Initial Glycosylation and Acidic pH in the Golgi Apparatus Are Required for Multimerization of von Willebrand Factor

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Abstract. Two conditions were identified that interfered with the complex polymerization process in biosynthesis of von Willebrand factor (vWF). Treatment of human umbilical vein endothelial cells with tunicamycin inhibited N-linked glycosylation of nascent vWF and the resulting pro-vWF monomers failed to dimerize. The single subunits accumulated in the endoplasmic reticulum and were neither processed further nor secreted. In the presence of a weak base (ammonium chloride or chloroquine), interdimer disulfide bond formation was inhibited in a dose-dependent manner. This process appeared therefore to be pH sensitive and likely to be initiated in the acidic trans-Golgi apparatus (Anderson, R. G. W., and R. K. Pathak, 1985, Cell, 40:635–643). The weak base had no obvious effect on the other processing steps, i.e. dimerization, complex carbohydrate formation and sulfation, and produced only slight inhibition of pro-sequence cleavage. On the other hand, the weak base interfered with the targeting of newly synthesized vWF into Weibel–Palade bodies, with all of the vWF being secreted constitutively and none stored in the Weibel–Palade bodies. In summary, initial glycosylation of the nascent vWF protein and low pH in the trans-Golgi apparatus were important conditions for the successful polymerization of human vWF. Genetic defects disrupting any one of these conditions could result in the phenotype of von Willebrand disease.

Von Willebrand factor (vWF) is an adhesive glycoprotein necessary for binding platelets to subendothelium after vascular injury (12, 23, 30). vWF is synthesized as a large precursor (260,000-mol-wt) that contains high mannosyl carbohydrate (17, 31). After dimer formation and transport to the Golgi apparatus, the carbohydrate is processed to the complex type, the propiece is cleaved, and interdimer disulfide bond formation begins (32). Carbohydrate processing is accompanied by an apparent increase in molecular weight of the precursor subunit to 275,000. These observed molecular weights of precursor subunits underestimate actual size by ~30,000–50,000, as determined by the size of vWF cDNA clones that span the vWF messenger RNA (9).

vWF is stored in the endothelial cells within specific organelles called Weibel–Palade bodies (34). Secreted vWF consists of a series of multimers that range in size from 0.5 to over 20 × 10⁶ daltons, depending upon the number of dimers that are disulfide bonded. In type IIA von Willebrand disease, only the large and intermediate size multimers are lacking (22), but patient plasma does not support efficient attachment of platelets to subendothelium (24), indicating that multimer size is important for vWF biological activity. As yet, the cellular defects that are responsible for incomplete polymerization of vWF in the type IIA von Willebrand disease are not known.

Materials and Methods

Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously (8, 34); only primary cultures were used. Cells were cultured in McCoy's 5A medium (Flow Laboratories, Inc., McLean, VA) containing 20% fetal bovine serum. Tunicamycin (Calbiochem-Behring Corp., San Diego, CA) was dissolved in dimethyl sulfoxide, chloroquine (Sigma Chemical Co., St. Louis, MO) in water, and ammonium chloride (Fisher Scientific Co., Rochester, NY) in culture medium. Control cells were treated with the solvent alone. In the case of tunicamycin, cells were pre-treated for ~14 h with the drug prior to metabolic labeling. For continuous metabolic labeling, cells were grown in medium containing [35S]methionine (25–40 μCi/ml, 18.7 Ci/mmol; Amersham Corp, Arlington Heights, IL). In one study, cells were labeled overnight with [35S]sodium sulfate (230 μCi/ml, 767 mCi/mmol; New England Nuclear, Boston MA) in medium containing dialyzed serum (20).

Antisera

Preparation and characterization of antisera against human vWF were as

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Ammonium Chloride Treatment of Cells for Fluorescence Microscopy

Cells freshly obtained from the umbilical vein were plated on glass coverslips; after 2 d, the medium was replaced with medium containing 5 mM ammonium chloride. The cells were grown in this medium for 5 d (with one medium change) before being processed for fluorescence microscopy (34). The pH of the culture medium containing ammonium chloride was between 7.4-7.6 in all experiments.

Pulse-Chase Experiments in the Presence of Tunicamycin

All media contained 1 μg/ml of tunicamycin, and incubations were at 37°C. Cells were preincubated overnight in medium containing fetal bovine serum and for 15 min in serum-free medium. Medium was then removed and the cells were covered with 1 ml serum-free medium containing 1.5 mCi [35S]methionine and incubated for 20 min. Cells were then rinsed in medium containing serum and incubated in this medium for varying lengths of time. Control cells were treated identically except that all media did not contain tunicamycin.

Purification of vWF

vWF was immunopurified as described previously (32) and samples were analyzed by gel electrophoresis after the radioactive count was determined.

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. (28). Purified vWF 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.

Electrophoresis Gels

SDS polyacrylamide gels were prepared as described by Laemml (14), 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.

Results

Effect of Tunicamycin on vWF Processing and Secretion

Culturing of human umbilical vein endothelial cells in the presence of 1 μg/ml tunicamycin, an antibiotic which prevents N-linked glycosylation by inhibiting the formation of the donor dolichol-sugar (27), had a profound effect on vWF processing and secretion (Fig. 1a). vWF purified from control cells contained similar amounts of precursor (260,000-mol-wt) and cleaved subunits (220,000-mol-wt) which are shown. Tunicamycin-treated cells contained precursor subunit but no cleaved subunit (220). Secretion of vWF was completely inhibited in tunicamycin-treated cells, in that the medium lacked the precursor (275) and cleaved subunits (220) that were secreted in control cultures. (b) Digestion of pro-vWF synthesized in the presence of tunicamycin with endoglycosidase H. vWF purified from cells treated in the presence (+) or absence of tunicamycin (−) was digested with endoglycosidase H (E). The 260,000-mol-wt precursor subunit (260) in control cells contains high mannose carbohydrate (32); therefore endoglycosidase H treatment produced a shift in its migration towards a smaller apparent molecular weight. The precursor synthesized in the presence of tunicamycin co-migrated with the endoglycosidase H-treated (260,000-mol-wt) precursor present in control cells, and no shift in migration occurred after enzymatic treatment. This indicates that tunicamycin inhibited the addition of N-linked carbohydrate. The 220,000-mol-wt subunit present in control cells is resistant to endoglycosidase H (32), since it contains mature complex-type carbohydrate.

The effect of tunicamycin on dimer formation was studied by pulse-chase experiments. Cells preincubated in the presence of 1 μg/ml of tunicamycin were labeled with [35S]methionine for 20 min. The cells were then chased in unlabeled medium for varying time intervals. Tunicamycin was present during the labeling and the chase periods. Purified vWF from treated and control cells was examined nonreduced on 4.5% acrylamide gels (Fig. 2). While the monomeric precursor in the control cells dimerized almost completely in the first hour from the onset of labeling, dimers were not formed in the tunicamycin-treated cells. It appears therefore that initial glycosylation of vWF precursor monomers is required for processing and secretion of vWF.

Effect of a Weak Base on the Processing of vWF

Endothelial cells were metabolically labeled for 3 d in the presence of varying concentrations of ammonium chloride to determine whether any of the processing steps in vWF biosynthesis are pH sensitive. vWF was purified from culture medium and lysed cells and analyzed nonreduced (Fig. 3) or reduced (Fig. 4a) by gel electrophoresis. Ammonium chloride inhibited interdimer disulfide bond formation in a dose-dependent manner (Fig. 3) that was similar to that produced by monensin (33). In contrast to monensin, however, carbohydrate processing and precursor cleavage were not significantly affected.
by ammonium chloride. vWF subunits produced by treated cells migrated at the same position as subunits from control cultures (Fig. 4a) and the secreted material was resistant to endoglycosidase H (Fig. 4b), indicating that these subunits contained complex-type carbohydrate. Sulfation of vWF (5) also was not inhibited by ammonium chloride (not shown).

The ratios of precursor to mature subunit in secreted vWF were quantitated by densitometric scanning. In three independent experiments, precursor cleavage was inhibited only 20% by 25 mM ammonium chloride, a concentration which completely inhibited multimerization of vWF dimers. Concentrations of 5 mM and higher depleted the endothelial cells of processed subunits (Fig. 4a) and small multimers (Fig. 3). The ratio of secreted to cell-associated vWF increased threefold in treated cells, duplicating the effect seen with monensin (33), and reflected in the loss of Weibel–Palade bodies (Fig. 5). It appears therefore that the multimerization of vWF and the formation of Weibel–Palade bodies from the Golgi apparatus (26) are pH dependent. The same results (not shown) were obtained with another weak base, chloroquine (10–50 μM), using shorter labeling periods because of the toxic effect of chloroquine on cells. The small multimers synthesized in the presence of 5 mM NH₄Cl contained fully processed subunits, but did not incorporate into endothelial cell extracellular matrix (Fig. 5), confirming prior observations that vWF multimer size is a crucial factor in this interaction (33).

**Figure 2.** The effect of tunicamycin on dimerization of vWF. Human umbilical vein endothelial cells were metabolically labeled for 20 min in the absence (−) or in the presence (+) of 1 μg/ml of tunicamycin and then chased in unlabeled medium. vWF was purified from the lysed cells and examined nonreduced on a 4.5% acrylamide gel after autoradiography. Numbers indicate time in hours from the onset of labeling. In control cultures, monomeric vWF formed dimers, while in tunicamycin-treated cells the subunit remained monomeric.

**Figure 3.** The effect of ammonium chloride on multimerization of vWF. Endothelial cells were metabolically labeled for 3 d in the presence of ammonium chloride. vWF was purified from the lysed cells and culture media and electrophoresed nonreduced on 2% agarose gels, autoradiographs of which are shown. Some of the 25 mM ammonium chloride–treated cells lifted prior to cell lysis and therefore less radioactivity was present in this sample. Ammonium chloride inhibited interdimer disulfide bond formation in a dose-dependent manner and the small multimers produced in its presence were not stored in the cells.

**Figure 4.** The effect of ammonium chloride on precursor cleavage and carbohydrate processing of vWF. (a) vWF purified from endothelial cells treated as in Fig. 3 was analyzed reduced on 5% polyacrylamide gels. The autoradiograph shows that the majority of the pro-vWF subunits were cleaved to the 220,000-mol-wt size in the presence of ammonium chloride and that the electrophoretic migration of the subunits did not change with increasing concentration of ammonium chloride. (b) Endothelial cells were labeled for three days in the presence of 25 mM ammonium chloride, and vWF purified from lysed cells and culture medium was treated with endoglycosidase H (E). Both of the secreted subunits (275 and 220) were endoglycosidase H resistant, indicating that the carbohydrate was processed to the complex type. As noted for untreated cultures in Fig. 1b, only the cellular precursor of 260,000-mol-wt was endoglycosidase H sensitive.
Figure 5. The effect of ammonium chloride on the distribution of vWF in the endothelial cell culture. Human umbilical vein endothelial cells were grown on glass coverslips for 5 d in the absence (a) or presence (b and c) of 5 mM ammonium chloride. The cells were permeabilized and stained by immunofluorescence with anti-vWF antiserum (a and b) and with antifibronectin antiserum (c). The Weibel-Palade bodies (arrowhead) were absent from the treated cells (b). The ammonium chloride-treated cells produced a fibronectin-containing extracellular matrix (c), but the small vWF multimers secreted by these cells (see Fig. 3) did not incorporate into this matrix (b). Bar, 20 μm.

Discussion

Importance of Initial Glycosylation on Dimerization and Intracellular Transport of Human vWF Subunits

The inhibition of N-linked carbohydrate addition to the growing polypeptide chain of vWF by tunicamycin appeared to have a profound effect on its subsequent fate. It is likely that the carbohydrate provides the vWF monomer with a conformation that favors dimerization. The presence of tunicamycin inhibited the formation of dimers entirely (Fig. 2) and no subsequent processing of the pro-vWF monomers took place, i.e., the pro-sequence was not cleaved and the monomers were not secreted. 220,000-mol-wt monomers are not found in untreated endothelial cells, nor are they secreted (32). It appears therefore, that only dimer molecules are recognized to be packaged for transport from the endoplasmic reticulum to the Golgi apparatus where processing continues. The hypothesis that tunicamycin causes the accumulation of monomeric vWF in the endoplasmic reticulum is further supported by strong perinuclear immunofluorescence staining and a gradual decrease in Weibel-Palade body number (11; also, our unpublished observations).

Tunicamycin inhibition of vWF transport and processing is specific to human endothelial cells, since precursor subunits are cleaved and secreted by bovine cells in the presence of tunicamycin (17). A tunicamycin-preincubation period of >3 h was necessary to inhibit N-linked glycosylation. When tunicamycin treatment was initiated simultaneous with metabolic labeling, both glycosylated and nonglycosylated precursor subunits were produced, and cleavage of the former to a 220,000-mol-wt subunit occurred (not shown). This could explain the observation by Hormia et al. of three distinct vWF bands in reduced samples of tunicamycin-treated human endothelial cells (11).

The presence of tunicamycin resulted in human endothelial cell cultures with the phenotype of severe von Willebrand disease, i.e. absence of secretion of vWF. Subtle changes in the vWF gene, translated into missing glycosylation sites, could produce a similar effect in vivo.

Acidic pH Is Necessary for Multimerization of vWF and for Formation of Weibel-Palade Bodies

Our previous study (33) on biosynthesis of vWF in the presence of the carboxylic ionophore monensin (29) demonstrated inhibition of all the processing steps localized to the Golgi apparatus. However, the exact mechanisms of this inhibition are not known. It has been shown that the Golgi vesicles contain a proton pump capable of creating a transmembrane pH gradient (10) and since monensin dissipates such a gradient (2, 4), some of the inhibitory effects of monensin might reflect its action on the pH of the Golgi cisternae. We therefore decided to compare the effect of a weak base (25) with that of monensin on vWF processing and secretion. The difference was striking in that monensin blocked all the Golgi-localized processing steps (33) while the weak base completely inhibited only the interdimer disulfide bond formation (Figs. 3 and 4).

It appears that monensin acts on the Golgi apparatus by at least two different disruptive mechanisms, only one of which is abolishment of a pH gradient that can be replicated by a weak base. Anderson and Pathak have demonstrated by immunocytochemical techniques that the trans-Golgi apparatus and the forming secretory vesicles are acidic (1). Thus, the pH-sensitive multimerization of vWF dimers (Fig. 3) can be localized to the trans-Golgi apparatus and possibly to the Weibel-Palade bodies. Two observations lead us to the conclusion that at least some multimerization takes place in the Golgi apparatus and that not all multimers are formed in the Weibel-Palade bodies. First, multimers larger than dimers are produced in the presence of 10^-8 M monensin (33) or 5 x 10^-3 M ammonium chloride (Fig. 3), but Weibel-Palade bodies are not found in cells treated in this manner (11) (Fig. 5). Second, while only one of the two pathways for secretion of vWF by endothelial cells involves the Weibel-Palade bodies (16), the protein secreted by both pathways is multimeric (Sporn L. A., V. J. Marder, and D. D. Wagner, manuscript submitted for publication). Conversion of high mannose to complex type carbohydrate is initiated in the central cisternae of the Golgi stack (7), consistent with the lack of inhibitory effect of ammonium chloride on this processing step (Fig. 4b).

The pH in the trans-Golgi apparatus is probably regulated...
by a proton pump (10). Any defects in its functioning would be reflected by incomplete multimerization of vWF, resulting in the phenotype of type IIA von Willebrand disease.

The effect that the weak base had on storage of vWF in the Weibel–Palade bodies was not entirely unexpected. There are two other examples where the presence of a weak base interferes with the targeting of proteins. Newly synthesized lysosomal enzymes are secreted instead of delivered to the lysosome in the presence of chloroquine (13) and ACTH is secreted through an electrogenic H+ pump in parallel to a chloride conductance. J. Cell Biol. 107:673–684.

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