Biosynthesis of von Willebrand Protein by Human Endothelial Cells: Processing Steps and Their Intracellular Localization

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ABSTRACT Biosynthesis of von Willebrand protein by human umbilical vein endothelial cells involved distinct processing steps marked by the presence of several intermediate molecular species. Examination of endoglycosidase H sensitivity of these intracellular intermediates indicated that the processing steps occurred in at least two separate cellular compartments. In the pre-Golgi apparatus (most probably the endoplasmic reticulum), the high mannose carbohydrates were added onto the precursor monomer chains and the 260,000-mol-wt monomers dimerized by interchain disulfide bond formation. The other processing steps have been localized to the Golgi apparatus and later compartments (e.g., Weibel-Palade bodies). High mannose carbohydrate was converted to the complex type, leading to the appearance of a larger precursor subunit of 275,000 mol wt. The 275,000-mol-wt species was not formed if carbohydrate processing was inhibited by the ionophore monensin. From the large pool of dimers of precursor subunits, the high molecular weight multimers were built. These dimer molecules appeared to have free sulfhydryls which might have been involved in the interdimer disulfide bond formation. Simultaneously with multimerization, the precursor subunits were cleaved to the 220,000-mol-wt form. The cleavage of the pro-sequence was not likely to be an absolute requirement for von Willebrand protein multimerization or secretion, as the 275,000-mol-wt precursor subunit was present in secreted high molecular weight multimers of the protein.

von Willebrand (vW)[1] protein is a large glycoprotein of complex multimeric structure held together by disulfide bonds (1, 2). Absence of or molecular defects in the protein may cause a bleeding disorder owing to inadequate binding of platelets at sites of injury (1, 3, 4). vW protein is synthesized by megakaryocytes (5) and by endothelial cells that are likely to be responsible for the presence of vW protein in blood and subendothelium. In the endothelial cells, vW protein is concentrated in organelles called Weibel-Palade bodies (6). Endothelial cells synthesize vW protein subunits first as large precursors of 260,000-mol-wt (7, 8), which undergo several processing steps including carbohydrate addition, polymerization by disulfide bond formation, and proteolytic cleavage to a mature subunit form of 220,000-mol-wt. Although the presence of high molecular weight (HMW) polymers of vW protein in cell lysates of trypsin-treated cells indicates that they form within the cells (7), the intracellular locations of the processing steps are not known. Pulse-chase experiments on human umbilical vein endothelial cells have demonstrated that cleavage of the intracellular precursor to the mature subunit is very slow. It is first observed 2 h after the onset of labeling and even after several hours of chase some uncleaved precursor protein is still present. This slow processing explains the relatively long lag period (2 h) between the onset of labeling and the secretion of radiolabeled vW protein (7).

We now describe the biosynthetic steps involved in the polymerization of vW protein and by following the carbohydrate processing, we also propose the subcellular organelles in which these steps probably take place. Our results were reported in part at the Ninth International Congress on Thrombosis and Haemostasis (9).

[1] *Abbreviations used in this paper:* DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HMW, high molecular weight; vW, von Willebrand.

MATERIALS AND METHODS

Cells and Culture Conditions: Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously (6, 10). Cells were cultured in McCoy's 5A medium (Flow Laboratories, Inc., McLean, VA) containing 20% fetal bovine serum. For continuous metabolic labeling, cells were grown in the presence of [^{35}S]methionine (25 μCi/ml, 12.3 Ci/mmol, Amersham Corp., Arlington Heights, IL).

Antisera: The preparation and characterization of antisera against human vW protein were as described previously (6, 11). Some experiments used antiserum of equally high quality purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Electrophoresis Gels: SDS polyacrylamide gels were prepared as described by Laemmli (12) and agarose horizontal slab gels were prepared using a solution of 2% agarose, 0.1% SDS in 0.05 M phosphate buffer at pH 7.0. Molecular weights were calibrated using the following markers: fibronectin (dimer 460,000, monomer 230,000); fibrinogen, 340,000; myosin, 200,000; β-galactosidase, 130,000; and phosphorylase *a*, 94,000. For two-dimensional gel electrophoresis, protein was first run nonreduced on a 2% agarose gel. The strip was then cut out and incubated with constant shaking for 10–20 min at room temperature in running buffer (12) that contained 50 mM dithiothreitol. It was transferred to the top of a 5% SDS polyacrylamide gel and overlayed with 0.1% agarose, 0.1 M dithiothreitol in running buffer before electrophoresis.

Purification of vW Protein: Cells were lysed as described previously (7) so that the final concentration of ingredients was that of the radioimmunoprecipitation assay buffer (13) used for washing the immunoprecipitate. The cell lysate or culture medium samples were then incubated for 1.5 h at room temperature with gelatin-Sepharose to remove fibronectin and other proteins that adhere nonspecifically. The gelatin-Sepharose was removed by centrifugation. Protein A-Sepharose CL-4B (30 mg/25 cm^2 flask) (Pharmacia Fine Chemicals, Piscataway, NJ) was preincubated at room temperature for 30 min with 100 μl anti-vW protein antiserum before it was added to samples. The incubation with the samples was for 1.5 h at room temperature. After extensive washing, protein A-Sepharose was boiled in electrophoresis sample buffer (12) and the supernatant was analyzed by gel electrophoresis.

Pulse-Chase Experiments: Pulse-chase experiments were performed as described previously (7).

Endoglycosidase H Digestion: The enzyme was a generous gift of Dr. P. W. Robbins (Massachusetts Institute of Technology) and was purified according to Tarentino et al. (14). Purified vW protein was diluted in 0.1 M Tris buffer, pH 5.8, so that the final concentration of SDS was <0.5%. Endoglycosidase H was added (3 μg/ml) and samples were incubated for 2 h at 37°C before analysis on gels.

Monensin Treatment: The effect of monensin on carbohydrate processing by cultured endothelial cells was assessed by the addition of monensin (Calbiochem-Behring Corp.) at a final concentration of 1–50 μM of culture medium.

Detection of Free Sulfhydryl Groups by Covalent Chromatography: Immunopurified vW protein was resuspended in 900 μl of 0.1 M Tris, pH 8 buffer, 1 mM EDTA and saturated with urea, and 800 μl was loaded on a 2-ml column of activated Thiol-Sepharose 4B (Pharmacia Fine Chemicals). After 4–6 h of incubation at room temperature the column was washed with 0.1 M Tris, pH 8 buffer, 1 mM EDTA, 8 M urea and then with this buffer containing 1 M NaCl. Disulfide bonds were broken with 10 mM dithiothreitol in the same buffer. In control experiments vW protein was incubated for 1 h at 37°C in the presence of 2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) before being loaded on the column.

RESULTS

Subunit Composition of Cellular and Secreted vW Protein

The subunit of human vW protein (220,000 mol wt) is first synthesized as a precursor of 260,000-mol-wt. This precursor accounts for about half of the total cellular vW protein. vW protein that is secreted into the culture medium also contains a small amount of slightly larger precursor species (275,000-mol-wt) (7). To see whether the precursor subunits were incorporated into the HMW multimers, we metabolically labeled endothelial cells for 3 d, purified vW protein from the cell lysate and culture medium, and examined the composition of the cellular and secreted multimers by two-dimensional gel electrophoresis (Fig. 1). The multimers were first separated in a 2% agarose gel and after reduction their subunit composition was analyzed in a 5% polyacrylamide gel used for the second dimension. Small amounts of the 275,000-mol-wt precursor subunit were present in all of the secreted multimers, representing 3.8% (SD = 2.6) of vW protein subunits in the intermediate and HMW multimers and slightly less in the dimers. The large pool of intracellular 260,000-mol-wt precursor was found mainly at the dimer position along with some processed 220,000-mol-wt subunits. As observed by others (15), very little precursor was seen in the HMW multimers associated with cells, but unlike the report by Lynch et al. (15), we found precursor subunits in multimers past the stage of dimer in secreted vW protein. This indicates that the cleavage of precursor was more complete in stored cellular vW protein than in the material secreted into the culture medium.

Polymer Formation

Pulse-chase experiments were performed to examine the biosynthetic steps involved in the formation of vW protein multimers. Umbilical vein endothelial cells were labeled for 30 min with [^{35}S]methionine and then incubated for various lengths of time in unlabeled culture medium. vW protein was purified from the cell lysates and culture medium and analyzed reduced on 5% SDS polyacrylamide gels (Fig. 2*a*) and nonreduced on 2% agarose gels (Fig. 2*b*). vW protein was first (0.5 h) synthesized as a monomeric precursor (260,000-mol-wt), and within 2 h after the onset of synthesis the monomer dimerized without the formation of 220,000-mol-wt subunits. From this large pool of precursor dimers, the HMW multimers were built slowly and multimers of all sizes were secreted into the culture medium (Fig. 2*b*). The composition of multimers of cellular vW protein from the 2–7-h pulse-chase samples was similar to that of multimers from long-term labeled cells (not shown), except that the intracellular dimer was almost exclusively composed of precursor subunits at the short chase times. The HMW multimers were composed of the 220,000-mol-wt subunit even in the 2-h pulse-chase time point, indicating that polymerization and cleavage occurred at about the same time.

Early secreted vW protein analyzed by two-dimensional gel electrophoresis (Fig. 3) contained a higher proportion (up to 40%) of precursor subunits in the intermediate and HMW multimers than was seen in long-term labeled cultures (Fig. 1). This indicates that young, poorly processed multimers were secreted into the culture medium and that cleavage of the precursor was not a requirement for polymer formation or for vW protein secretion.

Endoglycosidase H Sensitivity and Treatment with Ionophore Monensin

The cleavage of vW protein precursor to the 220,000-mol-wt form was first observed at ~2 h after the onset of labeling (7) (Fig. 2*a*), by which time essentially all vW protein precursor was dimerized. This was also the time when HMW multimers were formed (Fig. 2*b*) and vW protein was first detected in the culture medium (7) (Fig. 2*b*). Thus, four processes occurred: (*a*) dimerization of the 260,000-mol-wt precursor, followed by (*b*) polymerization of the dimer, (*c*) cleavage of precursor to the 220,000-mol-wt subunit, and (*d*) secretion of

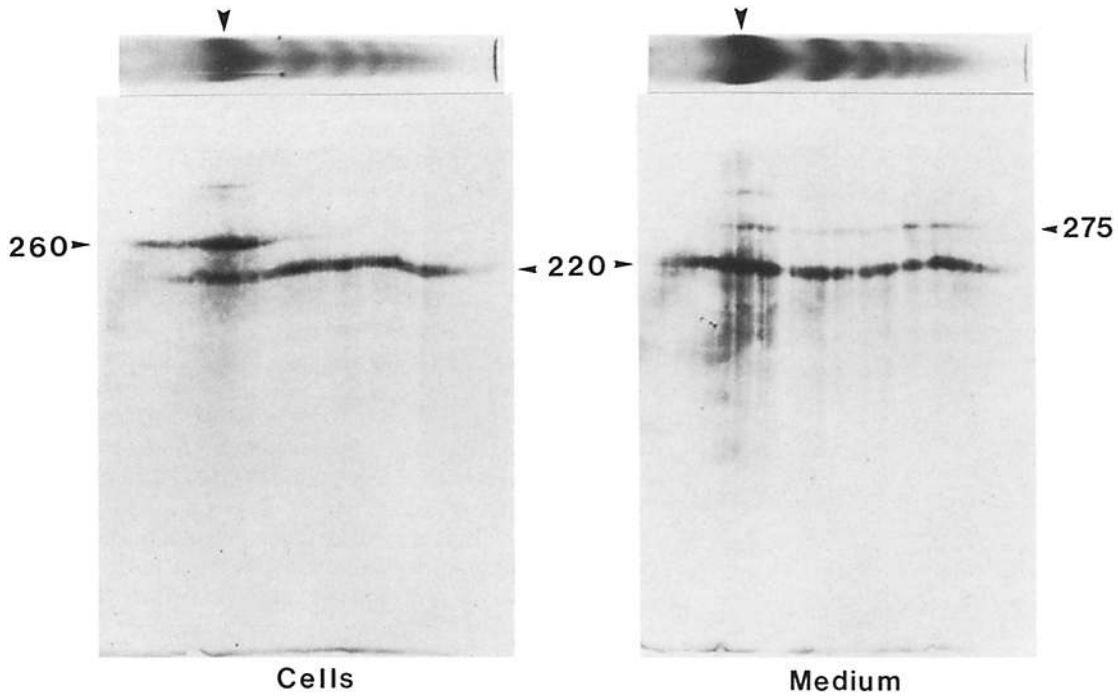

FIGURE 1 Subunit composition of vW protein analyzed by autoradiography after two-dimensional gel electrophoresis. Human umbilical vein endothelial cells were metabolically labeled for 3 d and vW protein was purified from the cell lysate and from the culture medium. In the first dimension (*top*), nonreduced vW protein was electrophoresed in a 2% agarose gel (*right* to *left*). The arrowhead indicates the position of the vW protein dimer; the HMW multimers are to the right. After reduction, the agarose strip was placed on top of the 5% polyacrylamide gel (as shown) and electrophoresis was performed from top to bottom to determine the subunit composition of the oligomers. The secreted vW protein contains small amounts of precursor subunit (*275*) in all polymers as well as in the dimer, while the precursor in the cells (*260*) is mostly present at the dimer position.

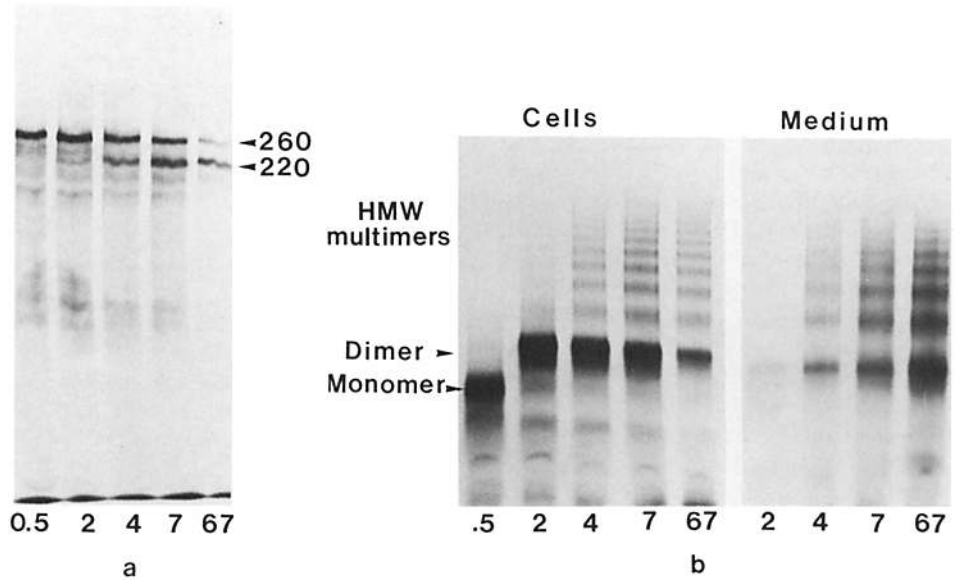

FIGURE 2 Pulse-chase experiment showing the cleavage of precursor, the formation of vW multimers, and their secretion into the culture medium. Cells were metabolically labeled with [^{35}S]methionine for 30 min and then chased in unlabeled medium. Numbers indicate the time in hours after the onset of labeling. Samples shown in *a* and *b* are from the same experiment. (*a*) Autoradiograph of a 5% SDS polyacrylamide gel of reduced vW protein purified from cell lysates. The 260,000-mol-wt precursor subunit (*260*) is gradually converted to the 220,000-mol-wt (*220*) size. (*b*) Autoradiograph of 2% agarose gels showing nonreduced vW protein purified from cell lysate and from culture medium. vW protein is initially synthesized as a monomer which forms dimers, from which the HMW multimers are built. The dimer and all multimers are secreted, but the monomer is not secreted.

vW protein. The following rationale and experiments were used to determine the location within endothelial cell in which the processing steps occur.

vW protein is known to be a glycoprotein with complex asparagine-linked side chains. These are added in a high mannose precursor form during translation in the rough endoplasmic reticulum, and processing of the high mannose form to the complex form takes place in the Golgi apparatus

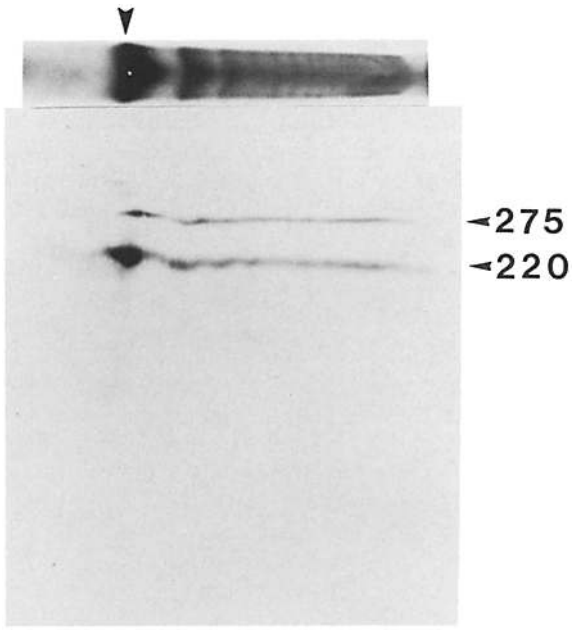

FIGURE 3 Subunit composition of early secreted vW protein. Cells were metabolically labeled for 30 min and then chased overnight in unlabeled medium. The secreted vW protein was purified and analyzed by two-dimensional gel electrophoresis as described in Fig. 1. In comparison to vW protein secreted over a period of 3 d (Fig. 1), the early secreted multimers have a significantly higher precursor subunit (275) content.

(16). Therefore, nascent vW protein before its transfer to the Golgi should be distinguishable from that in the Golgi apparatus or later compartments by the nature of its carbohydrate side chains The enzyme endoglycosidase H cleaves the chitobiosyl unit of the high mannose form but not that of the complex form (14, 17). Therefore, vW protein before Golgi processing will be endoglycosidase H sensitive whereas that in cellular compartments after processing will be resistant. Removal of the carbohydrate side chains produces a shift to a lower molecular weight form that is detectable on gels. In this way, it should be possible to determine which processing steps occur before and after entry into the Golgi apparatus.

vW protein was purified from 3-d metabolically labeled cells and culture medium and then subjected to endoglycosidase H digestion. The digested and control samples were analyzed reduced on a 5% SDS polyacrylamide gel (Fig. 4). This gel system resolves the cellular precursor of 260,000-mol-wt from uncleaved secreted precursor of 275,000-mol-wt and from 220,000-mol-wt cleaved subunits. As expected, all secreted subunits, whether 275,000 or 220,000-mol-wt, had been processed during passage through the Golgi complex and therefore were resistant to endoglycosidase H treatment. All of the 260,000-mol-wt cellular precursor was sensitive to endoglycosidase H, but the cellular 220,000-mol-wt subunits did not show a size change after endoglycosidase H treatment. This suggests that all of the cellular 260,000-mol-wt precursor was present in compartments before Golgi processing (e.g., rough endoplasmic reticulum), whereas all of the 220,000-mol-wt subunit was present in Golgi or later compartments. Analysis of the endoglycosidase H-treated samples and controls on 2% agarose gels (not shown) showed that only the cellular dimer migrated noticeably faster upon enzymatic treatment. This further supports the observation in Fig. 1 that cellular vW protein polymers were made of 220,000-mol-wt subunits, which were endoglycosidase H resistant (Fig. 4).

Processing of high mannose carbohydrate to the complex form is usually accompanied by an apparent increase in molecular weight on SDS polyacrylamide gels (18). To examine if the size difference of the cellular (260,000-mol-wt) and secreted (275,000-mol-wt) precursors was due to carbohydrate processing, we grew endothelial cells in the presence of the ionophore monensin (19, 20) which affects the carbohydrate processing of fibronectin (21) and other proteins. Monensin inhibited the conversion of high mannose to complex type of carbohydrate of vW protein as shown by the sensitivity of the secreted vW to endoglycosidase H (Fig. 5, right). The secreted precursor in monensin-treated cultures co-migrated with the cellular 260,000-mol-wt precursor of controlled cells (fig. 5, left), indicating that the size difference between the 275,000- and 260,000-mol-wt precursors was due to a Golgi-localized processing step, most likely the conversion of high mannose to complex type carbohydrate. Secreted, mature (cleaved) subunit was smaller in monensin-treated cultures compared with the 220,000-mol-wt subunit found in control cells and medium (Fig. 5). This is in agreement with the observation (Fig. 4) that the cellular and secreted 220,000-mol-wt subunits normally have complex type endoglycosidase H-resistant carbohydrate, which is replaced by an endoglycosidase H-sensitive form that migrates faster on gels when carbohydrate conversion is inhibited by monensin.

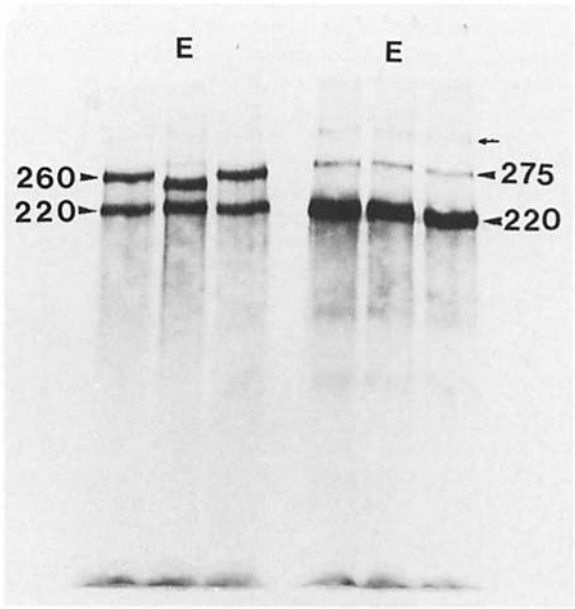

FIGURE 4 Endoglycosidase H digestion of vW protein from long-term labeled cultures. Human umbilical vein endothelial cells were metabolically labeled with [^{35}S]methionine for 3 d. Purified vW protein from cell lysate (*left*) and culture medium (*right*) was divided into three aliquots, one of which was digested with endoglycosidase H (*E*). All samples were reduced and submitted to electrophoresis on a 5% SDS polyacrylamide gel before autoradiography. The 260,000-mol-wt (*260*) intracellular precursor subunit migrated faster after endoglycosidase H digestion, while the larger 275,000-mol-wt (*275*) precursor species, whether secreted or in the cell lysate (trace amount), was resistant to endoglycosidase H. The enzyme had no effect on the migration of the cellular or secreted 220,000-mol-wt (*220*) subunit. The arrow indicates the position of a larger protein of unknown origin which appeared to associate with all of the secreted multimers (Fig. 1).

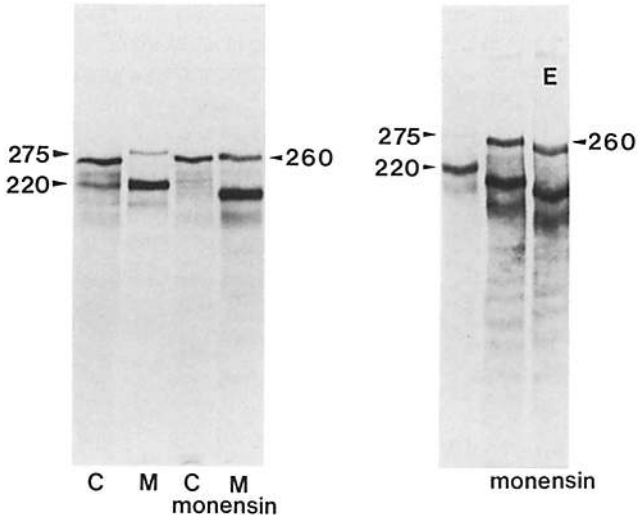

FIGURE 5 Effect of monensin on subunit size and endoglycosidase H sensitivity of vW protein. Autoradiographs of reduced 5% polyacrylamide gels. (*Left*) Cells were labeled metabolically in the absence or presence of 1 μM monensin (*monensin*). After 1 d, vW protein was purified from the cell lysate (*C*) and culture medium (*M*). The secreted precursor in monensin-treated cultures co-migrated with intracellular precursor of treated and control cells (*260*), while the secreted precursor of control cells was larger (*275*). (*Right*) Purified vW protein from culture medium of cells labeled (3 d) with or without monensin. Both precursor and cleaved subunits of vW protein secreted in monensin-treated cultures migrated faster than their control counterparts (as seen also at *left*). This was probably due to inhibition of carbohydrate processing of vW protein by monensin. The presence of high mannose carbohydrate on secreted vW protein from monensin-treated cultures was shown by the susceptibility of the protein to endoglycosidase H (lane *E*). Under normal conditions, secreted vW protein was endoglycosidase H resistant (Fig. 4).

A pulse-chase experiment was performed to examine endoglycosidase H sensitivity of the different intracellular species of vW protein (Fig. 6). The 260,000-mol-wt monomer, which was the major species labeled after 30 min (Fig. 2*b*), was endoglycosidase H sensitive (Fig. 6) indicating that it already contained high mannose type carbohydrate. At 2 h after onset of labeling, when the major species present was a dimer of the 260,000-mol-wt subunits (Fig. 2*b*), all of the material migrating at the 260,000-mol-wt position was still endoglycosidase H sensitive (Fig. 6). Thus, the precursor dimer contained carbohydrate of the high mannose type and it was formed before passage to Golgi apparatus, probably in the endoplasmic reticulum. At this 2-h time point, the larger precursor species of 275,000-mol-wt first became apparent in the cells and it did not change mobility after endoglycosidase H digestion. This precursor species, which contained complex type carbohydrate, might represent a transient form before the pro-sequence was cleaved. It was the only precursor species that was found in the culture medium (Figs. 1 and 4). The newly formed 220,000-mol-wt subunit that was present in the 2-h sample (Fig. 6) was entirely endoglycosidase H resistant, as was also observed in long-term labeled cultures (Fig. 4). This indicates that the pro-sequence cleavage leading to the 220,000-mol-wt subunit occurred after vW protein entered the Golgi complex, where its carbohydrates were processed to the complex type.

Free Sulfhydryl Groups

The presence of free sulfhydryls on precursor molecules could influence the polymerization reaction. We used an activated thiol-Sepharose 4B column, which covalently binds proteins containing free sulfhydryl groups, to determine if there were free sulfhydryls on intracellular vW protein. vW protein purified from long-term [^{35}S]methionine-labeled cell lysate was immediately submitted to chromatography. Approximately half of the material bound to the column and was eluted with 10 mM dithiothreitol. Pretreatment of the sample with DTNB blocked its binding to the column (Fig. 7). Analysis of the flow-through and dithiothreitol-eluted samples on polyacrylamide gels showed that the 260,000-mol-wt precursor bound preferentially, while most of the 220,000-mol-wt cleaved protein came in the flow-through (Fig. 7). Since the 260,000-mol-wt precursor was mostly present at the dimer position in continuously labeled cells (Fig. 1), it seems likely that intracellular precursor dimers contain free sulfhydryl(s) which might get oxidized or removed with the pro-sequence as the protein polymerizes.

DISCUSSION

vW protein is first synthesized as a monomeric polypeptide chain of 260,000-mol-wt (Fig. 2) which contains carbohydrate of the high mannose type and is therefore sensitive to endo-

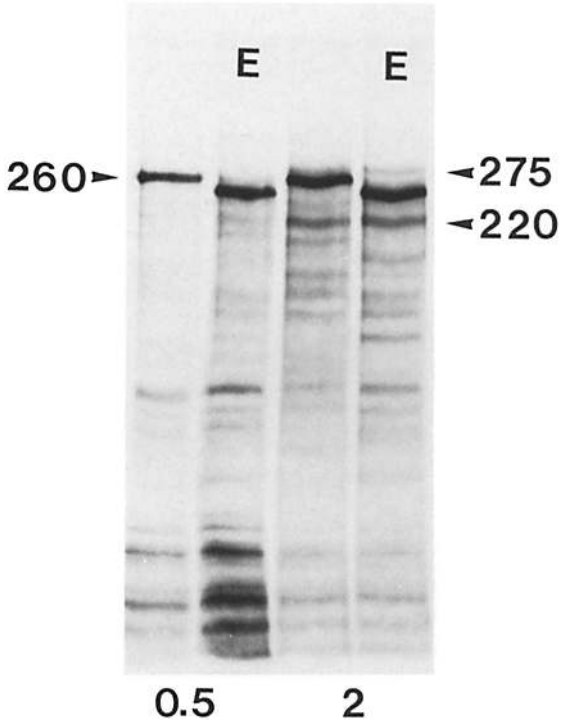

FIGURE 6 Susceptibility of early vW protein forms to endoglycosidase H digestion. vW protein was purified from a pulse-chase experiment. The cell lysate samples were divided in two aliquots, one of which was submitted to endoglycosidase H digestion (*E*). All reduced samples were analyzed by a 5% SDS PAGE, the autoradiograph of which is shown. Numbers indicate time in hours after the onset of labeling. The 260,000-mol-wt precursor subunit (*260*) was susceptible to the enzymatic digestion whether as the monomer (0.5 h) or dimer (2 h). Neither the 275,000- (*275*) nor the 220,000-mol-wt (*220*) subunit appearing in the 2-h sample changed migration after treatment with endoglycosidase H.

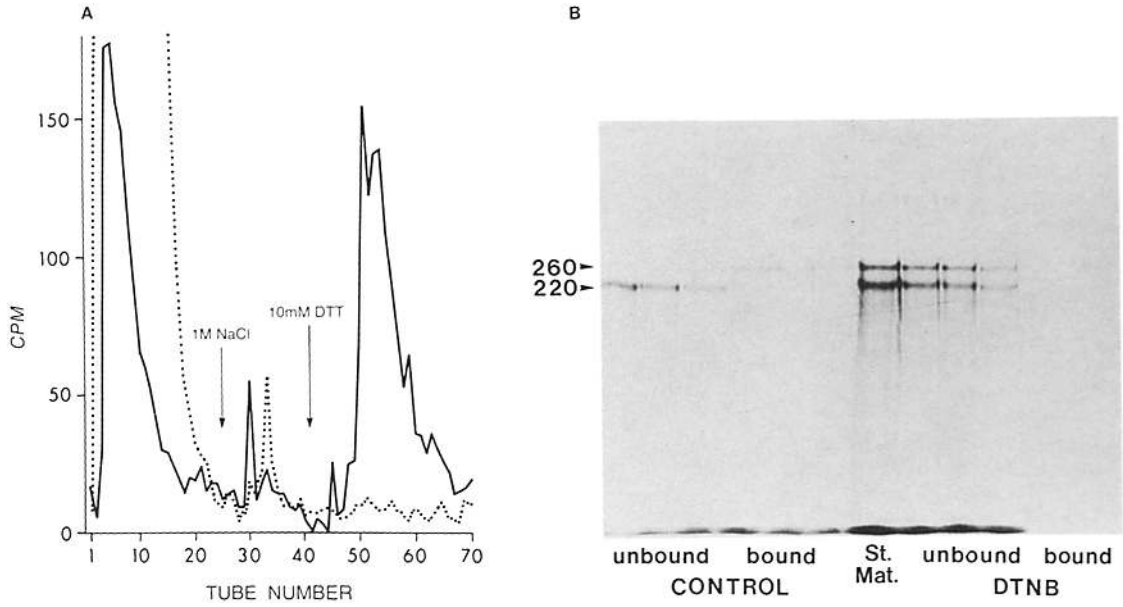

FIGURE 7 Detection of sulfhydryl groups in intracellular vW protein. vW protein purified from long-term labeled endothelial cells was divided into two parts, one of which was reacted with DTNB. The samples were loaded onto two identical activated thiol-Sepharose columns (*A*). The columns were first washed with buffer, then with buffer plus 1 M NaCl, and finally eluted with dithiothreitol (*DTT*). Approximately half of the control vW protein bound to the column (continuous line). By contrast, the sample with DTNB-blocked free sulfhydryl groups came off in the flow-through (dotted line). *B* is an autoradiograph of a 5% polyacrylamide gel that analyzed the reduced samples eluted from the columns. *St. Mat.* refers to the starting material of cellular vW protein that was loaded onto the columns. Samples from the control column are shown on the left, and those from the DTNB-treated column are on the right. In the control, the 220,000-mol-wt subunits (*220*) eluted mostly in the flow-through (*unbound*) and were partially separated from the 260,000-mol-wt subunits (*260*) that bound preferentially to the activated thiol-Sepharose column and had to be eluted with dithiothreitol (*bound*). In the DTNB-treated sample, neither 260,000- nor 220,000-mol-wt subunits bound to the column. This indicates that the 260,000-mol-wt subunits have free sulfhydryls that can be blocked by DTNB.

glycosidase H digestion (Fig. 6). The 260,000-mol-wt monomer dimerizes to form a large pool of precursor dimers (Fig. 2*b*). The dimers are also endoglycosidase H sensitive (Fig. 6), indicating that the first interchain disulfide bonds are formed before entering the Golgi apparatus, probably in the endoplasmic reticulum.

At ~2 h after the onset of labeling, when all 260,000-mol-wt monomers of vW protein have been converted to precursor dimers, three new processing steps are first observed: (*a*) multimer formation, (*b*) carbohydrate processing from the high mannose to the complex form associated with the appearance of a new precursor species of 275,000-mol-wt, and (*c*) cleavage of precursor to the 220,000-mol-wt subunit. All of the 220,000-mol-wt subunits are endoglycosidase H resistant (Fig. 6), which indicates that precursor cleavage follows carbohydrate processing. The 2-h delay in dimer processing and the change in endoglycosidase H sensitivity reflect the time required for the precursor dimer pool to move to the cellular compartment(s) where the enzymes for interdimer disulfide bond formation and precursor cleavage are located and from where the protein can be secreted. Because the carbohydrate processing enzymes are localized in the Golgi apparatus (16), processing of the precursor dimers probably begins in this organelle (Fig. 8). This is not surprising, since the Golgi complex contains enzymes responsible for prosequence removal of many small peptide hormones and other proteins such as ovalbumin (for review see references 22 and 23).

The observed compartmentalization in processing of vW protein suggests that two different mechanisms are responsible for disulfide bond formation in this complex protein. One is probably located in the endoplasmic reticulum and directs the formation of precursor dimers, and the other would be present in the Golgi apparatus and/or Weibel-Palade bodies and would form the interdimer bridges. This is reminiscent of the biosynthesis of secretory IgM where one enzyme, a disulfide interchange enzyme, is required for monomer IgM assembly (24) and a different enzyme, probably a sulfhydryl oxidase, is responsible for formation of the intermonomer bonds of IgM pentamers (25). The binding studies of intracellular vW protein to activated thiol-Sepharose (Fig. 7) indicate that free sulfhydryl(s) are present on the precursor dimer molecules and these are likely to be involved in the formation of HMW multimers of vW protein.

When the processing of carbohydrate from high mannose to complex form is inhibited by monensin, the secreted precursor co-migrates with the cellular (260,000-mol-wt) precursor and the 220,000-mol-wt cleaved subunit appears smaller than that present in untreated control media (Fig. 5). This indicates that the difference in migration on gels of the 275,000- and 260,000-mol-wt precursors is due to post-translational modifications, probably carbohydrate processing to complex type which can be inhibited by monensin, and that vW protein can be secreted even with such unprocessed carbohydrate.

Our observation of a higher precursor content in secreted vW protein purified after short labeling periods in comparison with long-term labeling (Figs. 1 and 3) is consistent with the recent description of two pathways of secretion of vW protein (26). The protein that is processed in the Golgi apparatus

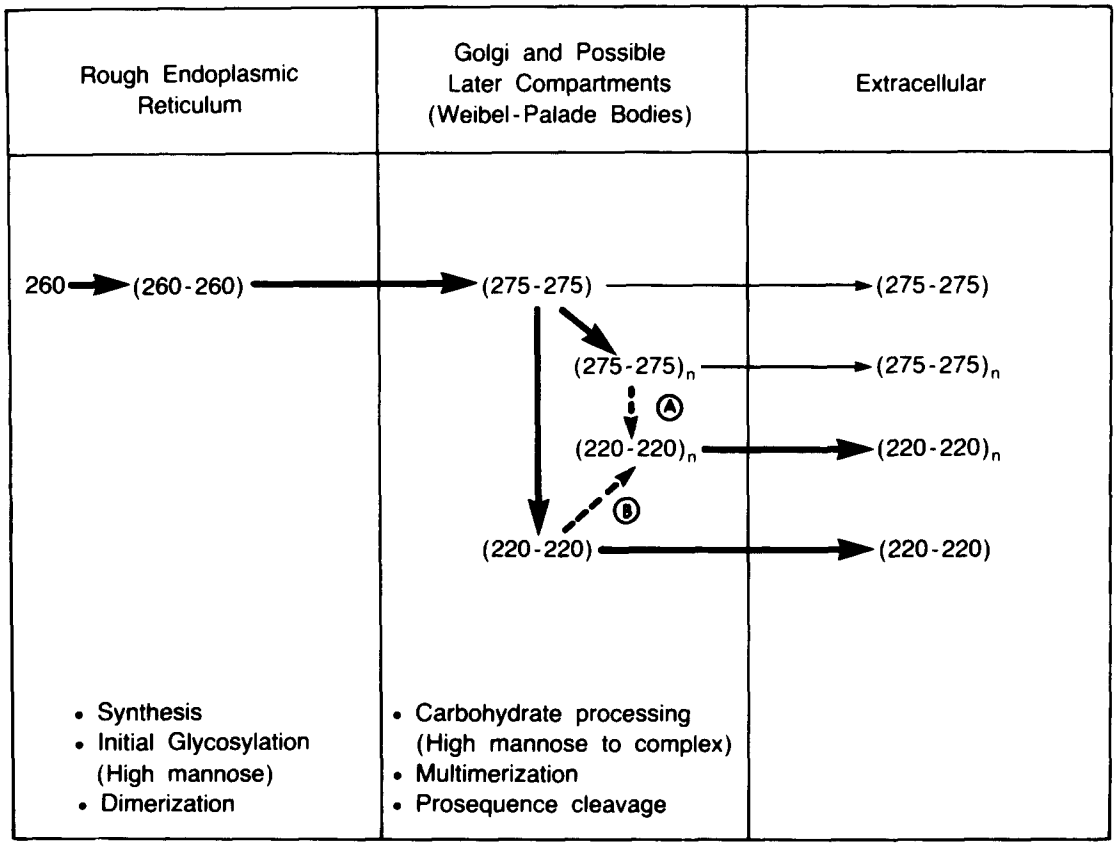

FIGURE 8 Schematic representation of the processing steps involved in von Willebrand protein biosynthesis and their proposed subcellular localization. 260,000-mol-wt precursor monomers are dimerized to form a large intracellular pool of precursor dimer (260,000-260,000). Since both species are endoglycosidase H sensitive, they reside in a pre-Golgi compartment, most probably the rough endoplasmic reticulum. Transport of the dimers to the Golgi apparatus is accompanied by an apparent increase in molecular weight from (260,000-260,000) to (275,000-275,000) and by acquiring resistance to endoglycosidase H. When processing of high mannose carbohydrates to the complex type is inhibited by monensin, the increase in molecular weight is not observed. Since multimerization and cleavage of the pro-sequence follow closely after carbohydrate processing, these steps appear to occur also in the Golgi apparatus, and likely continue in the Weibel-Palade bodies. Our data do not allow the determination of whether mature polymers $(220,000\text{-}220,000)_n$ are formed by cleavage of the pro-sequence from precursor polymers $(275,000\text{-}275,000)_n$ (pathway *A*) or by polymerization of (220,000-220,000) dimers (pathway *B*). While long-term labeled cultures secrete mostly 220,000-mol-wt subunits, early secreted von Willebrand protein contains significant amounts of 275,000-mol-wt precursor incorporated into dimers and HMW multimers. Only homologous dimers and multimers are shown in this figure, but the existence of mixed dimers (275,000-220,000) or their incorporation into polymers is not excluded.

could be secreted either directly by the first pathway as an incompletely cleaved moiety, or later as more completely processed protein from the Weibel-Palade bodies by the second pathway. Other cells are known to continue proprotein cleavage in their secretory granules (23, 27). Another explanation for the presence of lower precursor content after long-term labeling would be continued cleavage after protein secretion into the culture medium, as has been observed for rat haptoglobin (28). However, incubation of the short-term labeled medium for longer periods at 37°C in the absence of cells failed to show a change in precursor content (data not shown). In either case, the polymeric vW protein stored in the endothelial cells and released in response to injury contains negligible amount of precursor subunits, which raises a question whether the precursor subunits present in the secreted polymers have a biological function.

The role of the vW pro-sequence in the biosynthesis of vW protein is not known. Although only precursor vW protein forms dimers, this does not prove that the pro-sequence is required for dimerization. Fibronectin, which does not appear to have a proprotein sequence, still forms dimers rapidly in its endoglycosidase H-sensitive form (29). The pro-sequence may assist in aligning the precursor dimers in proper conformation for interdimer disulfide bond formation. This hypothesis could explain the many different sizes of vW protein. If the precursor ends are important for polymerization, then their premature removal could arrest the growth of the polymer. It is also possible that pro-sequence removal is necessary for proper packaging and concentration of the protein in the Weibel-Palade bodies, as is the case for insulin granules (30). This possibility is supported by the observation (Fig. 1) that cellular HMW polymers, which are probably present mostly in the Weibel-Palade bodies, contain few precursor subunits (15). On the other hand, removal of the pro-sequence is not a requirement for HMW polymers formation and secretion, as has been suggested by others (15), since uncleaved precursor subunits can be incorporated into the HMW polymers (Fig. 1), as shown most clearly by the early secreted polymers, which are rich in precursor subunits (Fig. 3).

To summarize (Fig. 8), our results indicate that the first polymerization step, namely dimer formation, occurs before the Golgi apparatus, probably in the endoplasmic reticulum.

The next polymerization step, interdimer disulfide bond formation, occurs after the dimers are transferred to the Golgi complex, where the carbohydrate is processed to the complex form. Free sulfhydryls on the dimer molecules might be involved in the interdimer polymerization reaction. HMW multimer formation appears to correspond in time to propeptide removal. Although HMW polymers of precursor subunits exist, it is not known if the cleavage of precursor necessarily follows multimerization. The role of the precursor sequence in the biosynthesis of vW protein and the fate of the propeptide after cleavage remain to be clarified.

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