Biogenesis of Weibel-Palade bodies in von Willebrand's disease variants with impaired von Willebrand factor intrachain or interchain disulfide bond formation

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ABSTRACT

Background
Mutations of cysteine residues in von Willebrand factor are known to reduce the storage and secretion of this factor, thus leading to reduced antigen levels. However, one cysteine mutation, p.Cys2773Ser, has been found in patients with type 2A(IID) von Willebrand's disease who have normal plasma levels of von Willebrand factor. We hypothesize that disruption of either intra- or interchain disulfide bonds by cysteine mutations in von Willebrand factor has different effects on the biogenesis of Weibel-Palade bodies.

Design and Methods
The effect of specific cysteine mutations that either disrupt intrachain (p.Cys1130Phe and p.Cys2671Tyr) or interchain (p.Cys2773Ser) disulfide bonds on storage and secretion of von Willebrand factor was studied by transient transfection of human embryonic kidney cell line 293. Upon expression of von Willebrand factor these cells formed endothelial Weibel-Palade body-like organelles called pseudo-Weibel-Palade bodies. Storage of von Willebrand factor was analyzed with both confocal immunofluorescence and electron microscopy. Regulated secretion of von Willebrand factor was induced by phorbol 12-myristate 13-acetate.

Results
p.Cys1130Phe and p.Cys2671Tyr reduced the storage of von Willebrand factor into pseudo-Weibel-Palade bodies with notable retention of von Willebrand factor in the endoplasmic reticulum, whereas p.Cys2773Ser-von Willebrand factor was stored normally. As expected, wild-type von Willebrand factor formed proteinaceous tubules that were seen under electron microscopy as longitudinal striations in pseudo-Weibel-Palade bodies. p.Cys2773Ser caused severe defects in von Willebrand factor multimerization but the factor formed normal tubules. Furthermore, the basal and regulated secretion of von Willebrand factor was drastically impaired by p.Cys1130Phe and p.Cys2671Tyr, but not by p.Cys2773Ser.

Conclusions
We postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds do not affect either the storage in Weibel-Palade bodies or secretion of von Willebrand factor, whereas mutations of cysteines forming intrachain disulfide bonds lead to reduced von Willebrand factor storage and secretion because the von Willebrand factor is retained in the endoplasmic reticulum.

Key words: cysteine mutations, secretion, storage, von Willebrand factor, Weibel-Palade body, von Willebrand's disease.

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The online version of this article has a Supplementary Appendix.

Introduction

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that facilitates platelet adhesion to an injured vessel wall and carries coagulation factor VIII in the circulation.[1] Aberrations in the function or concentration of VWF may lead to the most common human inherited bleeding disorder von Willebrand's disease (VWD).

The main source of plasma VWF is the endothelial cell which stores VWF in a unique organelle called the Weibel-Palade body (WPB). VWF is released from WPB by basal and regulated mechanisms.[2,3] During its biosynthesis, VWF undergoes extensive post-translational modifications that include formation of intrachain and interchain disulfide bonds. The interchain disulfide bonds bridge the cysteine knot domains ("tail-to-tail") and D3 domains ("head-to-head") of VWF to form dimers and multimers of up to 80 monomeric subunits.[1,4,5] Such interchain disulfide bond formation has recently been shown to be facilitated by the low pH seen in the *trans* Golgi and WPB.[6,7] Through a process called tubulation, VWF is organized at the *trans* Golgi into proteinaceous tubules that are stored in the WPB. Under an electron microscope VWF tubules appear as striations (if viewed longitudinally) or as hollow rings (if viewed in cross-section).[4,5]

VWF contains numerous cysteines[8] and a large number of mutations of cysteines have been identified in VWD patients with either quantitative (VWD types 1 and 3) or qualitative (VWD type 2) defects of VWF (ISTH-SSC VWF mutation database *www.vwf.group.shef.ac.uk/*). The mechanisms by which specific cysteine mutations lead to the different clinical phenotypes of VWD are largely unknown. Based on the expression data of cysteine mutations in VWF, we and others have shown that intracellular retention of VWF is a common mechanism underlying VWD with quantitative deficiencies of VWF.[9-12] Recently we showed that impaired WPB formation and reduced regulated secretion of VWF contribute to this intracellular retention.[13] However, cysteines p.Cys2771 and p.Cys2773, which are involved in interchain-disulfide bond formation at the cysteine knot domain,[14] have been implicated in type 2A(IID) VWD in patients with normal plasma VWF antigen level[15-17] suggesting normal storage and secretion. We, therefore, hypothesized that disruption of interchain disulfide bonds may not hamper VWF-induced biogenesis of WPB and secretion into the circulation whereas disruption of intrachain disulfide bonds does.

To address this hypothesis, the intracellular storage and secretion of p.Cys2773Ser-VWF mutant was compared with that of two other VWF mutants, p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF, in which intrachain disulfide bonds are disrupted. We found that disruption of intrachain disulfide bonds in VWF by mutations p.Cys1130Phe and p.Cys2671Tyr impairs WPB biogenesis whereas disruption of an interchain disulfide bond by p.Cys2773Ser does not affect WPB formation. Our findings also confirmed that VWF tubulation and storage in WPB are not dependent on C-terminal dimerization.

Design and Methods

Patients and mutations

The mutations investigated in this study had all been identified earlier in VWD patients. p.Cys1130Phe (c.3389G>T) was identified in a heterozygous state in patients originally diagnosed with type 1 VWD with moderately severe bleeding tendencies.[18,19] The phenotype of patients carrying p.Cys1130Phe has more recently also been classified as 2A(IIE).[20,21] p.Cys2671Tyr (c.8012G>A) was described in a compound heterozygous type 3 VWD patient with a deletion of the second allele.[22] p.Cys2773Ser (c.8318G>C) was identified in heterozygous type 2A(IID) patients.[17]

Plasmid constructs

Recombinant pSVH expression plasmids containing full length cDNA encoding either the wild-type human VWF (WT-VWF) or the p.Cys2671Tyr and p.Cys2773Ser VWF variants have been described before.[9] The full-length VWF cDNA fragments, obtained by EcoRI restriction of these pSVH-VWF plasmids, were cloned into the pCI-neo mammalian expression vector (Promega, Madison, WI, USA). Mutation p.Cys1130Phe was introduced into pCI-neo WT-VWF plasmid with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and verified by sequencing.

Cell culture and transfection

Cells of the human embryonic kidney cell line 293 (HEK293) were purchased from the American Type Culture Collection (ATCC, Rockville, USA) and cultured in minimum essential medium Eagle alpha modification (MEM-α, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μg/mL gentamicin (Invitrogen, Carlsbad, CA, USA). Using the FuGENE HD transfection reagent (Roche Diagnostics, Mannheim, Germany), 0.66 μg/mL plasmid constructs (final concentration in the medium; 0.33 μg/mL WT-VWF and mutant VWF plasmids for co-transfections) were transiently transfected into HEK293 cells according to the manufacturer's instruction.

Immunofluorescent staining and antibodies

Transfected cells were stained with immunofluorescent antibodies and analyzed with a Leica SL confocal laser scanning microscope with a 63X/1.40 NA oil objective, as previously described.[13] Monoclonal antibody CLB-RAg35 and polyclonal rabbit anti-human protein disulfide isomerase (PDI) antibody A66 (obtained from Prof. I. Braakman, Department of Chemistry, Utrecht University, Utrecht, The Netherlands) were used as primary antibodies against VWF and the endoplasmic reticulum (ER) marker PDI, respectively.[23,24] Alexa 488- and Alexa 594-conjugated secondary antibodies were purchased from Invitrogen. To quantify the extent of retention of VWF in the ER, we determined the percentage of cells expressing VWF which also showed retention of VWF in the ER by co-staining the VWF with PDI.

Transmission electron microscopy

Transfected cells grown on 35-mm Petri dishes were fixed and prepared for transmission electron microscopy as previously described.[13,25] Briefly, cells treated with osmium tetroxide and tannic acid were serially dehydrated with ethanol and embedded in Epon. The samples cut into thin sections of 70-100 nm were stained with uranyl acetate and lead citrate, and then analyzed with a Tecnai 12 at 120 kV equipped with a 4kx4k CCD camera (Model Eagle, Fei Company, The Netherlands).

Structural analysis of von Willebrand factor

Seventy-two hours after transfection cells were incubated with release medium [OPTIMEM1 medium, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.2% bovine serum albumin, pH 7.4] for 24 h. The conditioned media were electrophoresed through 1.6% agarose gel containing 1% sodium

dodecyl sulfate for multimer analysis. VWF multimers were visualized by western blotting as described elsewhere.[9] Electrophoresis and blotting were performed under non-reducing conditions. To analyze the processing of VWF, reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blotting were performed for the conditioned media and cell lysates. Cell lysates were prepared with Passive Lysis Buffer (Promega) supplemented with 100 μM phenylmethylsulfonyl fluoride (Roche) and the protease inhibitor cocktail Complete™ with EDTA (Roche). Samples were reduced using 20 mM dithiothreitol, separated by Novex 6% Tris-Glycine gel electrophoresis (Invitrogen), immunostained with polyclonal rabbit anti-human VWF conjugated to horseradish peroxidase (DAKO) and visualized with Supersignal WestFemto (Thermo Scientific, Rockford, IL, USA).

Basal and regulated secretion of von Willebrand factor

In the absence of stimulation, VWF is mainly secreted via the basal secretory pathway from WPB.[3] HEK293 cells are known to form pseudo-WPB that are similar to the WBP in endothelial cells.[13] Basal and regulated secretion of VWF was determined as previously described.[13] Briefly, 24 h after transfection, cells were incubated with fresh culture medium for 48 h and then the conditioned media and cell lysates were collected to determine basal secretion of VWF. To analyze the regulated secretion of VWF, 72 h after transfection cells were incubated for 60 min with release medium containing 160 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) or vehicle (dimethylsulfoxide). VWF:Ag in the media and cell lysates was determined by an enzyme-linked immunosorbent assay. For the calculation of regulated secretion, secreted VWF was expressed as a fraction of total VWF (amount in the medium plus lysate). Statistical analyses were performed with Student's t-test using GraphPad Prism software (version 4.02).

Results

Retention of von Willebrand factor in the endoplasmic reticulum

As shown in Figure 1, WT-VWF formed numerous elongated pseudo-WPB organelles. Each of the VWF mutants was able to form pseudo-WPB although these organelles were relatively short or more granular compared with those formed by WT-VWF. As indicated by the co-localization of VWF and the ER marker PDI, both p.Cys1130Phe and p.Cys2671Tyr led to considerable retention of synthesized VWF in the ER. In contrast, most of the p.Cys2773Ser-VWF was stored in pseudo-WPB similar to those observed for WT-VWF. Quantification of VWF storage showed that 60-80% of cells expressing p.Cys1130Phe or p.Cys2671Tyr displayed retention of VWF in the ER, whereas this was less than 10% for WT-VWF or p.Cys2773Ser-VWF (Figure 1). The retention of p.Cys1130Phe-VWF in the ER was reduced by co-transfection with WT-VWF. In this case the proportion of cells showing ER retention of VWF decreased from 83% to 26%. There was no reduction in ER retention of p.Cys2671Tyr-VWF upon co-transfection with WT-VWF. The different efficiencies in storage of VWF variants were also reflected by PMA-induced pseudo-WPB exocytosis (see section on *Secretion of VWF*).

Analysis of the cell ultrastructure with transmission electron microscopy revealed pseudo-WPB containing VWF tubules in cells expressing WT-VWF as well as each of the mutants (Figure 2A-E). The tubules formed by the three VWF mutants appeared normal compared to those formed by WT-VWF (Figure 2A).[25] This indicates that all

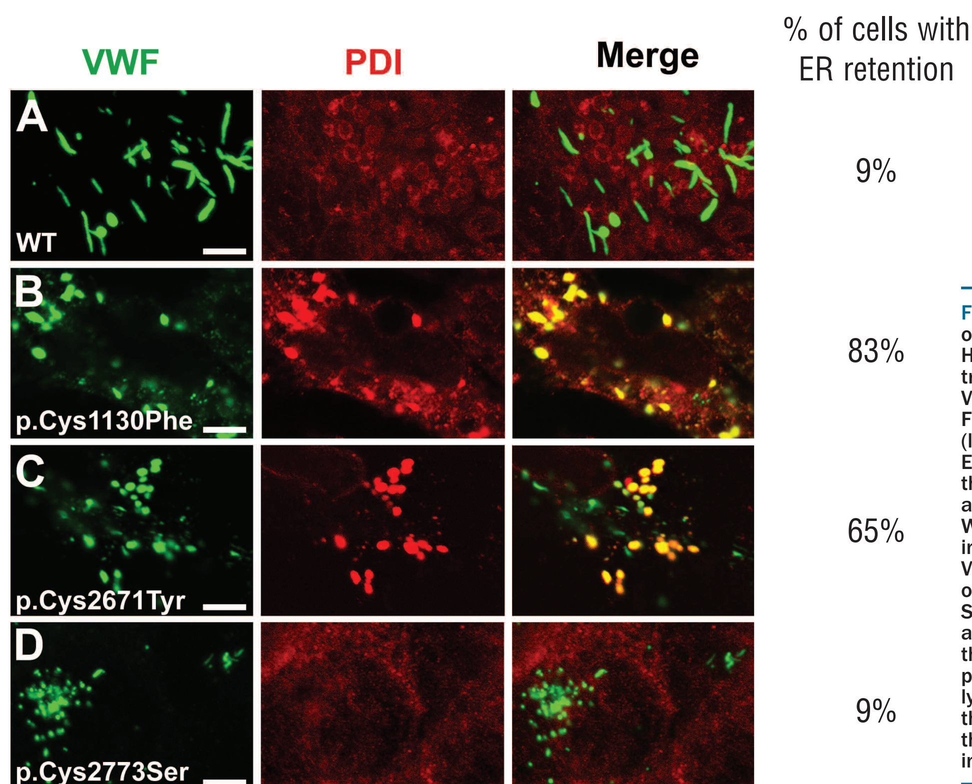

Figure 1. Intracellular localization of VWF variants in transfected HEK293 cells. HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. Fixed cells were stained for VWF (left panel, green) and for PDI (the ER marker, middle panel, red). In the right panel (merge of green and red channels), the pseudo-WPB show up in green (VWF staining only), and the ER containing VWF shows up in yellow as a result of double staining for VWF and PDI. Scale bar = 5 μm. Between 175 and 370 cells expressing each of the VWF variants from two independent experiments were analyzed and the percentage of cells that showed retention of VWF in the ER (cells displaying yellow) is indicated on the right.

three mutants were able to form pseudo-WPB although p.Cys1130Phe-VWF did so rarely. Furthermore, grossly dilated ER was frequently observed in cells expressing p.Cys1130Phe (Figure 2F) or p.Cys2671Tyr (Figure 2G). The ER morphology in cells expressing p.Cys2773Ser was normal (Figure 2H).

The retention of VWF mutants in the ER was further confirmed, using western blotting under reducing conditions, by evaluating the extent to which VWF was processed into its mature form. Secreted VWF consisted mainly of fully processed mature VWF subunits (Figure 3A). In the cell lysates, both mature subunits and uncleaved pro-VWF were detectable for all VWF variants (Figure 3B). However, p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF showed relatively much more uncleaved pro-VWF compared to WT-VWF and p.Cys2773Ser-VWF (Figure 3C), indicating that p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF were retained in the ER, and did not reach the Golgi apparatus where furin cleaves pro-VWF.[26-28]

Secretion of von Willebrand factor

WPB contributes to both the basal and regulated secretion of VWF.[3] To determine the effect of pseudo-WPB formation on the secretion of VWF mutants, both secretory pathways were examined. Basal secretion of VWF was defined as the secreted VWF:Ag level in the conditioned medium over 48 h (*Online Supplementary Table S1*). Compared to WT-VWF, both p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF showed significantly reduced basal secretion, which was apparent from a decrease in VWF level in the medium as well as from the decreased ratio of VWF:Ag in medium over lysate. In contrast, the basal secretion of the p.Cys2773Ser variant of VWF was normal, with a similar medium/lysate ratio as that of WT-VWF.

To mimic the heterozygous state of the mutations in carriers, cells were co-transfected with VWF mutants and WT-VWF at a 1:1 ratio. After this co-transfection with WT-VWF and mutant VWF, the basal secretion of VWF:Ag into medium was similar for all three mutants. However, the decreased ratio of VWF:Ag in medium over lysate observed for p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF indicates that the secretion of these VWF variants remained compromised in the heterozygous state (*Online Supplementary Table S1*). Furthermore, the absolute amount of VWF protein produced by WT-VWF or the three variants was similar (total VWF:Ag in the medium and lysates; about 100 mU VWF per 7×10^5 cells) for both single transfections and co-transfections,

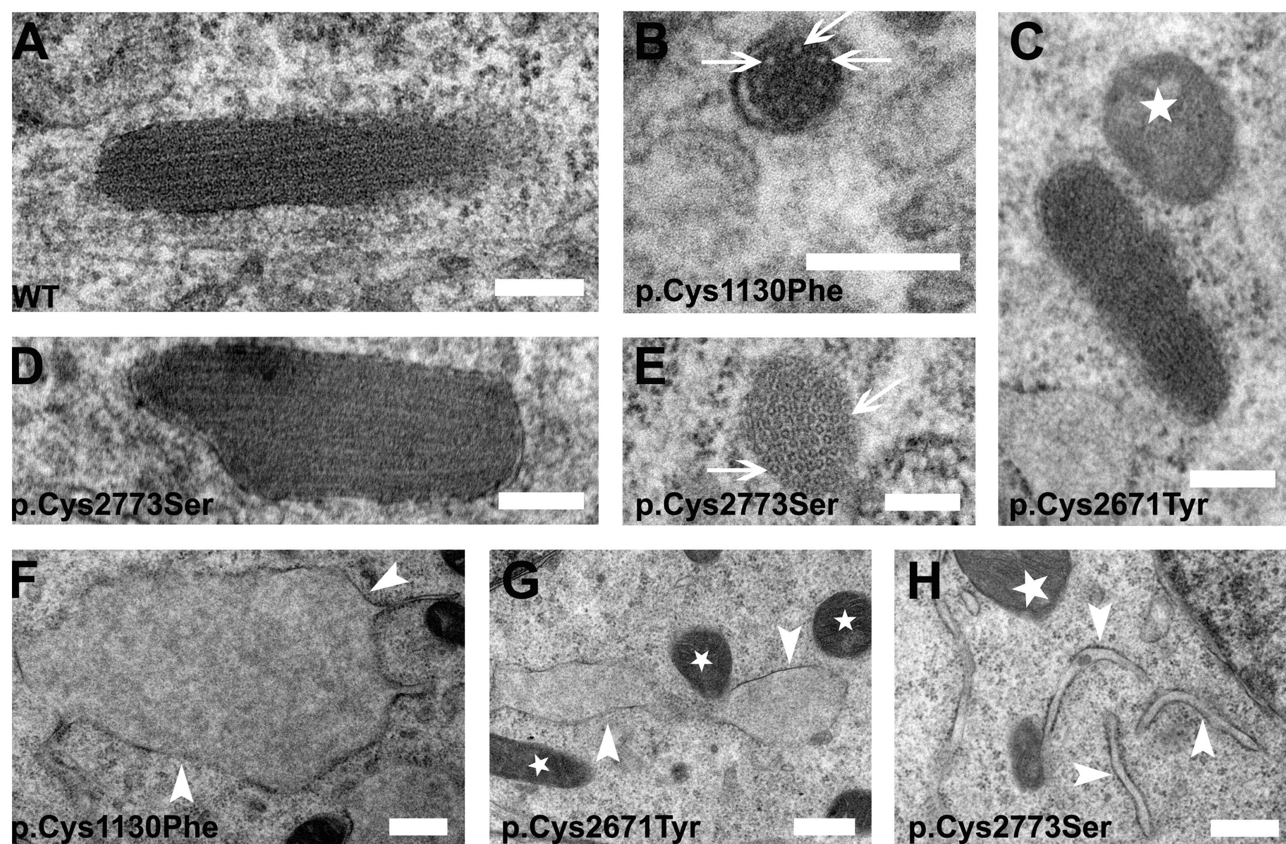

Figure 2. Ultrastructure of transfected HEK293 cells visualized by transmission electron microscopy. HEK293 cells were transiently transfected as indicated. Pseudo-WPB displaying VWF tubules were formed by WT-VWF (A) and the three mutants (B-E). Dilated ER was observed in cells expressing p.Cys1130Phe (F) or p.Cys2671Tyr (G) but not in cells expressing p.Cys2773Ser (H) or WT-VWF (*not shown*). Note that the VWF tubules were clearly visualized in the cross sections of pseudo-WPB (indicated by the arrows in panels B and E). The ER (indicated by the arrowheads) shown in panels F, G and H was imaged in the same cells shown in panels B, C and D, respectively. The stars in panels C, G and H indicate mitochondria. In panel A-E, scale bar = 200 nm; in panels F-H, scale bar = 500 nm.

confirming that the effects on WPB formation and ER retention of VWF (Figure 1) could not be explained by the expression level of the mutants. The secretion and total amount (medium plus lysate) of p.Cys2773Ser-VWF was higher than that of WT-VWF, although the difference was not statistically significant (*Online Supplementary Table S1*; 126.5 mU in single transfections and 117.7 mU in co-transfections *versus* 97.9 mU for WT-VWF per 7×10^5 cells), consistent with the normal to high plasma levels of VWF in the patients.[17] Rapid clearance *in vivo* was shown to account for the more severe phenotypes of patients with p.Cys1130Phe-VWF and p.Cys2671Tyr-VWF.[29]

The regulated secretion of VWF was analyzed by using PMA, which efficiently induces exocytosis of pseudo-WPB in HEK293 cells.[13] Incubation of HEK293 cells for 60 min with PMA increased the secretion of WT-VWF by 2.7-fold (12.2% of total VWF was secreted by PMA-stimulated cells *versus* 4.5% by control cells, Figure 4). Both p.Cys1130Phe- and p.Cys2773Ser-transfected cells showed good response to PMA stimulation although the total secretion of p.Cys1130Phe-VWF was much lower. Only a minimal increase in the secretion of p.Cys2671Tyr-VWF was observed after stimulation. Co-transfections with p.Cys2773Ser or p.Cys1130Phe produced a regulated secretion of VWF comparable to that of WT-VWF if judged by the fold increases, but to a much lower absolute level in the case of p.Cys1130Phe. Co-transfection of p.Cys2671Tyr only slightly increased the secretion of VWF during stimulation. These *in vitro* data for p.Cys1130Phe (co-transfection mimicking the heterozygous state) and p.Cys2671Tyr (single transfection mimicking the compound heterozygous state) are consistent with the response to DDAVP infusion in the patients with these mutations.[29,30] One may speculate that patients with p.Cys2773Ser will show a normal response to DDAVP; however, no patient was available for direct testing.

Multimeric analysis of secreted von Willebrand factor

We performed multimer analysis of the conditioned media (Figure 5). A full range of VWF multimers was present in the conditioned medium for WT-VWF and p.Cys2671Tyr-VWF, while p.Cys1130Phe-VWF showed relative loss of large multimers indicating that mutations close to the interchain disulfide bonds may have a minor but significant effect on VWF multimerization. p.Cys2773Ser-VWF mainly formed monomers and dimers. The appearance of double bands for the dimers was caused by the different mobility between C-terminal dimers and N-terminal dimers formed by p.Cys2773Ser.[17] The multimeric defects caused by p.Cys1130Phe and p.Cys2773Ser were only partly restored by co-transfection with WT-VWF. This is consistent with the multimer patterns in the patients.[17,19,20] Furthermore, the odd-numbered VWF multimers produced by co-transfection of p.Cys2773Ser with WT-VWF confirmed co-expression of both constructs in individual cells.

Discussion

Chemical modification and mutagenesis studies have demonstrated that cysteines p.Cys2771 and p.Cys2773 are involved in interchain disulfide bond formation and are equally essential for VWF dimerization.[14] Natural mutations in the two cysteines lead to essentially the same

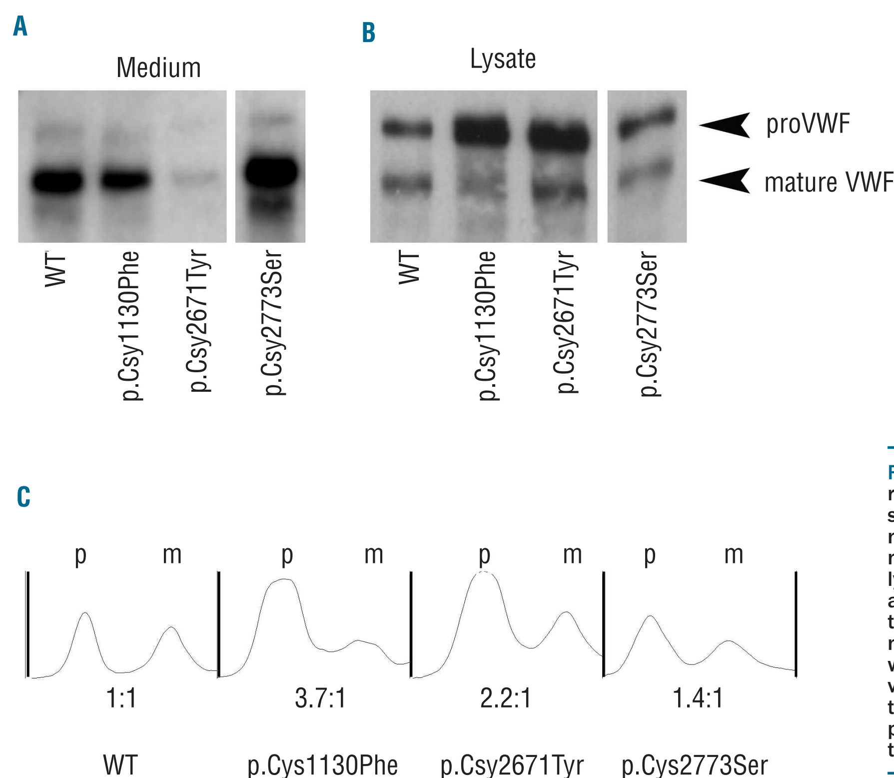

Figure 3. Subunit composition of VWF under reducing conditions. HEK293 cells were transiently transfected with WT-VWF or VWF mutants as indicated. VWF secreted into the medium (A: Medium) or VWF retained in lysate (B: Lysate) was reduced with DTT and analyzed with SDS-PAGE and western blotting. In panel C, the ratio of proVWF (p) and mature VWF subunit (m) in the cell lysate was analyzed with Image J (NIH software, version 1.44P). Note that in panels A and B the parts separated by the space between p.Cys2671Tyr and p.Cys2773Ser are from the same western blot.

clinical phenotype.[15-17] In addition, extensive studies have indicated that p.Cys1130 at the N-terminal and p.Cys2671 at the C-terminal are not involved in the interchain disulfide bonds but rather are involved in the intrachain disulfide bonds.[14,31,32] We, therefore, selected the three mutations p.Cys2773Ser, p.Cys1130Phe and p.Cys2671Tyr that were identified in our center[17,18,22] as exemplary mutants to examine the differential effects of interchain *versus* intrachain disulfide bonds on WPB biogenesis. p.Cys2773Ser-VWF was normally stored in and secreted from transfected HEK293 cells despite strongly reduced dimerization and multimerization. In contrast, p.Cys1130Phe and p.Cys2671Tyr resulted in strong retention of VWF in the ER and less VWF stored in pseudo-WPB, which is consistent with previous reports for other cysteine mutations that disrupt intrachain disulfide bonds in VWF.[13,33] Our data, therefore, suggest that VWF mutations that disrupt interchain disulfide bonds differ from mutations that disrupt intrachain disulfide bonds in the effect on WPB biogenesis.

The mutations that were analyzed in this study are located in different domains in VWF: p.Cys1130Phe in the D3 domain, p.Cys2671Tyr in the region immediately amino-terminal to the cysteine knot domain and p.Cys2773Ser in the cysteine knot domain itself. The different phenotypes of these mutations may be ascribed to the fact that they disrupt different domains of VWF but the differences in phenotypes may also be related to interchain *versus* intrachain defects. Several cysteine mutations, all involved in intrachain disulfide bonds, located in the D' (p.Cys788Arg), D3 (p.Cys1060Tyr, p.Cys1149Arg and p.Cys1225Gly) and cysteine knot (p.Cys2739Tyr and p.Cys2754Trp) domains showed very similar disruptive effects on the storage and secretion of VWF as p.Cys1130Phe and p.Cys2671Tyr.[13,33] This suggests that the effects are not domain-specific. Furthermore, p.Cys2739Tyr and p.Cys2754Trp in the cysteine knot domain are not stored properly, whereas p.Cys2773Ser in the same domain but involved in interchain disulfide bonds showed normal storage.[13] In addition, another mutation p.Cys1099Tyr in the D3 domain that disrupts interchain disulfide bonds between N-termini of VWF was identified in VWD type II C Miami patients characterized by normal plasma level of VWF.[31,34,35] Even stronger support for our hypothesis comes from the 2010 version of the ISTH-SSC VWF mutation database (*www.vwf.group.shef.ac.uk/*). It lists with 72 different mutations involving cysteine residues, 50 with loss of cysteines, 18 with gain of cysteines and 4 with premature stop codons (*Online Supplementary Table S2*). Among these mutations, 42 that predict the loss of intrachain disulfide cysteines are associated with low plasma levels of VWF, whereas five (p.Cys1099Tyr, p.Cys2771Ser/Tyr and p.Cys2773Arg/Ser) that predict the loss of interchain disulfide cysteines are associated with normal to relatively high plasma levels of VWF.[14-17,31,35] We, therefore, believe that the differences are related to differential effects of mutations involved in intrachain *versus* interchain disulfide bonds.

We propose a model to explain the differential effects of intrachain *versus* interchain disulfide bonds on WPB formation (Figure 6). Disruption of intrachain disulfide bonds in VWF may mainly disturb the conformation of VWF such that the ER quality control system prevents trafficking to the Golgi apparatus.[36] The retained VWF molecules either aggregate in the ER to lead to ER dilation (Figure 2F-G)[13,33] or undergo intracellular degradation.[13,37] Some mutant VWF molecules appear to escape the quality control system and are able to reach the Golgi apparatus. The extent to which this escape takes place appears to depend

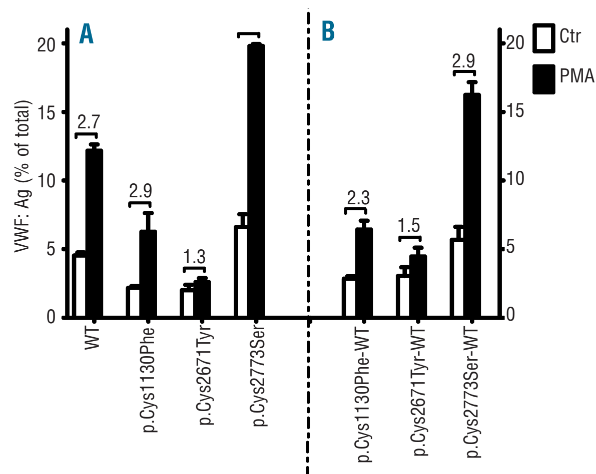

Figure 4. Regulated secretion of WT-VWF or VWF variants. HEK293 cells were transiently transfected with WT-VWF, p.Cys1130Phe, p.Cys2671Tyr and p.Cys2773Ser, respectively (Panel A). In panel B, p.Cys1130Phe, p.Cys2671Tyr or p.Cys2773Ser was co-transfected with WT-VWF at a 1:1 ratio. Seventy-two hours after transfection HEK293 cells were rinsed twice with the release medium and incubated at 37°C for 60 min in the release medium without (Ctr) or with 160 nM PMA (PMA). Each bar represents VWF secreted into the release medium as a fraction of total VWF (medium plus lysate) times 100%. The mean and SEM are based on three independent experiments in duplicate. The numbers above the bars indicate the fold increase of secreted VWF comparing the stimulated (PMA) and control (Ctr) samples.

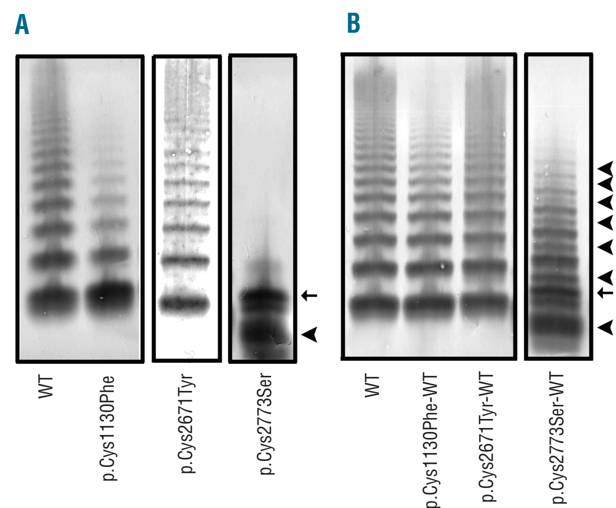

Figure 5. Multimer analysis of secreted VWF. Multimers are shown for single transfections (A) and co-transfections with WT-VWF at a 1:1 ratio (B). The N-terminal dimers and odd-numbered multimers formed by VWF p.Cys2773Ser are indicated by the arrow and arrowheads, respectively. In panel A, the multimers of VWF p.Cys2671Tyr are from a different gel, the other separated parts are from the same gel. In panel B, the separated parts are from the same gel. The multimer patterns of secreted VWF were analyzed by SDS-agarose gel electrophoresis and western blotting under non-reducing conditions.

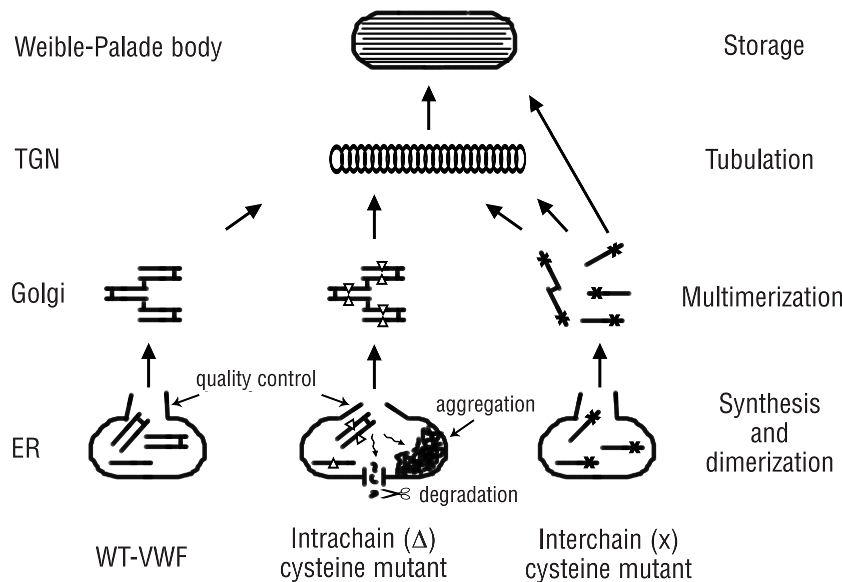

Figure 6. Schematic illustration of VWF intracellular trafficking. WT-VWF (left) is synthesized and dimerized in the ER, and passes the quality control in the Golgi apparatus to multimerize and tubulize therein, and is eventually packed into WPB. Some of the VWF mutants with impaired intrachain disulfide bond formation (middle) escape the quality control and are stored in WPB, the others are retained in the ER undergoing aggregation or intracellular degradation. VWF mutants with impaired C-terminal interchain disulfide bond formation (right) fail to form C-terminal dimers in the ER. The monomers can get through the ER to the Golgi and partly form N-terminal dimers which are tubulized and stored into WPB. The remaining monomers are presumably stored into WPB; however, whether they are incorporated into VWF tubules is unknown. This hypothesis may also apply to the mutants lacking N-terminal interchain disulfide bonds. TGN: *trans* Golgi network; ER: endoplasmic reticulum.

on the nature of the defects. Once the mutants get to the *trans* Golgi, where further processes of VWF are highly regulated by pH,[6,7,38] their effects are minimal unless the defects cause severe changes in VWF conformation. In contrast, disruption of interchain disulfide bonds such as that in the VWF C-termini does not hamper trafficking to the Golgi apparatus and WPB thereafter. C-terminal dimerization of VWF is not, therefore, required for WPB formation. According to the notion that N-terminal dimerization (i.e. multimerization) of VWF is not required for WPB formation,[39] the mutations that disrupt interchain disulfides in VWF N-terminal may not disrupt the biogenesis of WPB as well. It is thus tempting to speculate that unpairing all the five interchain disulfide bonds (two at the N-terminal and three at the C-terminal) should have minimal, if any, effect on the storage and secretion of VWF.

Tubulation is a key step in the storage of VWF into WPB. Recent studies provide emerging evidence in support of a hypothesis that VWF tubulation is regulated by the low pH in *trans* Golgi and WPB.[7,38,40,41] In a cell-free system with low pH as in *trans* Golgi, Huang and colleagues showed that truncated N-terminal dimers of VWF D'D3 domains together with VWF propeptide formed tubular structures by self-assembly.[38] The present data confirmed this finding for full-length VWF in a cell system. The VWF p.Cys2773Ser that forms N-terminal dimers[17] and partly remains as monomers showed normal tubulation (Figure 2E). The persistence of monomers raises two questions: (i) whether (and how) the monomers are stored into pseudo-WPB; and (ii) are these monomers able to form VWF tubules? As VWF is mainly secreted from WPB via basal and regulated pathways, the large amount of monomers that was detected in the medium of cells expressing p.Cys2773Ser (Figure 5A) suggests that VWF monomers are probably stored in the pseudo-WPB. Furthermore, immunofluorescent staining showed that very little VWF p.Cys2773Ser was localized in the ER (Figure 1D), indicating that the excessive monomers found in the lysate (*data not shown*) are indeed stored in the pseudo-WPB. It is unknown whether those monomers are able to form tubules in the *trans* Golgi network as it has been suggested that N-terminal dimerization is required for the tubulation of VWF in a cell-free system.[38] Strikingly, Zhou and colleagues observed a dimeric bouquet-like VWF complex formed at the acidic pH of the *trans* Golgi and WPB.[7] The C-terminal dimer of VWF was zipped up by the acidic pH from the cysteine knot to B1 domain (six domains) into a dimeric stem. This process was proposed to facilitate VWF tubulation.[7] As such, unpairing the interchain disulfide bonds at the cysteine knot domain, for example, by mutation p.Cys2773Ser, would unlock the start point of the zip. It is unknown whether the stability and/or conformation of the dimeric complex would be affected. As formation of the dimeric complex is highly pH-dependent, the p.Cys2773Ser monomers are likely to form the bouquet-like complex and, therefore, to incorporate into VWF tubules. In contrast, VWF mutations p.Cys2739Tyr and p.Cys2754Trp which cause severe conformational changes by defects in intrachain disulfide bonds led to disorganized tubular storage of VWF.[13] This suggests that the conformational changes in VWF may be more disruptive to VWF tubulation than no interchain pairing.

In conclusion, we postulate that natural mutations of cysteines involved in the formation of interchain disulfide bonds, p.Cys2771, p.Cys2773 and p.Cys2811 in the cysteine knot domain and p.Cys1099 and p.Cys1142 in the D3 domain, do not affect the storage and secretion of VWF, whereas mutations of cysteines forming intrachain disulfide bonds lead to reduced VWF storage and secretion due to ER retention.

Authorship and Disclosures

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