Mechanisms for Targeting of Proteins to Secretory Lysosomes of Haematopoietic Cells

Rosén, Hanna

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

Hanna Rosén, Jero Calafat, Lars Holmberg, and Inge Olsson

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 (VWFm) were investigated after expression in the 32D cell line. Normal proteolytic processing was observed for both proteins; however, the processing of VWFm was much slower and a large portion was unprocessed. Results from subcellular fractionation and immunoelectron microscopy confirmed that a part of VWF, but not VWFm, was targeted to lysosome-related granules. Partial constitutive secretion was also observed for all forms of VWF and VWFm. 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 hematopoietic 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-hematopoietic 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 hematopoietic 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 control

Corresponding author. Fax: +46-46-184493.
E-mail address: Inge.Olsson@hematologi.lu.se (I. Olsson).

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

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

Materials and methods

Cell sources. 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 interferon-α [18].

cDNA expression. Inserts of cDNA encoding VWF and VWF<sub>m</sub> [16] were excised from pVHE-VWF and pMT2-VWF<sub>m</sub> followed by ligation into pcDNA3.1(+) (Invitrogen) to create the expression vectors pcDNA3-VWF and -VWF<sub>m</sub>. The 32D cells were transfected (Gene Pulser II, BioRad) with electrical settings of 960V and 260V after addition of 15 μg plasmid to 4 × 10<sup>6</sup> cells in 400 μl complete medium. After electroporation, 5 × 10<sup>5</sup> cell/ml were incubated for 48 h to allow expression of the genetin resistance. A maximum of 5000 cells per well were then seeded in 100 μl complete medium with 1 μg/ml genetin in 96-well plates. The multimeric structure of the expressed VWF was determined by electrophoresis in 1.1% agarose in 1× TBE buffer in 1× TBE buffer. A range of multimers are seen with VWF but only the fastest moving band (the protomer) with VWF<sub>m</sub>. The 32D cells stably transfected with VWF and VWF<sub>m</sub> were fixed for 24 h in 2% paraformaldehyde in 0.1 M acetate and examined using a Philips CM 10 electron microscope.

Immunoblot analysis of VWF proteins. Western blot analysis was performed in a precast 4–12% Tris–glycine gel (Novex). The proteins were blotted onto Hybond membranes, that were incubated with the monoclonal VWF antibody DO314 (DAKO). Immunoblot analysis of 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 D0314 (DAKO).

Biosynthetic radiolabelling, immunoprecipitation, and subcellular fractionation were performed as previously described [19]. SDS-PAGE was performed in a precast 4–12% Bis-Tris 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 (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<sub>m</sub> (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-pro-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 3h, respectively.

![Fig. 1. Multimer analysis of VWF and VWF<sub>m</sub>.](image-url)
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 was slower than that of VWF. Mature VWF was first observed in the cell lysates after 3 h of radiolabel chase (Fig. 2B). Both pro-VWF and VWF 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

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**Fig. 2.** Processing of wild-type VWF and mutated VWF as detected with an antibody against VWF. 32D cells transfected with cDNA for wild-type VWF (A) and VWF with a mutation in the propeptide (B) were radiolabelled with 25 Ci/mL [35S]methionine/[35S]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 positions of pro-VWF, VWF, pro-VWFm, and VWFm are indicated to the right with arrows.

**Fig. 3.** Processing of wild-type VWF and VWF as detected with an antibody against the VWF propeptide: 32D cells transfected with VWF (A) and VWF (B) cDNA were radiolabelled with 25 Ci/mL [35S]methionine/[35S]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-VWFm, and the mutated propeptide (propeptide m) are indicated to the right with arrows. Numbers to the left are the values of molecular mass standards.

**Fig. 4.** Subcellular targeting of wild-type VWF and mutated VWF as detected with an antibody against VWF. 32D cells transfected with VWF (A) and VWF (B) cDNA were radiolabelled with 25 Ci/mL [35S]methionine/[35S]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-VWFm 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.
VWFm. 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-VWFm in most subcellular fractions without accumulation in the densest organelles upon radiolabelling (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 VWFm 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 membranes 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, VWFm 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 VWFm 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
did not affect the degradation of pro-VWFm during radiolabel chase (Fig. 7B). However, mature VWFm 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 VWFm 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...
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 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 VWFm 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 hematopoietic 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 VWFm mutant, in contrast to wild-type VWF, may also support a role for protein self-association in sorting. Even if pro-VWFm was processed to generate both the mature VWF and the mutated propeptide forms, pro-VWFm-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<sub>m</sub> was slower than for wild-type pro-VWF<sub>m</sub>, probably as a result of abnormal folding that slows ER-export. As the furin cleavage site is intact in pro-VWF<sub>m</sub>, this rules out defective cleavage to be the problem. In- tact 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 haemato poetic cells. The results also suggested that VWF<sub>m</sub> 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<sub>m</sub> in vivo in patients with a variant of von Willebrand's disease [39] and when expressed in COS-7 cells [16].

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