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Sorting of Von Willebrand factor to lysosome-related granules of haematopoietic cells

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Abstract

The aim of this work was to investigate sorting mechanisms of von Willebrand factor (VWF) when expressed in haematopoietic cells. The processing and sorting of both the wild-type VWF and a multimerization defective propeptide-mutant (VWF_m) were investigated after expression in the 32D cell line. Normal proteolytic processing was observed for both proteins, however the processing of VWF_m was much slower and a large portion was unprocessed. Results from subcellular fractionation and immunoelectron microscopy confirmed that a part of VWF, but not VWF_m, was targeted to lysosome-related granules. Partial constitutive secretion was also observed for all forms of VWF and VWF_m. Inhibition of acidification by chloroquine blocked VWF processing but allowed unprocessed pro-VWF targeting to dense organelles. In conclusion, our observations are consistent with VWF multimerization being of importance in cellular retention and targeting to lysosome-related organelles in haematopoietic cells, suggesting a role of protein aggregation for sorting in these cells.
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Keywords: Multimerization; Aggregation; Targeting; Secretory lysosome; Basophil

The von Willebrand factor (VWF) has an important role in hemostasis by mediating platelet adhesion to the injured subendothelium and by its function as a carrier protein in the protection of coagulation factor VIII. VWF is normally stored in the Weibel–Palade bodies of endothelial cells [1] and in platelet granules [2]. After endothelial cell biosynthesis, the VWF undergoes a series of processing steps including C-terminal dimerization, glycosylation, sulphation, N-terminal multimerization, and propeptide removal to finally becoming sorted for storage or secreted as high molecular weight multimers [3,4]. Furthermore, VWF can redirect coagulation factor VIII from a constitutive to a regulated secretory pathway by a chaperone-like mechanism [5]. VWF can also form storage granules when expressed in cells other than endothelial cells and megakaryocytes [6]. To determine the sorting role of multimerization, we have chosen to investigate the fate of VWF when constitutively expressed as an exogenous protein in haematopoietic cells.

Haematopoietic cells manufacture lysosome-related organelles [7] such as the azurophil granules of the neutrophil series that lack the lysosome-associated membrane proteins LAMP-1 and LAMP-2 [8,9] but carry the membrane protein CD63/LAMP-3 [10,11]. Sorting mechanisms are not unique for endogenous proteins in these cells inasmuch as gene transfected non-haematopoietic proteins may also be sorted for granule storage [12,13]. Protein aggregation has been suggested to play a role in secretory granule sorting in endocrine and neuroendocrine cells [14,15]. As the itinerary for retrieval may be dependent on protein self-association and aggregation, results from the naturally self-associating VWF could shed light on principles for cellular retrieval and sorting in haematopoietic cells. Stable expression of VWF cDNA constructs was assumed to result in the synthesis and translocation of the protein into the lumen of the ER and followed, after quality

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control, by export of normally folded protein to the Golgi complex. The protein may then undergo either constitutive secretion or lysosome-related organelle targeting.

The role of multimerization in sorting might be illustrated with a multimerization defective VWF mutant (VWF_m) [16]. We therefore asked whether both VWF and VWF_m could be targeted to lysosome-related organelles when constitutively expressed in haematopoietic cells such as the murine myeloblast-like 32D cell line [17].

Materials and methods

Cell culture. Murine myeloblast-like 32D c13 cells [17] were grown in Iscove's modified Dulbecco's medium (Gibco) with glutamax or IMDM with L-glutamine supplemented with 10% FBS (Gibco). WEHI-conditioned medium (30%) was added as a source of interleukin-3 [18].

cDNA expression. Inserts of cDNA encoding VWF and VWF_m [16] were excised from pSVH-VWF and pMT2-VWF_m followed by ligation into pcDNA3.1(+) (Invitrogen) to create the expression vectors pcDNA3-VWF and -VWF_m. The 32D cells were transfected (Genepulser II, BioRad) with electrical settings of 960 μF and 260 V after addition of 15 μg plasmid to 4×10^6 cells in 400 μl complete medium. After electroporation, 5×10^5 cells/ml were incubated for 48 h to allow expression of the geneticin resistance. A maximum of 5000 cells per well were then seeded in 100 μl complete medium with 1 mg/ml geneticin in 96-well plates. The multimeric structure of the expressed VWF was determined by electrophoresis in 1.1% agarose under non-reducing conditions [16]. The proteins were blotted onto Hybond PVDF membranes, that were incubated with the monoclonal VWF antibody M616 (DAKO), and the Alkaline Phosphatase substrate rabbit anti-mouse DO314 (DAKO).

Biosynthetic radiolabelling, immunoprecipitation, and subcellular fractionation were performed as previously described [19]. SDS–PAGE was performed in a precast 4–12% Tris–glycine gel (Novex). The peak activity of β-hexosaminidase and galactosyl transferase in subcellular Percoll fractions of 32D c13 cells was localized in fractions 2 and 6, respectively [13]. Polyclonal antibodies against human VWF (A0082) (DAKO A/S) and monoclonal antiserum against the propeptide of human VWF (BR5 and 8H10) from Dr. Ulrich Vischer were used.

Immunoelectron microscopy. 32D cells stably transfected with VWF or with VWF_m were fixed for 24 h in 2% paraformaldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM Hepes, 2 mM $MgCl_2$, and 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described [20]. 45-nm cryosections were cut at −120 °C using diamond knives (Drukker Cuijk, The Netherlands) in an ultracryomicrotome (Leica Aktiengesellschaft, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids [21]. The grids were placed on 35-mm petri dishes containing 2% gelatine. For double immunolabelling the procedure described by Slot et al. [22] was followed with 10- and 15-nm protein-A conjugated colloidal gold probes (EM Lab., Utrecht University, The Netherlands). The antibodies used were a rat monoclonal ID4B against mouse LAMP-1 (CD107a) (Pharmingen Leiden, The Netherlands) and a rabbit anti-VWF. After immunolabelling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined using a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For the controls, the primary antibody was replaced by the corresponding non-relevant rat and rabbit antiserum.

Results

Von Willebrand factor processing and secretion

Results from agarose electrophoresis under non-reducing conditions showed multimer formation of VWF, whereas no aggregates larger than the protomer were observed for VWF_m (Fig. 1).

Biosynthetic pulse-chase radiolabelling was used to investigate VWF processing. Immunoprecipitation was performed with two different antibodies, one that reacts with both the pro-VWF and mature VWF (anti-VWF), and another that reacts with pro-VWF and released VWF propeptide but not the mature VWF (anti-pro-VWF). Immunoprecipitation with anti-VWF of 1 h pulse cell lysates showed a major band corresponding to pro-VWF (Fig. 2A). During the radiolabel chase mature VWF was also observed. In addition, secreted mature VWF was observed, but secreted pro-VWF was barely detectable (Fig. 2A). However, the results from immunoprecipitation with anti-pro-VWF confirmed that pro-VWF was also secreted (Fig. 3A). Densitometric analyses after 1 and 3 h of radiolabel chase showed the ratio between the cellular pro-VWF and mature VWF to be 1.3 and 1.1, respectively (data not shown). This indicates that more than half of the pro-VWF produced was processed to mature VWF. Furthermore, the ratio between intra- and extra-cellular mature VWF was 13.5 and 1.8 at the same time points. As extracellular VWF increased even further with time, approximately half the mature VWF generated was estimated to be constitutively secreted. Thus, half of the VWF generated was retained and half secreted. The propeptide released during radiolabel chase was present in both the cells and the medium (Fig. 3A). Densitometric data from the radiolabel chase revealed the ratio between intra- and extra-cellular propeptide to be 3.4 and 2.4 at 1 and 3 h,

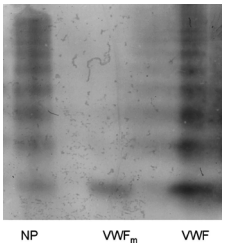

Fig. 1. Multimer analysis of VWF and VWF_m. Medium from 32D cells transfected with cDNA for wild-type VWF and VWF_m with a mutation in the propeptide was analysed by electrophoresis in 1.1% agarose under non-reducing conditions. A range of multimers are seen with VWF, but only the fastest moving band (the protomer) with VWF_m. The control to the left consists of VWF multimers from normal plasma (NP).

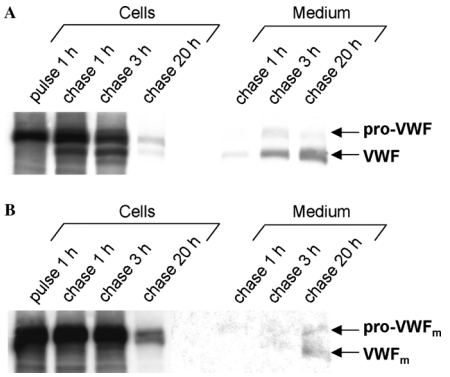

Fig. 2. Processing of wild-type VWF and mutated VWF_m as detected with an antibody against VWF. 32D cells transfected with cDNA for wild-type VWF (A) and VWF_m with a mutation in the propeptide (B) were radiolabelled with 25 μCi/mL [^{35}S]methionine/[^{35}S]cysteine for 1 h followed by chase of the radiolabel for up to 20 h. At depicted time points, 20×10^6 cells and medium were removed, immunoprecipitated with anti-VWF, and analysed by SDS–PAGE. The fluorogram of medium in B was overexposed to increase the visibility of the secreted forms. The positions of pro-VWF, VWF, pro-VWF_m, and VWF_m are indicated to the right with arrows.

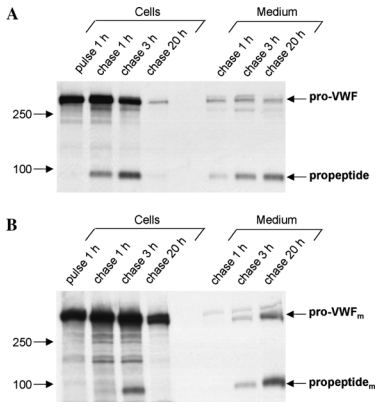

Fig. 3. Processing of wild-type VWF and VWF_m as detected with an antibody against the VWF propeptide. 32D cells transfected with VWF (A) and VWF_m (B) cDNA were radiolabelled with 25 μCi/mL [^{35}S]methionine/[^{35}S]cysteine for 1 h followed by chase of the radiolabel for up to 20 h. At depicted time points, 20×10^6 cells and medium were removed, immunoprecipitated with antibodies against the VWF propeptide, and analysed by SDS–PAGE. The positions of pro-VWF, the propeptide, pro-VWF_m, and the mutated propeptide ($propeptide_m$) are indicated to the right with arrows. Numbers to the left are the values of molecular mass standards.

respectively (data not shown). Again, an additional increase in the extracellular propeptide with time was noticed (Fig. 3A), indicating that approximately half the generated propeptide was secreted. Thus, cellular retention and constitutive secretion appeared to be similar for both the mature VWF and the released propeptide. The processing of VWF_m was slower than that of VWF. Mature VWF_m was first observed in the cell lysates after 3 h of radiolabel chase (Fig. 2B). Both pro-VWF_m and VWF_m were secreted during radiolabel chase (Figs. 2B and 3B). Cleaved mutated propeptide was also secreted in a similar manner to the wild-type (Fig. 3B). Densitometric analyses of data from Fig. 3B indicated that all the released propeptide was secreted (data not shown), consistent with a lack of propeptide targeting to lysosome-related organelles.

Von Willebrand factor targeting

The VWF propeptide is known to be involved in the multimerization process [23,24]. We therefore investigated whether the targeting to lysosome-related organelles was similar for VWF and multimerization defective

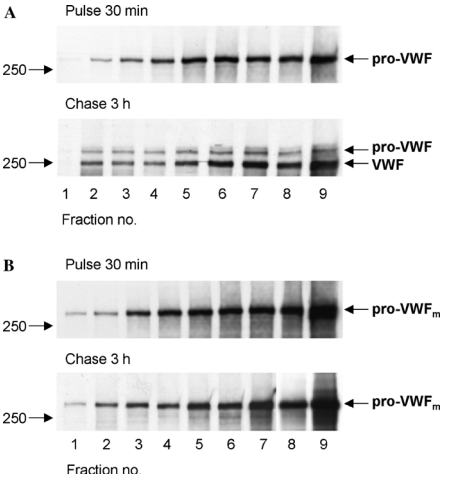

Fig. 4. Subcellular targeting of wild-type VWF and mutated VWF_m as detected with an antibody against VWF. 32D cells transfected with VWF (A) and VWF_m (B) cDNA were radiolabelled with 25 μCi/mL [^{35}S]methionine/[^{35}S]cysteine for 30 min followed by chase of the radiolabel for 3 h. At these time points, 100×10^6 cells were subcellular fractionated on a Percoll gradient [19]. The fractions were immunoprecipitated with anti-VWF and analysed by SDS–PAGE. The positions of pro-VWF, VWF, and pro-VWF_m are indicated to the right with arrows. The peak activity of β-hexosaminidase (lysosome-related organelles) and galactosyl transferase (Golgi) was localized in fractions 2 and 6, respectively [13]. Numbers to the left are the values of molecular mass standards.

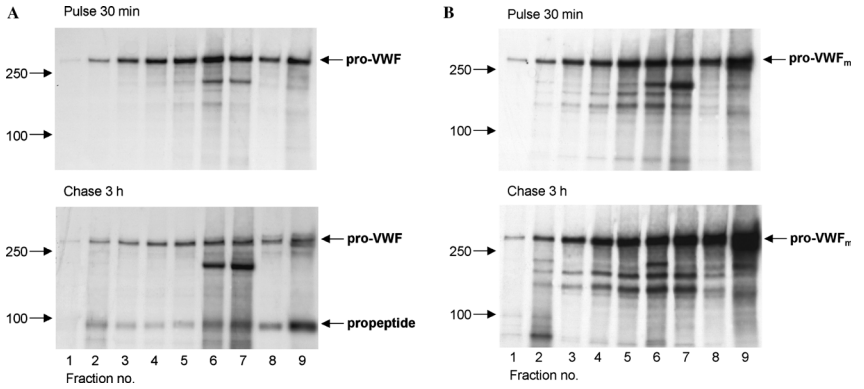

Fig. 5. Subcellular targeting of wild-type VWF and VWF_m as detected with an antibody against the VWF propeptide. 32D cells transfected with VWF (A) and VWF_m (B) cDNA were radiolabelled with 25 μCi/mL [^{35}S]methionine/[^{35}S]cysteine for 30 min followed by chase of the radiolabel for 3 h. At these time points, 100×10^6 cells were subcellular fractionated on a Percoll gradient [19]. The fractions were immunoprecipitated with antibodies against the VWF propeptide and analysed by SDS–PAGE. The positions of pro-VWF, the propeptide, and pro-VWF_m are indicated to the right with arrows. The peak activity of β-hexosaminidase (lysosome-related organelles) and galactosyl transferase (Golgi) was localized in fractions 2 and 6, respectively [13]. Numbers to the left are the values of molecular mass standards.

VWF_m. The cells were subcellularly fractionated after 30 min radiolabelling and 3 h subsequent radiolabel chase. Proteins were immunoprecipitated with anti-VWF (Fig. 4) or anti-pro-VWF (Fig. 5). After 30 min, radiolabelled pro-VWF was concentrated into fractions corresponding to ER and Golgi elements (Fig. 4A). Upon radiolabel chase, a slight accumulation of mature VWF was observed in the densest granule-containing fractions (Fig. 4A). The finding that VWF was present in all fractions after the radiolabel chase implied that pro-VWF cleavage to generate VWF occurred in non-granule organelles, presumably in the TGN [3]. Furthermore, the data suggested that a portion of the released propeptide was transported to the densest organelles (Fig. 5A) while another portion was constitutively secreted as seen in Fig. 3. The relative distribution of VWF (Fig. 4A) and released propeptide (Fig. 5A) in the subcellular fractions was compared by densitometry after 3 h radiolabel chase. The data showed a similar distribution for both VWF and free propeptide (not shown).

Subcellular fractionation experiments of radiolabelled cells showed pro-VWF_m in most subcellular fractions without accumulation in the densest organelles upon radiolabel chase (Figs. 4B and 5B). Cleaved, mutated propeptide was not observed, a finding that could be explained by the slow processing with only a minor release of mutated propeptide during a 3-h experiment (see Fig. 3B). Thus, our results are consistent with slow export of VWF_m from the ER and subsequent constitutive secretion and inability to target lysosome-related organelles. If granule targeting requires aggregate formation, the lack of multimer formation may have prevented this targeting and resulted in constitutive secretion by default.

Results from immunoelectron microscopy using double immunogold labelling in 32D cells transfected with VWF revealed colocalization between VWF and the lysosomal marker LAMP-1 [25,26] (Fig. 6A). Aggregate-like VWF labelling was associated with the lumen of multivesicular bodies corresponding to the lysosome-related organelles of the cells, indicating that some VWF was targeted to these organelles. On the other hand, VWF_m was not identified in the multivesicular bodies/granules of cells transfected with the gene for this protein (Figs. 6B–D). However, clusters of the gene product were observed to some extent in swollen ER (Figs. 6C and D). Unsuccessful efforts were made to detect pro-VWF in lysosome-related organelles using anti-pro-VWF (data not shown). In conclusion, VWF but not VWF_m was targeted to lysosome-related organelles of 32D cells.

Von Willebrand factor turnover

Both pro-VWF and VWF were degraded during radiolabel chase (Fig. 7A). When organelle acidification was blocked with chloroquine, two effects were observed. First, chloroquine inhibited the processing of pro-VWF into VWF, indicating pH-dependency for this step (Fig. 7A). Second, chloroquine inhibited the degradation of pro-VWF (Fig. 7A). The amount of secreted protein was unaffected by chloroquine although secreted pro-VWF predominated (Fig. 7A). In contrast, chloroquine

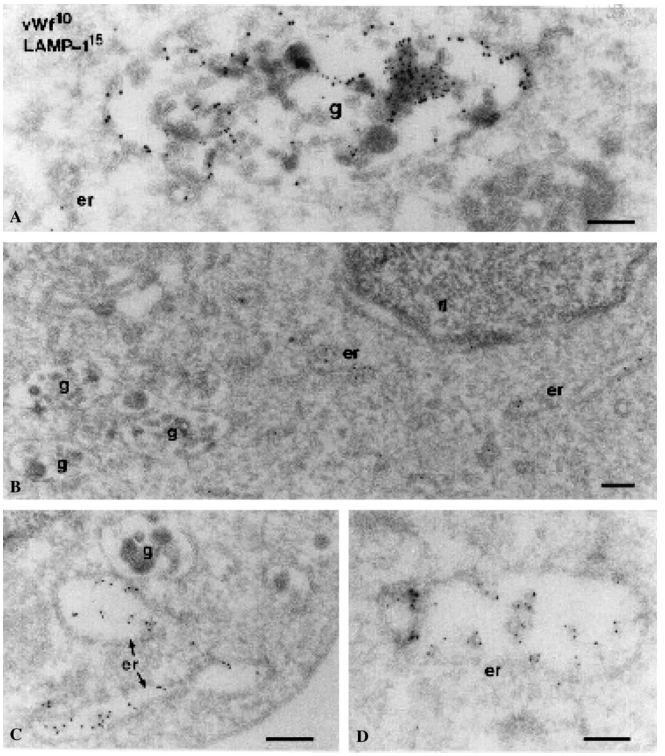

Fig. 6. Intracellular localization of wild-type and mutated von Willebrand factor. Ultrathin cryosections from 32D cells transfected with VWF (A) and VWF_m (B–D) were double labelled in (A) with rabbit anti-VWF followed by protein A gold (10 nm) and rat anti-LAMP-1, followed by rabbit anti-rat IgG and protein A gold (15 nm); in (B–D) cells were labelled with rabbit anti-VWF and protein A gold (10 nm). In (A), an area of a cell is shown with a large multivesicular body/granule (g) double labelled with LAMP-1 (15 nm-gold) on the membrane and VWF (10 nm-gold) in the lumen, mainly in a large aggregate; some labelling is present in the endoplasmic reticulum (er). (B) Large area of a cell at low magnification showing labelling for VWF_m in the endoplasmic reticulum (er) and no labelling in the multivesicular bodies/granules (g). Nucleus (n). (C,D) Higher magnification of areas from other cells showing swollen endoplasmic reticulum (er) containing VWF_m protein arranged in clusters. Bars, 200 nm.

did not affect the degradation of pro-VWF_m during radiolabel chase (Fig. 7B). However, mature VWF_m was not observed, suggesting that chloroquine inhibited its production (Fig. 7B).

The subcellular distribution of radiolabelled pro-VWF was not affected by chloroquine (Fig. 7C). The processing inhibition was not complete in this experiment because the chloroquine was added after pulse radiolabelling (Fig. 7C), but complete processing inhibition was observed when chloroquine was present during the whole experiment (Fig. 7B). Even if the VWF formation was blocked, the subcellular distribution of pro-VWF was similar in the presence or absence of chloroquine. This suggested that the pro-VWF accumulation in the densest fractions corresponding to the lysosome-related organelles might occur without a requirement for acidification and VWF formation.

The VWF and VWF_m stability was not affected by 10 μM of the proteasomal inhibitor lactacystin, suggesting the degradation of these proteins not to involve the proteasome (data not shown).

Discussion

Expression of VWF cDNA has been achieved previously in non-endothelial cell types [6,24,27–30], and now in the present work with haematopoietic cells. VWF-containing granules have been observed after VWF cDNA expression in endocrine and neuroendocrine cells

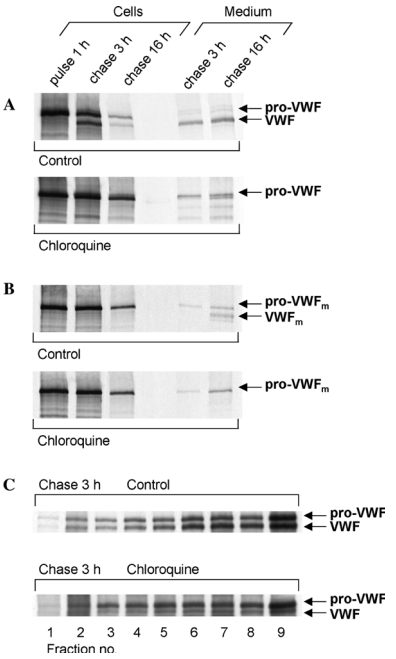

Fig. 7. Chloroquine effect on processing, degradation, and subcellular distribution of VWF and VWF$_m$. 32D cells transfected with VWF (A) and VWF$_m$ (B) were incubated with 25 μCi/mL [^{35}S]methionine/[^{35}S]cysteine for 1 h, followed by chase of the radiolabel for up to 16 h without (control) or with 100 μM chloroquine. At depicted time points, 20×10^6 cells and medium were removed, immunoprecipitated with anti-VWF, and analysed by SDS–PAGE. The positions of pro-VWF, VWF, pro-VWF$_m$, and VWF$_m$ are indicated to the right with arrows. (C) Cells transfected with VWF cDNA were radiolabelled with [^{35}S]methionine/[^{35}S]cysteine for 30 min followed by chase of the radiolabel for 3 h without (control) or with 100 μM chloroquine, after which 100×10^6 cells were subjected to subcellular fractionation on a Percoll gradient [19]. The fractions were immunoprecipitated with anti-VWF and analysed by SDS–PAGE. The positions of pro-VWF, VWF, pro-VWF$_m$, and VWF$_m$ are indicated to the right with arrows. The peak activity of β-hexosaminidase (lysosome-related organelles) and galactosyl transferase (Golgi) was localized in fractions 2 and 6, respectively [13].

[6], as well as in monkey kidney CV-1 cells [31]. VWF multimer assembly is a prerequisite for dense-core organelle biogenesis in endothelial cells [4], but not a general requirement for VWF granule targeting since targeting was observed in AtT-20 cells of a VWF variant with almost complete loss of N-terminal multimerization [32]. Most of the VWF was processed intracellularly, but some pro-VWF was secreted unprocessed indicating it had not undergone cleavage. However, much more VWF than pro-VWF was secreted. The correlation between processing and secretion indicated that furin-catalysed cleavage of the propeptide was followed by secretion of both the mature VWF and the propeptide, similar to that seen in endothelial cells [3,33]. The rate of secretion of pro-VWF$_m$, mature VWF$_m$, and released mutant propeptide was also slower. The furin-cleavage site is not affected in the VWF$_m$ and the portion of the protein that escaped ER retention and degradation was obviously secreted. Thus, multimer assembly was not necessary for constitutive secretion.

The conclusion that VWF is targeted to lysosome-related organelles was based on the assumption that subcellular fractionation detected a shift in density as these organelles were formed. However, this density shift is not always easily detectable since the organelles may have a wide density distribution. Therefore, immunoelectron microscopy data were used to substantiate the evidence for lysosome-related VWF organelle targeting and lack of VWF$_m$ targeting. A major question addressed in this paper was whether multimerization/aggregation played a role in lysosome-related organelle sorting. Aggregation is thought to be an important step in protein sorting to regulated secretory granules of endocrine cells [14,34]. In the latter, proteins are routed to vesicles within the TGN (sorting-for-entry) whereupon processing and segregation is completed in the mature granules (sorting-for-retention) [35,36]. VWF targeting into Weibel–Palade bodies has been postulated to depend on VWF multimerization [31], and VWF-containing granules seem to form only in cells that synthesize polymerized VWF [37]. Therefore, it is possible that VWF multimerization is also important in sorting-for-entry to lysosome-related organelles of haematopoietic cells when expressing VWF. However, dimeric VWF can also be targeted to storage organelles [6,38], indicating multimerization not to be necessary for targeting. A similar relative distribution of both mature VWF and released propeptide in the subcellular fractions suggested that propeptide could be non-covalently associated with VWF after cleavage. Propeptide has been shown to bind to mature VWF [3] and suggested to navigate sorting-for-entry by non-covalent association with VWF in AtT-20 cells [32]. Some cells may therefore possess targeting receptors or chaperone proteins that direct VWF to storage compartments, and sorting mechanisms might be cell-type specific.

The lack of lysosome-related organelle targeting of the multimerization-defective VWF$_m$ mutant, in contrast to wild-type VWF, may also support a role for protein self-association in sorting. Even if pro-VWF$_m$ was processed to generate both the mature VWF and the mutated propeptide forms, pro-VWF$_m$-derived VWF was not detectable in lysosome-related organelles of 32D cells. Furthermore, in contrast to non-mutated propeptide, all released mutated propeptide was constitutively secreted (Fig. 3B). It should be noted that the processing

of pro-VWF_m was slower than for wild-type pro-VWF, probably as a result of abnormal folding that slows ER-export. As the furin cleavage site is intact in pro-VWF_m, this rules out defective cleavage to be the problem. Intact propeptide, necessary for multimer assembly, might be a prerequisite for VWF targeting and storage in the lysosome-related organelles. The uncleaved VWF propeptide may function as an intramolecular chaperone during the final folding prior to multimerization [32].

In conclusion, our observations are consistent with VWF multimerization being of importance in cellular retention and sorting-for-entry to lysosome-related organelles of haematopoietic cells. The results also suggested that VWF_m may be prevented from targeting because of improper folding and therefore become constitutively secreted. The role for multimerization/aggregation in protein sorting is supported by the lack of multimerization of VWF_m in vivo in patients with a variant of von Willebrand's disease [39] and when expressed in COS-7 cells [16].

Acknowledgments

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