Von Willebrand factor biosynthesis, secretion & clearance: connecting the far ends

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Abstract

To understand the placement of a certain protein in a physiological system and the pathogenesis of related disorders, it is not only of interest to determine its function but also important to describe the sequential steps in its life-cycle, from synthesis to secretion and ultimately its clearance. Von Willebrand factor (VWF) is a particularly intriguing case in this regard because of its important auxiliary roles both intra- and extracellular that implicate a wide range of other proteins: its presence is required for the formation and the regulated release of endothelial storage organelles, the Weibel-Palade bodies (WPBs), while VWF is also a key-determinant in the clearance of coagulation factor VIII. Thus, understanding the molecular and cellular basis of the VWF life-cycle will help us to gain insight into the pathogenesis of von Willebrand disease, to design alternative treatment options to prolong the factor VIII half-life and to delineate the role of VWF and co-residents of the WPBs in the pro-thrombotic and pro-inflammatory response of endothelial cells. In this review, an update on our current knowledge on VWF biosynthesis, secretion and clearance is provided and we will discuss how they can be affected by the presence of protein defects.
**Introduction**

Von Willebrand factor (VWF) is a multimeric glycoprotein present in blood plasma, the subendothelial matrix as well as in storage granules in endothelial cells (Weibel-Palade bodies) (WPBs) and platelets (α-granules). Although a series of novel functional properties of VWF has recently been proposed, the protein is mostly known for its contribution to the hemostatic process: it mediates platelet-adhesion and -aggregation at sites of vascular injury and carries coagulation factor VIII (FVIII) in the circulation. Patients lacking VWF manifest a severe hemorrhagic phenotype, originating from defective formation of platelet-rich thrombi and a secondary deficiency of FVIII impairing the generation of a fibrin network. Functional and/or quantitative deficiencies of VWF are known as von Willebrand disease (VWD), a disorder affecting 0.01-1% of the population. Quantitative deficiencies of VWF result from changes in biosynthesis, secretion and/or clearance of the protein. In this review, we will provide an overview of the current knowledge on each of these processes and we will discuss how they can be affected by the presence of protein defects.

**Part I: Basics of von Willebrand factor biosynthesis**

*Primary structure:* VWF is produced in endothelial cells and megakaryocytes as a single pre-pro-polypeptide of 2813 amino acids. The primary sequence of VWF was reported in 1986, and rapidly the presence of repeating domain structures within the protein was recognized. The different domains are arranged in the order: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK, with the D1-D2 domains representing the propeptide and the remainder corresponding to the mature VWF subunit (Fig. 1). Progress in structural and bio-informatical analysis of protein structures has recently led to a re-assessment of the mosaic architecture of VWF, proposing the following domain structure (Fig. 1): D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK. This analysis revealed that the D-domains consist of various distinct structures: The D1-, D2- and D3-domains each contain a VW-domain, a C8-fold, a trypsin inhibitor-like (TIL)-structure and an E-module. The VWF-domain and C8-fold are both absent in the D'-domain, while the D4-domain lacks the E-module, but contains a unique subdomain, designated D4N. Another important change relates to the C-terminal part of the protein, in which the B-C-domain region is replaced by six consecutive C-domains.
**Disulfide bridging:** VWF sequence contains an unusually high content of Cysteine residues (8.3%), a percentage 4-fold higher than the average in human proteins.⁶ Concerning the incorporation of these Cys-residues in disulfide bridging, there have been apparent contradictory observations. Some reports indicate that there is no or little free thiols present in VWF.⁷⁸ In contrast, mass-spectrometrical analysis of purified plasma-derived or recombinant VWF revealed the presence of several unpaired cysteines (positions 889, 898, 2448, 2451, 2453, 2490, 2491, 2528 and 2533; Fig. 2).⁹ A recent study by Shapiro et al. revealed that these apparently free cysteines are essential for proper folding and secretion of the protein.⁶ In their model, all cysteines participate in disulfide bonding but they do so in a sequential manner during biosynthesis. Furthermore, some of these pairs are sensitive to disulfide reduction and could therefore be converted into free thiols once secreted. Such flexibility would allow VWF to use the oxidation-state of the cysteines to modify its functional properties.⁹¹

**Multimerisation:** The pairing of cysteines within VWF is not limited to a single subunit. During synthesis, two pro-VWF subunits first engage into a covalent connection, involving the formation of three inter-chain cysteine-pairs located in the C-terminal Cysteine Knot (CK)-domains.¹¹ Importantly, the structural location of these three inter-molecular pairs protects them from disulfide reduction, ensuring the long-term stability of the dimeric conformation of VWF.¹² Second, pro-VWF dimers are linked covalently via inter-chain disulfide bridging involving cysteines of the D₃ domain. For this process the presence of the propeptide (D₁-D₂ domains) and D' domain is crucial for two reasons. First, the propeptide and D' domain serve to properly align the pro-VWF dimers thereby facilitating D₃ domain-mediated inter-dimeric cross-linking ("zipper-model"). Second, the propeptide may catalyze disulfide formation between D₃ domains via its protein disulfide isomerase activity, which locates to the CxxC-motifs at positions 159-162 and 521-524.¹³ Noteworthy, the propeptide does not need to be attached to the D'D₃-region to exert its cross-linking activity, as expression of the propeptide in trans proved sufficient to support VWF multimerization.¹⁴ The propeptide is indeed able to bind to the D'-D₃ region, particularly under conditions found in the cellular environment (low pH, low NaCl, high CaCl₂).¹⁵,¹⁶ The multimerization process generates a heterogeneous pool of differentially sized multimers that contain between 2 and more than 60 subunits.¹⁶,¹⁷

**Glycosylation:** During synthesis, VWF is subject to various post-translational modifications, including furin-mediated separation of the propeptide and
glycosylation. Crucial to VWF life-cycle, glycosylation starts in the early phase of synthesis. Indeed, within the endoplasmic reticulum, the enzyme oligosaccharyltransferase mediates the attachment of a core 14-saccharide unit to asparagine residues within the developing polypeptide chain. Different studies revealed that the pro-VWF subunit carries 17 N-linked carbohydrate structures: 4 being located in the propeptide and 13 within the mature subunit. Further along the synthesis pathway, the N-linked glycans undergo maturation, while ten O-linked glycans are also added. Detailed analysis of VWF by various groups unveiled an immense variation among these carbohydrate structures, particularly among the N-linked glycans (>300 structures identified). A number of interesting features are worth highlighting: 1) Sialylation: The majority (>90%) of the glycan structures are capped by sialic acid structures. Moreover, part of the O-linked glycans is characterized by bi- and tri-sialic acids structures, indicating that these structures are capped with two rather than a single sialic acid residue. 2) Sulfation: 5 sites for N-linked glycans (p.Asn1515, p.Asn2223, p.Asn2290, p.Asn2400 & p.Asn2790) are preceded by Pro-Xxx-Arg/Lys/His motifs, favoring terminal sulfation. Metabolic labeling and mass-spectrometrical analysis have confirmed the presence of sulfated glycan residues in VWF. The functional importance of these sulfated residues remains to be elucidated. 3) Blood group determinants: Both N- and O-linked glycans can carry ABO(H) blood group carbohydrate determinants. These determinants are present on the glycans of the mature subunit but not on those of the propeptide. It is estimated that approximately 13% of the N-linked glycans (i.e. 1-2 per subunit) and 1% of the O-linked glycans (i.e. 1 per 10 subunit) harbor these blood group determinants.

Another point of interest is the different glycosylation pattern described for endothelial- and platelet-VWF. Platelet-VWF contains about 50% fewer sialic acids and lacks the blood group A-antigen and B-antigen structures, while the H-antigen is normally present.

Part II: Basics of von Willebrand factor storage & secretion

Storage-granule formation: Following synthesis, VWF is transported to storage organelles in both megakaryocytes/platelets (α-granules) and endothelial cells (WPBs). WPBs and α-granules differ from each other in their dependency on VWF for their formation: α-granules can form in the absence of VWF, whereas the generation of WPBs is strictly VWF dependent. Given this VWF-dependency, we
will focus on the formation of WPBs rather than α-granules in the remainder of this paragraph (Fig. 3).

Although the field is in constant evolution, our understanding of how WPBs are formed and how VWF is packed in these organelles has dramatically improved recently, thanks to the newest electron microscopical imaging techniques (for reviews see: 29, 30). Whether the process of WPBs formation initiates in the Golgi-apparatus or the Trans-Golgi network is still a matter of debate. What is generally accepted is that organelle formation requires VWF multimers to be assembled into a helicoidal structure or tubule (see next paragraph), a step that allows a 100-fold compaction of VWF. In a very elegant study, the Cutler group recently demonstrated that the basic size of the future organelle is already pre-determined in the Golgi.31 Indeed, in this compartment, the size of the VWF structure, whether already organized in tubules or not, cannot exceed the size of the Golgi-ribbon structural subunit or ministack, i.e. 0.5 microns, leading to the notion of a “length unit”. Later on, in the continuous lumen of the Trans-Golgi network, the VWF cargo originating from different ministacks can be co-packaged together into forming organelles that will lead to WPBs of sizes varying from 0.5 to 5 microns (with sizes incrementing by 0.5 microns).31 At this step, the VWF tubules induce membrane protrusions from the Trans-Golgi network, leading to vesicles budding off and formation of immature WPBs.31, 32 Another aspect of WPBs formation that has been reported relates to the possibility of homotypic fusion of immature WPBs, a process that could contribute to their heterogeneity.32

At the molecular level, a number of players have been identified, many of them being involved in classical secretory granule formation. For VWF packaging into nascent organelles, a clathrin coat, the adaptor protein AP-1 and Discs Large-1 (Dlg1), appear necessary, probably to provide a scaffold and generate the typical rod-shaped or cigar-like structure of the WPBs (Fig. 4).33-35 In this regard, it should also be noted that electron tomography analysis has shown that some of the WP-tubules deviate from the rod-shaped structure in that they are twisted or truncated.36 Finally, after being released from the Trans-Golgi network, WPBs undergo a maturation step while being anchored on filamentous actin via a triple protein complex containing Rab27a, myosin and Rab27a-interaction protein (MyRIP) and myosin Va (MyoVa).37

**VWF packaging in WPBs:** The tubular structure of WPBs is a reflection of how VWF is folded in these organelles. The slightly acidic environment in the WPBs favors self-assembly of VWF subunits via inter-molecular interactions within the propeptide/D'-D3 domains.38 This self-assembly results in the formation of long right-handed...
helical tubules, the interior of which comprises the propeptide/D'-D3 domains. The remainder of the protein (i.e. the A1-CK region) sticks out of this helical architecture, and determines the regular spacing between the helical tubules that are characteristic of the WPBs. Recent work from the Springer group has shown that under acidic conditions the dimeric A1-CK region folds into an intertwined bouquet-like structure, with the domains being aligned in a side-by-side manner.

Insight into the packaging organization of VWF in the WPBs is not only of relevance to explain the typical morphology of these organelles, but may also be helpful in future studies to explain how WPBs co-residents are incorporated. Indeed, many other proteins, mostly involved in inflammation or hemostasis, have been identified in the WPBs besides VWF and its propeptide, such as P-selectin, interleukin-8, osteoprotegerin, angiopoietin-2 and in a selected subset of endothelial cells also FVIII. Incorporation of some of these residents (like interleukin-8 and tissue-type plasminogen activator) results from a random inclusion process. In contrast, other co-residents like P-selectin, Galactins and osteoprotegerin have been shown to directly interact with VWF, which may facilitate their targeting to the WPBs.

Interestingly, except VWF, WPBs do not all contain the same cargo, a feature that also seems to be true for platelet α-granules. Furthermore, the presence of some co-residents can potentially alter the process of WPBs-formation. Elegant work by the Mertens/Voorberg group revealed that both the shape and length of the WPBs is dramatically affected by the presence of FVIII: disturbance in the tubular structure leads to round or pear-shaped granules instead of the usual rod-shaped appearance.

How WPBs proteins modulate the well-defined VWF-driven helical tubular structure is an enigma that deserves to be solved in order to better understand how VWF regulates the storage and secretion of these co-residents.

**Basal and regulated secretion of VWF:** Whereas α-granules release VWF predominantly upon platelet activation, endothelial cells combine basal and regulated release of WPBs contents. A number of studies have tried to elucidate the mechanism by which VWF is released from endothelial cells and how the balance between basal- and regulated-release of VWF is determined and only a brief summary will be provided here (for reviews see 40,50). There have been opposite views as to whether the majority of VWF released from endothelial cells originates from constitutive or regulated secretory pathways. However, it is fair to say that the currently accepted view is that VWF is mostly released constitutively from WPBs (Fig. 4). When present in the endothelial cytoplasm, WPBs dance around in an undirected manner. Such movements will
eventually drive single WPBs to the cellular periphery, allowing them to fuse with the plasma membrane and release their contents into the extracellular space (blood or subendothelium). Circulating VWF mostly originates from this random fusion mechanism. However, in some instances, fusion of WPBs with the plasma membrane results in the selective release of WPBs co-residents (interleukin-8, eotaxin-3), whereas VWF and its propeptide are retained within the cell (Fig. 4).55 This unexpected observation (referred to as "lingering kiss") can be explained by a sudden rise in pH of the WPB (de-acidification) due to exposure to the extra-cellular environment.55 This de-acidification provokes a collapse of the pH-dependent tubular structure of the WPBs, thereby deforming the VWF-dependent helical structure and preventing secretion of VWF.

Basal release of single WPBs is probably insufficient to produce the long endothelial cell-anchored VWF bundles that recruit platelets, as such strings are only observed upon endothelial stimulation in vitro and in vivo.56,57 Massive release of WPBs is indeed part of the multiple reactions occurring upon endothelial stimulation. From a macroscopic point of view, three steps can be distinguished: (1) WPBs center to the perinuclear area, an event that is more or less pronounced, depending on the stimulation trigger.54 (2) the formation of VWF-enriched patches is observed, probably representing fusion of multiple WPBs and forming a secretory pod.54,58 (3) bundles of assembled VWF multimers are released. These bundles are highly prothrombotic in that they efficiently promote platelet adhesion.56,57

At the molecular level, the exocytosis process appears as a complex multistep process and only part of the essential players involved in the sequential steps (tethering, docking, priming and fusion) have been identified. Among these, the implication of a series of intracellular proteins (including but not limited to: RalA, Rab3, Rab27a, Rab15, Rab33a, Rab37, Munc13-4, Munc18c, Slp4a, Annexin A2-S100A10, syntaxin-binding protein 1, syntaxin-4, VAMP-3, G-proteins) has been documented based on the finding that deletion or mutation of these proteins modulates constitutive and/or regulated release of VWF.37,59-67

Recently, an unexpected and novel type of regulation of VWF release has been reported by Torisu and colleagues.68 Not only did they observe that WPBs are often in close proximity of autophagosomes but they also detected the presence of VWF in these organelles.68 In vitro or in vivo inhibition of autophagy led to decrease WPBs release, evidenced by lower basal levels of VWF, a lower response to epinephrine-induced WPBs release and a reduction in high molecular weight multimers combined with an increased bleeding time in mice. This new set of information thus suggests that VWF release is a combined process involving WPBs and autophagosomes and

follow-up studies aiming to understand how both types of organelles collaborate in this exocytosis process are required.

**Part III: Basics of von Willebrand factor clearance**

**VWF clearance:** Following its release in the circulation, VWF is ready to function as molecular carrier (*i.e.* for FVIII, osteoprotegerin, galectins and several other proteins) and recruiter of platelets upon vascular injury. However, VWF is similar to other plasma proteins in that its circulatory life-span is limited. Plasma proteins are sensitive to physical changes (oxidation, proteolysis, glycation, etc.), which may alter their functional properties and regulatory mechanisms are in place to eliminate “old” plasma proteins from the circulation. These mechanisms may be unique to each protein, dependent on the need for protein renewal in the circulation.

Following application of therapeutic plasma-derived VWF concentrates, the half-life of VWF antigen in humans is approximately 16 hours. These therapeutic preparations are prepared from large plasma pools, and are thus not representative of the circulatory half-life of VWF in individuals. Indeed, when analyzing the half-life of endogenous VWF following desmopressin-treatment, a large variation is observed between individuals, ranging from 4.2 to 26 hours. The main determinant for this inter-individual variation most likely originates from different glycosylation patterns. In particular, the presence of blood group ABO(H)-structures appears to explain a large portion of this variation. Individuals with blood group non-O display a longer VWF half-life after desmopressin-treatment than individuals with blood group O, which may explain the average 25% higher VWF levels in individuals with blood group non-O.

This inter-individual variation may also explain the large variation in the half-life of FVIII in hemophilia A-patients. VWF functions as a carrier-protein for FVIII and therefore FVIII-half-life may vary with the individual VWF half-life of the patient. This possibility is supported by the notion that FVIII half-life is dependent on blood group and correlates with pre-infusion VWF antigen levels. Moreover, FVIII half-life can be accurately predicted for each patient using an algorithm based on blood group and pre-infusion levels of VWF and of VWF propeptide.

**Cellular basis of VWF clearance:** Our knowledge on the mechanism by which VWF is eliminated from the circulation is predominantly based on cellular and murine models. The use of *VWF*-deficient mice first allowed the identification of tissues that are responsible for the clearance of VWF. The indication that the majority of VWF is targeted to the liver, indicated that VWF is cleared via an active regulatory
mechanism rather than via passive elimination.\textsuperscript{5} Interestingly, when taking into account the size of the different organs, the spleen appeared as efficient as the liver in taking up VWF.\textsuperscript{80} In order to identify cells that are involved in VWF clearance, immuno-histochemical analysis of liver and spleen were analyzed, revealing that VWF principally co-localizes with macrophages (Fig. 5).\textsuperscript{80} This co-localization was confirmed in alternative experiments in which the chemical depletion of macrophages resulted in increased VWF survival and elevated levels of endogenous VWF.\textsuperscript{80,81} Finally, experiments using human macrophages (primary or THP-1-derived) established that VWF is bound and endocytosed by macrophages.\textsuperscript{80,82} The finding that macrophages play an important role in the basal clearance of VWF does not exclude the participation of other non-macrophage cells in this process.

\textit{Molecular basis of VWF clearance:} The identification of macrophages as VWF-eliminating cells prompted us to examine how VWF uptake was mediated. Macrophages may internalize proteins randomly via receptor-independent macropinocytosis, but it is unclear whether this is also true for VWF. In contrast, a number of receptors have been identified that interact with VWF, indicating that receptor-mediated endocytosis of VWF plays an important role in this regard (Fig. 5).

The first VWF receptor that was identified is the asialoglycoprotein-receptor, also known as the Ashwell-receptor.\textsuperscript{83} However, since the majority of the VWF glycans are sialylated, this receptor is not expected to play a major role in the regular clearance process of VWF. Rather, its relevance could become apparent when VWF molecules are hypo-sialylated, for instance upon pathogen infection or upon reduced activity of sialytransferase enzymes.\textsuperscript{83,84}

A second receptor recently identified is the lipoprotein-receptor LRP1, which was previously identified as a receptor for FVIII.\textsuperscript{82,85,87} Importantly, VWF binds to LRP1 only when exposed to shear stress, thereby mimicking the flow-dependency of the VWF-platelet interaction.\textsuperscript{84,85} Indeed, VWF needs to unfold in order to be recognized by this receptor, although lower shear forces are needed compared to VWF-platelet interactions. The physiological relevance of macrophage LRP1 in basal VWF clearance is illustrated by the increased endogenous VWF levels and increased survival of VWF in mice that are selectively deficient for LRP1 in macrophages.\textsuperscript{85} In addition, polymorphisms in the LRP1 gene are associated with VWF plasma levels.\textsuperscript{88,89}

A third potential receptor is Siglec-5, a receptor present on macrophages that specifically interacts with sialic acid-residues.\textsuperscript{90} Although over-expression of Siglec-5
in mouse liver reduces VWF levels, its relevance in the clearance pathways of VWF remains uncertain.

Finally, the Lillicrap-group identified CLEC4M as a receptor for VWF. Interestingly, genome-wide association studies had previously identified the CLEC4M gene as a determinant of VWF plasma levels, pointing to a physiologically relevant relation between CLEC4M and VWF. However, whether CLEC4M modulates VWF clearance or affects VWF levels via an alternative mechanism remains to be determined. It should further be noted that CLEC4M is selectively expressed on sinusoidal endothelial cells, suggesting a potential role for these cells in VWF removal from the circulation.

Taken together, several potential VWF receptors have been identified, but so far only LRP1 appears to play a significant role in basal clearance of VWF. Since LRP1-deficiency prolongs VWF half-life not more than two-fold, it seems conceivable that other, so far unidentified receptors contribute to VWF clearance as well.

**Part IV: Connecting the far ends: VWF cysteines in VWF life-cycle**

The sequential steps of VWF synthesis, packaging into WPBs, secretion and removal from the circulation highlight the complexity of the VWF life-cycle. It is not surprising therefore that mutations in the VWF molecule may affect one or more of these steps. In this last part of the review, we would like to illustrate how these steps are modulated in the context of VWD-related mutations, thereby focusing on cysteine-mutations, given that cysteines play an important role in the proper folding and maturation of the protein.

*Cysteine mutations and WPBs formation:* Cysteines contribute to the intrinsic folding of individual domains, tail-to-tail dimerization and head-to-head multimerization. Mutations provoking the (dis)appearance of cysteines may affect each of these processes. But how do such mutations affect WPBs formation? Intuitively, one would predict that impaired dimerization or multimerization would prevent the formation of WPBs. However, no general rule can be applied to such situations as exemplified by three Cys-mutations located in the CK-domain (p.Cys2739Tyr, p.Cys2754Trp and p.Cys2773Ser). These mutations impair C-terminal dimerization and consequently only N-terminal dimerization occurs, preventing the formation of long multimers. Interestingly, each mutant has a different effect on WPBs formation: no WPBs are formed with mutation p.Cys2739Tyr, whereas round organelles are produced with mutation p.Cys2754Trp. In contrast, normal rod-shaped WPBs are visible for mutant p.Cys2773Ser, demonstrating that WPBs may form even in the absence of
multimerization. Interestingly, a broad analysis of several types of mutations further revealed that the opposite is also true, i.e., the presence of VWF multimers is no guarantee for proper WPBs formation. Based on the available information, two processes seem of critical importance for WPBs formation: (1) correct arrangement of the propeptide/D'-D3 domains to form the core of the helical tubule, independent of whether they engage in disulfide bridging and (2) assembly of the remainder of the domains (A1-CK) into the side-by-side bouquet structure. Distortion of this bouquet structure may compromise the alignment of the tubules that make up the interior of WPBs.

**Cysteine mutations and secretion:** Apart from WPBs formation, cysteine mutations may also affect VWF secretion. Again, these effects seem mutation specific, with some mutants being secreted normally (e.g., mutant p.Cys1225Gly), whereas others are retained within the cells (e.g., p.Cys1149Arg), despite normal WPBs formation. In a large study concerning VWD-type 1 patients, 5 out of 15 mutations involving the (dis)appearance of a cysteine, were associated with the absence or a partial response to desmopressin, further illustrating the heterogeneous effect of cysteine mutations on VWF secretion. How these mutants modulate secretion is unclear. They may be associated with an abnormal recruitment of components of the secretory machinery. Alternatively, misfolding of the proteins due to the cysteine mutations may alert quality control mechanisms that maneuver the mutated proteins to the intracellular degradation pathway.

**Cysteine mutations and clearance:** Despite intracellular quality control systems, small or large amounts of mutated proteins may escape the cell, including those with cysteine mutations. Are such mutants then cleared similarly to normal VWF? In the last several years, 35 different mutations have been associated with increased clearance of the protein. Interestingly, 14 of those (40%) involve a cysteine, indicating that cysteine mutations are over-represented in this group of mutations. Three examples thereof have previously been described in detail by our group; p.Cys1130Phe, p.Cys1149Arg and p.Cys2671Tyr. Patients with these mutations are characterized by increased VWF-propeptide/VWF-antigen ratios and a reduced survival of VWF-antigen following desmopressin treatment. In addition, recombinant variants display a reduced survival in a murine model. The mechanism by which these mutants are cleared more rapidly is currently unknown. Mutations may induce enhanced binding to the regular clearance receptors, such as LRP1. Indeed, we observed that mutant VWF/p.Cys1130Phe binds to LRP1 without the need for shear stress (Wohner & Lenting, unpublished observation). Alternatively, presence of
mutations may provoke binding to clearance/scavenger receptors otherwise unable to recognize VWF. Studies in this direction are currently ongoing, and will provide more insight into how VWD-related mutations are associated with increased clearance of VWF.

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Legends:

Figure 1: Schematic representation of the old and new domain arrangement of VWF.
The molecular architecture of VWF is characterized by the presence of distinct domain structures. The top schema represents the arrangement of five different domain structures according to the original analysis of the VWF sequence (reviewed by Pannenkoek & Voorberg). The numbering of the domain boundaries has been used in our laboratory in the previous years. The middle schema shows the domain organization as has recently been proposed by Zhou et al. One striking difference with the original domain structure is the replacement of the B1-3 - C1 - C2 domain region by 6 homologous C-domains. In addition, their analysis revealed that the D-domains consist of various independent structures, which are highlighted in the lower schemas. The D1, D2 and D3 domains each contain a VW-domain, a trypsin inhibitor-like (TIL)-structure, a C8 fold and an E module. The D’ region lacks the VW domain and C8 fold. The D4 domain lacks the E module, but instead comprises a unique sequence designated D4N. (Figure adapted from Rauch et al.)

Figure 2: Overview of relevant cysteine residues in VWF.
The VWF protein sequence contains 234 cysteine residues, representing 8.3% of the total amino acid composition, a number that is 4-fold higher than the average in human proteins. Cysteines contribute to the folding of the domains that characterize the VWF structure. Some of these cysteines form disulfide bridges during synthesis, but are susceptible to reduction following secretion. Consequently, they are converted into free thiols (positions 889, 898, 2448, 2451, 2453, 2490, 2491, 2528 & 2533). Other cysteines engage in inter-subunit disulfide bonding (positions 2771, 2773 & 2811 in the CK-domain and positions 1142, 1222, 1225 & 1227 in the D3 domain). N-terminal multimerization involves disulfide isomerase activity located in the D1 and D2 domains (motif 159-162 and 521-524). Given their importance for the VWF structure, it is not surprising that mutations of cysteines may affect different stages of the VWF life-cycle. Examples hereof (red boxes) include mutations affecting multimerization, storage in WPBs, secretion and clearance.

Figure 3: Biosynthesis and packaging of VWF in WPBs.
The biosynthesis of VWF distinguishes a series of sequential steps that ultimately lead to its incorporation into endothelial storage organelles, the WPBs. Step 1: During synthesis of the VWF pro-polypeptide chain in the endoplasmic reticulum, intraprotein cysteine bonding occurs to facilitate folding of the individual domains. Subsequent tail-to-tail inter-protein disulfide bridge formation involving the C-terminal CK domains allows the formation of pro-dimers. Furthermore, the first building blocks for N-linked glycosylation are coupled to the growing polypeptide chain. Step 2: Upon arrival in the Golgi apparatus, the presence of a slightly acidic
pH and relatively high Ca\textsuperscript{2+} concentration promote the organization of the prodimers into a dimeric bouquet structure, in which the dimers are aligned into a side-by-side manner. Moreover, this environment favors multimerization via disulfide bridging that couples adjacent N-terminal D3 domains, a process that is catalyzed by the propeptide. While the multimerization process takes place, the expanding multimer organizes into a right-handed helical structure, allowing a 100-fold compaction of the protein. In this helical structure, the propeptide (D1-D2 domains) and the D'-D3 domains form the wall of the hollow tube. The remainder of the VWF protein (A1-CK domains) protrudes outward from the helical architecture, occupying the space between the tubules that characterize the electron-microscopic images of WPBs. VWF tubules assemble into so-called ministacks that represent the first WPB-like structure. During the passage of VWF through the Golgi, maturation of the N-linked glycans proceeds while also O-linked carbohydrate structures are added to the protein. 

**Step 3:** An important gap in our knowledge of WPB-formation is the location of the proteins that co-reside with VWF in this organelle. For example, FVIII is known to interact with the D'D3 region, suggesting that FVIII may locate to the inner core of the helix. In contrast, osteoprotegerin (which binds to the A1 domain) and Galectins-1 and -3 (which bind to VWF glycans) are more likely to be present in the inter-tubular space. 

**Step 4:** In the Trans-Golgi network, co-packaging of VWF-containing ministacks promotes maturation and formation of larger WPBs. In addition, furin mediates the proteolytic separation of the propeptide from the mature VWF subunits. Of note, under the slightly acidic conditions present within the Trans-Golgi network, the propeptide remains associated with mature VWF. Multimer analysis of endothelial VWF has revealed the presence of very large VWF multimers that exceed the size of multimers found in plasma.

**Figure 4: Schematic representation of VWF secretion from endothelial cells.**

Following its synthesis and packaging in WPBs, VWF follows a complex pathway allowing intra-endothelial storage combined with basal and regulated secretion. In recent years, many aspects of the molecular machinery that regulates these processes have been identified and this figure provides a schematic overview of the essential elements. 

**Step 1:** The formation of VWF-containing ministacks and subsequent WPBs requires the presence of clathrin coat, the adaptor protein AP-1 and Disc large-
1. **Step 2:** WPBs move around randomly within the endothelial cell along microtubules driven by a so far unidentified kinesin/dynein-complex. **Step 3:** Subsequently, WPBs adhere onto the filamentous actin network via a triple protein complex involving Rab27a, Myosin Va (MyoVa) and Myosin Va & Rab27a interacting protein (MyRIP). WPBs attract also a series of other proteins involved in the secretory machinery, including Rab3, Rab15, Rab27a, Rab37, Munc13-4, and Sip4a. **Step 4:** Secretion of the WPB content is preceded by sequential steps of tethering, docking and priming before fusion with the cellular membrane. Specific protein complexes are involved in each of these steps (see boxes). **Step 5:** three types of secretion can be distinguished: (A) Basal secretion, in which a single WPB fuses to the cellular membrane and releases its contents (both VWF and other WPB proteins). (B) On rare occasions, exposure to the extra-cellular environment causes a rapid de-acidification of the organelle provoking the pH-dependent tubular VWF structure to collapse. Consequently, VWF is retained within the remainder of the WPB, whereas other WPB proteins (such as interleukin-8) are secreted into the circulation. This process is referred to as a lingering kiss. (C) Upon agonist-induced endothelial stimulation, multiple WPBs aggregate and might eventually fuse into a large secretory vesicle, referred to as secretory pods. This results in the release of massive amounts of VWF multimers. These multimers assemble into the long bundles (up to several hundred microns) that consist of multiple multimers. These bundles are highly thrombogenic as they efficiently recruit platelets. To reduce the thrombogenic potential of the platelet-decorated VWF strings, the action of ADAMTS13 (and also of plasmin under certain conditions) is required. *This figure has been inspired by figures presented elsewhere.*

**Figure 5: Potential clearance pathways for VWF**

VWF circulates as a globular protein, with the majority of its glycan structures being well sialylated. In this form, VWF is recognized by two different receptors that could mediate the removal of VWF from the circulation: CLEC4M on endothelial cells and Siglec-5 on macrophages. Shear stress-induced unfolding of VWF is associated with the exposure of interactive site(s) for LRP1. Indeed, in *vitro* and in *vivo* experiments have confirmed the involvement of LRP1 in the uptake of VWF in macrophages. Certain VWD-related mutations (*e.g.*, the VWD-type 1 Vicenza mutation p.R1205H and the VWD-type 2B mutations p.R1306Q and p.V1316M) provoke exposure of LRP1 interactive sites in the absence of shear stress, which could perhaps explained the accelerated clearance of these mutants. It is unknown whether binding of mutant VWF is limited to macrophage LRP1 (as seems to be the case for wt-VWF) or whether interactions also include LRP1 on other cell types, like hepatocytes or even other yet
unidentified receptors. De-sialylation of VWF exposes terminal galactose-residues, allowing efficient interaction with the asialoglycoprotein-receptor (ASGPR) on macrophages and hepatocytes. Of note, the clearance receptors responsible for the discordant clearance of blood group O and non-O VWF have not been identified yet. *This figure is reprinted from Casari et al. with permission.*
- disulfide bonding
- C-terminal tail-to-tail dimerization
- initiation N-linked glycosylation
- assembly into dimeric bouquet structure

- N-terminal head-to-head multimerization
- maturation N-linked glycans & O-linked glycosylation
- formation of VWF-containing tubules

- packaging into ministacks (small WPBs)
- incorporation of co-residents

- propeptide cleavage
- co-packaging VWF containing ministacks (WPB maturation)
Figure 4

C) Agonist-induced release:
- Multiple WPBs fuse
- VWF multimers assemble into platelet-binding strings

B) Lingering kiss (VWF retained in WPBs)

A) Basal secretion (VWF + other WPBs proteins)

Tethering:
- Rab27a
- Phospholipase D
- RabA ~ GTP
- Exocyst

Docking:
- Rab27a
- Slp4a
- STXBP1
- VAMP3
- Munc18-c
- Syntaxin-4

Priming:
- Rab27a
- Munc-13-4
- VAMP-3
- SNAP23
- Munc18-c
- Syntaxin-4
- Annexin A2

Golgi

Fusion of ministacks
VWF & other WP-body proteins
Rab37
Rab27a - Rab15 - Munc13-4
Rab27a - Rab3b/d - Slp4a
kinesin-dynein
microtubules
F-actin

AP1/clathrin Coat/Dlg1
VWF-containing ministack (0.5 micron)

Secretory pod
Platelet-decorated VWF strings
Figure 5

- globular VWF
- sialylated
- de-sialylated
- unfolded & mutant VWF
- CLEC4M
- Siglec-5
- LRP1
- Endothelial cell
- Macrophage
- ASGPR
- Hepatocyte
- mutant VWF?
Von Willebrand factor biosynthesis, secretion & clearance: connecting the far ends

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