Composition of the von Willebrand Factor Storage Organelle (Weibel–Palade Body) Isolated from Cultured Human Umbilical Vein Endothelial Cells

B. M. Ewenstein,* M. J. Warhol,‡ R. I. Handin,* and J. S. Pober‡

‡Department of Pathology and *Division of Hematology, Brigham and Women's Hospital, and the ‡Departments of Pathology and *Medicine, Harvard Medical School, Boston, Massachusetts 02115

Abstract. von Willebrand factor (VWF) is a large, adhesive glycoprotein that is biosynthesized and secreted by cultured endothelial cells (EC). Although these cells constitutively release VWF, they also contain a storage pool of this protein that can be rapidly mobilized. In this study, a dense organelle fraction was isolated from cultured umbilical vein endothelial cells by centrifugation on a self-generated Percoll gradient. Stimulation of EC by 4-phorbol 12-myristate 13-acetate (PMA) resulted in the disappearance of this organelle fraction and the synchronous loss of Weibel–Palade bodies as judged by immunoelectron microscopy. Electrophoretic and serologic analyses of biosynthetically labeled dense organelle fraction revealed that it is comprised almost exclusively of VWF and its cleaved pro sequence. These two polypeptides were similarly localized exclusively to Weibel–Palade bodies by ultrastructural immunocytochemistry. The identity of the dense organelle as the Weibel–Palade body was further established by direct morphological examination of the dense organelle fraction. The VWF derived from this organelle is distributed among unusually high molecular weight multimers composed of fully processed monomeric subunits and is rapidly and quantitatively secreted in unmodified form after PMA stimulation. These studies: (*a*) establish that the Weibel–Palade body is the endothelial-specific storage organelle for regulated VWF secretion; (*b*) demonstrate that in cultured EC, the VWF concentrated in secretory organelles is of unusually high molecular weight and that this material may be rapidly mobilized in unmodified form; (*c*) imply that proteolytic processing of VWF involved in regulated secretion takes place after translocation to the secretory organelle; (*d*) provide a basis for further studies of intracellular protein trafficking in EC.

VON Willebrand factor (VWF)[1] is a large, adhesive glycoprotein synthesized by vascular endothelium (Jaffe et al., 1973; Jaffe et al., 1974) and megakaryocytes (Nachman et al., 1977). In plasma, it serves as a stabilizing "carrier" protein for factor VIII with which it circulates as a complex (Weiss et al., 1977). In blood vessels, VWF serves as a platelet-subendothelial "molecular bridge" in the initiation of the hemostatic plug at the site of vessel wall damage (Stel et al., 1985; Turitto et al., 1985). Although the precise nature of this bridging remains to be defined, binding to both vascular subendothelium as well as to purified collagens and to platelet membrane glycoprotein Ib has been demonstrated in a number of studies (Kao et al., 1979; Meyer and Baumgartner, 1983; Houdijk et al., 1985). In plasma, VWF circulates as a series of disulfide-linked heterogeneous multimers with apparent molecular masses of 0.4–20 × 10^6, assembled chiefly from M_r 220,000 subunits (Ruggeri and Zimmerman, 1980). A number of clinical observations suggest that the hemostatic potency of VWF is at least partially proportional to multimer size (Zimmerman et al., 1983). The existence of a physiologically important storage pool of VWF is suggested by the observations that several stimuli including exercise, adrenalin (Prentice et al., 1972), and desmopressin acetate (Ruggeri et al., 1982) rapidly increase plasma VWF levels and ameliorate clinical bleeding.

Cultured endothelial cells (EC) secrete VWF in both a constitutive (Jaffe et al., 1973) and regulated manner. A number of agents, including thrombin, calcium ionophore A23187, and 4-phorbol 12-myristate 13-acetate (PMA) can induce the rapid release of preformed VWF (Loesberg et al., 1983; de Groot et al., 1984). This regulated secretion of VWF by EC, observed in vitro, may be the mechanism by which VWF is rapidly mobilized in vivo. Reinders et al. (1984) reported the isolation of a VWF-containing organelle from human umbilical vein endothelium that was not present in cells pretreated with PMA. This organelle was postulated to be identical to the Weibel–Palade body (Weibel and Palade,

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1. *Abbreviations used in this paper*: EC, endothelial cells; VWF, von Willebrand factor.

1964), an endothelial cell-specific, membrane-bound organelle previously shown by immunohistochemical techniques to contain VWF (Wagner et al., 1982; Hormia et al., 1984; Warhol and Sweet, 1984). This conclusion was inferred from the coincident disappearance of characteristic granular immunofluorescent staining for VWF thought specific for Weibel-Palade bodies. However, vascular endothelial cells from a number of sources, including bovine aorta and human adipose tissue capillaries, lack identifiable Weibel-Palade bodies yet show granular staining patterns (Schwartz, 1978; Kern et al., 1983). Therefore, the association of the Weibel-Palade body with regulated secretion of VWF can only be rigorously established at the ultrastructural level.

The primary structure of plasma VWF has been ascertained by classical protein sequencing techniques (Titani et al., 1986). Recently, several groups have reported the cloning of VWF cDNA from EC (Ginsburg et al., 1985; Lynch et al., 1985; Verweij et al., 1985; Sadler et al., 1985) and the complete sequence of the VWF transcript has been obtained (Bonthron et al., 1986). Comparison with the plasma protein sequence predicts that the primary VWF translational product contains an amino terminal "pre" or signal sequence and an adjacent M_r 100,000 "pro" sequence, identical with a plasma protein previously called VWF antigen II (Fay et al., 1986). VWF antigen II is also biosynthesized and released by cultured EC (McCarroll et al., 1985). The predominant subunit size of VWF secreted by cultured EC is M_r 220,000. An intracellular precursor form of M_r 260,000 has been identified as the earliest biosynthetic intermediate in both human and bovine EC lysates (Wagner and Marder, 1983; Lynch et al., 1983). This molecular species is known to undergo a series of posttranslational modifications including dimerization, interchain disulfide formation, carbohydrate processing, removal of the M_r 100,000 pro sequence, and multimerization of the dimers. Incomplete cleavage of the propolypeptide leads to the release of small quantities of M_r 275,000 subunits that are also incorporated into multimers (Wagner and Marder, 1983; Lynch et al., 1986). The intracellular localization of VWF processing has been inferred from pulse-chase experiments in the presence of metabolic inhibitors (Wagner and Marder, 1984). By these methods, it was shown that the cleavage of proVWF to its mature form occurs in the Golgi and "later compartments". However, these experiments, which analyzed unfractionated EC lysates, could not differentiate between processing of VWF destined for constitutive as opposed to regulated secretion.

To ascertain the form of VWF involved in regulated secretion, we have isolated a biosynthetically labeled dense organelle from cultured human umbilical vein endothelial cells. The isolated organelle was found to be ultrastructurally indistinguishable from Weibel-Palade bodies as previously described in intact cells. Immunochemical analysis of the protein contained in this cell fraction revealed that it is comprised almost exclusively of VWF and the cleaved VWF pro sequence, and by ultrastructural immunocytochemistry, both proteins were shown to colocalize in Weibel-Palade bodies. The VWF derived from this organelle is distributed among unusually high molecular weight multimers composed of fully processed monomeric forms. Furthermore, we demonstrate the rapid and quantitative release of both proteins into culture medium following PMA stimulation and the concomitant loss of Weibel-Palade bodies from the treated cells.

Materials and Methods

Cell Source and Culture

Endothelial cells were isolated from 1-4 human umbilical vein segments by collagenase digestion as previously described (Gimbrone, 1976) and cultured in Medium 199 (Biofluids, Rockville, MD) containing 20% heat-inactivated FCS (Gibco, Grand Island, NY) supplemented with penicillin (125 U/ml), streptomycin (125 μg/ml), and 2 mM L-glutamine. Cells were passaged under the conditions of Thornton et al. (1983), additionally supplementing the medium with porcine heparin (100 μg/ml) from Sigma Chemical Co. (St. Louis, MO) and endothelial cell growth factor (50 μg/ml) from Meloy Laboratories, Inc. (Springfield, VA). The plastic substratum (either T75 flasks or 10-cm petri dishes, both from Corning Glass Works, Corning, NY) was coated with gelatin (Difco Laboratories, Inc., Detroit, MI) before plating. Cells in these experiments were passaged one to three times from primary cultures. For radiolabeled experiments, EC were maintained for 3 d in the above medium supplemented with L-[^{35}S]cysteine (0.5 Ci/ml; 1,000 Ci/mmol) (New England Nuclear, Boston, MA), then chased for 3 h with unlabeled medium. Where indicated, confluent cultures were rinsed three times with Hanks' balanced salt solution (HBSS) (Gibco), then treated with 100 nM PMA (Sigma Chemical Co.) in fresh medium. For analysis of radiolabeled proteins, the 3 d conditioned medium, the medium conditioned during PMA or mock-PMA treatments (releasates), and the cell layer were separately collected.

Subcellular Fractionation

Cells were harvested by treatment with trypsin-EDTA (Gibco) diluted 1:1 with HBSS minus calcium and magnesium (3 ml/plate) for 2 min at 37°C. The resultant cell suspension was transferred to tubes containing an equal volume of soybean trypsin inhibitor (1 mg/ml; Cooper BioMedical, Inc., Malvern, PA) in an isotonic buffer containing 20 mM Tris HCl (pH 7.2), 0.2 M sucrose, and 1 mM EDTA. In general, 2-6 million cells were pelleted at 600 *g* for 10 min, resuspended in 1 ml fresh buffer, and subjected to 25-30 strokes in a ground-glass Dounce homogenizer (made by Kontes Glass Co., Vineland, NJ) at 4°C. Nuclei and cellular debris were removed by centrifugation at 600 *g* for 10 min. The resultant "precleared" supernatant was further fractionated on Percoll. In experiments directed toward morphological characterization of isolated fractions, the number of endothelial cells was increased to 40-60 million. This larger number of cells appeared to diminish the resolution of the gradient, but was necessary to obtain sufficient material for preparation of thin sections (see below).

Percoll gradient centrifugation was carried out in a SS24 rotor using a centrifuge (model RC-5B; Sorvall Instruments Division, DuPont Co., Newton, CT). A 10-ml Oakridge tube (Nalge Co., Div. of Sybron Corp., Rochester, NY) was filled with 8.5 ml of a suspension containing 50% Percoll (Pharmacia, Uppsala, Sweden), 0.25 M sucrose, pH 7.2, and a 0.2 ml cushion of 2.5 M sucrose was carefully underlayered. The precleared cell homogenates (0.8 ml) were carefully layered on top then banded through a self-generated density gradient for 65 min at 40,000 *g*. Fractions (0.8 ml) were manually collected from the top of the gradient. The average density of each fraction was determined by weighing. Radiolabeled proteins were extracted from gradient fractions by the addition of an equal volume of a buffer containing 2% NP-40, 25 mM NaCl, 25 mM Tris-HCl (pH 7.4), 4 mM EDTA, 4 mM phenylmethyl sulfonyl fluoride, 2 mM *N*-ethylmaleimide, and 2 mM iodoacetic acid (all from Sigma Chemical Co.).

Inhibition ELISA

VWF antigen was quantitatively measured by an inhibition ELISA using an avidin-biotin-peroxidase detection system. Microtiter plates were coated overnight at 4°C with 50 ng of highly purified human VWF, washed, and then incubated for 2 h at 20°C with 100 μl of affinity-purified rabbit anti-VWF antiserum that had been preincubated with known concentrations of purified VWF protein (standards) (Loscalzo and Handin, 1984) or VWF-containing media or cell lysate (unknowns). After incubation overnight at room temperature, plates were washed and treated sequentially with biotinylated second antibody (goat anti-rabbit IgG) and with avidin-biotin-peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). After washing, the plates were treated with the chromogenic substrate *o*-phenylenediamine 0.5 mg/ml in 140 μl of phosphate/citrate buffer (pH 5.0) containing 0.025% H_2O_2. Color development was stopped after 10-15 min by the addition of 40 μl 2M H_2SO_4 and the A_{490} was determined on a Minireader II (Dynatech Laboratories, Inc., Alexandria, VA). VWF antigen concentra-

tions determined in this assay were linear over a range of 1–25 ng/0.1-ml aliquots.

Protein Electrophoresis

SDS PAGE of reduced proteins was carried out on slab gels of either 5% acrylamide or linear gradients of 4–13% acrylamide using the buffer system of Laemmli (1970); all reagents were from BioRad Laboratories (Richmond, CA). Fixed and stained gels were equilibrated with Autofluor (National Diagnostic, Inc., Somerville, NJ) for 1 h before drying and autoradiography. Agarose slab gel electrophoresis was carried out on a water-cooled, horizontal electrophoresis cell (BioRad Laboratories). Gels consisting of 2% agarose (Standard Low m_r from BioRad Laboratories), 0.1% SDS, 0.05 M phosphate buffer (pH 7.0) were run for 3.5 h at 100 V (electrode buffer 0.1 M phosphate buffer, pH 7.0 with 0.1% SDS), then fixed, stained, and equilibrated with Autofluor and dried before autoradiography.

Immunoprecipitation

Radiolabeled proteins were isolated by precipitation with 1–10 μl of mouse monoclonal antibody (4°C, 16 h) followed by Sepharose-bound rabbit anti-mouse Ig (20°C, 2 h) (Miles-Yeda Ltd. Rehovot, Israel). For quantitative immunoprecipitation, rabbit anti-mouse gamma globulin (Cappel Laboratories, Inc., Cochranville, PA), which was previously incubated with sepharose-bound protein A (Sigma Chemical Co.), was used in place of the aforementioned rabbit anti-mouse reagent. Precipitates were washed four times with 0.1 M Tris HCl pH 8.0, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 mM EDTA and 2 mM phenylmethylsulfonyl fluoride, and then eluted by boiling for 2 min in SDS PAGE sample buffer containing 1% 2-mercaptoethanol. Anti-VWF antibody was purchased from Cappel Laboratories, Inc.; anti-fibronectin antibody (Anti-FN Human, type I) was purchased from Calbiochem (341646; La Jolla, CA). Five separate murine monoclonal antibodies to the VWF pro sequence (anti-proVWF, Fay et al., 1986) were kind gifts of Dr. P. Fay (University of Rochester, New York).

Electron Microscopy

Confluent 10-cm petri dishes of EC were treated for various times with 100 nM PMA as described above, rinsed twice with 5 ml of HBSS, then rapidly fixed in situ with 3% paraformaldehyde, 0.1% glutaraldehyde (vol/vol) for 10 min at 20°C. The cells were harvested by gentle scraping and pelleted for 10 s in an Eppendorf centrifuge (model 5414; Brinkmann Instruments Co., Westbury, NY).

The pellets were embedded using a Lowicryl K4M (Balzers, Hudson, NH) protocol (Roth, 1982). After thin sectioning, VWF and proVWF were identified by immunochemical staining with either rabbit anti-human VWF (Dako Corp., Santa Barbara, CA) or mouse monoclonal antibody (anti-VWF and proVWF, as above), rabbit anti-mouse Ig (Dako Corp.), and staphylococcal protein A (Sigma Chemical Co.) -colloidal gold as previously described (Warhol and Sweet, 1984). The number of Weibel-Palade bodies in various EC preparations was quantitated by visually counting the number in 300 separate sectioned cell profiles for each group by two different "blinded" observers.

For electron microscopy of isolated Percoll gradient fractions, aliquots of these fractions were subjected to recentrifugation of 100,000 *g* for 1 h in an ultracentrifuge (model L5-50; Beckman Instruments, Inc., Palo Alto, CA). Biological material was carefully aspirated away from the packed Percoll, fixed in 3% paraformaldehyde, 0.1% glutaraldehyde for 1 h at 20°C, and repelleted. To aid in microsectioning, the fixed organelles were overlayered with 10% gelatin before osmium postfixation. Samples were embedded in Poly/bed 812 (Polysciences, Inc., Warrington, PA), sectioned, then double stained with uranyl acetate and lead citrate.

Results

Effect of PMA on VWF Release and Endothelial Cell Ultrastructure

PMA is thought to activate protein kinase C in a number of cell types (Nishizuka, 1984) and has been shown to rapidly increase the amount of VWF released by cultured human endothelial cells (Loesberg et al., 1983; Reinders et al., 1985). To determine whether the action of this agent could be correlated with the Weibel-Palade content of cultured endothelium, confluent cells were treated for 0, 15, and 30 min with 100 nM PMA and fixed in situ. Untreated cells contained numerous Weibel-Palade bodies that clearly stained with rabbit anti-human VWF visualized with colloidal gold whereas PMA-treated cells were largely devoid of such bodies. Quantitative analysis of multiple cells revealed a >90% reduction in Weibel-Palade bodies at 15 min and a virtual absence of these structures 30 min posttreatment (Table I). To correlate VWF release during this time period, aliquots of culture medium from PMA- and mock-treated cells were assayed for VWF by inhibition ELISA. The quantity of VWF released as a consequence of secretagogue stimulation was determined by subtracting the VWF released constitutively (by mock-treated cells) from the total VWF released by PMA-treated cells. A dramatic increase of VWF release over constitutive levels was noted in stimulated cells during the first 30 min of treatment. In experiments carried out to 6 h (not shown), the subsequent rate of continued release of VWF from PMA-treated cells was seen to parallel that of mock-treated endothelial cell cultures.

Table I. Effects of PMA on Endothelial Cell Weibel-Palade Body Content and VWF Release

		Time		
		0 min	15 min	30 min
Weibel-Palade bodies*	PMA	25	2	0
VWF in culture medium‡	PMA	0	1,301	2,158
	Mock	0	191	290
	Difference	0	1,110	1,868

* Total number of organelles seen in 100 cells. Average of two blinded observers. Total VWF (nanograms) detected in conditioned culture medium of cells treated with 100 nm PMA. A background of 43 ng/flask was subtracted from each time point.

‡ Total VWF (nanograms) detected in conditioned culture medium of mock-treated cells. A background of 55 ng/flask was subtracted from each time point.

Interestingly, a number of cells examined after 15 min of PMA treatment showed events suggestive of membrane fusion and degranulation at the cell surface (Fig. 1 *B*). At 30 min posttreatment, a small number of vacuolated structures could be discerned that labeled for VWF (Fig. 1 *C*). The genesis of these structures and their relationship to Weibel-Palade bodies is unknown.

Percoll Gradient

When radiolabeled endothelial cells were subfractionated on a self-generated Percoll gradient, the bulk of radioactivity was recovered in a cytosolic (ρ = 1.02) and in a buoyant (ρ = 1.05) organelle fraction (Fig. 2 *A*). The profile of radioactivity was found to closely parallel that of total protein as measured by a quantitative Coomassie Blue assay (Bradford, 1976; data not shown). Pretreatment of the endothelial cells with PMA did not materially alter the profile of either radioactivity or total protein. Analysis by inhibition ELISA of gradient fractions from untreated cells revealed the presence of two distinct peaks of VWF activity (Fig. 2 *B*). The more buoyant fraction (ρ = 1.05) corresponded to the second peak of radioactivity while a denser, broad fraction (ρ = 1.10), containing the bulk of intracellular VWF, was found in gradient fractions containing comparatively little radioactivity.

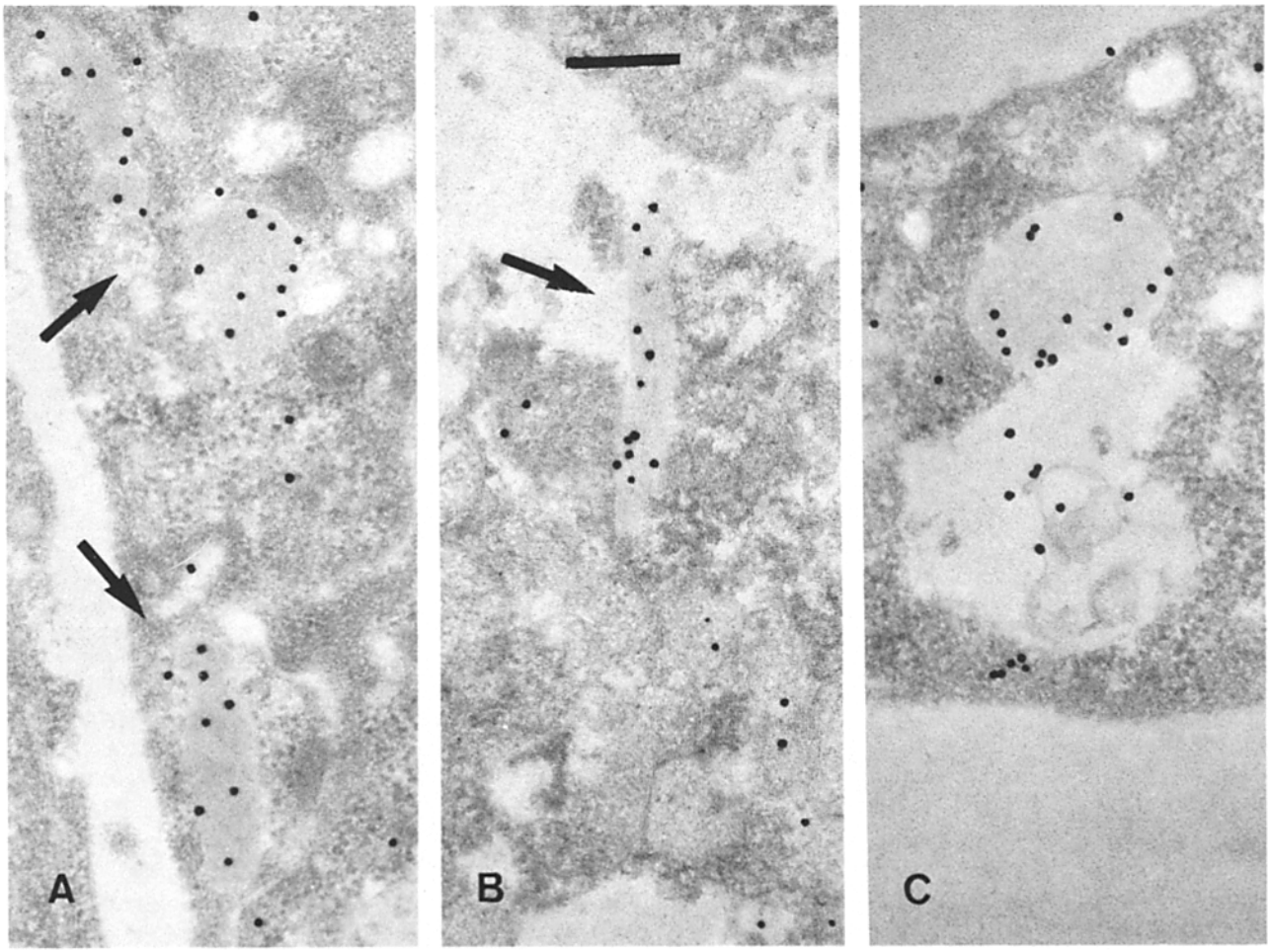
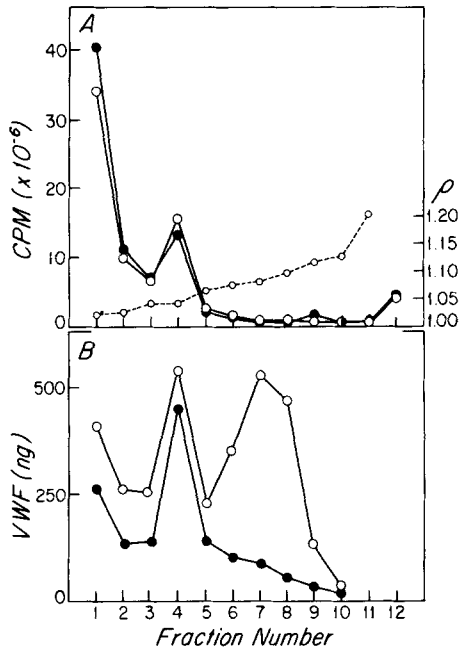


Figure 1. (*A*) Control endothelial cell cultures labeled with rabbit polyclonal anti-VWF. A representative endothelial cell contains three Weibel-Palade bodies labeled with the anti-VWF (*arrows*). (*B*) An endothelial cell 15 min after treatment with PMA. The number of Weibel-Palade bodies seen is markedly reduced (see Table I). One residual body labeled with anti-VWF seems to be in the process of being extruded from the cell (*arrow*). (*C*) An endothelial cell 30 min after treatment with PMA. No definite Weibel-Palade bodies are identified. Rare cells contain faintly electron dense granular material labeled with anti-VWF within cytoplasmic vacuoles. Bar, 150 nm.



In contrast, homogenates of cells pretreated with PMA exhibited a nearly quantitative loss of this denser VWF-containing peak.

To better characterize these fractions, unlabeled endothelial cells were similarly fractionated for morphological examination. Electron microscopic analysis of the buoyant organelle fraction (Fig. 3 *A*) revealed the presence of a heterogeneous population of subcellular organelles including rough endoplasmic reticulum, plasma membrane, and

Figure 2. Percoll gradient fractionation of [^{35}S]cysteine-labeled endothelial cell homogenates. (*A*) Cells were labeled for 3 d then subjected to a 30-min treatment with 100 nM PMA (●——●) or mock treatment (○——○). After trypsinization, treated or untreated cells were homogenized and subjected to Percoll gradient centrifugation (40,000 *g*; 65 min) as described in Materials and Methods. Fractions (0.8 ml) were manually collected from the top of the gradient and the total radioactivity in each fraction calculated from the amount of radioactivity in 10-μl aliquots. (*B*) The total VWF content of fractions 1–10 was determined by inhibition ELISA of 50-μl aliquots of detergent-solubilized fractions, symbols as in *A*. Average density (○ - - ○) was determined by the weighing of aliquots.

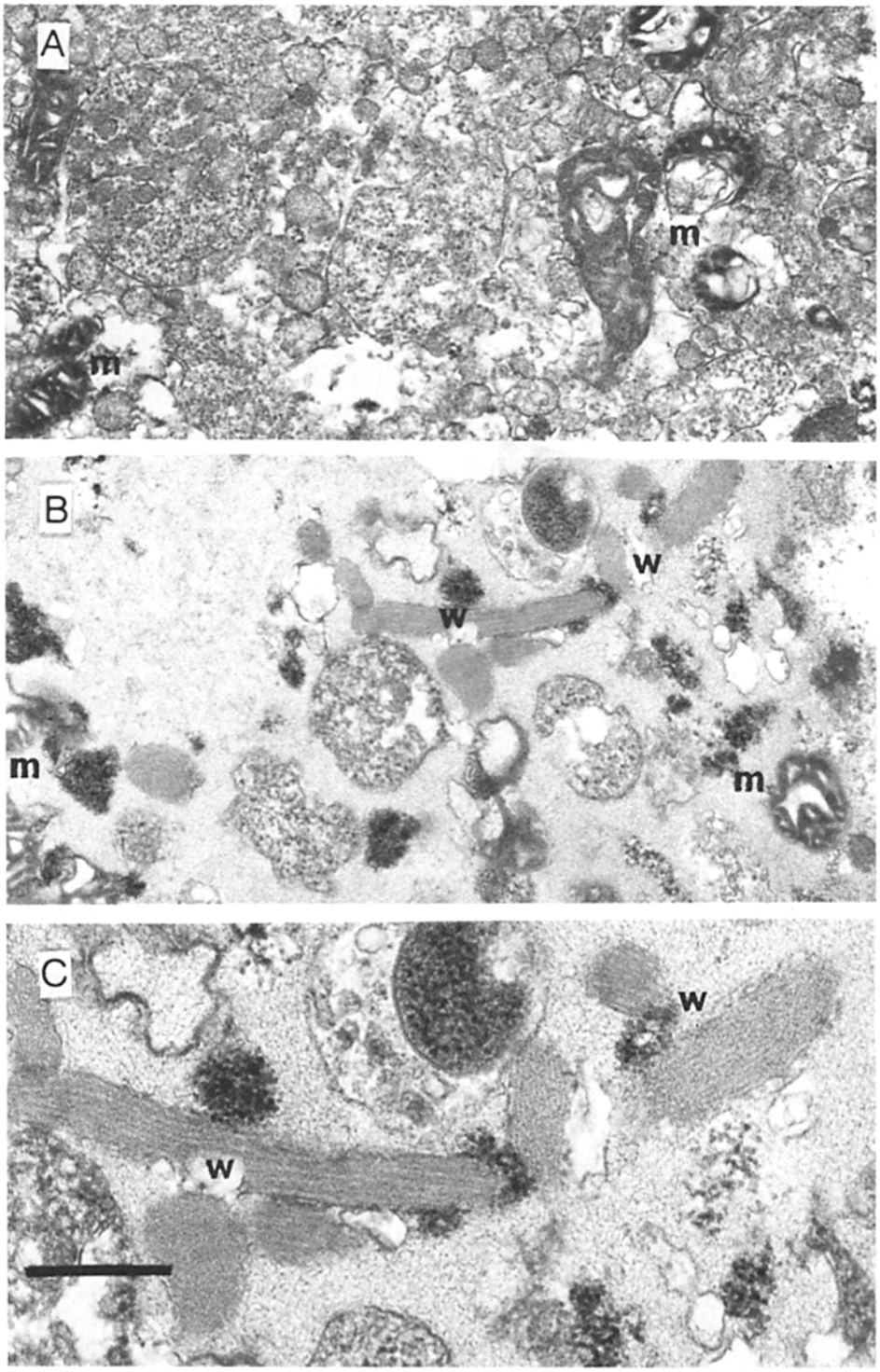


Figure 3. Transmission electron micrographs of Percoll fractions of endothelial cell homogenates. (*A*) The buoyant organelle fraction consists of a heterogenous population of organelles. (*B*) The dense organelle fraction consists principally of organelles of the size and lamellar substructure typical of endothelial cell Weibel–Palade bodies. In this overloaded gradient, mitochondria were found to be the predominant contaminating organelle. (*C*) The same field as *B*, magnified ×2. *m*, Mitochondria; *w*, Weibel–Palade body. Bar, 100 nm.

possibly Golgi apparatus. In contrast, the denser fraction (Fig. 3, *B* and *C*) was seen to consist largely of organelles that were morphologically indistinguishable from Weibel–Palade bodies seen in intact untreated cells. In this overloaded gradient, the principal contaminating organelles were mitochondria.

Analysis by SDS PAGE (Fig. 4 *A*) of fractions 1–10 from untreated labeled endothelial cells revealed that the denser fraction consisted principally of two proteins of M_r 220,000 and 100,000, respectively. Both proteins were selectively depleted from homogenates of pretreated cells (Fig. 4 *B*). Longer exposures (up to 10 times that shown) failed to reveal the presence of any other protein that was similarly enriched in this fraction.

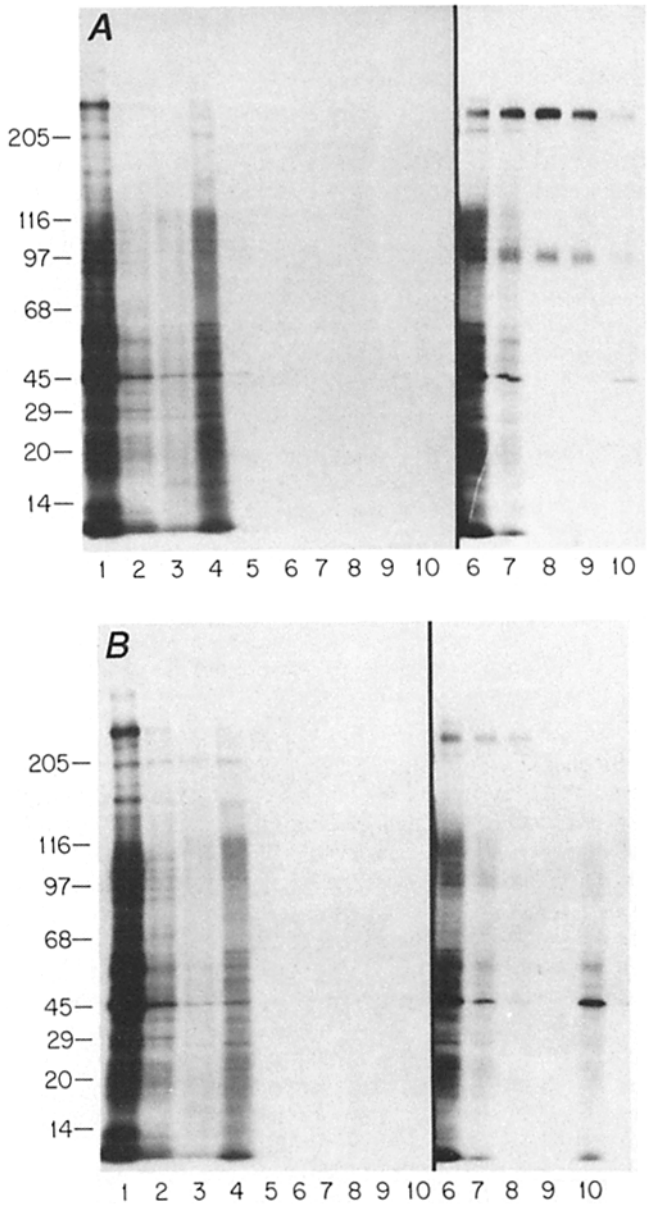


Figure 4. Analysis by SDS PAGE of Percoll gradient fractions of [^{35}S]cysteine-labeled endothelial homogenates. (*A*) Mock-treated (control) cells and (*B*) PMA-treated cells were analyzed on different sides of the same slab gel. 10-μl aliquots of Percoll gradient fractions (from Fig. 2 *A*) were subjected to SDS PAGE (4–13% acrylamide) and analyzed by autofluorography. Initial fluorographs (lanes *1–10, left*) were obtained at 16 h. Longer exposures (5 d) of lanes *6–10* (*right*) were obtained to better visualize the contents of the dense organelle fractions. Numbers on left indicate $M_r \times 10^{-3}$.

Serological Identification of the Principal Proteins of the Dense Organelle

To identify the principal protein constituents of the isolated Weibel–Palade bodies, [^{35}S]cysteine-labeled dense-fraction extracts, PMA releasates, and 3-d conditioned medium were subjected to immunoprecipitation using either anti-VWF (Fig. 5, lanes *b–d*) or anti-proVWF (Fig. 5, lanes *e–g*) monoclonal antibodies. VWF isolated from all three sources showed an identical electrophoretic mobility with M_r 220,000. Similarly, anti-proVWF antibodies identified the cleaved M_r 100,000 pro sequence in the dense fraction and in both media sources. Repetition of these experiments on several occasions failed to reveal the presence of any uncleaved (i.e., M_r 275,000) forms. Thus, all of the immunoreactive material identified by the anti-proVWF appears to be in the form of cleaved pro sequence. Parallel precipitation with monoclonal anti-fibronectin antibody (Fig. 5, lanes *h–j*) demonstrated the presence of biosynthetically labeled fibronectin in 3-d medium but not in the dense organelle fraction nor in PMA releasates. Furthermore, both VWF and the proVWF polypeptide could be quantitatively depleted from labeled dense-fraction extracts using monoclonal antibodies (Fig. 6), implying that each band represents only a single polypeptide.

Ultrastructural Localization of VWF and Its Cleaved Pro Sequence

Ultrastructural localization of VWF and its cleaved pro sequence was carried out using Lowicryl fixation and indirect immunochemical staining with protein A-colloidal gold. Because both polypeptides had been previously demonstrated in the dense organelle fraction isolated in the biosynthetic experiments, it was expected that the same proteins should be identifiable in Weibel–Palade bodies by immunoelectron microscopy. Indeed, indistinguishable staining of every Weibel–Palade body was observed using monoclonal antibodies against VWF and proVWF, respectively (Fig. 7, *A* and *B*).

Comparison of Dense Organelle Proteins and PMA-induced Releasates

To characterize the VWF released during PMA induced secretion, mock-induced and PMA-induced releasates were compared by coelectrophoresis with material obtained from dense organelle fractions derived from mock-treated and PMA-treated endothelial cells. Under reducing conditions on SDS polyacrylamide gels (Fig. 8 *A*), the two previously identified bands corresponding to VWF and the cleaved pro sequence were observed both in the dense fraction of mock-treated cells and in the releasate from PMA-treated cells. In contrast, a much smaller quantity of VWF was noted in the releasate from untreated cells. Under nonreducing conditions on 2% SDS agarose gels (Fig. 8 *B*), a series of large molecular weight forms of VWF was observed both in the dense fraction and in the PMA-induced releasate. The distribution of VWF multimers from either source was virtually identical and thus appears not to have been altered in the process of secretion. The multimers were of strikingly high molecular weight compared with those seen in VWF isolated from 3-d conditioned medium (Fig. 8 *B*, lane *e*). In addition, a number of constitutively secreted proteins of lower molecular weight were seen in conditioned media from both PMA-treated and mock-treated cells.

Discussion

Human vascular endothelial cells in culture biosynthesize VWF and secrete this protein in both a constitutive and regulated manner. We have isolated a radiolabeled dense organelle fraction from cultured human EC and have shown that is the source of VWF released by treatment with PMA (Fig. 8).

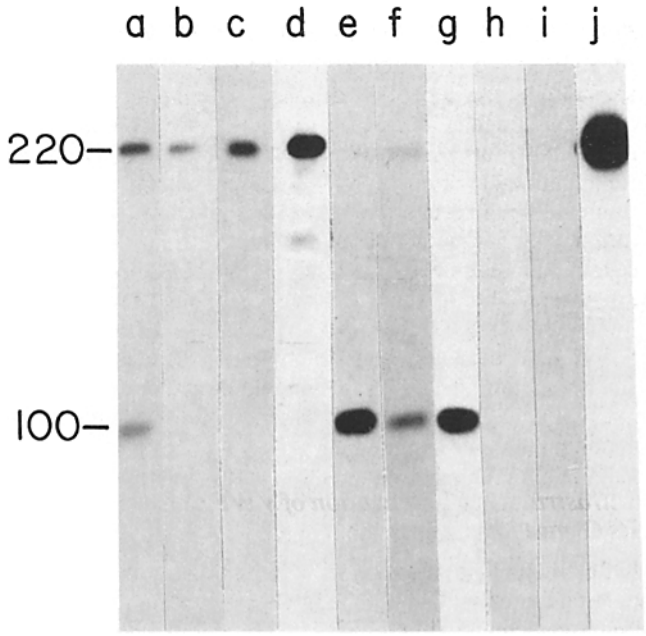


Figure 5. Serologic identification of the principal proteins of purified [^{35}S]cysteine-labeled Weibel–Palade bodies. The detergent-solubilized organelle (Fig. 2 *A*, fractions *7* and *8*) was analyzed by SDS PAGE (5% acrylamide; lane *a*) and compared with immunoprecipitates of VWF (lanes *b–d*), proVWF (lanes *e*, *f* and *g*) or fibronectin (lanes *h–j*) that were prepared from detergent extracts of the dense organelle (lanes *b*, *e*, and *h*), from PMA releasates (*c*, *f*, and *i*), and from 3-d conditioned medium (lanes *d*, *g*, and *j*). All samples are from the same labeled cell preparation, but immunoprecipitates from each fraction were subjected to SDS PAGE on replicate slab gels. Numbers on left indicate $M_r \times 10^{-3}$.

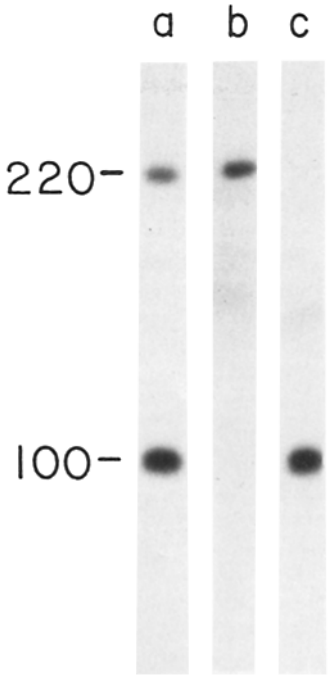


Figure 6. Quantitative immunoabsorption of mature VWF and cleaved proVWF polypeptide from purified [^{35}S]cysteine-labeled Weibel–Palade bodies. The detergent solubilized dense organelle was analyzed by SDS PAGE (5% acrylamide, lane *a*) and compared with supernatants obtained by prior immunoabsorption with monoclonal antibodies to either proVWF (lane *b*) or mature VWF (lane *c*). Immune complexes were removed by the addition of a rabbit anti-mouse antiserum previously bound to protein A-Sepharose beads. See Materials and Methods for details.

The data presented in this study demonstrate an identity between the dense organelles isolated by Percoll subcellular fractionation and Weibel–Palade bodies seen by immunoelectron microscopy. Specifically, electron microscopic analysis of the dense VWF-rich peak on Percoll gradients revealed a predominance of organelles morphologically indistinguishable from Weibel–Palade bodies. In addition, we observed a temporal relationship after PMA stimulation of the disappearance of Weibel–Palade bodies as judged by morphometric analysis, the loss of the dense VWF-rich peak on Percoll gradient, and the accumulation of VWF in culture medium. The established identity between the protein content of the dense organelle fraction and the PMA-releasates of [^{35}S]cysteine-labeled cells strongly suggests that these organelles are secretory in nature. This latter conclusion is in concordance with previous observations of Weibel–Palade bodies fusing with outer cell membranes in stimulated toad aorta (Fujimoto, 1982) and extra-alveolar vessels from lungs of rabbits (McNiff and Gil, 1983). Our ultrastructural results confirm previous inferences based upon the loss of characteristic immunofluorescent staining of VWF after stimulation with PMA, thrombin, and calcium ionophore A23187 (Reinders et al., 1984; Hormia et al., 1984; Sporn et al., 1986). Our results predict either that vascular endothelial cells that lack Weibel–Palade bodies should not be capable of regulated secretion of VWF or that the Weibel–Palade body is not the only organelle involved in regulated VWF secretion.

Biochemical analysis of isolated Weibel–Palade bodies indicate that these organelles contain only two principal polypeptides, identified immunochemically as processed VWF (M_r 220,000) and the cleaved VWF pro sequence (M_r 100,000) (Figs. 3 and 4). Both polypeptides were localized to the Weibel–Palade body by ultrastructural immunocytochemistry. The apparently stoichiometric quantities of these two proteins in the same organelle implies that proteolytic cleavage occurs after proVWF (M_r 275,000) is translocated into the storage organelle. An alternative explanation is that mature VWF and its cleaved pro sequence remain noncovalently associated and are thus sorted together. However, the two proteins clearly separate under the conditions used for immunoprecipitation (Fig. 4). A third possibility is that both proteins are separately sorted into the same organelle by a process that effectively excludes all other proteins; this explanation appears far less likely but cannot yet be formally ruled out. If cleavage occurs after sorting, then the secretory organelle must contain a specific protease. This prediction is under investigation.

A number of clinical and laboratory observations suggest that the adhesive potency of VWF is at least partially related to the degree of multimerization (Zimmerman et al., 1983). Sporn et al. (1986) observed the release of only very large multimers from EC stimulated by either the calcium ionophore A23187 or thrombin. The purification of metabolically labeled dense organelles permits a direct analysis of VWF multimers contained within Weibel–Palade bodies. As shown in Fig. 8, the VWF present within this organelle exists as an array of high molecular mass multimers that upon reduction were seen to consist entirely of M_r 220,000 subunits. Only trace quantities of dimer could be appreciated and very high molecular mass species could clearly be discerned. This is in marked contrast to the pattern observed in VWF isolated from 3-d culture medium by us and others (Lynch et al., 1986; Sporn et al., 1986). Analysis of the latter material consistently shows a predominance of dimeric and tetrameric

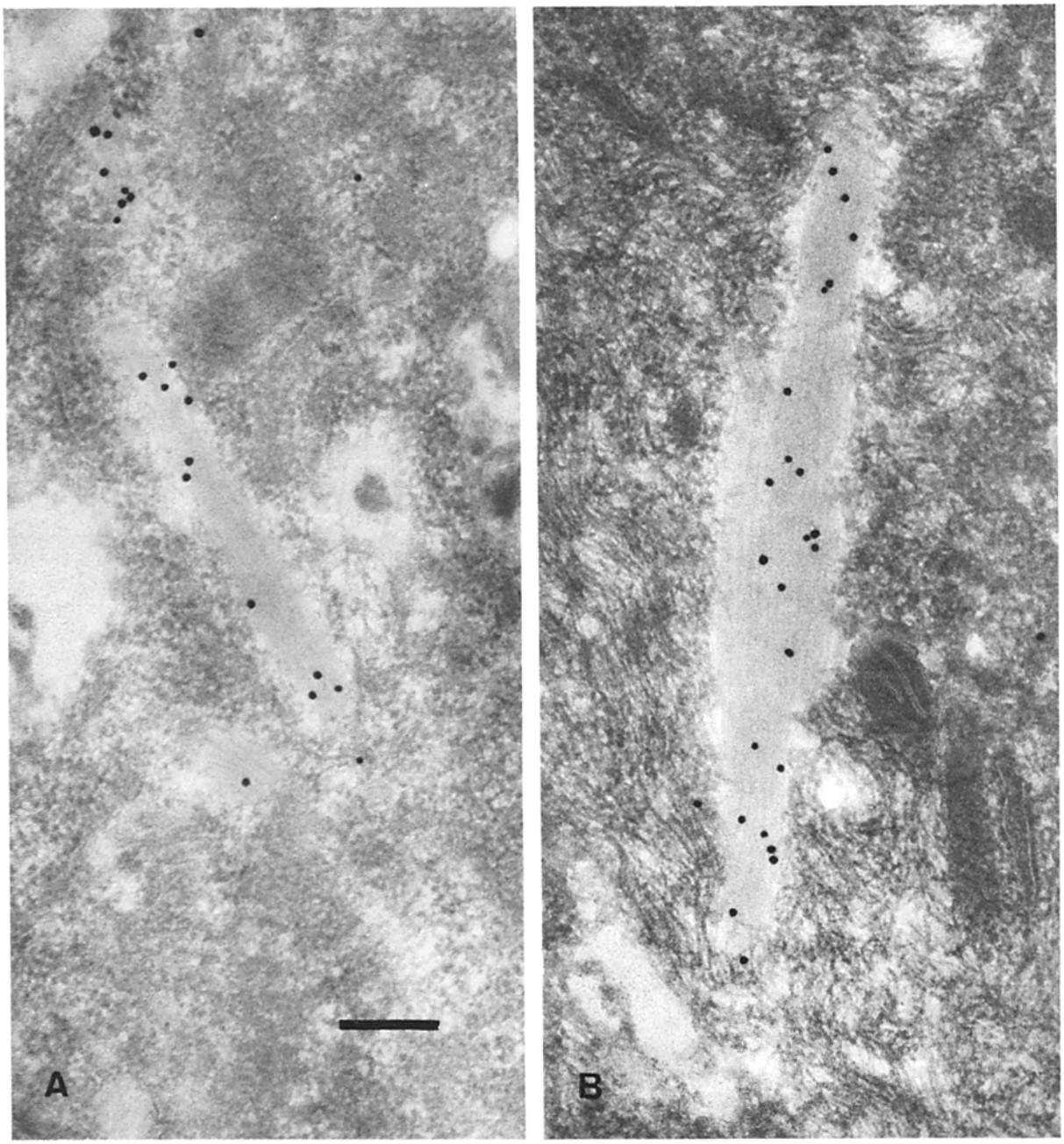


Figure 7. (*A*) An endothelial cell in culture labeled with murine monoclonal anti-VWF. All Weibel–Palade bodies identified were labeled. A representative Weibel–Palade body is seen in the center of the photograph. (*B*) An endothelial cell in culture labeled with a mixture of five monoclonal antibodies directed against proVWF sequences. All the Weibel–Palade bodies in all cells were also labeled with this

Percoll gradient, and the accumulation of VWF in culture

species and often fails to demonstrate appreciable quantities of larger forms. These differences further support the concept of separate intracellular pathways for VWF destined for storage and regulated release on the one hand and constitutive secretion on the other. The factors that lead to the high degree of multimerization within the storage organelle are, at present, unknown. One possibility is that VWF in the storage granule is composed of protomers that are in some way biochemically different than those secreted along the constitutive pathway. Alternatively, and more likely, intraorganelle conditions such as protein concentration and redox potentials predispose to the formation of large multimers (Loscalzo et al., 1985). Finally, stimulation by PMA results in the release of VWF with a molecular weight distribution identical to that seen in isolated dense organelles suggesting that the process of regulated secretion per se does not alter the multimeric pattern of VWF.

The physiologic significance of a storage pool of high molecular weight VWF remains to be elucidated. It seems reasonable to suppose, however, that locally high concentrations of thrombin, a known endothelial secretagogue, might be transiently achieved at the site of vascular injury. This

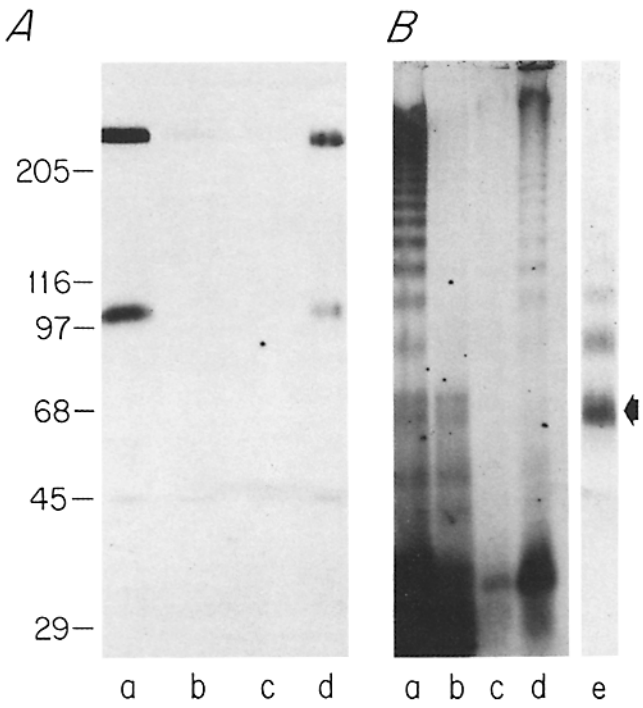


Figure 8. Comparison of dense organelle proteins and PMA-induced releasates of [^{35}S]cysteine-labeled endothelial cells. (*A*) SDS PAGE (4–13% acrylamide) and fluorography of releasates of (*a*) PMA-treated and (*b*) mock-treated cells and of dense organelles from (*c*) PMA-treated and (*d*) mock-treated cells. All samples were subjected to disulfide reduction with 1% 2-mercaptoethanol (100°C, 2 min) before electrophoresis. Numbers on left indicate $M_r \times 10^{-3}$. (*B*) SDS 2% agarose electrophoresis of the same samples under nonreducing conditions (lanes *a–d*) compared with VWF immunoisolated from 3-d conditioned medium from [^{35}S]cysteine-labeled cells analyzed on a replicate gel. (<) indicates electrophoretic mobility of VWF dimer.

might in turn lead to an amplification of the hemostatic response by the regulated secretion of highly adhesive VWF and a consequent increase in platelet binding. In addition, the elaboration of tissue factor on the surface of endothelial cells in response to inflammatory mediators such as tumor necrosis factor or interleukin-1 (Bevilacqua et al., 1984, 1986; Nawroth and Stern, 1986) may lead to increased local levels of thrombin. The subsequent increase of regulated VWF secretion may serve to amplify the "procoagulant" effects of these agents.

Most types of regulated secretory cells contain within them vesicles with electron-opaque, dense cores of highly concentrated secretory product surrounded by a unit membrane (Kelly, 1985). In contrast, the Weibel–Palade body of the endothelial cell contains within its "core" a number of lamellar structures embedded within a dense matrix (Burri and Weibel, 1968). Because VWF and its cleaved pro sequence are the major constituents of the Weibel–Palade body, it is reasonable to assume that these proteins will make a substantial contribution to the unique morphology of these organelles. Little is known of the tertiary structure of the pro VWF polypeptide. Ohmori et al. (1982) and Slayter et al. (1985) analyzed the structural characteristics of purified plasma-derived VWF by electron microscopy and determined that the protein takes the form of thin coils consisting of end-to-end multimers of a morphologically identifiable protomer. The lamellar fine structure of Weibel–Palade bodies may represent small bundles of highly polymerized filamentous VWF. The secretion of these large multimers appears to be accomplished by the direct fusion of the Weibel–Palade body with cell plasma membrane in response to external stimuli. The distinctive morphology of this secretory organelle may thus simply be a consequence of the unique molecular structure of its stored protein.

The large degree of enrichment of VWF and its cleaved pro sequence in the dense organelle peak with respect to other intracellular proteins is apparent from the analysis of radiolabeled Percoll gradient fractions (Fig. 3). No other cysteine-containing proteins were found to be similarly enriched in these organelles. This is in agreement with work of Reinders et al. (1985) which demonstrated that neither immunoreactive thrombospondin nor fibronectin were present in isolated organelles from endothelial cells. As these two proteins colocalize with VWF in the α-granules of platelets (Holt and Niewiarowski, 1985), their absence in the VWF-rich, endothelial organelles suggests different intracellular trafficking patterns in the two cell types. In addition to its effects on VWF secretion, thrombin stimulates cultured endothelium to release both tissue-type plasminogen activator and platelet-derived growth factor (Levin et al., 1984; Ross et al., 1986). Both these proteins contain a large percentage of cysteine residues and platelet-derived growth factor colocalizes with VWF in platelet α-granules, yet neither protein was clearly evident in SDS polyacrylamide gel analyses of the dense organelle fraction. Thus, either both products are stored elsewhere in endothelial cells or are present in Weibel–Palade bodies in concentrations below that which could be detected by these methods. It is also striking that we detect no specific structural proteins associated with the secretory organelle using our labeling protocol ([^{35}S]cysteine for 3 d in complete medium). Such proteins may be cysteine free or of relatively low abundance. A preliminary experiment using [^{35}S]methionine- and cysteine-labeled cells also failed to reveal enrichment of any other polypeptides; additional efforts to identify structural or other secretory constituents of the Weibel–Palade body are in progress.

Endothelial cells direct newly synthesized VWF along at least two different intracellular pathways. The information responsible for such sorting must reside both in the VWF protein itself and in endothelial subcellular structures. Defects either in the primary structure of VWF or in the endothelial cell secretory machinery could lead to defective regulated secretion of high molecular weight VWF. In fact, such a deficiency of highly multimerized VWF, in plasma and/or platelets is observed in patients with type II variants of von Willebrand's disease. The isolation of VWF storage vesicles as reported here, in conjunction with the synthesis of appropriate VWF cDNA constructs and the development of more highly efficient endothelial cell transfection techniques, will permit further delineation of endothelial cell sorting signals.

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