Von Willebrand factor processing

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Keywords

von Willebrand factor, multimer biosynthesis, disulfide bonds, glycosylation

Summary

Von Willebrand factor (VWF) is a multimeric glycoprotein essential for primary haemostasis that is produced only in endothelial cells and megakaryocytes. Key to VWF's function in recruitment of platelets to the site of vascular injury is its multimeric structure. The individual steps of VWF multimer biosynthesis rely on distinct posttranslational modifications at specific pH conditions, which are realized by spatial separation of the involved processes to different cell organelles. Production of multimers starts with translocation and modification of the VWF prepropolypeptide in the endoplasmic reticulum to produce dimers primed for glycosylation. In the Golgi apparatus they are further processed to multimers that carry more than 300 complex glycan structures functionalized by sialylation, sulfation and blood group determinants. Of special importance is the sequential formation of disulfide bonds with different functions in structural support of VWF multimers, which are packaged, stored and further processed after secretion. Here, all these processes are being reviewed in detail including background information on the occurring biochemical reactions.

Schlüsselwörter

von-Willebrand-Faktor, Disulfidbrücken, Glykosylierung, Multimersynthese

Zusammenfassung

Der von-Willebrand-Faktor (VWF) spielt eine zentrale Rolle in der primären Hämostase. Grundvoraussetzung hierfür ist seine multimere Struktur, die die effiziente Rekrutierung von Thrombozyten an die verletzte Gefäßwand ermöglicht. Die einzelnen Schritte der VWF-Multimersynthese bestehen aus verschiedenen posttranslationalen Modifikationen, die nacheinander bei bestimmten pH-Bedingungen ablaufen müssen. Deshalb finden sie in unterschiedlichen Zellorganellen statt. Zunächst werden die VWF-Monomere im endoplasmatischen Retikulum zu Dimeren verknüpft und vorglykolisiert. Im Golgi-Apparat werden diese zu Multimeren zusammengefügt, die nach abgeschlossener Modifikation mehr als 300 komplexe Zuckerstrukturen tragen, welche durch Sialylierung, Sulfonierung und mit Blutgruppenantigenen funktionalisiert wurden. Von besonderer Wichtigkeit ist die sequenzielle Ausbildung von Disulfidbrücken, die verschiedene Aufgaben in der Strukturgebung der VWF-Multimere besitzen. Die Multimere werden spiralisiert, gelagert und nach Sekretion weiter modifiziert. Dieser Artikel liefert einen detaillierten Überblick über diese Prozesse, inklusive Grundlageninformationen zu den biochemischen Reaktionen.

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Von-Willebrand-Faktor-Prozessierung

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Von Willebrand factor (VWF) is a multimeric glycoprotein that is best known for its essential roles in haemostasis but more and more functions and contributions to pathological processes are being discovered. The highly complex multi-domain structure results in the remarkable functional diversity of VWF (reviewed in 1).

Besides the best understood functions in recruitment of platelets under conditions of arterial shear stress and its role in secondary haemostasis, as a carrier protein for coagulation factor VIII (FVIII), VWF

- is involved in arterial (2) as well as venous thrombosis (3) and stroke (4–6),
- negatively regulates angiogenesis (7),
- stimulates smooth muscle cell proliferation (8, 9),
- contributes to inflammatory processes (10–12) and to apoptosis of platelets (13), and
- interacts with osteoprotegerin (14) thereby influencing physiological bone remodeling and tumor cell apoptosis (15).

The key to VWF's function in recruitment of platelets to the site of vascular injury is its multimeric structure, meaning that VWF is not secreted into the circulation as a single molecule (monomer) but as covalently connected multi-molecule entities, called multimers. These multimers are highly complex protein structures that have to be produced stepwise in a sequential manner during which distinct posttranslational modifications have to occur in a specific order and at defined pH conditions. This is realized by spatial separation of the involved processes to different cell organelles. Posttranslational modification of the resulting multimers further varies depending on whether they were produced in endothelial cells or in megakaryocytes.

In this article, all steps involved in VWF multimer processing are being reviewed, starting with VWF monomer translation in the cytoplasm and ending with multimer degradation in the circulation. All occurring biochemical reactions are described in detail and focus is laid on providing background information regarding the basic biochemical principles that underlie protein modification and functionalisation.

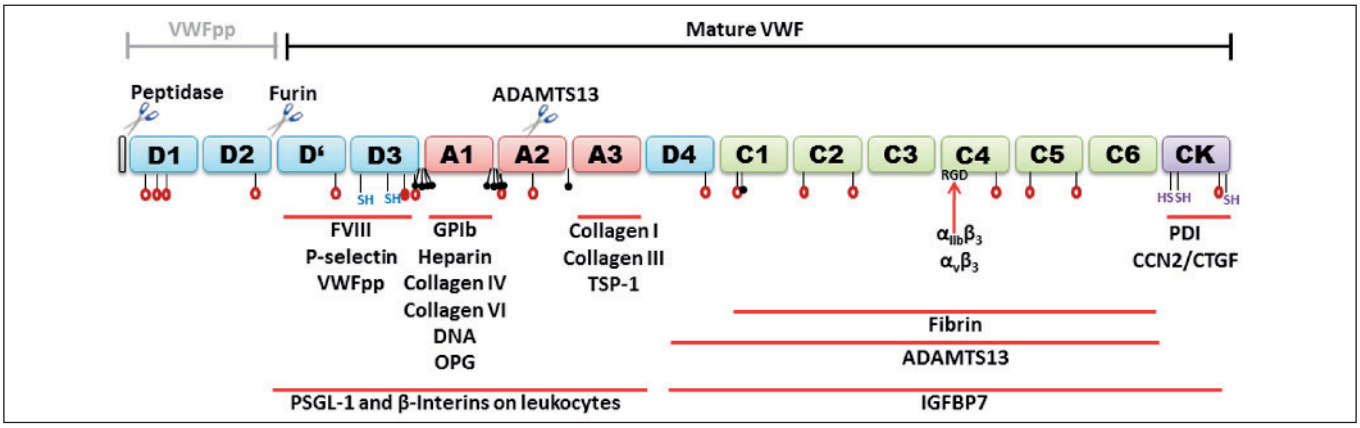

Fig. 1 Schematic presentation of the VWF domain structure. The VWF propeptide (VWFpp) is framed by a grey line, the mature VWF by a black line. Scissors label cleavage sites for indicated proteases. N-glycosylation sites obeying the consensus sequence N-X-S/T are indicated by red/white lollipops, the N-X-C sequon is marked by a red lollipop. O-glycosylation sites are indicated by black lollipops. Cysteine residues essential for dimerisation are indicated by violet SH-groups in the CK domain, cysteines used for disulfide bonds that facilitate multimerisation are indicated by blue SH-groups in the D3 domain. Red lines mark binding sites for the indicated proteins. FVIII: factor VIII; GPIb: glycoprotein Ib; OPG: osteoprotegerin; PSGL-1: P-selectin glycoprotein ligand-1; TSP-1: Thrombospondin-1; $\alpha_{IIb}\beta_3$: integrin $\alpha_{IIb}\beta_3$; $\alpha_v\beta_3$: integrin $\alpha_v\beta_3$; ADAMTS13: a disintegrin and metalloprotease with thrombospondin type 1 repeats; IGFBP7: insulin like growth factor binding protein 7; PDI: protein disulfide isomerase; CCN2/CTGF: connective tissue growth factor. Functions of these interactions reviewed in (1).

Cytoplasm

VWF's function in platelet recruitment requires secretion into the circulation. Thus, VWF needs to enter the secretory pathway. Such proteins typically possess N-terminal hydrophobic signal sequences, which guide them to the membrane of the endoplasmic reticulum (ER). After entering the ER lumen their journey through the cell is exclusively realized by vesicular transport, making crossing the ER membrane the only membrane translocation event such proteins require for entering the secretory pathway. Key to this process is the signal recognition particle (SRP) that delivers the nascent secretory protein together with the associated ribosome to the ER protein translocon (Sec61 complex) by recruitment to its cognate receptor (SR) that is presented on the ER membrane.

When the ribosome nascent chain complex (RNC) binds the Sec61 complex, nascent protein synthesis continues directly into the ER lumen. During translocation signal peptidase (SPase) can associate with the secretory protein and cleave the signal peptide from the nascent chain (16). Protein folding is also initiated during translocation by oligosaccharyltransferase (OST) that N-glycosylates the protein thereby laying ground for disulfide bond formation as described below.

VWF Primary Structure

VWF is produced exclusively in endothelial cells and megakaryocytes as a single prepropolypeptide consisting of 2813 amino acids (aa). The first 22 aa represent the signal peptide that guides the synthesizing ribosome to the ER membrane. The nascent VWF chain emerging into the ER lumen possesses a multi-domain structure that was recently re-annotated after detailed bioinformatical and structural analysis, suggesting the following domain structure: D1-D2-D'D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK (▶Fig. 1) (17).

While the D1-D2 domains represent a propeptide (741 aa, VWFpp) that is cleaved off by furin, the mature VWF subunit contains the domains D'D3 through CK (2050 aa). When folded into their tertiary structure, VWF domains harbor several functional binding and cleavage sites (see ▶Fig. 1 and Conclusion).

The primary aa sequence of VWF contains a strikingly high percentage (8.3%) of cysteine residues that is 3.7-fold higher than in the average of human proteins (18, 19). The crucial importance of these cysteine residues for VWF multimer structure, biosynthesis and self-assembly will be discussed below in the appropriate paragraphs.

Endoplasmatic reticulum

Glycosylation and folding by intramolecular disulfide bonds

Protein folding is governed by chaperons, which are proteins that assist in the folding of another protein without being part of its final structure (20). Whereas single-domain proteins undergo folding in the cytoplasm after their translation is complete and they have been released from the ribosome, most multi-domain proteins, such as VWF, fold co-translationally while emerging into the ER from the ribosome. This sequential folding of one domain after the other prevents the formation of unproductive intermediates resulting from non-native interactions between the different folding domains (20).

The folding and modification process is started by N-linked glycosylation. In general, the enzyme oligosaccharyltransferase (OST) attaches a preformed 14-saccharide core unit ($Glc_3Man_9GlcNAc_2$, consisting of 3 Glucose [Glc], 9 Mannose [Man] and 2 N-acetylglucosesamine [GlcNac] molecules) to the amino group ($-NH_2$) of asparagine residues within specific consensus sequences in the primary structure of the nascent peptide chain (21) (▶Fig. 2a).

Each pro-VWF monomer contains 16 N-glycosylation sites that obey the

N-glycosylation consensus sequence N-X-S/T (Asn- any aa – Ser or Thr). These sequences are located at Asn99, Asn156, Asn211, and Asn666, in the propeptide (22), and at Asn857, Asn1231, Asn1515, Asn1574, Asn2223, Asn2290, Asn2357, Asn2400, Asn2546, Asn2585, Asn2635 and Asn2790 in mature VWF (red/white lollipops, ▶Fig. 1). Another consensus sequence is the N-X-C (Asn- any aa – Cys) glycosylation sequence, that represents about 1% of all efficiently used sites. VWF possesses 9 of these sequons (Asn367, Asn819, Asn847, Asn1147, Asn2209, Asn2415, Asn2526, Asn2531, Asn2625).

Out of these 9 asparagine residues it has only been shown for Asn1147 (red lollipop, ▶Fig. 1) that it is actually used as an N-glycosylation site (23, 24).

To promote correct protein folding, membrane bound glucosidases I and II remove the two terminal glucose residues from the core unit and the resulting mono-glucosylated $Glc_1Man_9GlcNAc_2$ structure becomes a binding motif for the ER membrane localized chaperone protein calnexin (CNX) (▶Fig. 2b) or the soluble calreticulin (CRT) (25–27). Subsequently, a complex is formed consisting of the substrate protein, and either CRT or CNX, plus the protein disulfide isomerase isoform A3 (ERp57). ERp57 is a thiol oxidoreductase that catalyzes the formation of disulfide bridges between the sulfur atoms at the thiol group (-SH) of cysteine residues, thus leading to formation of intramolecular disulfide bonds that facilitate protein folding (28).

For VWF it has been shown that mutations in the propeptide prolong VWF interaction with CNX and ERp57 resulting in ER retention of the VWF mutant (29) that is most likely due to activation of the cell's unfolded protein response. These data confirm that CNX and ERp57 are responsible for the formation of disulfide bonds that determine VWF folding.

In this step it is of paramount importance that some very specific cysteine residues remain unpaired because they need to be available for

- C-terminal intermonomer disulfide bonds mediating dimerisation (Cys2771, Cys2773 and Cys2811; ▶Fig. 1) (30, 31),
- N-terminal interdimer bonds connecting dimers to multimers (Cys1099 and Cys1142; ▶Fig. 1) (32, 33) , and
- intermultimer bridges for VWF cross-linking after secretion (Cys889, Cys898,

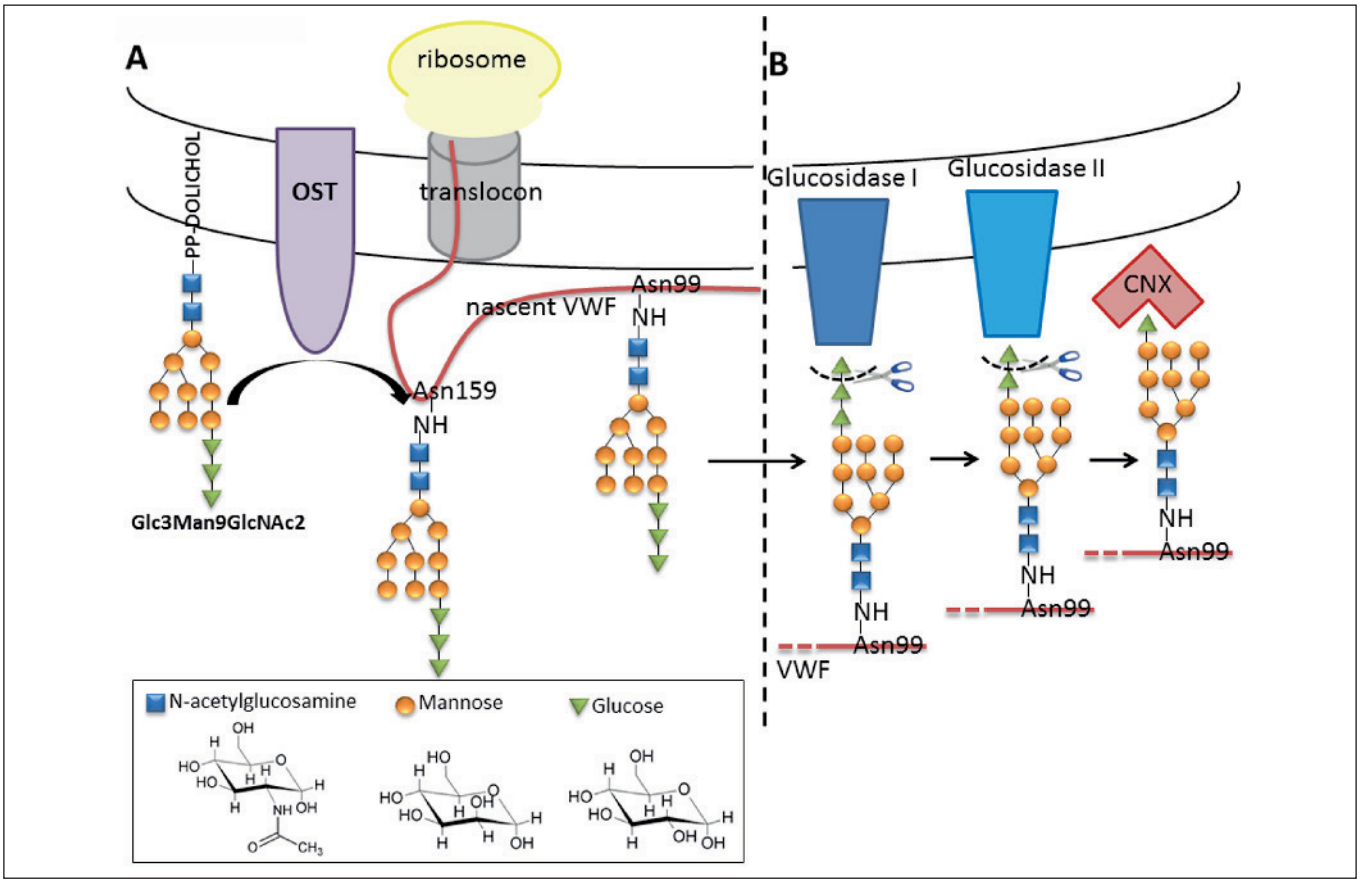

Fig. 2 Schematic presentation of N-glycosylation in the endoplasmatic reticulum (ER), shown exemplary for the VWF N-terminus.
(**A**) The protein folding and modification process is started by N-linked glycosylation by the enzyme oligosaccharyltransferase (OST) that attaches a preformed 14-saccharide core unit ($Glc_3Man_9GlcNAc_2$) to the amino group of asparagine residues within specific consensus sequences in the primary structure of the nascent peptide chain. Here, exemplary Asn99 and Asn159 of VWF.
(**B**) To promote correct protein folding, membrane bound glucosidases I and II remove the two terminal glucose residues from the core unit and the resulting monoglucosylated $Glc_1Man_9GlcNAc_2$ structure becomes a binding motif for the ER membrane localised chaperone protein calnexin (CNX).

Cys2448, Cys2451, Cys2453, Cys2490, Cys2491, Cys2528, and Cys2533) (19).

All other cysteine residues were shown to be part of a folding disulfide bond. For some of them the partner cysteine in the bridge has been determined (19, 30, 33, 34).

If the folding process is incorrect the protein becomes re-glucosylated by the protein UDP-glucose glycoprotein glucosyltransferase 1 (UGGT1) (6), redirecting it to CNX/CRT binding for proper folding. After successful folding, the final glucose residue is removed by glucosidase II, resulting in release from CRT or CNX and transit through the ER into the Golgi. But before VWF can continue its journey to the Golgi, dimerisation has to occur.

VWF dimerisation via intermonomer disulfide bonds

Dimerisation is facilitated by disulfide bond formation between cysteine residues Cys2771, Cys2773 and Cys2811 in the C-terminal cystine-knot (CK) domain (▶Fig. 1) (30, 31). The ER with a pH of 7.4 and the presence of several thiol oxidoreductases is the ideal environment for the formation of disulfide bonds.

We recently showed that protein disulfide isomerase isoform A1 (PDIA1) is the protein that catalyzes dimerisation (35). Employing different high-resolution methods (Stochastic Optical Reconstruction Microscopy [STORM] and Atomic Force Microscopy [AFM], Microscale Thermophoresis [MST], and Fluorescence Correlation Spectroscopy [FCS]), we found independent evidence that PDI directly binds to VWF only in its CK domain. Combined with docking and MD simulation data as well as colocalisation studies of von Willebrand disease-associated VWF mutants these results allowed proposing the following mechanism of VWF dimerisation (▶Fig. 3):

After an initial, probably electrostatic, association of PDI with VWF, N-terminally of Ser2772, PDI can only interact with Cys2771 and Cys2773. First, oxidized PDI forms a disulfide bond between its catalytic residue Cys36 and either Cys2771 or Cys2773 in the CK domain of one VWF monomer (▶Fig. 3a). Then PDI catalyzes the formation of the first disulfide bond between the Cys2771 of one monomer with the Cys2773' of the other monomer (Cys2771–2773') (▶Fig. 3b) or vice versa (2771'-2773). PDI is then released in its reduced form (A, out).

Second, a new oxidized PDI molecule (▶Fig. 3a, in) binds (▶Fig. 3b) and formation of the second disulfide bond is catalyzed (▶Fig. 3c). These two disulfide bridges stabilize the CK dimer and the CK C-termini can now assemble into a β-sheet conformation (▶Fig. 3c), which brings Cys2811 and Cys2811' together close enough to form the third disulfide bond Cys2811–2811' (▶Fig. 3d).

> The simulation data indicate that the formation of the last bond is not necessarily catalyzed by PDI. It might also be formed by a spontaneous process or through catalysis by a different enzyme or a small molecule, e.g. glutathione.

We hypothesize that disulfide bond Cys2811–2811' forms as a protective cover that shields Cys2771 and Cys2773 from reduction by PDI, thereby ensuring VWF dimerisation to be irreversible (35). These data are in agreement with the previous conclusion of Springer's group that the dimer interface is suited to resist hydrodynamic force and disulfide reduction because the cystine-knots in each monomer flank the inter-chain disulfides and their presence in β-structures with dense backbone hydrogen bonds creates a rigid, highly crosslinked interface (31).

One wonders how nature ensures that exactly those cysteine residues that will later be needed to be unpaired in the Golgi are not becoming disulfide bridged during the process of folding and dimerisation. The restriction of PDIA1 binding to the CK-domains seems to answer the latter question. Furthermore, we recently showed that absence of certain cysteine residues seems to compromise the selectivity of PDI binding to the CK domain, leading to misfolding of the protein and the formation of VWF-mutant-PDI clusters in the ER (36).

> These data indicate that PDI binding is only then restricted to the CK domain when VWF is folded correctly.

How modification of Cys2771 and Cys2773 by ERp57 is prevented and preference is given to PDI to exclusively bind to these two cysteine residues is an open question and subject of ongoing investigation.

Concerning the cysteine residues that need to be available for multimerisation the group of Sadler found an astounding mechanism that protects them from involvement in folding mediating bonds in the ER and thus prepares them for later multimerisation in the Golgi (32, 37) (see below).

Golgi apparatus

Modification of N-linked saccharides and O-glycosylation

Upon entering the Golgi, VWF undergoes massive glycosylation until almost 20% of its final mass result from N- and O-linked oligosaccharide chains (38).

In the cis or medial cisternae of the Golgi apparatus mannosidases modify the N-linked saccharides that were attached in the ER, to the $Man_5GlcNAc_2$ form. In the medial cisternae, N-acetylglucosaminyltransferase and fucosyltransferase can add GlcNac and fucose to the saccharide structure. Production of „complex" glycans is finalized in the trans Golgi cisternae by addition of galactose and sialic acid residues by galactosyl- and sialyl-transferases, respectively (21).

In contrast to N-linked glycosylation, there is no consensus sequence directing O-glycosylation to reliably predict this post-translational modification. Since O-glycosylation occurs in the Golgi on a readily folded protein only amino acids with hydroxyl groups (-OH) exposed on the surface of the protein can be modified (39). The biosynthesis of all O-GalNAc glycans is always initiated by the transfer of N-acetylgalactosamine (GalNAc) (structure shown in ▶Fig. 4a) from UDP-GalNAc directly to a serine or threonine residue. The generated O-glycosidic bond is formed between the amino acid residue and the C1 atom of the sugar (▶Fig. 4a). This is the simplest O-glycan, called Tn antigen (boxed in ▶Fig. 4a), because it often is antigenic.

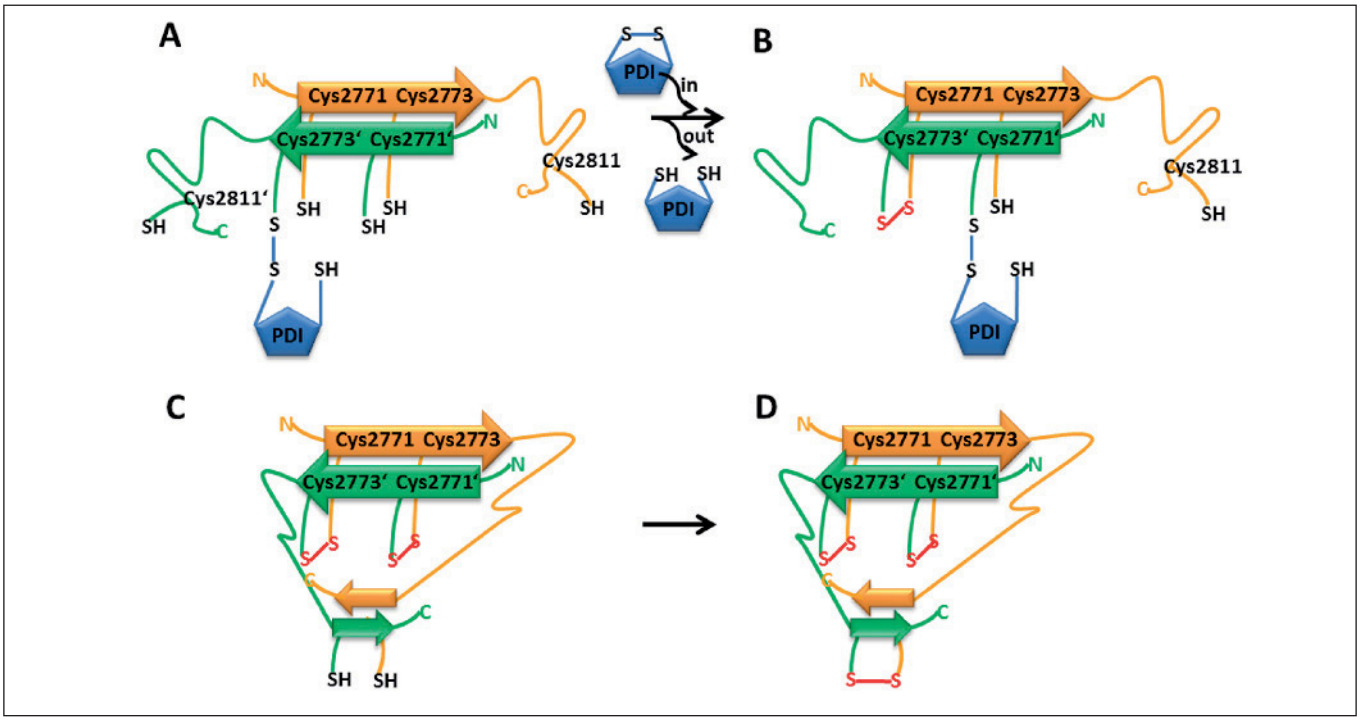

Fig. 3 Proposed mechanism of VWF dimerization (modif. from 31). (**A**) Oxidized PDI initially establishes a disulfide bond between its catalytic residue Cys36 and either Cys2771 or Cys2773 in the VWF monomer. (**B**) Then PDI catalyzes the formation of the first disulfide bond (either Cys2771–2773' or 2771'-2773). PDI is released in its reduced form (out) and a new oxidized PDI molecule (in) binds to (**C**) catalyze the formation of the second disulfide bond. Now the CK dimer is stabilized and the CK C-termini can assemble into a β-sheet conformation, which brings Cys2811 and Cys2811' together close enough to form the last disulfide bond Cys2811–2811' (**D**).

Addition of more sugars produces O-glycan core structures. The most common O-GalNAc glycan is Galβ1–3GalNAc that is named core 1 O-GalNAc glycan because it represents the core of many longer, more complex structures. It is also antigenic and therefore termed T antigen (structure shown in ▶Fig. 4a). The core 2 structure contains a branching N-acetylglucosamine attached to core 1 (▶Fig. 4b) (40). The N-linked glycosylation process is finalized by sialyltransferases that produce sialic acid caps. O-linked glycans can carry bi-sialic and tri-sialic acid structures.

Sialylation is a posttranslational modification that terminates oligosaccharide chains of glycoproteins and glycolipids. Sialic acids are the N- or O-substituted derivatives of neuraminic acid, a monosaccharide with a nine-carbon backbone. The most common member of this group is N-acetylneuraminic acid (Neu5Ac or NANA, boxed in ▶Fig. 4c) (41).

Detailed analysis of VWF's N-linked glycome revealed a vast variety of more than 300 different carbohydrate structures. Complex-type, mainly bi-antennary (2 branches), N-glycans dominated, but also traces of high-mannose and truncated structures were found. A remarkably high variety of tri- and tetra-antennary N-glycans were observed in smaller abundance and the presence of LacNAc (N-Acetyl-D-Lactosamine) extensions was detected (23, 42).

Using Edman degradation 10 putative O-glycosylation sites within VWF were suggested, 4 of which are located N-terminally of the A1 domain (T1248, T1255, T1256, S1263) and 6 C-terminally (T1468, T1477, S1486, T1487, T1679, and T2298) (black lollipops, ▶Fig. 1) (43). Studies on VWF O-glycosylation by Samor et al. (44, 45) and Canis et al. (46) revealed that 62–70 % of the O-linked sugars are represented by the disialylated T antigen (T Ag; NeuAc(α2–3)Gal-(α1–3)-[NeuAc(α2–6)] GalNAc; structure shown in ▶Fig. 4c), with one sialic acid residue, α2–3-linked to galactose, and the second one, α2–6-linked to N-acetylgalactosamine (GalNAc).

Further, disialosyl groups and core 2 O-glycans as well as blood group antigens were identified in a lower percentage.

Canis et al. (46) showed that all 10 putative O-glycosylation sites in VWF are indeed occupied and the composition of the structures was described in detail: The disialyl core 1 O-glycan was determined to be the major structure at all sites, whereas the core 2 O-glycans are enriched at the glycosylation sites C-terminally of the A1 domain. They further identified O-glycans that have not been described in previous reports, such as sulfated core 2 O-glycans and the Tn antigen.

Blood group determinants

In endothelial cells, in which VWF is synthesized, the fucosyltransferase FUT1 competes with sialyltransferase. The expression of FUT1 in endothelial cells explains the presence of blood group determinant on VWF because this enzyme adds fucose to the end of the chain creating the H-antigen of blood group O that is composed

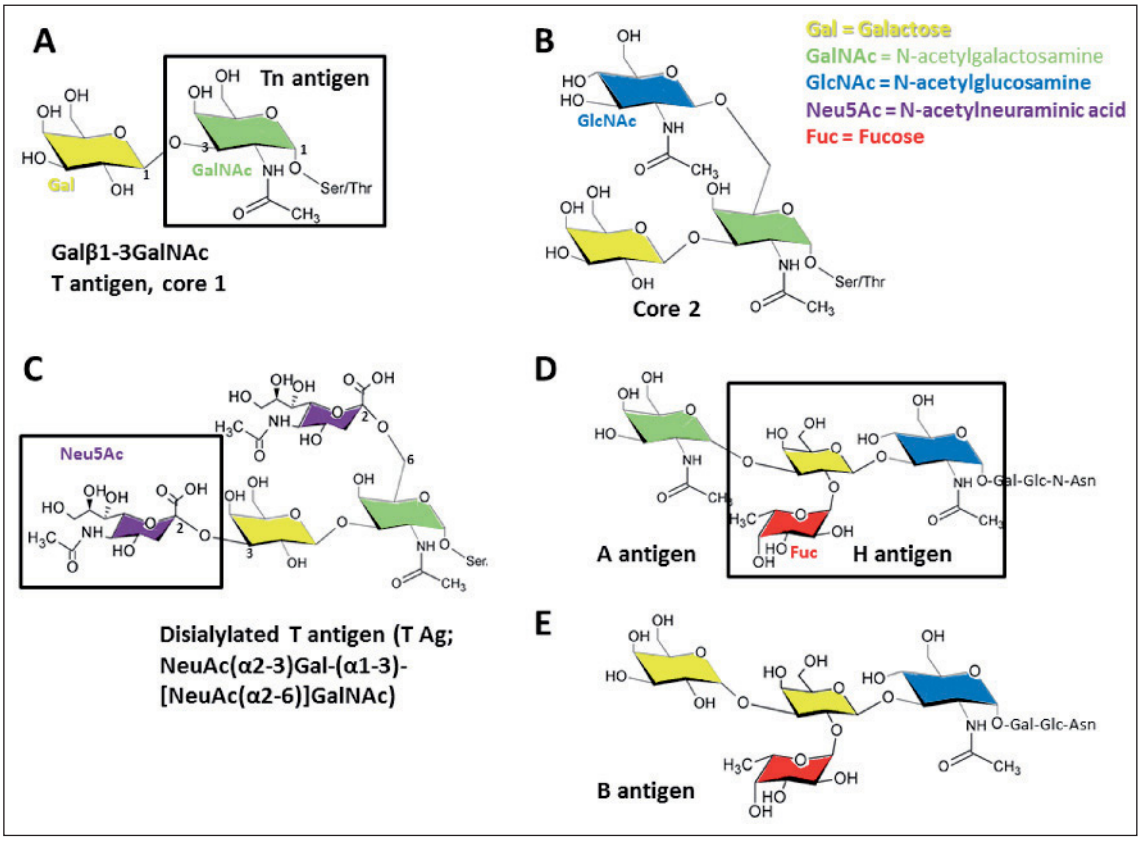

Fig. 4
Structures of indicated O-glycans and blood group antigens. Galactose is shown in yellow, N-acetylgalactosamine in green, N-acetylglucosamine in blue, N-acetylneuraminic acid in violet and fucose in red.

of N-acetylglucosamine–galactose–fucose (boxed in ▶Fig. 4d) coupled to a glucose-galactose disaccharide bound to an Asn residue. In individuals with blood group A or B the H-antigen can then further be modified by the A transferase (α1,3-N-acetylgalactosaminyltransferase) or the B transferase (α1,3-galactosyltransferase) that attach N-acetylgalactosamine (GalNAc) (=A antigen; ▶Fig. 4d) or galactose (=B antigen; ▶Fig. 4e), respectively (42).

The glycoproteomics data of Canis et al. showed that N-glycans substituted with blood group H antigen are present on all but one of the VWF N-glycosylation sites. The most heavily fucosylated site is Asn2635, on which N-glycans carry the H antigen on approximately 16% of its antennae (23). Blood group determinants have also been identified at O-glycosylation sites T1468, T1477, S1486, and T1487 (24).

Sulfation

As early as 1876 it has been known that biomolecules can contain sulfate groups (47) but it took about 80 years to gain first insights into the mechanism of sulfation by discovery of the active sulfate donor, 3-phosphoadenosine-5-phosphosulfate (PAPS) (48). The enzymes catalyzing the transfer of a sulfuryl group (-SO3) from PAPS to a variety of amine and hydroxy substrates are called sulfotransferases (STs) (49) (▶Fig. 5a).

During VWF processing in the Golgi, not the cytosolic STs, but the membrane-associated STs are responsible for VWF sulfation. Carew et al. (50) showed that N-linked sulfation occurs in the N-terminal part of VWF, at asparagine residues Asn1147 and Asn1515.

By employing a modern MS-based methodology, a pool of approximately 10 mono-sulfated N-glycans was identified by Canis et al. (23) showing that sulfation occurs at bi- and tri-antennary structures containing at least one fucose and one NeuAc residue. From these MS/MS data, they concluded that VWF sulfation occurs mainly on the sialylated LacNAc antennae.

> The importance of VWF antennae sulfation has still to be determined. It has been speculated that it might have implications in recognition by specific glycan binding proteins (23).

VWF multimerisation via interdimer disulfide bonds

VWF multimerisation is yet another disulfide bond formation. This time N-terminal interdimer disulfide bridges are formed. But the environment in the Golgi with an acidic pH of 6.2 and absence of oxidoreductases is detrimental to disulfide bond formation.

> VWF brings along its own oxidoreductase that is activated by acidic pH conditions.

The VWF propeptide contains two CXXC motifs which is the signature consensus sequence of active sites in the oxidoreductase family. Both motifs are composed of the amino acids Cys-Gly-Leu-Cys. The first is located in the D1 domain at positions 159–162 and the second in the D2 domain at 521–524 (51). Purvis et al. (37) showed

that a transient disulfide-linked complex between the propeptide and the D'D3 region of VWF is formed already in the ER. Oxidoreductases form such complexes in the first step of disulfide bond formation catalysis between one of the cysteine residues in the substrate protein and one cysteine in their own active site (as also shown for PDI in Figure 2).

The same group has reported later that Cys1099 and Cys1142 are the amino acids that form the multimerizing disulfide bridges between dimers (32). It is thus likely that the intermediate exhibits disulfide bonds between one or both CGLC sequences in the D1 and D2 domain with Cys1099 and Cys1142 in the D'D3 domain thereby protecting these cysteines from getting involved in intermonomer bonds during protein folding.

To ensure finalisation of this process after folding and dimerisation, the CGLC motifs are kept inactive in the ER by neighboring histidine residues and become activated exclusively after entry into the Golgi.

> Histidine is the only amino acid that possesses a pK_a value (6.0) close to the pH in the Golgi (6.2). Thus, no other amino acid can sense the difference in pH between the ER and Golgi.

This is due to the fact that 50 % of the acid molecules are dissociated (deprotonated) at $pH = pK_a$ and can thus act as a buffer by release or uptake of protons under changing pH conditions. Because the pH is the negative logarithm to base 10 of the proton concentration ($pH = -\log c(H^+)$) a rise in the pH to 7.4 reduces the H^+ concentration, prompting histidine to release protons. Ergo, only histidine is stronger protonated at 6.2 than at 7.4.

Dang et al. (52) showed that protonation of His395 and His460, both located N-terminally of the D2 CGLC sequence, are essential for the multimerisation process. Replacement of His395 with Lys or Arg (which are protonated to roughly the same extent at both pH 6.2 and 7.4) prevented multimer assembly, suggesting that reversible protonation of this His residue is essential. In contrast, replacement of His460 with Lys or Arg preserved normal multimer assembly, whereas amino acids without the possibility to carry a positive charge (Leu, Met, Gln) did not, indicating that the function of His460 depends primarily upon protonation.

These data suggest that His395 might be responsible for the activation of the multimerisation at low pH and His460 contributes to substrate coordination. Upon activation the propeptide then catalyzes formation of the Cys1099–1099' and Cys1249–1249' disulfide bridges producing an exponential size distribution (53) of VWF multimers that contain 2 to more than 40 dimers. In the trans-Golgi network (TGN), the protease furin cleaves the D1–D2 propeptide (54, 55) from the mature VWF but the two remain non-covalently bound until the release of VWF into the circulation (56). This association is crucial for the arrangement of VWF into tubules.

VWF tubule packaging in the TGN

VWF tubules have solely been detected in the TGN indicating this cisternae to be the origin of these helical VWF structures (57). Studies by the groups of Sadler and Cutler (58, 59) shed light on the mechanism of tubule assembly by showing that the VWF D1–D2 propeptide and D'D3 domain dimers are sufficient for tubule formation.

The core of the tubule is built by a right-handed helix containing ≈4.2 repeating units per turn. These units each consist of the D1-D2 propeptide positioned between the two D3 domains of one dimer (58). The association between these domains is already formed in the ER representing the

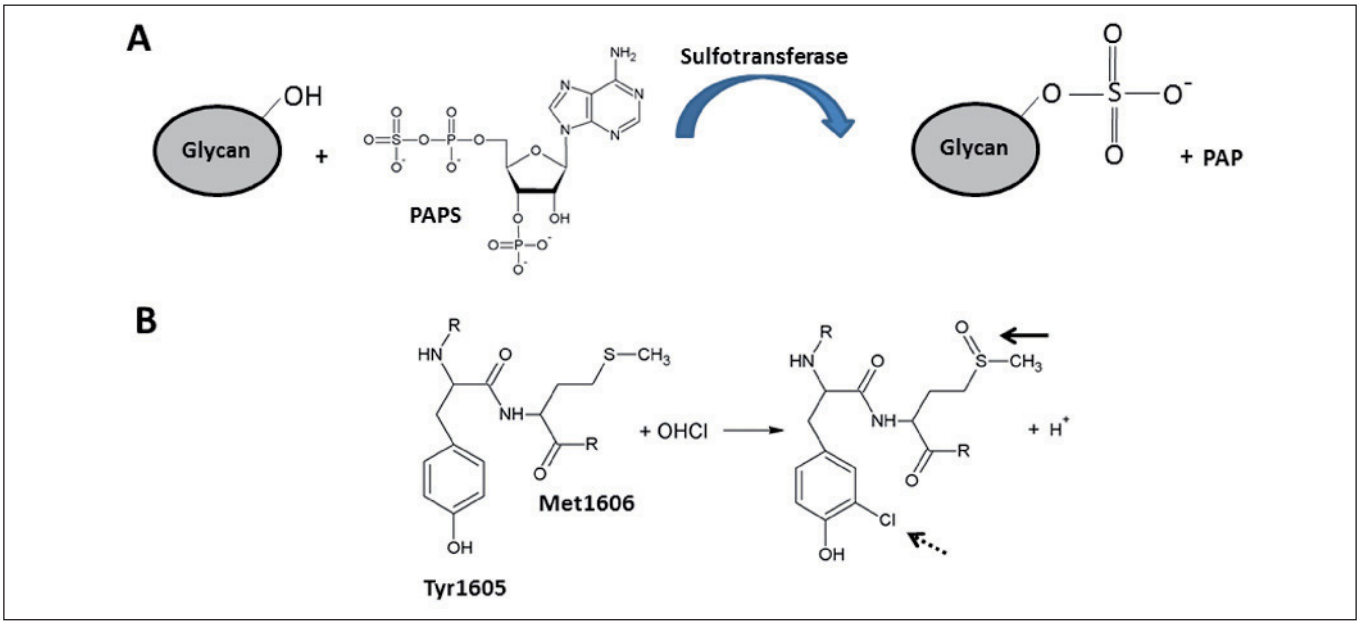

Fig. 5 Mechanisms of sulfation and oxidation. (**A**) Glycan structures are sulfated by sulfotransferases that transfer the sulfuryl group from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to one of their hydroxyl groups. (**B**) Amino acids can be oxidized. For VWF it has been shown that the ADAMTS13 cleavage site can be oxidized by OHCl at Met1606 to the sulfoxide. Weak conversion of Tyr1605 to chlorotyrosine was also detected.

above described transient disulfide-linked propeptide-D'D3-complex that prevents N-terminal intermonomer disulfide bonding in the ER (37). The low pH and high Ca^{2+} concentration (56, 59) in the Golgi increases the affinity of binding between D1-D2 and D'D3, which facilitates intersubunit disulfide bond formation by juxtaposing two D3 domains.

The growing multimers then organize into helical tubules in the TGN. Springer's group (60) showed that the C-terminal fragments within a dimeric subunit zip up into a dimeric bouquet at acidic pH forming a stem. These stems presumably decorate the outside of a tubule and comprise the matrix that later surrounds the denser tubules within Weibel–Palade bodies (WPB's) (58).

Zenner et al. (57) revealed stalk-like connections of the proximal ends of WPB's with the Golgi, suggesting that VWF tubules initiate WPB formation by pushing the Golgi membrane outward. Recently Eikenboom's group performed state of the art correlative light and electron microscopy as well as electron tomography to investigate WPB biogenesis in time providing more information on how immature WPB's recruit more VWF. The novel observation that clusters of non-tubulated VWF material are added to newly forming WPB's provided evidence for a novel mechanism of WPB formation and WPB growth (61). They hypothesize that WPB formation initially starts with the aggregation of VWF multimers in specialized Golgi areas that evolve into WPB's in which a suitable pH and calcium concentration is created to initiate tubule formation (58, 61).

The Golgi membrane, together with AP-1 and clathrin, initiates the formation of elongated WPB's by creating a matrix to guide parallel VWF tubule formation (61, 62).

Weibel palade bodies

By homotypic fusion small WPB's merge and become large WPB's (63, 64). The developing WPB remains multiply connected with the Golgi to allow further addition of VWF and other cargo proteins until WPB formation is completed. Maturation is accompanied by loss of the clathrin/AP-1 coat (62), aftiphilin, and γ-synergin, (65) as well as decrease of the pH to 5.4 (66) allowing condensation of mature WPB contents for compactness.

Additionally to VWF, WPB's contain a growing list of cargo proteins (reviewed in (67)). Release of the WPB cargo can be mediated by secretagogues, such as thrombin, histamine, epinephrine and vasopressin that induce exocytosis by different mechanisms (68):

- lingering-kiss exocytosis (69),
- single WPB exocytosis (70), and
- multigranular exocytosis leading to homotypical fusion of WPB's to secretory pods (reviewed in 71 and 72).

In circulation

Three different pathways of VWF secretion from endothelial cells have been described so far: Additionally to the above mentioned stimulated secretion, WPB's can further release VWF by basal, non-stimulated secretion (73).

> How much VWF, which was not stored in WPB's, is released via a third, constitutive pathway has been controversially discussed.

While an early study suggested that about 90% of VWF is secreted through the constitutive secretory pathway (74), it has later on been described that the percentage of VWF set free by this pathway is insignificant (75).

The Cutler group recently provided a detailed analysis of the relative amounts and multimeric states of VWF released through all three known secretory pathways. Since endothelial cells have two differentiated surfaces (apical, facing the vessel lumen and basolateral, facing the subendothelial extracellular matrix) they further included the polarity of VWF secretion to provide a comprehensive picture of VWF release (76). They found that constitutively secreted VWF is composed of mainly low molecular weight (LMW)-VWF multimers and targeted exclusively basolaterally. Continuous basal secretion from WPB's without stimuli, releases mostly ultra-large (UL)-VWF multimers together with a prominent dimer band. In line with previous studies they found this VWF accumulating in a 2:1 ratio apically:basolaterally (77, 78). Regulated, stimulated VWF release led to exclusively apically secretion of UL-VWF multimers (76).

Within the vessel hydrodynamic force induced by the blood flow leads to stretching of UL-VWF into haemostatically active strings (79) that fulfill VWF's well described role in primary haemostasis by recruiting platelets to the site of vascular injury. The flow induces secreted VWF to move from the site of exocytosis to the edge of the cell where VWF is anchored before forming strings (80, 81). The anchoring of VWF is supported by P-selectin and integrin αvβ3 (82, 83) but further proteins may be involved (81).

It has been shown that UL-VWF further forms rolling aggregates with platelets independent of activation and signalling at high shear rates of 10 000–20 000 s^{-1} (84). Above the threshold of 20 000 s^{-1} even platelets are no longer required for formation of aggregates formed by self-assembly of UL-VWF (85, 86).

To prevent vessel occlusion, VWF size regulation is provided by the activity of the protease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats) (87, 88). Several pathological conditions have been described that arise from the inability of ADAMTS13 to cleave UL-VWF:

- In thrombotic thrombocytopenic purpura (TTP) ADAMTS13 activity is highly reduced by autoimmune antibodies that inhibit its proteolytic activity (89).
- Mutations that reduce levels and/or activity of ADAMTS13 result in the hereditary form of TTP (90), also called the Upshaw-Schulman-Syndrome (91, 92).
- But also infectious diseases such as malaria (93, 94) and haemolytic uremic syndrome (HUS) (95) are associated with pathological accumulation of UL-VWF multimers that, furthermore, promote cancer-associated platelet aggregation in malignant melanoma (96).

Multimer biosynthesis is completed in WPB's but VWF processing does not end with its secretion. For example can high shear flow induce exposure of the above mentioned unpaired cysteines leading to covalent association via interchain disulfide bonds (97, 98). This lateral self-association was suggested to align multiple VWF A1 domains thereby increasing binding avidity and bond strength for platelet GPIb (98, 99) and collagen type III (98).

Within blood vessels, ADAMTS13 cleaves VWF upon shear-induced elongation of its A2 domain (mechanism reviewed by 100). Interestingly, the Tyr^{1605}-Met^{1606} peptide bond, which is the cleavage site for ADAMTS13, can be oxidized by hypochlorous acid (HOCl), a reactive oxygen species that is produced upon neutrophil activation under inflammatory conditions. The López group showed that Met^{1606} can indeed be oxidized to the sulfoxide, whereas only a small percentage of Tyr^{1605} is converted to chlorotyrosine (▶Fig. 5b). Cleavage by ADAMTS13 was strongly inhibited by this oxidation, indicating this modification can have a prothrombotic effect during inflammation (101).

Further modifications might also be involved in VWF clearance since it was shown that presence of sialic acids is crucial to the survival of the VWF protein in the circulation (102) indicating enhanced clearance upon desialylation or hyposialylation. These conditions can be the result of reduced activity of sialyltransferase ST3Gal-IV (103) or increased desialylation upon pathogen infection or due to aging and/or diet (1, 104, 105).

In platelets

Platelets contain VWF (106) stored in α-granules that accounts for about 25% of the total amount of VWF antigen (VWF:Ag) present in normal platelet-rich plasma (106–108). Platelet-VWF is already produced in megakaryocytes (109). If VWF biosynthesis in these platelet precursor cells differs from that in endothelial cells and if platelets are capable of VWF *de novo* biosynthesis remains incompletely understood. Platelet and endothelial VWF show the same binding affinity for type I collagen and O-linked sialic acid as well as H antigen expression are comparable.

But a number of distinct differences between platelet-VWF and VWF derived from endothelial cells have been described:

- Multimer gel analyses showed that platelet-VWF contains more UL-VWF multimers (110–112).
- McGrath et al. reported that the glycosylation profile of platelet-VWF differs significantly from that of plasma-VWF (113): The A and B antigens are not expressed on platelet-VWF. This finding is surprising because it has been shown that megakaryocytes and platelets do contain acetylgalactosaminyltransferases as well as galactosyltransferases (114) which are responsible for production of the A and B antigen, respectively. Furthermore, platelet-VWF exhibits a reduction of total sialic acid expression by about 55% which is mostly due to a specific reduction in sialic acid expression on the N-glycans of platelet-VWF (113).
- Williams et al. showed that compared to plasma-VWF, platelet-VWF exhibits a significantly increased binding affinity for GPIIb/IIIa, and a decreased affinity for GPIb (115).

If platelet-VWF is also able to bind FVIII is not yet entirely clear. Ectopical expression of FVIII in megakaryocytes leads to colocalisation of VWF and FVIII in α-granules (116–118). This finding suggests that platelet-VWF might be able to bind FVIII thereby targeting the factor to these storage organelles. But expression of FVIII in VWF-deficient platelets resulted in some storage in α-granules indicating that VWF is not absolutely essential for FVIII storage (119). More recent studies on a novel recombinantly expressed form of VWF (rVWF), which is similar to platelet-derived VWF due to its glycosylation pattern, described that this rVWF exhibits binding (120) and stabilisation (121, 122) of FVIII similar to plasma-VWF. Thus, further studies are necessary to finally determine if FVIII binding by plasma-derived VWF differs from that of platelet-VWF.

Conclusion

The biosynthesis of VWF multimers is a highly complex series of different processes which require flawless execution. It is thus prone to disturbances due to mutations in VWF that give rise to defects in processing, storage and secretion resulting in von Willebrand disease (VWD).

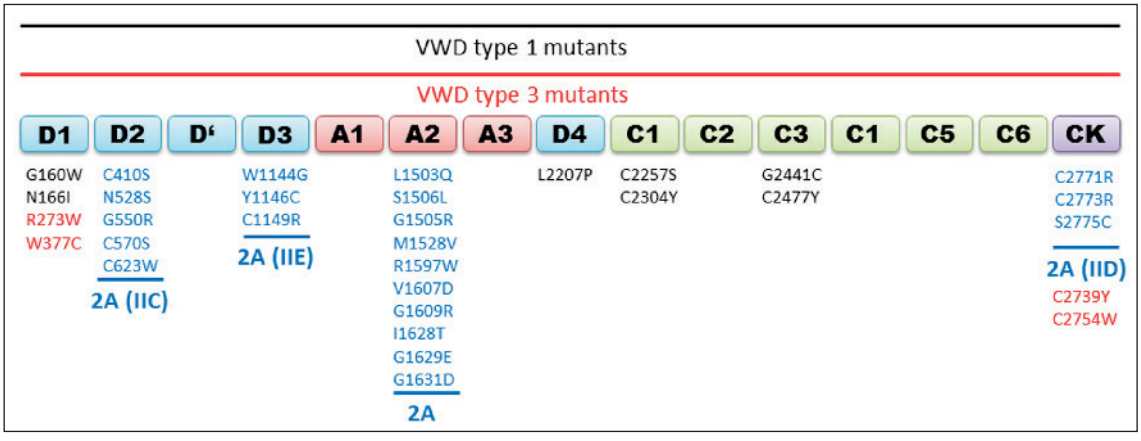

Fig. 6 Location of some exemplary von Willebrand factor (VWF) mutations which disturb VWF processing.

A vast variety of VWF mutations have been identified so far that disrupt one or more steps in the maturation of VWF multimers. A few examples are listed in ▶Fig. 6:

- Type 1 mutations, leading to reduced VWF:Ag levels, have been found across all domains of VWF.
- Intracellular retention due to disturbed processing seems to be a common mechanism for type 1 VWD pathogenicity and has been demonstrated for at least 7 missense mutations scattered across VWF domains (Figure 6, black) (123).
- Subtype 2A is the most common among type 2 VWD variants and is induced by missense mutations in specific VWF domains.

Depending on the mutated domain different single or combined mechanisms result in a defect in processing that leads to loss of high and sometimes intermediate molecular weight multimers:

a) CK domain (impaired dimerisation) (124–126),
b) D3 domain (impaired multimerisation and retention in WPB's) (124, 127),
c) D2 domain (impaired multimer assembly, ER retention) (124, 128–132),
d) domains A1 and A2 (enhanced susceptibility to cleavage by ADAMTS13 and/or intracellular retention) (133–135).

The molecular defects responsible for VWD type 3 cause a quantitative deficiency of VWF. Mutational defects include large gene deletions, transcriptional defects, frameshift mutations and generation of premature stop codons as well as missense mutations. These defects can be found across the complete VWF gene. However, for some missense mutations processing defects have been reported that lead to cellular retention of the mutant protein (136–139).

Summarizing, correct processing is key to VWF's function in primary haemostasis.

But the disruption of VWF structure can not only induce VWD but could also disturb other processes that involve VWF. The different domains of VWF contain binding sites for a variety of proteins (binding sites and proteins shown in ▶Fig. 1, functions reviewed in 140). Thus, a perfect tertiary structure of VWF multimers is essential for all domains to function properly. The D'D3 domains contain the binding sites for FVIII and P-selectin. Platelet glycoprotein GPIbα binds to the A1 domain which also contains binding sites for collagen types IV and VI and osteoprotegerin (OPG). The A1 domain was also shown to be the potential binding site for interaction with DNA from neutrophil extracellular traps (141). Before ADAMTS13 can cleave the shear elongated A2 domain, it binds shear-independently to the D4-C6 domains (100). An Arg-Gly-Asp (RGD) sequence that mediates binding to platelet integrin $\alpha_{IIb}\beta_3$ and integrin $\alpha_v\beta_3$ of endothelial cells is located in the C4 domain (141–146). The region encompassing D'D3-A3 has been shown to provide binding sites for leukocyte receptors involved in rolling (P-selectin glycoprotein ligand-1 [PSGL-1]) and stable (β2-integrins) adhesion (147). The A3 domain provides binding sites for collagen types I and III and thrombospondin-1 (TSP-1) which was suggested to control VWF multimer size by reducing the multimerizing disulfide bonds in the circulation (148). Domains D4-CK mediate interaction of insulin like growth factor binding protein 7 (IGFBP7) and VWF in WPB's (149). Thrombin binds in the C1-C6 domains (150). The CK domain binds PDI, that performs dimerisation, and binding of the CCN family 2/connective tissue growth factor (CCN2/CTGF) to the CK domain might be important for VWF's role in angiogenesis (151).

Conflict of interest

The author declares that there is no conflict of interest.

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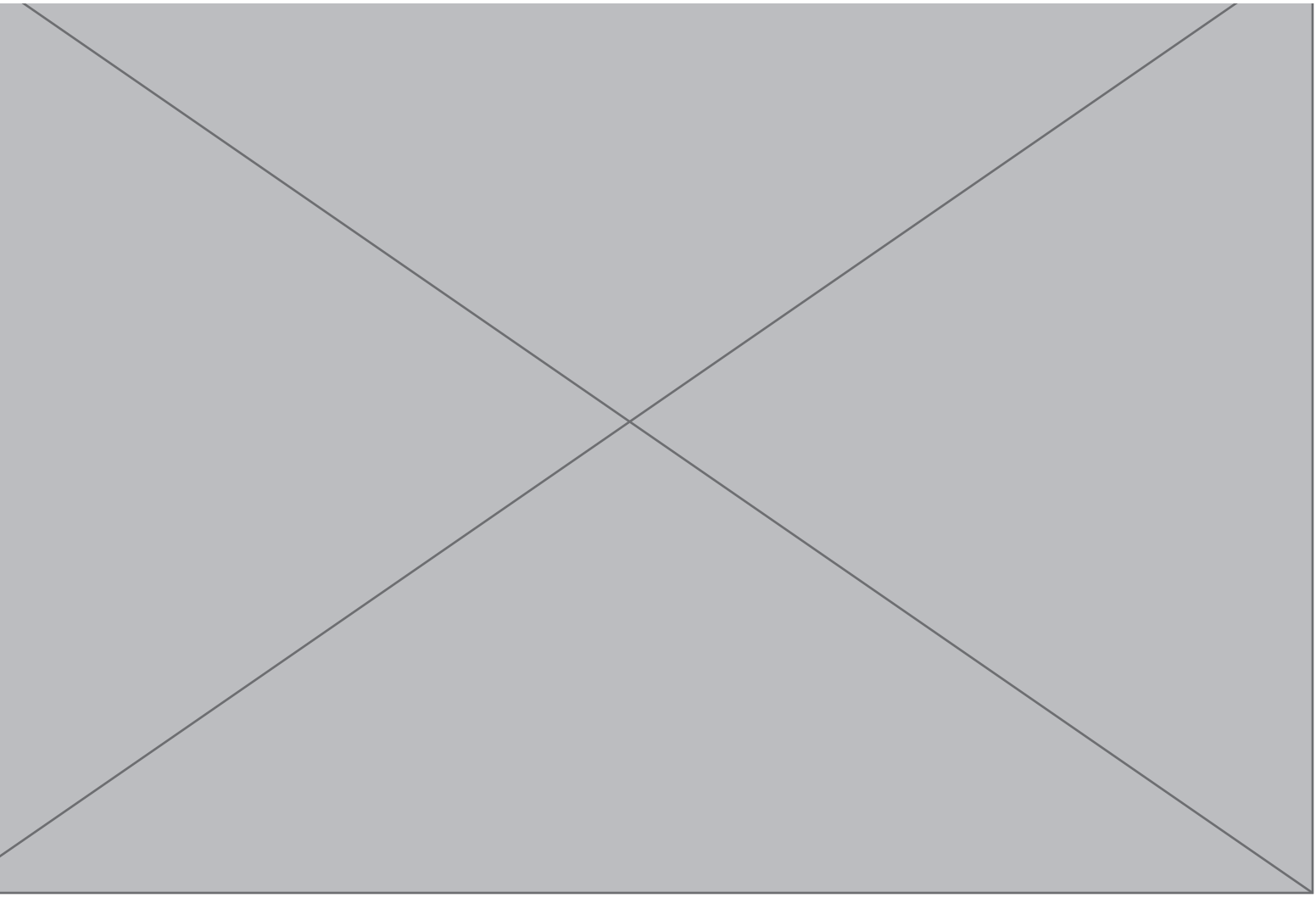

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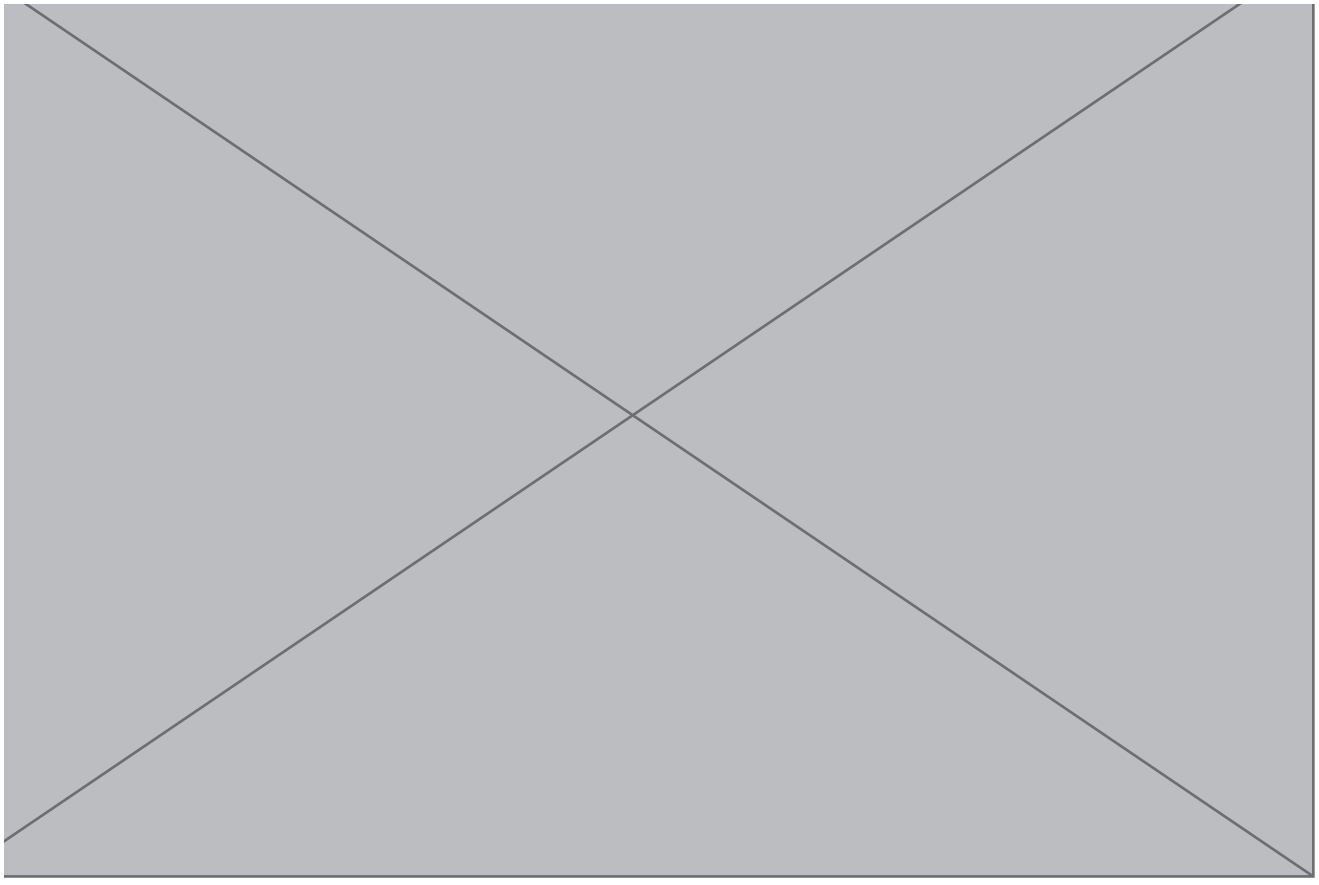

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