at -80°C.

Antisera. Antisera monospecific for vW AgII were prepared as described, using vW AgII derived from outdated normal human platelets (6). Absorption of antisera with plasma from an individual with severe von Willebrand's disease (no detectable vW AgII or vWf) yielded antisera that gave a single precipitin arc on crossed immunoelectrophoresis (6).

The IgG fraction of antisera to vW AgII was isolated after ammonium sulfate precipitation, DEAE ion exchange chromatography, and immunopurification on a column of Sepharose 4B with which vW AgII had been coupled using cyanogen bromide (5).

Antisera to human fibronectin or fibrinogen were used in crossed immunoelectrophoresis (Fig. 6). These antisera were the generous gifts of Dr. M. Mosesson and Dr. D. Amrani, Mount Sinai Medical Center, Milwaukee, WI. Polyspecific rabbit antisera to vWf, immunopurification of antibodies to vWf by affinity chromatography, and methods used in obtaining monoclonal antibodies to vWf are described elsewhere (5, 20).

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as previously described (5) using 0.9% agarose in buffer containing per liter 7.2 g Tris base, 3.0 g barbituric acid, and 13.1 g sodium barbital, pH 8.5. First dimension electrophoresis of up to six 100-µl samples was performed on a single platelet at 4 mA/cm at room temperature. Lanes containing antigen were excised and transferred to separate plates for second dimension electrophoresis into

agarose containing the appropriate antibodies. Gels were washed in several changes of 1% NP40, 10 mM benzamidine HCl, 0.15 M NaCl, pressed, dried, and autoradiographed at -80°C with Kodak X-Omat RP film (5). Radiolabeling of immunoprecipitates formed on crossed immunoelectrophoresis was done by (a) using endogenously labeled endothelial cell proteins (7); (b) forming immunoprecipitates with antibodies with which ¹²⁵I had been coupled, using the chloramine T method (21); or (c) using ¹²⁵I-labeled plasma protein that had been partially purified on Biogel A15 as described (5).

Immunofluorescent labeling of endothelial cells. Intracellular localization of vWf and vW AgII was determined by double label indirect immunofluorescence. Cells were grown on 4 cm² glass coverslips precoated with fibronectin. Purified fibronectin was the generous gift of Dr. D. Amrani and Dr. M. Mosesson, Mount Sinai Medical Center, Milwaukee, WI. Cells were fixed and permeabilized with 5% acetic acid in 95% ethanol. Incubations with primary and secondary antisera were for 30 min at 4°C, followed by rinsing in PBS (0.05 M phosphate, 0.15 M NaCl, pH 7.4). Antibodies to vWf were murine monoclonals (AVW1, AVW2, and AVW3) (20). The antibodies to vW AgII were immunopurified from polyclonal rabbit IgG as described above. To further remove any antibody to vWf, the immunopurified IgG was absorbed with 0.2 vol vWf from void-volume fractions of purified vWf, gel-filtered over 4% agarose. Absorption was overnight at 4°C.

Fluorescein-conjugated goat anti-rabbit IgG was the second antibody used to label polyclonal rabbit IgG directed against vW AgII. Rhodamine-conjugated rabbit anti-mouse IgG was the second antibody used to label murine monoclonal IgG directed against vWf. For the double label experiments presented here, antibodies to both antigens were added to the same cell preparation. Respective second antibodies were likewise added to the same cells. Fluorescein was visualized by excitation at 489 nm; rhodamine at 554 nm. Both conjugated second antisera were preabsorbed against interspecies cross-reacting material.

Molecular weight estimations using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS polyacrylamide gels were prepared using standard methods as described previously (22). Immunoprecipitates were obtained from crossed immunoelectrophoresis gels or from antigen/antibody mixtures immobilized on protein A coupled with Sepharose 4B (14, 23). Immunoprecipitates generated in agarose by crossed immunoelectrophoresis were washed in cell lysis buffer that contained proteolytic inhibitors and autoradiographed at 4°C. The precipitin arcs were cut from the gels using the autoradiographs as templates. The excised immunoprecipitates were transferred into 100 µl SDS-PAGE loading buffer that contained 0.1 M Tris, pH 6.8, 2% SDS, 20% glycerol, 0.1% bromophenol blue, and for reduced samples, 20% β-mercaptoethanol. Samples in loading buffer were melted in boiling water and immediately layered on SDS-PAGE gels (23).

Immunoprecipitates were isolated from antigen/antibody mixtures using protein A Sepharose 4B. Antigen/antibody mixtures were incubated for 30 min. at room temperature and then added to protein A Sepharose 4B for 1 h. PMSF was added to each washing buffer immediately before use. Protein A Sepharose beads were washed before use in 0.1 M phosphate buffer, pH 8.6, containing 0.15 M NaCl, 0.1% bovine serum albumin (BSA), 0.5% NP40, and 0.02% NaN₃. After coupling protein A with antigen/antibody complexes, beads were washed in 100-fold excesses of the following buffers. The initial wash was in 1.0 M NaCl consisting of 0.1% NP40 buffered to pH 8.6 with 0.05 M Tris HCl. The next seven washes were in the PBS-BSA-NP40 buffer, pH 8.6, used to wash the protein A Sepharose initially. The next two washes were in 0.1 M NaCl consisting of 0.1% NP40 buffered to pH 6.8 with 0.5 M Tris HCl, followed by two washes in 0.5 M LiCl, and then two washes in deionized water. This regime resulted in no further release of radioactivity into washing buffers. Washed immunoprecipitates bound to protein A beads were subsequently prepared for SDS-PAGE as described for immunoprecipitates contained in agarose.

Ultracentrifugation. Samples of 100 µl of endogenously labeled endothelial cell lysates were centrifuged for 20 min at 100,000 g at

4°C in 4.8 × 19.9 mm cellulose nitrate tubes, using an airfuge (Beckman Instruments, Inc., Fullerton, CA). Three aliquots of 33 μl were removed sequentially from the top, using a micropipette, taking care that no mixing occurred. Samples were analyzed by crossed immunoelectrophoresis and compared with uncentrifuged lysates.

Results

Molecular weight of vW AgII and endothelial cell vWf. The molecular weight of vW AgII was estimated using SDS-PAGE for samples of both endothelial cell protein and plasma protein isolated from commercial concentrate. vW AgII from both sources demonstrated a molecular weight of 74,000 nonreduced and 92–98,000 reduced. The reduced and nonreduced molecular weights of plasma vW AgII are shown in Fig. 1. The molecular weights of endothelial cell vW AgII are shown in Fig. 2. The larger molecular weight identified upon reduction suggests significant intramolecular disulfide bonding.

vWf immunoprecipitated with monoclonal antibodies from endothelial cell lysates yielded two bands of ³⁵S-labeled vWf. Molecular weights of 220,000 and 260,000 were determined for these subunits (data not shown). These data are in agreement with estimates for the precursor (260,000 mol wt) and subunit forms previously described for vWf (13, 14).

Identification of an endothelial cell complex between vW AgII and vWf. The characteristic reaction of nonidentity that is seen for vW AgII and vWf derived from plasma, serum, platelet releasate, and platelet lysate (in the presence of PMSF alone or PMSF and leupeptin [20 μg/ml]) (4, 5) is also seen with endothelial cell supernate (data not shown). In contrast to this pattern, the results of crossed immunoelectrophoresis of [³⁵S]methionine-labeled endothelial cell lysate against antisera monospecific for vW AgII is shown in Fig. 3. A second, less

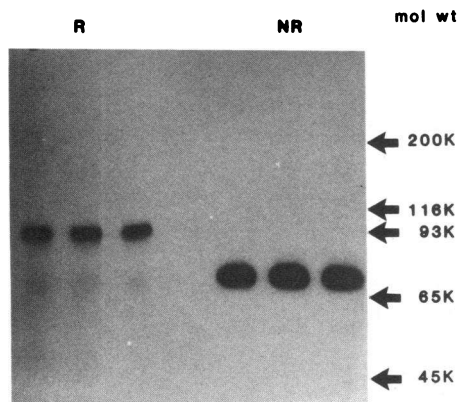


Figure 1. Molecular weight estimates of plasma vW AgII by SDS-PAGE. vW AgII from three different commercial Factor VIII concentrates was partially purified by gel filtration through Biogel A15 and radiiodinated. Labeled vW AgII was subjected to crossed immunoelectrophoresis into unlabeled antisera to vW AgII. Immunoprecipitates were visualized by autoradiography, excised, and subjected to SDS-PAGE. Samples of vW AgII from the three commercial Factor VIII concentrates were electrophoresed in the presence (left lanes) or absence (right lanes) of β-mercaptoethanol. Molecular weight markers (arrows) were stained with Coomassie blue, and the radiolabeled vW AgII protein was detected by autoradiography. Molecular weight estimates for vW AgII are 98,000 reduced and 74,000 nonreduced, suggesting intramolecular disulfide bonding. K, ×1,000; R, reduced; NR, nonreduced.

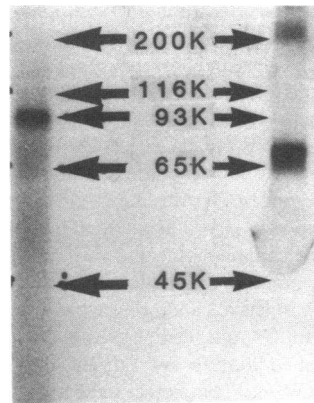


Figure 2. Molecular weight estimation of endothelial cell vW AgII. SDS-PAGE was performed as in Fig. 1. Endothelial cell proteins were endogenously labeled with [³⁵S]methionine and the cell lysate subjected to crossed immunoelectrophoresis into unlabeled antisera to vW AgII, and then analyzed on an SDS-PAGE in the presence (left lanes) or absence (right lanes) of β-mercaptoethanol. Molecular weight markers were visualized with Coomassie blue, while radiolabeled protein was

detected by autoradiography. Molecular weights for vW AgII of 92,000 reduced and 74,000 nonreduced were estimated and are in agreement with those obtained from plasma vW AgII. The upper band on the right of >200,000 mol wt is not vWf and its identity remains unknown. K, ×1,000.

anodic peak is present that shows an immunochemical reaction of complete identity with vW AgII. This less anodic peak can be distinguished from vWf after antisera to vWf is incorporated into the upper third of the crossed immunoelectrophoresis gel. The results showing vW AgII complex and vWf precipitin arcs are shown in Fig. 4. The vWf that is not complexed to vW AgII reacts with the anti-vWf antibody present in the upper one-third of the gel.

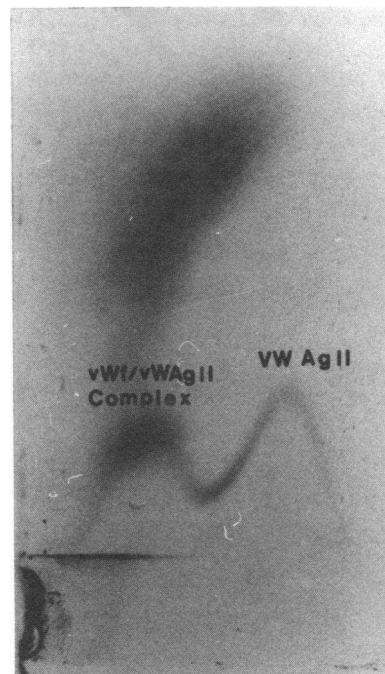


Figure 3. Crossed immunoelectrophoresis of endothelial cell lysate. Endogenously labeled endothelial cell lysate was analyzed using crossed immunoelectrophoresis. The second-dimension gel contained unlabeled antisera to vW AgII. Immunoprecipitates were visualized on an autoradiograph, which is presented in this figure. An immunochemical reaction of complete identity is seen between vW AgII and the less anodic peak.

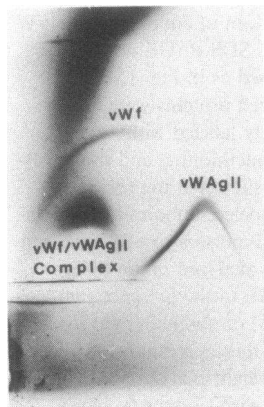


Figure 4. Crossed immunoelectrophoresis of endothelial cell lysate. Endogenously labeled endothelial cell lysate was analyzed as in Fig. 3. The autoradiograph is presented. Antiserum to vW AgII was present in the intermediate gel in the second dimension. Antiserum to vWf was present in an agarose strip placed anodally to the agarose containing the anti-vW AgII. Thus immunoprecipitation of free vWf was delayed and occurred anodally to vW AgII, demonstrating free vWf in addition to vW AgII and the vWf/vW AgII complex in these samples.

To demonstrate further that the complex in endothelial cells is comprised of both vWf and vW AgII antigenic determinants, we preincubated endothelial cell lysate with antisera monospecific for human fibrinogen, fibronectin, or vWf. The samples were then subjected to crossed immunoelectrophoresis, and the results are shown in Fig. 5. Only antisera to vWf removed the high-molecular-weight complex (hereafter termed vWf/vW AgII complex), indicating that this complex contained vWf epitopes, and that this reaction was specific since the other two antisera did not alter the vWf/vW AgII complex or vW AgII. This was further confirmed using nonradiolabeled endothelial cell lysate and radioiodinated monoclonal antibodies to vWf, termed AVW1, AVW2, and AVW3 (20). Radioiodinated monoclonal antibodies to vWf were specifically incorporated into both vWf and the vWf/vW AgII complex (Fig. 6) without labeling vW AgII. Of 14 monoclonal antibodies to vWf thus far tested, none shows cross-reactivity with vW AgII.

When washed platelets were lysed in the presence of PMSF and leupeptin, the vWf/vW AgII complex was not present on analysis by crossed immunoelectrophoresis (data not shown). Further, when endothelial cells were lysed in the absence of

PMSF and leupeptin, vWf/vW AgII complex was not stable and was lost from crossed immunoelectrophoresis patterns (Fig. 7). Plasma vWf and vW AgII do not exist as a complex regardless of whether protease inhibitors are present or not.

When endogenously labeled vWf/vW AgII complex was excised from crossed immunoelectrophoresis gels and analyzed by SDS-PAGE, vW AgII demonstrated a molecular weight of 98,000. Although molecular weight estimates above 200,000 decrease in accuracy, vWf demonstrated a molecular weight of ~30–50,000 more than that of plasma vWf when the two forms of this antigen were reduced and compared on the same gel by SDS-PAGE (Fig. 8).

Intracellular localization of vW AgII in the endothelial cell.

Endothelial cells were permeabilized and labeled with rabbit antisera, monospecific for vW AgII, and monoclonal mouse IgG, specific for vWf. Fluorescein-conjugated second antibody demonstrated significant fluorescent labeling of vW AgII in the perinuclear region and strong labeling of organelles previously identified as Weibel-Palade bodies (17) (Fig. 9 A). Rhodamine-conjugated second antibody displayed an identical pattern for labeling of vWf in these organelles, indicating colocalization of these two antigens (Fig. 9 B).

Samples of endothelial cell lysate were ultracentrifuged to determine whether the vWf/vW AgII complex might be insolubilized organelle material as seen in immunofluorescent-labeled cells. Ultracentrifugation of 100 μ l of endothelial cell lysate at 100,000 *g* did not alter the crossed immunoelectrophoresis pattern of vWf, vW AgII, or vWf/vW AgII complex. Neither the quantity nor the mobility of vWf/vW AgII complex differed in samples taken from the top or the bottom of the centrifuge tube. Thus the vWf/vW AgII complex was not caused by the electrophoresis of subcellular particulate matter.

Discussion

These data show that within the endothelial cell, vW AgII forms a soluble complex with vWf (Fig. 3). Crossed immuno-

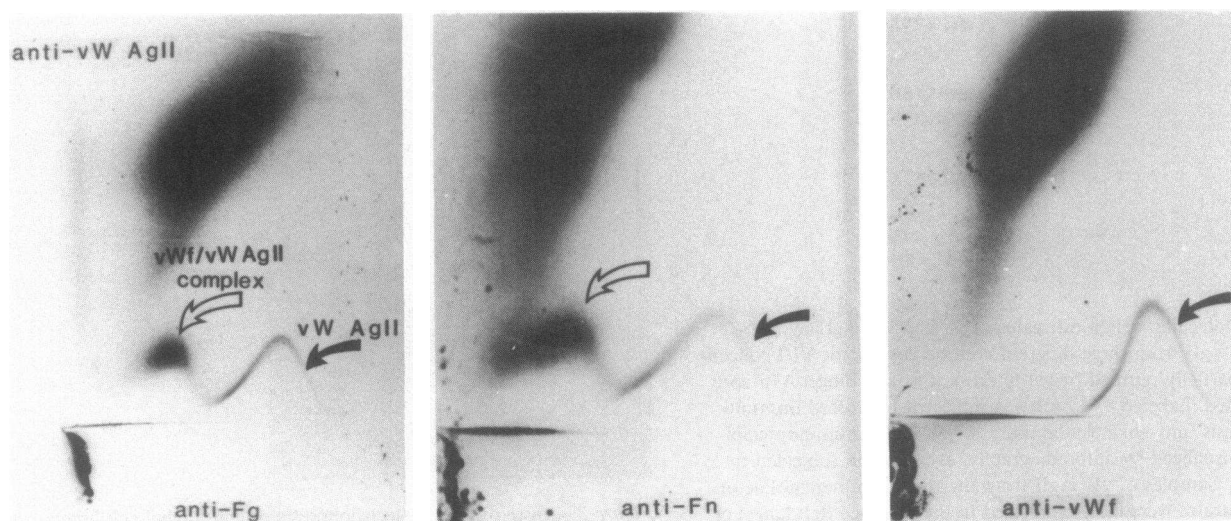


Figure 5. Inhibition of the vWf/vW AgII complex entry into the first dimension of crossed immunoelectrophoresis. Antisera to large adherent glycoproteins, fibrinogen (Fg), fibronectin (Fn), or vWf were mixed with endogenously labeled endothelial cell lysate before separation of proteins in the first dimension. Crossed immunoelectropho-

resis was performed as in Fig. 4, with autoradiographs presented in the composite. Antisera to vWf, but not Fg or Fn, completely inhibited the entry of vWf/vW AgII complex into the first-dimension gel, demonstrating the presence of vWf in this complex.

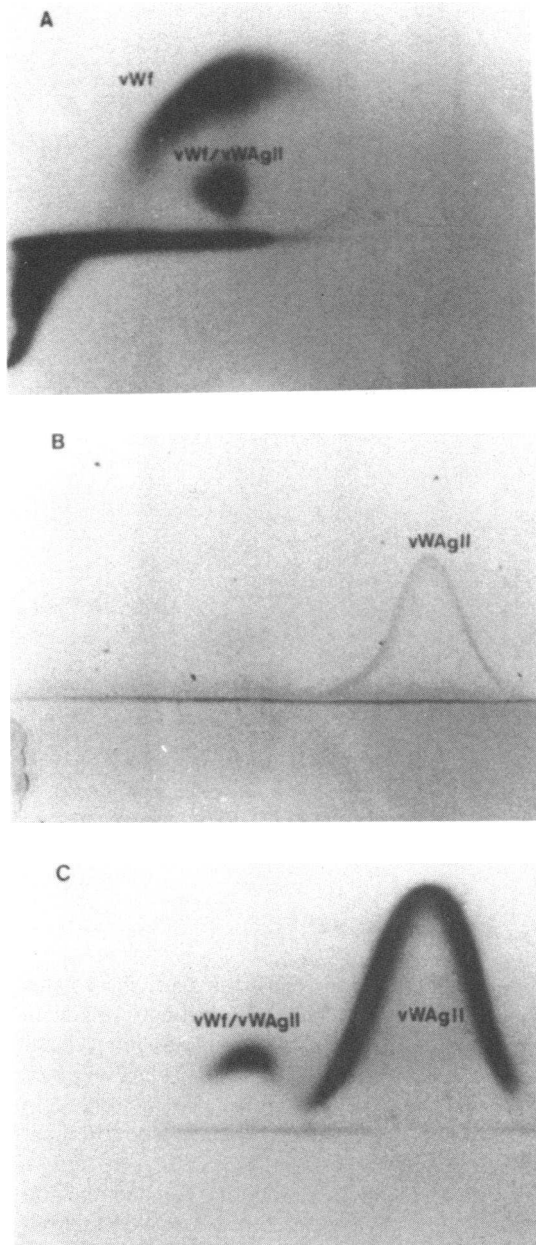


Figure 6. Localization of vWf epitopes in the vWf/vW AgII complex. Crossed immunoelectrophoresis was performed, as described in Fig. 5. Nonradiolabeled endothelial cell lysate was separated in the first dimension and then electrophoresed into unlabeled antisera to vW AgII containing radiolabeled monoclonal IgG (AVW1) directed against vWf (2×10^6 counts/plate). Radiolabeled antibody directed against vWf precipitated with free vWf and the vWf/vW AgII complex (A). vW AgII was visualized on the same slide by Coomassie blue staining (B). The same sample, assessed using radioiodinated antibodies to vW AgII, demonstrated the presence of vW AgII epitopes in the vWf/vW AgII complex (C).

electrophoresis patterns of endothelial cell lysates against antisera to vW AgII demonstrate that vW AgII forms immunoprecipitin lines of complete identity with the complex. The complex can be shown to contain both vW AgII and vWf by using crossed immunoelectrophoresis during which antibodies to vWf both specifically radiolabel the complex (Fig. 6) and

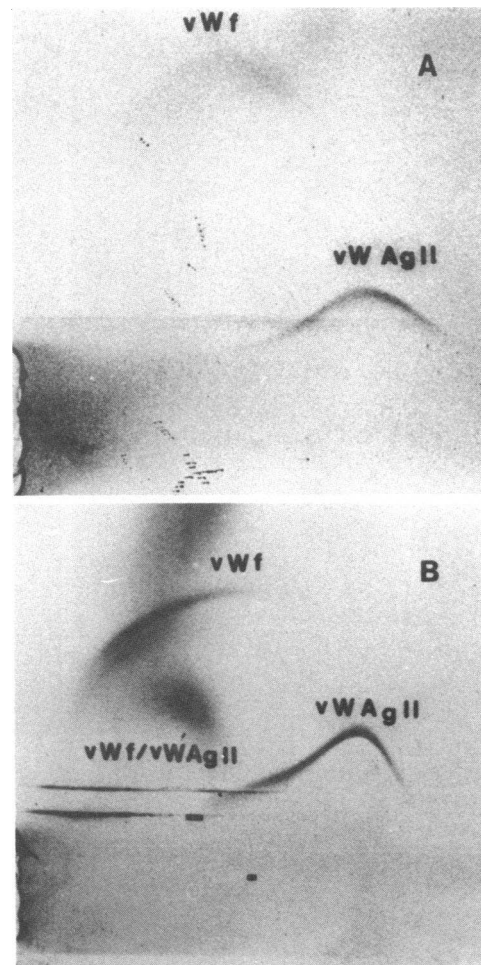


Figure 7. Crossed immunoelectrophoresis of endogenously labeled endothelial cell lysate harvested in the absence (A) or presence (B) of 1 mM PMSF. With PMSF, the vWf/vW AgII complex is detected by this method. In the absence of PMSF, the complex is undetectable, suggesting a susceptibility to serine protease activity.

remove it (Fig. 5) from immunoprecipitin patterns generated with antisera against vW AgII. Large adherent glycoproteins do not appear to play a role in the formation of this complex. Using crossed immunoelectrophoresis, the vWf/vW AgII complex can also be shown to be distinct from free vWf (Figs. 4

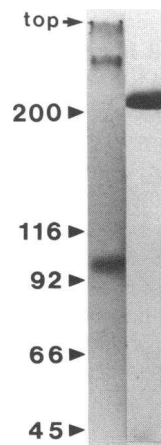


Figure 8. Molecular weight estimations of endothelial cell vWf and vW AgII present in the complex. Endogenously labeled endothelial cell lysate was subjected to crossed immunoelectrophoresis. The gel was washed, autoradiographed, and immunoprecipitin arcs excised and subjected to SDS-PAGE with β -mercaptoethanol. Molecular weight markers were visualized with Coomassie blue. Radiolabeled proteins were detected by autoradiography. The left lane demonstrates the reduced vWf and vW AgII complex. The molecular weight estimate for vW AgII was 98,000 and for vWf, 260,000. The mobility of reduced plasma vWf is shown on the right (220,000 mol wt). All measurements are $\times 1,000$.

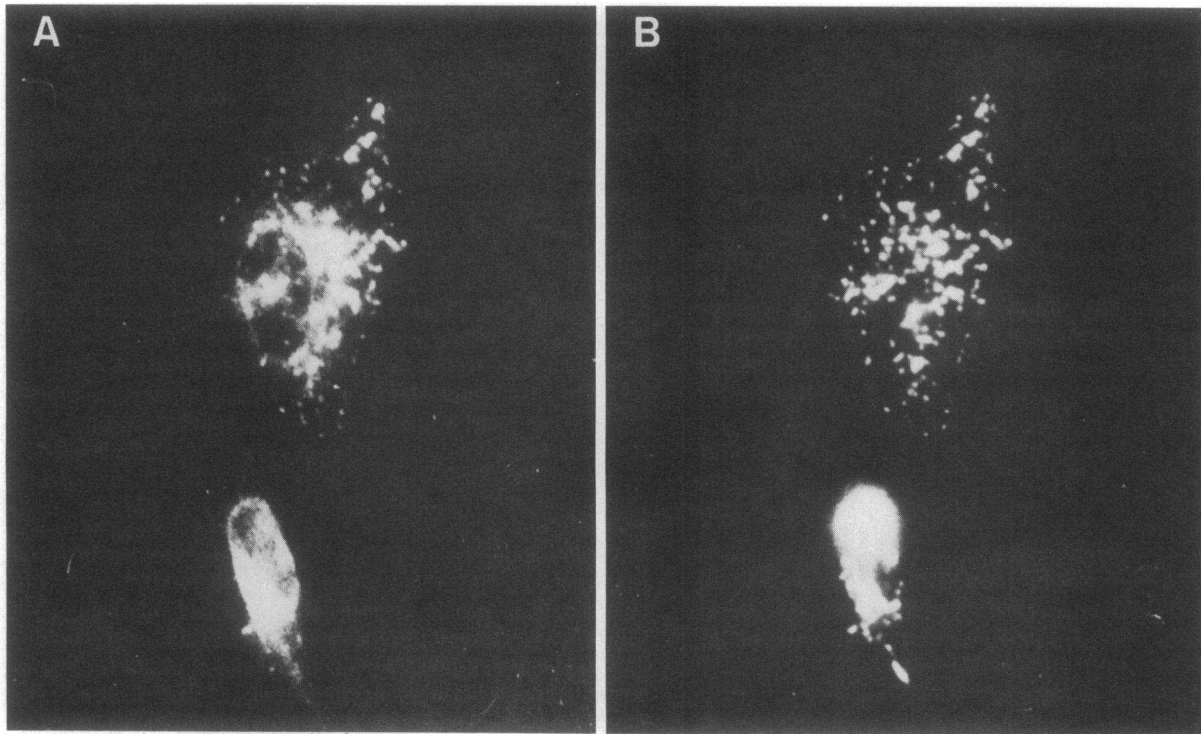


Figure 9. Immunofluorescent staining of vWf and vW AgII in endothelial cells. Double label immunofluorescence localization of vWf and vW AgII was performed by incubating fixed endothelial cells with murine monoclonal antisera to vWf and immunopurified rabbit IgG to vW AgII. Fluorescence labeling was with fluorescein-conju-

gated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG. Perinuclear fluorescein and fluorescence in organelles may be seen with fluorescein localization of vW AgII (A). Rhodamine fluorescence colocalized vWf to the same organelles (B).

and 5) by incorporating precipitating antisera to vWf into a second strip of agarose above that containing antibodies to vW AgII (Fig. 5).

The vWf/vW AgII complex appears to be sensitive to serine proteases as it is not stable in the absence of PMSE, an inhibitor directed against this class of proteases (Fig. 7).

If vW AgII and vWf were both present in Weibel-Palade bodies, then insoluble material from the detergent lysis of these organelles might account for an apparent complexing of vW AgII with vWf. However, the lack of sedimentation of vWf/vW AgII complex by ultracentrifugation indicated that this complexing did not occur.

[³⁵S]Methionine-labeled immunoprecipitates of vWf and vW AgII demonstrate synthesis of these molecules in endothelial cells. Reduction of the complex with SDS and β -mercaptoethanol yields ³⁵S-labeled bands on SDS-PAGE that agree in molecular weight with radiolabeled vWf and vW AgII produced by independent methods (14) (Figs. 1, 2, and 8).

Even though vW AgII is separated from the vWf protein before secretion and may be found free within endothelial cells, molecular weight estimates do not support the possibility that vW AgII is the 40,000-mol-wt fragment released from the 260,000-mol-wt vWf precursor (14). Likewise, no very large precursor forms of vWf have been reported other than the 260,000-mol-wt precursor to vWf (14, 24). This does not exclude, however, the possibility that vW AgII could be a precursor piece of vWf, since molecular weight estimates above 200,000 generated by extrapolation decrease in accuracy in the high-molecular-weight ranges.

Observations presented here resolve, in part, the enigma of the absence of two immunochemically distinct proteins in a single genetic disorder. Their relationship was intracellular rather than extracellular. Our recent work (6, 7) has suggested a fundamental association between these two molecules. In vivo release by DDAVP, for example, results in a concomitant release of vW AgII and vWf (7). Like vWf, vW AgII is synthesized in cultured human umbilical vein endothelium (7). Levels of circulating vW AgII are linearly related to levels of vWf in normal individuals and in individuals with Type I von Willebrand's disease (6). However, in individuals with Types IIA or IIB von Willebrand's disease, in which the multimeric composition of vWf is abnormal, the levels of vW AgII are not decreased along with those of vWf but are within normal limits (6). The complex between vW AgII and vWf in endothelial cells demonstrates this association. Future studies, however, will be necessary to characterize the significance of the vWf/vW AgII complex in the synthesis of vWf and, perhaps, in the pathophysiology of von Willebrand's disease.

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