Type 1 von Willebrand disease mutation Cys1149Arg causes intracellular retention and degradation of heterodimers: a possible general mechanism for dominant mutations of oligomeric proteins

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Some families affected by von Willebrand disease type 1 show high penetrance with exceptionally low von Willebrand factor (VWF) levels. Previously, a mutation associated with this dominant phenotype, Cys1149Arg, was found to decrease the secretion of coexpressed normal VWF, and the mutation was proposed to cause intracellular retention of pro-VWF heterodimers. To demonstrate heterodimer formation, a model was developed in which subunits could be distinguished immunologically and by size. Recombinant VWF lacking domain A1 (dA1), A3 (dA3), or both (dA13) was secreted efficiently as a full range of multimers. Cotransfection of Cys1149Arg and dA13 resulted in the secretion of multimeric VWF containing about 250 kd (Cys1149Arg) and about 210 kd (dA13). Cell lysates contained pro-VWF forms of Cys1149Arg and dA13. Immunoprecipitation with an antidomain A1 antibody recovered both subunits in heterodimers, and subunit ratios were consistent with random dimerization. Similar results were obtained for cotransfection of Cys1149Arg and dA1. Normal VWF has a Cys1149-Cys1169 intrachain bond. When cotransfected with normal VWF, Cys1149Arg or the double mutant Cys1149Arg+Cys1169Ser caused a similar decrease in VWF secretion, suggesting that an unpaired Cys1169 does not explain the intracellular retention of Cys1149Arg. VWF Cys1149Arg was not secreted from BHK cells but was degraded intracellularly within about 4 hours, and the proteasome inhibitor lactacystin delayed its clearance more than 16 hours. Thus, dominant von Willebrand disease type 1 may be caused by heterodimerization of mutant and normal subunits in the endoplasmic reticulum followed by proteasomal degradation in the cytoplasm. A similar dominant negative mechanism could cause quantitative deficiencies of other multisubunit proteins. (Blood. 2001;98:2973-2979)

Introduction

von Willebrand disease (VWD) is a bleeding disorder with an estimated prevalence of about 100 per million, similar to or higher than that of hemophilia A.[1] Most patients with VWD have "type 1" disease,[2] which is characterized by a decreased level of structurally and functionally normal von Willebrand factor (VWF) and usually exhibits autosomal dominant inheritance. Type 1 VWD can be divided into 2 subgroups. The more common form shows variable penetrance and variable severity even within the same family, whereas a less common subset of patients has high penetrance with a clearly dominant inheritance pattern and exceptionally low VWF levels. The VWF levels in these families may be less than 15% of normal, which is much lower than expected for heterozygous VWF deficiency and suggests that their mutant VWF subunits inhibit the incorporation of normal subunits into secreted multimers.

Heterozygous mutations frequently impair the function of oligomeric proteins, and the complex biosynthesis of VWF offers several targets for such an effect.[2,3] In the endoplasmic reticulum (ER), pro-VWF subunits form disulfide-linked dimers through C-terminal "cystine knot" motifs. The pro-VWF dimers are transported to the Golgi apparatus, where the propeptides are removed and additional disulfide bonds are formed between D3 domains near the N-termini of the mature subunits. The product is a gigantic multimeric protein, often containing more than 40 subunits and exceeding 10 000 kd in mass. Mutant subunits can impair the progress of normal VWF subunits through this pathway by interfering with any of several steps. For example, many mutations have been identified that interfere with multimer assembly or stability, causing the loss of large multimers that characterizes VWD type 2A.[4,5]

Despite the relatively high prevalence of type 1 VWD, the genetic cause remains elusive in most families. The few mutations known to cause dominant VWD type 1 are within the D3 domain, near the site of the N-terminal intersubunit disulfide bonds. One family from the Netherlands had 3 affected members with VWF levels of 10% to 15% of normal, associated with the mutation Cys1149Arg (numbered from the initiation codon, or Cys386Arg if numbered from the N-terminus of the mature subunit).[6] Two unrelated Italian patients with a similar phenotype had the mutation Cys1130Phe,[6] and another patient from Hungary had a mutation at the same residue, Cys1130Tyr.[7] Families from Italy, Germany,[8] and Hungary[7] have been identified with the mutation Arg1205His as the cause of VWD "Vicenza."[9] The phenotype has been described as type 1 or type 2M because the multimers are normal or supranormal in size but their function in vitro is variably normal or

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decreased. The apparent clustering of these mutations within the D3 domain suggests they may act through a shared mechanism.

We previously reported that the mutation-associated Cys1149Arg caused the intracellular retention of pro-VWF subunits in the ER of transfected cells and markedly reduced the secretion of mutant VWF. Furthermore, when the Cys1149Arg subunit was coexpressed with recombinant normal VWF, the level of normal VWF secretion was decreased.[6] Our working hypothesis was that the normal and mutant subunits formed heterodimers that, like homodimers of Cys1149Arg, were retained in the ER. Such a mechanism would explain the dominant negative effect of the Cys1149Arg mutation on VWF secretion. However, the single amino acid difference between the normal and mutant subunits prevented the direct demonstration of heterodimer formation.

We therefore developed a model system in which Cys1149Arg subunits could be distinguished from a variant of VWF that could act as a surrogate for normal subunits. The deletion of domains A1 (dA1) and A3 (dA3) proved to be compatible with efficient synthesis and secretion of highly multimerized VWF. The subunits bearing these deletions were easily resolved from full-length subunits by gel electrophoresis and were not recognized by monoclonal antibodies against the A1 domain. The results obtained substantiate several features of the proposed model for how dominant negative VWD mutations can cause a highly penetrant and relatively severe form of VWD type 1.

Materials and methods

Plasmid construction

The sequence of all constructs was verified by dideoxy sequencing (Sequenase Kit, USB, Cleveland, OH). Plasmids pSVHVWF1, pSVHVWF1.1, and pSVHCys1149Arg were described previously.[6,10] Plasmid pSVHCys1169Ser was constructed[6] using polymerase chain reaction (PCR)–based mutagenesis to alter the TGT codon for Cys1169 to TCT, encoding Ser1169. Plasmid pSVHCys1149ArgCys1169Ser was constructed by the same method using pSVHCys1149Arg as the template.

The disulfide loop of the VWF A1 domain was deleted as follows. Plasmid pGEM-4ZNK[11] was used as the template for 2 PCR reactions. The first reaction employed primers T7 (TCT AAT ACG ACT CAG TAT AGG GAG AC) and C1353a (CTG AGG CGC CGT AGA AAT CGT GCA ACG GCG GTT C; *Nar*I site underlined), and the product was digested with *Hind*III and *Nar*I. The second reaction employed primers C1458 (ATG CGG CGC CGA CCT TGC CCC TGA AGC CCC TCC T; *Nar*I site underlined) and 227a (CCG ATC CTT CCA GGA CGA ACG CCA), and the product was digested with *Nar*I and *Nco*I. The purified fragments were ligated with the 2.7 kilobase (kb) *Hind*III-*Nco*I fragment of pGEM-4ZNK. In the product pGEM-4ZNKdA1, the hexanucleotide *Nar*I site, GGCGCC, replaced the 561 base pairs that encode Cys1353-Cys1458 of the mature VWF subunit. The *Ngo*MI-*Kpn*I fragment of pSVHVWF1.1 was replaced with the mutant *Ngo*MI-*Kpn*I fragment of pGEM-4ZNKdA1, yielding the plasmid pSVHVWFdA1.

The disulfide loop of the VWF dA3 was deleted in a similar fashion. The 1924–base pair *Kpn*I-*Sac*I fragment of pSVHVWF1 was cloned into pGEM-4Z (Promega, Madison, WI) to yield plasmid pGEM-4ZKS. Two PCR reactions were designed such that an *Ngo*MI site (GCCGGC) replaced the sequence encoding Cys1686-Cys1872. The product fragment was cloned into pGEM-4ZKSG to generate plasmid pGEM-4ZKSdA3. The mutated *Kpn*I-*Sac*I fragment of pGEM-4ZKSdA3 was ligated with the 7.9 kb *Eco*RV-*Kpn*I fragment and the 1.6 kb *Sac*I-*Eco*RV fragment of pSVHVWF1.1 to generate plasmid pSVHVWFdA3.

Deletion of both disulfide loops of dA1 and dA3 was accomplished by a 3-fragment ligation of the *Kpn*I-*Sac*I fragment of pGEM-4ZKSdA3, the 7.4 kb *Eco*RV-*Kpn*I, and 1.6 kb *Sac*I-*Eco*RV fragments of pSVHVWFdA1 to generate the plasmid pSVHVWFdA13.

Expression of recombinant VWF

293T human kidney cells[12] or COS-7 monkey kidney cells were transiently transfected in 6-well plates with pSVHVWF1, pSVHCys1149Arg, pSVHCys1149ArgCys1169Ser, pSVHVWFdA1, or pSVHVWFdA13 using a calcium phosphate method[13] and culture conditions described previously.[14] Cells were washed in phosphate-buffered saline 24 hours after transfection and grown an additional 48 hours in 1 mL serum-free medium (Opti-MEM I, Life Technologies, Gaithersburg, MD). Conditioned media and cell lysates (0.5 mL) were collected as described.[4] The lysis buffer contained 0.6% Triton X-100, 10 μg/mL aprotinin (Sigma, St Louis, MO), and 100 μg/mL phenylmethylsulfonyl fluoride (Sigma) in 0.1 M Tris-HCl, pH 8.0.

Stable expression of recombinant normal VWF and Cys1149Arg was achieved by cotransfecting Fur4BHK cells (a BHK cell line expressing recombinant human furin) with pSV2neo and either pSVHVWF1 or pSVHCys1149Arg. Cells were cultured in Opti-MEM I containing geneticin (500 μg/mL), and clones expressing normal VWF or Cys1149Arg were selected.

Protein gel electrophoresis and immunoassay

Analysis of recombinant VWF multimers was performed by sodium dodecyl sulfate (SDS)–agarose gel electrophoresis and Western blotting as previously described.[15] VWF multimers were visualized with rabbit polyclonal horseradish peroxidase–labeled antihuman VWF antibody P0226 (Dako, Denmark) and the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Gel electrophoresis of recombinant VWF was performed as described[16,17] on SDS–5% polyacrylamide gels (Bio-Rad, Hercules, CA). Proteins were transferred by electroblotting onto a polyvinylidene difluoride membrane (Bio-Rad), and VWF was detected as described for VWF multimers.

Recombinant VWF antigen concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) method[16] using rabbit polyclonal antihuman VWF antibody 082 and rabbit polyclonal horseradish peroxidase–labeled antihuman VWF antibody P226 (Dako).

Immunoprecipitation and analysis of recombinant VWF

293T cells were cotransfected with Cys1149Arg and either dA1 or dA13 (dA1 and dA3) as above. Recombinant VWF was immunoprecipitated[18] overnight at 4°C with 5.8 μg antibody 082 or 4 μg antibody B710 [19] and 7.5 μL (packed volume) protein A–Sepharose or protein G–Sepharose in 1 mL of 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 0.02% sodium azide, 1% Nonidet P-40, and 0.1% SDS. Immunoprecipitates were analyzed on SDS polyacrylamide gels as described above. The exposed films (Kodak XAR-5) were scanned, and bands were quantitated using NIH Image software version 1.61. Calculations were based on the following considerations, which use the example of cotransfection with Cys1149Arg and dA13.

Transfected cells may express Cys1149Arg and dA13 at different levels. The fractions of total VWF subunits represented by Cys1149Arg and dA13 are a and b, respectively, and $a + b = 1$. Polyclonal antibody 082 immunoprecipitates all pro-VWF dimers. After gel electrophoresis under reducing conditions and quantitation of bands corresponding to Cys1149Arg and dA13 subunits, the ratio Cys1149Arg/dA13 $_{082}$ is equal to a/b.

If pro-VWF subunits dimerize randomly in the ER, the proportions of the possible dimers are given by $(a + b)^2 = a^2 + 2ab + b^2$ where a^2 is the fraction of Cys1149Arg homodimer, $2ab$ is the fraction of dA13-Cys1149Arg heterodimer, and b^2 is the fraction of dA13 homodimer. Monoclonal antibody B710 is specific for dA1[19] and immunoprecipitates dimeric species that contain the Cys1149Arg subunit. The dimers recovered correspond to species $a^2 + 2ab$ (equation 1). Each a^2 species contains 2 Cys1149Arg subunits, and each $2ab$ species contains 1 Cys1149Arg and 1 dA13 subunit. Therefore, the number of Cys1149Arg subunits recovered is proportional to $2a^2 + 2ab$, and the number of dA13 subunits recovered is proportional to $2ab$. If dimerization is random, the expected ratio of Cys1149Arg/dA13 $_{B710}$ is Cys1149Arg/dA13 $_{B710} = (2a^2 + 2ab)/2ab = a/$

$b + 1$. A tendency to avoid heterodimerization would increase the ratio Cys1149Arg/dA13$_{B710}$, and a tendency to prefer heterodimerization would reduce the ratio Cys1149Arg/dA13$_{B710}$ toward the limiting value of unity.

Pulse-chase labeling

Fur4BHK cell lines expressing recombinant normal VWF or Cys1149Arg were cultured to 70% to 90% confluence in 6-well plates, washed with phosphate-buffered saline, and incubated in cysteine-free and methionine-free medium for 1 hour. The medium then was supplemented with 100 μCi (3.7×10^6 becquerel) of ^{35}S-labeled cysteine plus methionine (ICN Pharmaceuticals, Costa Mesa, CA) for 30 minutes, after which the cells were washed with phosphate-buffered saline and incubated in Opti-MEM I medium containing a 10-fold increased concentration of unlabeled cysteine and methionine. Conditioned media and cell lysates were prepared at the indicated times, and VWF was immunoprecipitated with antibody 082 as described[18] except that a preclearing step without antibody was added. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) as described above. Gels were fixed, treated with Amplify (Amersham, Cleveland, OH), dried, and exposed to Kodak XAR-5 film at −80° C. If lactacystin (10 μM) was used, it was present during the 1-hour preincubation with cysteine-free and methionine-free medium as well as the pulse labeling and chase periods.

Results

Deletion of the A1 or A3 domain does not interfere with VWF biosynthesis

Mutations in one VWF allele can have a dominant effect and determine the properties of multimeric VWF provided that mutant subunits interact with normal subunits at the stage of dimer formation in the ER or at the stage of multimer assembly in the Golgi apparatus. Such a dominant negative mechanism has been proposed for certain missense mutations that cause a severe and highly penetrant form of VWD type 1.[6] However, a physical interaction between such mutant and normal subunits has not been demonstrated directly.

This limitation was addressed by constructing versions of VWF that can be distinguished from full-length subunits based on their size and immunologic properties (Figure 1). In construct dA1, most of the VWF A1 domain was deleted by replacing amino acid residues Cys1353-Cys1458 with a Gly-Ala dipeptide. In construct dA3, most of the A3 domain was deleted by replacing Cys1686-Cys1872 with an Ala-Gly dipeptide. The 2 deletions were combined in construct dA13. When expressed by transient transfection of COS-7 cells, all 3 constructs allowed the assembly and secretion of VWF multimers (Figure 1A). These results are consistent with previous studies demonstrating that other deletions affecting dA1 or dA3 were compatible with the assembly and secretion of VWF multimers.[20,21] The deleted subunits were easily distinguished from normal VWF by gel electrophoresis under reducing conditions (Figure 1B). As expected, dA1 or dA3 was about 22 kd and dA13 was about 45 kd smaller than full-length VWF. The apparently normal biosynthetic processing of these constructs suggests they should be useful to study their intracellular association with other VWF subunits that bear distinct mutations.

Pro-VWF subunits form heterodimers

To determine whether heterodimerization of pro-VWF can occur, 293T cells were cotransfected with plasmids encoding the deleted subunit dA13 and the mutant full-length subunit Cys1149Arg (Figure 2). Control experiments showed that dA13 was immunoprecipitated from cell lysates or conditioned medium using a polyclonal antibody to VWF. Also, monoclonal antibody B710, which recognizes an epitope within dA1, recognized construct Cys1149Arg but not construct dA13, as required. When both constructs were coexpressed in the same cells, immunoprecipitation of cell lysates with antibody B710 led to the recovery of both Cys1149Arg and dA13 pro-VWF subunits. Multimer gel electrophoresis under nonreducing conditions confirmed that cell lysates contained dimers and no multimers (data not shown). These results demonstrate the formation of heterodimers in the ER.

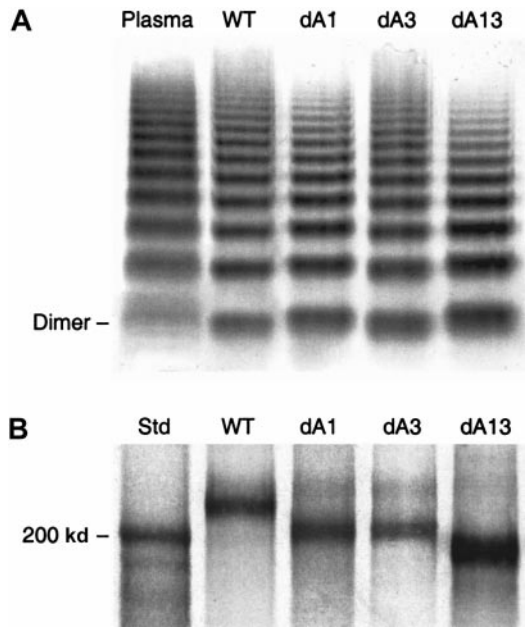

Figure 1. Electrophoretic analysis of VWF constructs dA1, dA3, and dA13. Recombinant normal human VWF (WT) and variants with deletions of dA1, dA3, or both dA1 and dA3 (dA13) were expressed in transiently transfected COS-7 cells, and conditioned medium was collected for analysis. (A) The multimer distribution of plasma vWF (plasma) and recombinant VWF preparations were compared by SDS–1.5% agarose gel electrophoresis under nonreducing conditions. The position of the smallest multimer, an approximate 500 kd dimer, is indicated. (B) VWF subunits and a 200 kd standard protein (M) were analyzed by SDS-PAGE on a 5% polyacrylamide gel under reducing conditions. The masses of the subunits are about 250 kd (WT), about 225 kd (dA1 or dA3), and about 205 kd (dA13).

Although Cys1149Arg is secreted with low efficiency in transiently transfected 293T cells,[6] immunoprecipitation of concentrated conditioned medium with B710 was able to recover some mature processed forms of both Cys1149Arg and dA13 (Figure 2, lane 8). The ratio of Cys1149Arg to dA13 is much more than 1:1 in this immunoprecipitate, which reflects the poor ability of Cys1149Arg subunits to form multimers.[6] Because of this defect, most Cys1149Arg subunits would be found in homodimers and very small multimers, accompanied by relatively few dA13 subunits. Consequently, immunoprecipitation with B710 should recover mainly Cys1149Arg subunits from conditioned medium of cells transfected with both Cys1149Arg and dA13, as observed in this experiment.

Dimerization of pro-VWF appears to be a random reaction

The efficiency of heterodimerization was assessed by densitometric analysis of pro-VWF species recovered from cell lysates. Polyclonal antibody 082 immunoprecipitates all VWF species present in the cells (data not shown) and therefore permits the determination of the ratio of Cys1149Arg to dA1 (Figure 3A) or dA13 (Figure 3B), which may vary depending on the efficiency with

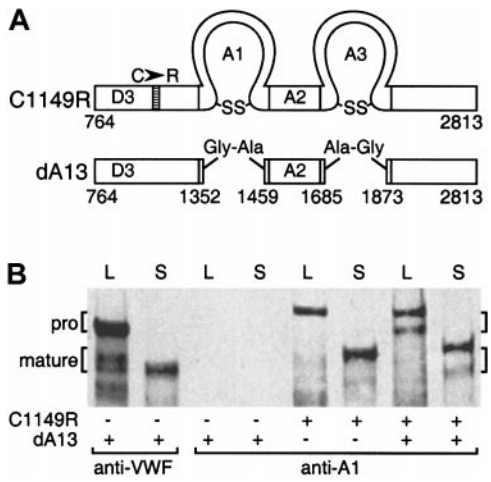

Figure 2. Formation of pro-VWF heterodimers. (A) Schematic structure of VWF constructs Cys1149Arg and dA13. Selected structural domains of VWF are labeled (A1, A2, A3, D3). The hatched and labeled segment of Cys1149Arg represents the substitution of Cys1149 to Arg in the D3 domain. Numbers below the structures indicate the positions of amino acid residues numbered from the initiation codon of pre-pro-VWF. In dA13, Gly-Ala replaces amino acid residues 1353-1458, and Ala-Gly replaces amino acid residues 1686-1872. (B) Analysis of VWF dimers by SDS-PAGE; 293T cells were transfected with plasmids encoding Cys1149Arg and dA13 as indicated. Cell lysates (L) and conditioned medium (S) were collected and immunoprecipitated with polyclonal anti-VWF antibody 082 (anti-VWF) or monoclonal anti-A1 domain antibody B710 (anti-A1). Samples were analyzed by SDS-PAGE on a 5% polyacrylamide gel under reducing conditions, and VWF was detected by Western blotting. The positions of intracellular pro-VWF and mature VWF subunits are indicated by brackets. The masses of the subunits are about 350 kd (pro-VWF Cys1149Arg), about 310 kd (pro-VWF dA13), about 250 kd (mature Cys1149Arg), and about 210 kd (mature dA13).

which the subunits are expressed in a given experiment. Monoclonal antibody B710 recognizes only dimers containing the Cys1149Arg subunit, and immunoprecipitation with B710 excludes homodimers of dA1 or dA13. Immunoprecipitation with excess B710 recovers about 50% of the total Cys1149Arg subunits in lysates (data not shown). B710 is sensitive to conformation,[19] and this result suggests that some A1 domains in the ER do not have a native structure.

A random dimerization mechanism predicts that the ratio Cys1149Arg/dA1 or Cys1149Arg/dA13 recovered with B710 will increase by 1.0 (equation 2) compared with the values obtained with antibody 082. The observed ratios are close to these expectations (Figure 3). This result eliminates limiting models of exclusive heterodimer formation and exclusive homodimer formation and is consistent with random dimerization of pro-VWF species in the ER. Some deviation from purely random dimerization cannot be ruled out because Cys1149Arg subunits were not quantitatively recovered in the B710 immunoprecipitates.

Cys1169 is not required for intracellular retention of mutant Cys1149Arg

In normal VWF, an intrasubunit disulfide bond[22] connects Cys1149 to Cys1169, and the mutation Cys1149Arg leaves Cys1169 potentially unpaired, which could prevent exit from the ER.[23,24] To determine whether the thiol group at Cys1169 contributes to the intracellular retention of Cys1149Arg subunits, constructs with the substitutions Cys1149Arg, Cys1169Ser, or both were prepared for cotransfection with normal VWF (Figure 4). As reported previously,[6] coexpression of Cys1149Arg decreased the secretion and increased the intracellular concentration of VWF. The mutation Cys1169Ser and the double mutation Cys1149Arg+Cys1169Ser had a similar phenotype. Notably, adding the second mutation Cys1169Ser did not reduce the severity of the Cys1149Arg phenotype. Therefore, the thiol group of Cys1169, which may be unpaired when Cys1149 is mutated, does not appear to account for the intracellular retention of Cys1149Arg.

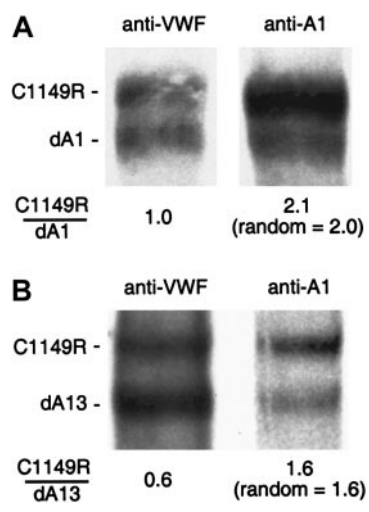

Figure 3. Densitometric analysis supports random intracellular dimerization. The 293T cells were transfected with plasmids encoding (A) Cys1149Arg and dA1 or (B) Cys1149Arg and dA13. Cell lysates were prepared and immunoprecipitated with polyclonal anti-VWF antibody 082 (anti-VWF) or monoclonal anti-A1 antibody B710 (anti-A1). After SDS-PAGE on a 5% polyacrylamide gel and Western blotting, the bands corresponding to pro-VWF species were quantitated as described under "Materials and methods." The ratios of the larger subunit (Cys1149Arg) to each of the smaller subunits (dA1 or dA13) are indicated. For each experiment, the value in parentheses is the ratio predicted for random dimerization, per equation 2, for immunoprecipitation with anti-A1 antibody B710 that is specific for Cys1149Arg. The observed ratios are similar to the predicted ratios.

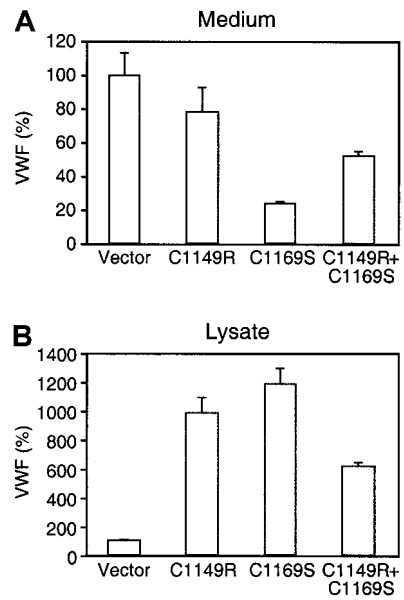

Figure 4. VWF mutations Cys1149Arg, Cys1169Ser, and Cys1149ArgCys1169Ser have similar phenotypes. The 293T cells were cotransfected with plasmid pSVH-VWF1 encoding normal VWF (3 μg) and 6 μg plasmid corresponding to each of the indicated constructs. VWF concentrations in conditioned medium (A) and cell lysate (B) were determined by ELISA and expressed as a percentage normalized to the values for control cells cotransfected with pSVHVWF1 and vector plasmid. Each bar represents the mean ± SD of 2 independent experiments; 100% corresponds to 304 ng/mL for medium and 34 ng/mL for lysate.

Pro-VWF Cys1149Arg is degraded by the proteasome

Proteins that cannot escape the ER may be transported to the cytoplasm for proteolytic degradation by the proteasome.[25] To investigate the fate of intracellular pro-VWF dimers, a pulse-chase experiment was performed in the presence and absence of lactacystin, a specific proteasome inhibitor[26] (Figure 5). In Fur4BHK cells expressing normal VWF, secretion of VWF was detected by 4 hours of chase and was complete within 16 hours. Intracellular pro-VWF species disappeared from the cells over a similar time (Figure 5A). Lactacystin delayed the secretion of VWF and prolonged its clearance from the ER. In contrast to these results for normal VWF, cells expressing Cys1149Arg did not secrete detectable amounts of VWF, and the intracellular cohort of labeled molecules was degraded almost completely by 4 hours (Figure 5B). The complete failure of Cys1149Arg secretion seen in this pulse-chase experiment was confirmed by ELISA on conditioned medium (data not shown), indicating that stably transfected BHK cells retain these mutant VWF subunits even more efficiently than transiently transfected 293T cells (Figure 2). Lactacystin delayed the removal of mutant pro-VWF Cys1149Arg subunits to more than 16 hours, indicating that the destruction of intracellular Cys1149Arg involved retrograde transport into the cytoplasm and proteasomal degradation.

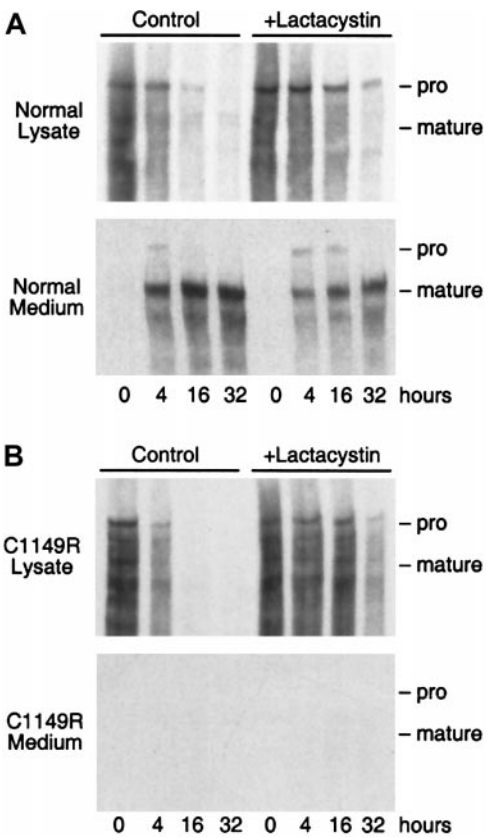

Figure 5. Effect of lactacystin on the synthesis of normal VWF and Cys1149Arg. BHK cells expressing normal VWF (A) or Cys1149Arg (B) treated without (control) or with lactacystin were pulse-labeled with [^{35}S]cysteine and [^{35}S]methionine for 30 minutes and chased in unlabeled complete medium for the times indicated. VWF was immunoprecipitated from cell lysates and conditioned medium for analysis by SDS-PAGE on 5% polyacrylamide gels and autoradiography as described under "Materials and methods." The positions of pro-VWF (about 350 kd) and mature VWF subunits (about 250 kd) are indicated at the right.

Discussion

Although the molecular mechanism of type 1 VWD remains elusive in most families, there seems to be clear heterogeneity among type 1 VWD patients. Some families have low penetrance and a moderate decrease in VWF levels to 30 to 45 U/dL in affected members, whereas other families exhibit complete penetrance and a more severe decrease in VWF, often to 10 to 15 U/dL. The VWF mutation Cys1149Arg was identified in one family with highly penetrant VWD type 1, and this mutation was shown to dominantly suppress the secretion of normal VWF when coexpressed in 293T cells. To explain this phenotype, the mutant subunit was proposed to form heterodimers with normal VWF that were retained intracellularly and degraded. However, we could not determine whether a physical association of mutant and normal subunits occurred in the ER as well as in the Golgi apparatus, and heterodimer formation was not demonstrated directly.[6]

To study the mechanism responsible for dominant negative VWD type 1, we developed a model system in which 2 "alleles" could be distinguished. VWF biosynthesis is tolerant of certain large deletions, as shown by the efficient assembly and secretion of multimers composed of subunits lacking dA1 or dA3.[20,21] The combined deletion of both dA1 and dA3 also was compatible with apparently normal multimer secretion (Figure 1A), and the larger deletion facilitates the differentiation of full-length and deleted subunits upon gel electrophoresis (Figure 1B). These properties enabled constructs lacking the A1 domain to serve as surrogates for a normal VWF "allele" that could be distinguished from full-size Cys1149Arg subunits by their smaller mass and lack of reactivity with anti-A1 domain antibodies. When both Cys1149Arg and dA13 subunits were expressed in 293T cells, immunoprecipitation of cell lysates with antibody B710 resulted in the recovery of both full-length Cys1149Arg and deleted dA13 pro-VWF subunits, proving that they form heterodimers within the ER (Figure 2).

VWF subunits encoded by 2 alleles must be synthesized on different polyribosomes, and heterodimerization in the ER might be disfavored by the spatial separation of these translation events. However, densitometric analysis of subunit ratios indicates that pro-VWF dimerization is random (Figure 3). The site of interchain disulfide bond formation is within the extreme C-terminal 90 amino acid residues of the VWF subunit,[27] and for this reason dimerization probably is not cotranslational but may occur instead after the release of polypeptide chains into the lumen of the ER. In addition, the intracellular retention and degradation of Cys1149Arg subunits presumably involves the persistent association of chaperones with misfolded segments near the N-terminus of the polypeptide, but this process does not impair the dimerization activity of the cystine knot domain at the C-terminus.

Random dimerization of pro-VWF implies that one-half of all dimers will be heterodimers, and the retention of all mutant homodimers and heterodimers in the ER would reduce the secretion of VWF to 25% of the total synthesized. If the plasma concentration were proportional to synthesis, the corresponding mean plasma VWF level would average 25 U/dL. Patients with the heterozygous Cys1149Arg mutation have even lower VWF levels of 10 to 15 U/dL, and the cause of the more severe reduction is not known.[6] The normal range of plasma VWF is broad and is influenced by several factors, such as ABO blood type and hormonal status. For example, the mean VWF levels in people of blood type O is 75 U/dL, with a range (± 2 SD) of 36 to 157 U/dL.[28] Thus, combining the mutation Cys1149Arg and blood type

O might lower the VWF level to the range observed for patients with the Cys1149Arg mutation. However, this explanation does not seem to apply, because unaffected family members had VWF levels of 83 to 86 U/dL and ABO blood type did not correlate with VWF level among the affected family members.[6] An alternative mechanism to produce lower than predicted VWF levels could be proposed if a small fraction of dimers containing mutant subunits escaped from the ER and were incorporated into multimers in the Golgi apparatus. Escaped malfolded proteins apparently are retrieved from the distal secretory pathway,[29,30] and the return of hetero-oligomers to the ER might remove additional normal subunits without substantially changing the size distribution of the final secreted multimers.

This model predicts that when dimers escape from the ER, the subsequent biosynthetic steps should be normal, including multimerization in the Golgi, storage in Weibel-Palade bodies, secretion, and intravascular survival. In keeping with this model, the VWF multimer pattern of affected patients was normal and administration of DDAVP caused a marked elevation of plasma VWF concentration into the normal range (data not shown).

Unpaired cysteines are known to prevent the intracellular transport of many proteins and might contribute to the phenotype of VWF Cys1149Arg. For example, treatment of cells with reducing agents causes the reversible accumulation of several proteins within the ER, including influenza virus hemagglutinin,[31] albumin, and the asialoglycoprotein receptor,[32] but has no effect on α_1-antitrypsin, which lacks disulfide bonds.[32] Reducing agents also cause the retention of VWF in the ER of endothelial cells.[33] Immunoglobulin M cannot leave the ER until a C-terminal cysteine residue of the heavy chain forms a specific disulfide bond, and mutation of the cysteine relieves this block.[24] A similar mechanism has been described for the unassembled subunits of acetylcholinesterase.[23] These observations suggest that the intracellular retention of VWF Cys1149Arg could be explained by the exposure of an unpaired thiol at Cys1169, because Cys1149 normally forms an intrachain disulfide bond with Cys1169.[22] However, mutating both of these cysteines did not rescue the biosynthetic defect of Cys1149Arg (Figure 4). Therefore, the intracellular retention of Cys1149Arg may not depend on the presence of an unpaired Cys1169 and could involve a less direct effect of the mutation on protein folding.

The ER retention and degradation of pro-VWF heterodimers raises interesting questions about quality control mechanisms for protein synthesis. Newly translated proteins are recognized by resident ER chaperones and are retained until they fold properly.[34] The signals for the detection of misfolding are not known, but at least some appear to be "local," and a large protein can have some misfolded domains that continue to bind certain chaperones and other folded domains that do not.[35] Mutant VWF Cys1149Arg appears to fit this model because the mutation near its N-terminus causes ER retention without impairing the dimerization activity of the cystine knot domain at its C-terminus, more than 1500 amino acids and about 30 nm distant.[36,37] Most retained cellular proteins are degraded by proteasomes in the cytoplasm, and the process probably involves the reduction of disulfide bonds, polypeptide unfolding, and retrotranslocation through a pore in the ER membrane.[34] The stabilization of Cys1149Arg by the proteasome inhibitor lactacystin (Figure 5) is consistent with this mechanism, and the dominant phenotype of the heterozygous Cys1149Arg mutation suggests that a localized folding defect in the mutant subunit is sufficient to ensnare the disulfide-linked normal subunit as well. At some point in the retrotranslocation process, the subunits may be separated by the reduction of intersubunit disulfide bonds. If so, by then both subunits appear to be marked by an unknown mechanism for degradation.

The dominant negative mechanism proposed for heterozygous VWF Cys1149Arg may apply more generally to other multimeric proteins. If a mutation causes the ER retention of a subunit without preventing its assembly with normal subunits, the entire complex could be retained and degraded. These conditions may be more likely to be fulfilled for proteins with intersubunit disulfide bonds and several independently folded domains, so that a structure remote from a site of covalent oligomerization mediates ER retention. As a potential example among hemostatic proteins, fibrinogen has the polypeptide composition $(\alpha\beta\gamma)_2$, and autosomal dominant forms of hypofibrinogenemia could include additional instances of this mechanism, particularly among patients with missense mutations that impair subunit synthesis[38,39] or cause intracellular fibrinogen to accumulate.[39,40] The same mechanism might enable heterozygous collagen mutations to reduce the secretion of triple-helical collagen, thereby contributing to the pathophysiology associated with missense mutations in collagen α 1(I) or collagen α 1(II), which cause dominant osteogenesis imperfecta[41] or hypochondrogenesis,[42] respectively. As in the case of VWF, testing this mechanism for other proteins will require methods to distinguish mutant and normal subunits so that their intracellular association can be detected.

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