Cyclosporin A Impairs the Secretion and Activity of ADAMTS13 (A Disintegrin and Metalloprotease with Thrombospondin Type 1 Repeat)*†§

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Background: Immunosuppressive drug cyclosporin A (CsA) is a potent inhibitor of cyclophilin B (CypB) function.

Results: CsA treatment leading to reduction in CypB levels is associated with decreased secretion of ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeat).

Conclusion: CypB function and levels affect the secretion of ADAMTS13.

Significance: A novel mechanistic explanation for CsA-induced thrombotic thrombocytopenic purpura in transplant patients is suggested.

The protease ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 repeat) cleaves multimers of von Willebrand factor, thus regulating platelet aggregation. ADAMTS13 deficiency leads to the fatal disorder thrombotic thrombocytopenic purpura (TTP). It has been observed that cyclosporin A (CsA) treatment, particularly in transplant patients, may sometimes be linked to the development of TTP. Until now, the reason for such a link was unclear. Here we provide evidence demonstrating that cyclophilin B (CypB) activity plays an important role in the secretion of active ADAMTS13. We found that CsA, an inhibitor of CypB, reduces the secretion of ADAMTS13 and leads to conformational changes in the protein resulting in diminished ADAMTS13 proteolytic activity. A direct, functional interaction between CypB (which possesses peptidyl-prolyl cis-trans isomerase (PPIase) and chaperone functions) and ADAMTS13 is demonstrated using immunoprecipitation and siRNA knockdown of CypB. Finally, CypB knock-out mice were found to have reduced ADAMTS13 levels. Taken together, our findings indicate that cyclophilin-mediated activity is an important factor affecting secretion and activity of ADAMTS13.

The large number of proline residues in ADAMTS13 is consistent with the important role of cis-trans isomerization in the proper folding of this protein. These results altogether provide a novel mechanistic explanation for CsA-induced TTP in transplant patients.

The von Willebrand factor (VWF)3 is a key player in the regulation of primary hemostasis during blood clotting (1). At sites of injury, where the shear forces are high, VWF exists in its elongated form, thus allowing for platelet binding and initiation of blood coagulation. Platelet aggregation under these conditions is a cause for concern as it leads to thrombosis. The enzyme ADAMTS13, a member of the ADAMTS (a disintegrin and metalloprotease with thrombospondin type 1 repeat) metalloprotease family plays a pivotal role in counteracting such platelet aggregation under normal conditions (2). The importance of ADAMTS13 in this process has been evident from the clinical effects of ADAMTS13 deficiency, which leads to thrombotic thrombocytopenic purpura (TTP). The ADAMTS13 precursor consists of 1427 amino acid residues (with a predicted molecular mass of 153,604 Da). However, the protein undergoes extensive maturation in endoplasmic reticulum (ER), is heavily glycosylated (3), and migrates during gel electrophoresis as a polypeptide of ~170 kDa. It has been shown that O-fucosylation occurs at 6 TSP1 repeats of ADAMTS13 and is required for its secretion (4), similarly, N-glycosylation appears to be critical for the secretion of ADAMTS13 and may affect its folding and consequently protein proteolytic activity (5). It should be noted that the protein-folding machinery of the ER consists of three major classes of proteins: (i) foldases, (ii) molecular chaperones, and (iii) the oligosaccharride processing enzymes as well as lectin chaperones calnexin and calreticulin, which all act in concert and are

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also involved in protein quality control (6). Among the foldases, the peptidyl-prolyl cis-trans isomerases (PPIs) are important, particularly in large proteins with significant numbers of proline residues. The isomerization of peptidyl-prolyl bonds is a rate-limiting step during protein folding and would spontaneously occur at a rate too slow to support efficient protein folding in the cell (7). Catalysis of proline cis-trans isomerization is therefore often a necessary step required for accurate protein folding in vivo. Mammalian cells contain three classes of PPIs: parvalins, cyclophilins (Cyps), and FK506-binding proteins (8). ADAMTS13, harboring 118 prolines, contains a higher number of proline residues in comparison with human proteins (9). It is therefore plausible that PPIases may play an important role in the folding and maturation of this protein. Cyclophilins, most notably cyclophilin A and B (CypA and CypB), constitute an important family of PPIases (8, 10). The immunosuppressive drug cyclosporin A (CsA) is a potent inhibitor of the PPIase activity of CypA and CypB (11). The CsA-mediated inhibition of PPIase is, however, independent of the mechanism by which CsA acts as an immunosuppressive drug.

Long-term clinical observations (for over two decades) indicate that there could be a link between CsA usage and TTP. Indeed, numerous reports suggest that the use of CsA in transplant patients may be associated with TTP (12–16), although some other reports reveal no interrelationship between CsA treatment and TTP development and found no influence of CsA treatment on ADAMTS13 levels (17). Nevertheless, it has been demonstrated that reduced activity and/or deficiency of ADAMTS13 can clinically manifest as TTP (18). We have therefore decided to check whether there could be a link between CsA usage and decreased levels of ADAMTS13 and investigate the direct effects of CsA treatment on the synthesis and function of recombinant human ADAMTS13 expressed in HEK293 cells. Using this cellular system, we have found that the levels of secreted ADAMTS13 are reduced in cells grown in the presence of CsA. Co-immunoprecipitation analysis and the use of siRNA against CypB revealed a direct interaction of CypB and ADAMTS13. Finally, we were able to demonstrate that plasma from CypB knock-out mice contains reduced levels of endogenous ADAMTS13. Taken together, our results strongly suggest that CypB is an important factor in the proper maturation and secretion of ADAMTS13.

EXPERIMENTAL PROCEDURES

Cell Lines, Cell Culture, and Mice Blood Plasma Used in this Study—The human HEK293 (ATCC, Manassas, VA), kidney embryonic cells, and KB 3.1 cells (modified HeLa cells) were grown as previously described (19), after transfections cells were incubated with Opti-MEM media (all materials from Invitrogen). Plasma from CypB knock-out (Ppib−/−) or littermate control mice was collected by tail vein bleeding, followed by centrifugation for 5 min at 13,000 × g at 4 °C. Mouse embryonic fibroblast (MEF) cells were obtained from the same mouse line and grown in the same conditions as the human HEK293 cells.

Plasmid DNA—pcDNA3.1 empty vector (Invitrogen) and pcDNA4–ADAMTS13 (a gift from Dr. Evan Sadler, Washington University Medical School, St. Louis, MO) carrying the liver wild-type (WT) form of ADAMTS13 (NM_139025.2) were used to transfect HEK293 cells.

Treatment of Cells with Immunosuppressive Drugs Cyclosporin A, FK506, Rapamycin, or Control Drug, PKC412, a Serine/Threonine and Tyrosine Kinase (PKC) Inhibitor—Stock solutions of 10 mM CsA (Calbiochem, San Diego, CA), 10 mM FK506, 10 mM rapamycin or PKC412 (all from Sigma), a serine/threonine and tyrosine kinase (PKC) inhibitor, were dissolved in dimethyl sulfoxide (DMSO; Sigma). Drugs were diluted to final concentrations of 10 and 20 μM in cell culture media 4 h post-transfection. Untreated cells received a volume of DMSO equivalent to the volume of the drug used for the highest treatment, not exceeding 1% of the total volume of the media.

Treatment of Cells with ALLN, the Cysteine Protease Inhibitor—Five hours before harvest, ALLN (N-acetyl-Leu-Leu-norleucinal) (Sigma), the cysteine protease inhibitor, was added into culture at a final concentration of 10 μg/ml. Untreated cells received DMSO as above.

Isolation of RNA—Total RNA was isolated from CsA-treated and untreated cells using the Qiagen RNaseasy Kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s protocol with an additional 15-min on-column DNase incubation step and quantified using the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE). The integrity of RNA was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) with a Eukaryote Total RNA Nano assay.

Quantification of ADAMTS13 mRNA Expression Using Real-time Quantitative RT-PCR—The ADAMTS13 mRNA expression levels were measured using real-time quantitative RT-PCR LightCycler RNA Master SYBR Green Kit and LightCycler II instrument (Roche Applied Science). The following PCR primers for ADAMTS13 and the reference gene (GAPDH), respectively (designed using the LightCycler Probe Design 2 software (Roche Applied Science)) were used: ADAMTS13 forward, 5′-TCACAGGCAACCTCACCTCG-3′; ADAMTS13 reverse, 5′-CGGCACCTGCGGTTAC-3′; GAPDH forward, 5′-CCTTTGACCTGAATCACAT-3′; GAPDH reverse: 5′-ACGATAACCAAGTTGTCAAGGAT-3′. Crossing points for each transcript were determined using the second derivative maximum analysis with an arithmetic baseline adjustment. Crossing point values were normalized to the respective crossing point values for the reference gene.

Preparation of Cell Lysate and Concentrated Medium—Collected media was concentrated 24-fold using 30-kDa cut-off Centricon concentrating vials and 10-kDa cut-off Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA). Harvested cells were washed with 5 ml of chilled PBS lysed with buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 μg PMSF, and 1 tablet of Protease Inhibitor Mixture (Roche Applied Science) per 10 ml of buffer). The samples were stored at −20 °C. The total protein was measured using Bradford protein assay (Bio-Rad).

Quantification of Intracellular and/or Secreted Forms of ADAMTS13 and Cyclophilin B—Expression of ADAMTS13 and cyclophilin B was assessed and quantified using Western blotting. Thirty μg of total protein from cell lysates and concentrated media samples were mixed with loading buffer and
heated at 95 °C for 10 min, sonicated for 10 min, and further separated on a 3–8% Tris acetate SDS and/or 12% BisTris gels (Invitrogen). Anti-V5 (Invitrogen), anti-cyclophilin B, and anti-Hsp70 (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-mouse IgG HRP (Invitrogen) were used for Western blotting. Plasma samples from mice were processed under nonreducing sample buffer conditions and directly loaded on 10% BisTris gels (Invitrogen). Detection of ADAMTS13 was performed using anti-ADAMTS13 (Novus Biologicals, Littleton, CO) and anti-rabbit IgG HRP (Rockland, Gilbertsville, PA). Detection of endogenous ADAMTS13 by SDS-PAGE analysis is not possible (20).

**Measurement of ADAMTS13 Protease Activity Using FRET-VWF73 Assay**—ADAMTS13 activity was measured using fluorescence resonance energy transfer substrate-von Willebrand factor 73 (FRET-VWF73) (Peptide International, Osaka, Japan). Varying amounts of FRET-VWF73 (0, 0.4, 1.0, 2.0, and 5 μM final concentrations) were added to the reaction buffer containing 5 mM Tris-HCl, 25 mM CaCl$_2$, and 0.005% Tween 20 (all from Sigma) at pH 6 to a final volume of 25 μl. Separately, 5 μl of each media sample was added to 0.5 μl of 3% sodium citrate (Sigma) to make a final volume of 25 μl. The two 25-μl mixtures were combined on a 96-well white plate. The fluorescence was measured using a GeminiMax plate reader every 5 min for 1 h with mixing at an excitation of 340 nm and emission of 450 nm. Each sample was done in triplicate. Also, an acute control was added to verify that CsA did not interact with the active site of ADAMTS13. It included untreated cell media to which CsA was added to a final concentration equal to the highest concentration tested in that experiment at reaction time added right before FRETs assay. The specific activity was calculated using ADAMTS13 Western blotting quantitation data.

**Trypsin Digestion**—ADAMTS13-cell lysates (total protein 200 μg/80 μl of reaction) from untreated and 20 μM CsA-treated cells were digested with increasing concentrations of trypsin (Sigma) for 3 min at 37 °C (0, 0.00025, 0.0005, and 0.001 mg/ml, diluted in 100 mM Tris-HCl (Invitrogen)). ADAMTS13 was analyzed by Western blotting using anti-V5 antibody as described earlier.

**Measurement of ADAMTS13 Using Flow Cytometry**—The ADAMTS13 transfection and CsA treatment were as described above. The cells were harvested after a 20-h incubation and tested by flow cytometry analysis. Briefly, the cells were permeabilized and incubated with ADAMTS13-specific monoclonal antibodies (WH10, WH22-22-1A, and W668X6-1) (21), Tag-specific antibody V5 (Invitrogen), or isotype antibody at 10 μg/ml concentrations for 30 min at 37 °C. After washing the cells with PBS and 0.1% bovine serum albumin buffer, tubes were incubated with Alexa 488 goat anti-mouse IgG secondary antibody (Invitrogen) at 37 °C for 30 min. The cells were then analyzed using the BD Biosciences FACS calibur, and the data were analyzed using FlowJo software.

**Co-immunoprecipitation**—The interaction of ADAMTS13 with cyclophilin B was also detected by immunoprecipitation followed by Western blotting analysis. Extracts from HEK293 ADAMTS13-transfected cells with and without CsA treatment (250 μg of protein in RIPA buffer composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate), were incubated with 0.1 mM protease inhibitor (4-(2-aminoethoxy)benzenesulfonyl fluoride, Sigma), and 10 μg of primary antibody (anti-V5) for 3 h at 4 °C under agitation. Protein A beads (Abcam, Cambridge, MA) (80 μl) were added after washing 3 times with RIPA buffer and then samples were incubated overnight at 4 °C under rotary agitation. Samples were centrifuged, washed with RIPA buffer, and separated on 12% BisTris gel (Invitrogen). Proteins were transferred onto nitrocellulose membrane (Invitrogen) and probed with anti-V5 and anti-cyclophilin B antibodies.

**Comparative Modeling**—Comparative modeling of the ADAMTS13 structure was performed using the three-dimensional PSSM algorithm developed by Kelley and Sternberg (21) and Swiss-Pdb Viewer version 4.0.2 using one- and three-dimensional sequence profiles coupled with secondary structure and solvation potential information. PSSM E-value (the score or “expectation value” of the match; “% Certainty”) was used to compare different model structures and the structures with the lowest E-value were chosen for further analysis (22). Two model fragments comprising ADAMTS13 residues 77–470 and 674–1254 (out of the 1427 total residues) were built with the help of PyMOL version 0.98 and/or Swiss-PDB Viewer version 4.0.2 and the prolines were visualized (van der Waals radii of the side chains were shown).

**RESULTS**

**Treatment of HEK293 Cells with Immunosuppressive Compounds Results in Reduced Secretion of ADAMTS13, in the Absence of Changes in mRNA Levels**—To address the effects of CsA treatment on expression of recombinant ADAMTS13 we used the experimental system similar to those previously used by other investigators, aiming to address the effects of CsA on the synthesis and activity of, e.g. synapsin (23), Kir2.1 (24), creatine transporter (25), and/or sodium calcium exchanger (19, 27). Following these studies, we used transient transfections and applied CsA in the reported range of concentrations (0–20 μM), which also reflects the concentration of drug circulating in the blood of patients undergoing CsA treatment (28). Transfection experiments revealed that 24 h post-transfection, the levels of secreted ADAMTS13 were significantly reduced upon CsA treatment (>90%). Treatment with other commonly used immunosuppressive drugs, FK506 and rapamycin, affected intracellular or extracellular levels of ADAMTS13 to a lesser extent (Fig. 1A). Immunosuppressive properties of CsA as well as FK506 are attributed to their ability to inhibit calcineurin, a serine-threonine phosphatase required for cytokine induction in response to stimulation of T cells. Rapamycin does not inhibit calcineurin, but inhibits mammalian target of rapamycin instead, providing a different mechanism of immune suppression (29, 30). However, in contrast to CsA, FK506 and rapamycin are not able to impair ADAMTS13 secretion and maturation substantially (Fig. 1A). This suggests that the calcineurin-mediated signaling pathway is likely not being involved in modulation of ADAMTS13 secretion and that the CsA effect on ADAMTS13 secretion may be attributed to its ability to inhibit PP1ase activity of cyclophilins. No changes in secreted ADAMTS13 levels were also detected in cells treated with a control drug PKC412 (Fig. 1A), which does not interact.
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Figure 1. ADAMTS3 mRNA and protein expression levels under treatment of various immunosuppressive drugs. A. HEK293 cells were transfected with ADAMTS3 cDNA and treated with the highest volume of DMSO used for these drugs. Intracellular (lysate) and extracellular (media) levels of ADAMTS13 were monitored by Western blotting using anti-V5 antibody, which can tag only the recombinant ADAMTS13. Untreated cells (left lane) are transfected cells treated with the highest volume of DMSO used for these drugs. Corresponding quantifications for ADAMTS3 mRNA expression were plotted. Corresponding quantifications for ADAMTS3 mRNA expression were plotted. B–E. Real-time PCR analysis of ADAMTS3 mRNA expression in control and CsA-treated HEK293 cells transfected with ADAMTS3 cDNA. Relative amounts of ADAMTS3 mRNA were based on crossing-point analysis. The data represent the average of six independent experiments and the error bars depict the S.E. Two-tailed p value was calculated. F. HEK293 cells were transfected with ADAMTS3 cDNA plasmid and treated with various concentrations of CsA: 0.1, 0.5, 1, 2, 5, and 20 μM. Intracellular and extracellular levels of ADAMTS13 were monitored by Western blotting using anti-V5 antibody. Untreated cells (left lane) are transfected cells treated with the highest volume of DMSO used for CsA. Band intensities of ADAMTS13 and CypB were plotted. Corresponding quantifications for ADAMTS13 mRNA expression were plotted. Corresponding quantifications for ADAMTS3 mRNA expression were plotted. G. Hsp70 protein expression with and without treatment with 20 μM CsA. Untreated cells (left lane) are transfected cells treated with the highest volume of DMSO used for CsA.
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Cyclophilins (31). Intracellular and extracellular Hsp70 levels were also shown to be unchanged in cells treated with CsA (supplemental Fig. S1D). In addition, no changes in intracellular levels of ADAMTS13 were observed, suggesting a post-translational mechanism of CsA action (Fig. 1A, note that the same number of cells was used and the same total protein amount was loaded in each lane). To further test this hypothesis we determined the levels of ADAMTS13 mRNA in control cells and those treated with 20 μM CsA and found them to be similar (Fig. 1B), consistent with previous reports (32). We note that CsA is known to interact with cyclophilins (CypA and CypB) (11), as opposed to FK506 and/or rapamycin, which are known to interact with FK506-binding protein (33, 34). We therefore examined the relationships between changes in secreted levels of ADAMTS13 and the changes in the levels and/or activity of cyclophilins (and specifically the secreted CypB) under increasing CsA concentrations. Our results indicate that with increasing CsA concentrations, secreted ADAMTS13 levels progressively decrease, whereas secreted CypB levels increase until they reach a plateau at ~5 μM CsA (Fig. 1C), therefore suggesting that ADAMTS13 may be a substrate of CypB recognition and activity, subsequently affecting proper ADAMTS13 maturation.

**CsA Disrupts the Interaction between the PPIase Cyclophilin B and ADAMTS13**—To assess possible interaction between CypB and ADAMTS13, we carried out immunoprecipitation experiments. In our transient expression system the ADAMTS13 is tagged with the V5 peptide; hence we have used the anti-V5 antibody during immunoprecipitation of lysates from control cells and those grown in the presence of CsA. Following immunoprecipitation the samples were subjected to Western blotting with anti-ADAMTS13 and anti-CypB antibodies, respectively (Fig. 2A). When the anti-V5 antibody was omitted during immunoprecipitation, neither ADAMTS13 nor CypB could be detected (Fig. 2A, lane 1). However, both ADAMTS13 and CypB were detected in CsA untreated cells (Fig. 2A, lane 2), suggesting a likely interaction between the two proteins. However, in cells treated with 10 and 20 μM CsA (Fig. 2A, lanes 3 and 4) only ADAMTS13 could be detected further suggesting that CsA treatment disrupts the interaction between these two proteins. Western blotting in the absence of immunoprecipitation is also shown (Fig. 2A, lanes 5–7). We note that both CypB and ADAMTS13 are detected in lysates from untreated cells and those treated with CsA. This suggests that CsA does not affect CypB synthesis, but likely affects the interaction between CypB and ADAMTS13. Based on these findings, we hypothesize that CsA may affect maturation and secretion of ADAMTS13, possibly through its ability to inhibit proline cis-trans isomerase activity of CypB.

To further assess the extent to which CsA-mediated reduction in ADAMTS13 expression levels is mediated by CypB, we knocked down CypB expression using siRNA. It should be noted that efficient down-regulation of CypB expression by more than 50% with cyclophilin B-specific siRNA resulted in cell death (not shown). However, moderate abrogation of CypB expression (~35%) did not substantially affect cell survival, but resulted in about 75% reduction in intracellular ADAMTS13 expression levels (Fig. 2B). Scrambled siRNA showed no effect, and staining for β-actin demonstrated that similar levels of total protein were analyzed (Fig. 2B). This result suggests that intracellular ADAMTS13 expression is mediated by CypB.
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FIGURE 3. Specific activity of intracellular ADAMTS13 obtained from control cells and those treated with CsA. ADAMTS13 activity was monitored with FRETS, using the synthetic FRETS-VWF73. ADAMTS13-mediated proteolysis was monitored as change in fluorescence over time. Lysates used in the assay contained equal amounts of the ADAMTS13 protein as determined by protein estimation and immunoblot analysis. The graph depicts specific activity in the presence of increasing concentrations of FRETS-VWF73. Intracellular ADAMTS13 was obtained from the lysates of control cells (square) and those grown in the presence of 20 μM CsA (circle), or control in which CsA was added to the reaction (triangle). V_max and K_m values were calculated using GraphPad Prism software. V_max values were statistically significant as determined by one-way analysis of variance (p = 0.0002) and post hoc Tukey’s test shows a statistically significant difference (p < 0.05) between treated and untreated, and treated and acute control V_max values. K_m values were not statistically significant (p = 0.055). n = 3; mean ± S.E.

Number, Location, and Frequency of Proline Residues in ADAMTS13—Schematic of the ADAMTS13 domain structure as well as ADAMTS13 structural models of two fragments and a structure of ADAMTS13 fragment that has been solved by crystallography (PDB code 3GHM) highlight the location and increased occurrence of proline residues along the ADAMTS13 polypeptide chain (supplemental Fig. 2, A and B). The total number of proline residues in ADAMTS13 is 118, thus constituting 8.3% of all the amino acids in this protein, whereas the average proline usage in vertebrate proteins is about 5% and in human proteins ~6.17% (9).

Treatment of ADAMTS13 Overexpressing HEK293 Cells with CsA Results in a Protein Product with Reduced Proteolytic Activity—The results presented above suggest that CsA treatment affecting CypB activity could lead to aberrant ADAMTS13 folding. This could result in impaired function of the ADAMTS13 or reduced levels of the protein due to ER-associated degradation of misfolded molecules. Either scenario would result in inefficient proteolysis of the VWF protein and TTP-like symptoms. We therefore measured ADAMTS13 activity in cells that were grown in the presence of CsA for 48 h. Treatment of cells with CsA decreases the V_max and increases the K_m of ADAMTS13 (Fig. 3). It is important to note that the acute treatment of the lysate with 20 μM CsA had no effect on ADAMTS13 activity (Fig. 3, Acute Control). Thus, CsA has no direct antagonistic effect on ADAMTS13-mediated cleavage of FRETS-VWF73, whereas prolonged treatment of cells with CsA results in a protein with significantly impaired activity, leading to its reduced affinity for its substrate. These results suggest that ADAMTS13 is likely being misfolded in cells grown in the presence of CsA.

Treatment of Cells with CsA Produces ADAMTS13 with Altered Conformation—If the PPIase activity of CypB is indeed necessary for the correct folding of ADAMTS13, then the protein synthesized in the presence of active CypB will possess a different conformation relative to the protein synthesized under the conditions in which CypB activity was compromised (i.e. inhibited by CsA treatment). One way to compare different conformations of the same protein is to compare the susceptibility of the protein conformers to proteolytic cleavage and the pattern of limited proteolysis fragments (e.g. after trypsin digestion). We have found that ADAMTS13 synthesized in the absence of CsA begins to show significant degradation at 0.0005 mg/ml of trypsin (Fig. 4A). However, ADAMTS13 synthesized in the presence of 20 μM CsA is considerably more resistant to trypsin and can be readily detected at 0.0005 and 0.001 mg/ml of trypsin (Fig. 4A, this experiment was repeated 8 times). To further study the conformational effects of ADAMTS13 protein expression in the presence CsA treatment, we used different conformation sensitive antibodies (21, 35). The ADAMTS13-transfected positive cells were gated based on V5 antibody and the same gate was applied to isotype as well as ADAMTS13-specific antibodies. As expected we did not see much difference with V5 antibody upon CsA treatment but about 30 ~ 45% of lower expression was observed with WH10, WH2-22-1A, and W688X6-1 antibodies (Fig. 4B). Interestingly, we have seen the higher expression in the presence of PEP4-5B1 antibody (Fig. 4B). These results suggest that the ADAMTS13 protein produced in untreated HEK293 cells and cells treated with CsA may be indeed conformationally distinct. Impaired folding of ADAMTS13 in the presence of CsA would likely result in the clearance/degradation of the misfolded protein via the ER-associated degradation system (36). This suggestion is consistent with the observations above, revealing reduced secretion of ADAMTS13 in the presence of CsA, because misfolded protein has likely been cleared by the quality control machinery of the ER, which actively monitors the folding status of its cargo (37). We therefore also tested the accumulation of intracellular ADAMTS13 in cells treated with CsA, in the absence and/or presence of ALLN (N-acetyl-Leu-Leu-norleucinal) (the cysteine protease inhibitor). ALLN is used here to block proteasomal degradation. Increased levels of ADAMTS13 were observed (about 2.5-fold) when cells were co-treated with CsA and ALLN (Fig. 5).

Reduced Levels of ADAMTS13 in Plasma from CypB Knockout Mice—Next, we examined whether reduced CypB levels may also impact ADAMTS13 expression in vivo in an animal model. ADAMTS13 levels in plasma samples obtained from CypB-deficient mice (Ppib−/−) (38) lacking both copies of the gene for CypB, Ppib, were compared with those in the CypB-expressing mice strain (Ppib+/+). To assure that similar total protein levels were loaded on the gel, silver staining was performed on exactly the same samples, which were loaded on SDS-PAGE Western blotting (Fig. 6A). We have found significantly lower levels of ADAMTS13 in the plasma of Ppib−/− mice (Fig. 6B), with a p value of 0.0037 (4 repeats of different mice plasma). These results provide in vivo evidence that CypB is essential in the ADAMTS13 maturation process. ADAMTS13 mRNA and protein levels in other tissues of these mice, in particular, MEF cells show similar trends (Fig. 6, D and E).
DISCUSSION

The expression, correct folding, and secretion of functional ADAMTS13 into circulating blood is vital for preventing the clinical effects of ADAMTS13 deficiency, TTP. ADAMTS13 is a large, complex protein that is a subject of extensive co- and post-translational modifications (4, 5). The role of foldases and PPIases in the folding and secretion of ADAMTS13 has not been investigated in detail, thus far. The aminoacyl-proline cis-trans interconversion plays a critical role in the protein three-dimensional structure formation and protein processing and is a rate-limiting step during protein folding (39). It is plausible that PPI may play an important role in ADAMTS13 function. The unique structure of proline leads to an exceptional conformational rigidity of this amino acid. Prolines are considered
secondary structure “breakers” and are often found at the edges of α-helices and β-sheets, as also seen in the ADAMTS13 model (supplemental Fig. S2B). In addition, for many proteins, proper isomerization of the peptide bond preceding the proline is essential for proper protein folding. Although the cis-trans interconversion of the amino acylproline amide bond can occur spontaneously, the process is relatively slow. In biological systems, therefore, this process is accelerated by the PPIases (40).

In this study we present evidence demonstrating that CypB, which belongs to an ubiquitous family of immunophilins (the PPIases), is associated with the secretion of active ADAMTS13. The pharmacological inhibition of the CypB-mediated PPIase activity by the immunosuppressive drug CsA has been extensively characterized since the anti-HIV activity of this drug was reported in 1988 (41). The binding of CsA to CypA and CypB results in a ternary complex with calcineurin, which inhibits its phosphatase activity, thus preventing signal transduction during the activation cascade of T-cells (42). This is the molecular basis of the immunosuppressive action of CsA. Two separate domains in the undecapeptide CsA involved in binding to CypA/CypB and calcineurin can be discerned in the crystal structure of CsA bound to its ligands (43). It is now evident that the immunosuppressive activity of CsA can be separated from its binding affinity to cyclophilins and the consequent inhibition of the PPIase activity of this protein (39). Treatment of HEK293 cells expressing human ADAMTS13 with CsA shows similar levels of intracellular ADAMTS13 but lower extracellular levels compared with untreated cells (Fig. 1A). However, the levels of ADAMTS13 mRNA are comparable in both control and CsA-treated cells (Fig. 1B). In addition, we found that FK506 did not cause the same effect, indicating that this was not due to inhibition of calcineurin. It is therefore plausible that CsA perturbs the synthesis of ADAMTS13 through its inhibition of the CypB-mediated catalysis of the
aminoacyl-proline cis-trans interconversion. The reduced production of ADAMTS13 in cells treated with CsA are likely to be the result of clearance of the misfolded ADAMTS13 via the ER-associated degradation system (36) as suggested by our experiments utilizing ALLN (the cysteine protease inhibitor), which demonstrates slightly increased intracellular ADAMTS13 levels following the block of the degradation system (Fig. 5). We also do not exclude the possibility that altered folding may also lead to altered post-translation modifications of the protein.

A large body of literature supports the view that enzymatic catalysis of cis-trans isomerization plays an important role in in vivo protein folding, thus generating biologically active proteins (for reviews, see Refs. 10 and 44). We have therefore studied the enzyme kinetics of ADAMTS13 expressed in control cells and those treated with CsA (Fig. 3). We have found that treatment of cells for 48 h with CsA results in a >14-fold decrease in the activity of ADAMTS13. However, the observation that acute exposure to CsA has no effect on ADAMTS13 activity (Fig. 3) is consistent with the premise that treatment of cells with CsA during protein synthesis results in a misfolded protein product with altered conformation. Given the frequency of proline residues found in ADAMTS13 and their positioning at critical locations in the structure (supplemental Fig. S2), it is unlikely that a correctly folded and functionally active ADAMTS13 can be generated in the absence of PPIase activity (45). These results suggest that ADAMTS13 may be a substrate of CypB and that treatment with CsA may disrupt the interaction between CypB and ADAMTS13 and therefore interfere with the process of ADAMTS13 secretion, possibly affecting its proper folding in ER.

It is important to note that among TTP patients, the most common defect is impaired ADAMTS13 secretion (46–49). We have found that although the levels of intracellular ADAMTS13 were not significantly affected by treatment with CsA, such treatment resulted in almost complete loss of the secreted ADAMTS13 (Fig. 1, A and C). Limited tryptic digestion of ADAMTS13 generated in control cells and those treated with CsA show a clear difference in susceptibility to proteolysis of ADAMTS13, produced under these two different conditions. These experiments suggest that treatment with CsA may lead to conformational changes in the protein. Although there are clear evidences demonstrating that most cyclophilins are PPIases (40), and thus may play an important role in protein folding, it has also been suggested that cyclophilins may also act as chaperones, binding to peptide sequences containing proline residues (50–52). Interestingly, studies that support this view also show that CsA impairs the chaperone activity of the cyclophilin and thus the secretion of the target protein (53). It is conceivable that CypB plays an auxiliary role as a molecular chaperone that traffics through the entire secretory pathway in association with ADAMTS13. In support of this view, we demonstrate a direct interaction between ADAMTS13 and the CypB by co-immunoprecipitation experiments and note that this interaction is disrupted by CsA treatment (Fig. 2, A and C). We finally show that ADAMTS13 levels are significantly reduced in the plasma of CypB-deficient mice (Fig. 6).

Thus, the results presented in this study offer a potential mechanistic explanation for a vexing clinical outcome in transplant patients treated with the immunosuppressant CsA. Even if infrequent, transplantation-associated TTP is a devastating syndrome, and patients almost always require intensive support and have characteristically poor outcomes. Clinical studies and case reports going back almost 25 years have demonstrated that the use of immunosuppressive drugs are associated with the development of TTP in many cases of bone marrow transplantation (54). Occurrences of TTP in association with CsA treatment have also been reported following transplants of solid organs such as liver (55, 56), kidney (57), and lung (58, 59). These studies are carried out in extremely sick transplant patients with multiple clinical symptoms and thus it is difficult to estimate the precise incidence of TTP (60) that therefore may be underreported in the literature. However, the results presented here suggest that altered ADAMTS13 folding and secretion can contribute to the development of TTP in patients treated with CsA. The concentrations of CsA used in the present study are in line with, or even lower than those used in numerous other studies employing cellular models (23, 24, 27).

Moreover, the pharmacokinetics of commercial CsA formulations are recognized to be variable, with deviations in Cmax, trough levels, and area under the curve even with a standard dosing strategy (61). Thus a temporary excursion in blood levels outside the conventional therapeutic range is a distinct possibility in any patient receiving CsA therapy.

A number of clinical reports stand at odds with the data presented here, demonstrating that both the level and activity of plasma ADAMTS13 does not significantly change following immunosuppressive therapy with CsA (15, 62, 63). Undoubtedly, more in-depth clinical studies of levels and activity of ADAMTS13 in patients showing early signs of TTP following CsA treatment are needed to clearly delineate the clinical relationship between CsA, ADAMTS13, and TTP. Moreover, there are multiple other clinical observations and questions that to date have not been fully explained and addressed. For example, why does only a subset of patients treated with CsA develop TTP? What are the exact cellular and physiologic mechanisms underlying the manifestation of TTP in transplant patients not receiving CsA? It is worth noting that in addition to the reports of TTP in transplant patients treated with CsA, an apparently contradictory set of clinical studies support the prophylactic use of CsA in patients at risk for recurrences of TTP (64). The data from these studies indicate that the improvement in clinical outcomes following CsA treatment occurs in patients with idiopathic TTP (65–67). Acquired idiopathic TTP is attributed to autoantibodies that neutralize ADAMTS13 activity (26).

In these patients the positive clinical outcome may be attributed to the immunosuppressive property of CsA, i.e. to the formation of CsA-cyclophilin A/B complex inhibiting the serine-threonine phosphatase activity of calcineurin essential for cytokine induction. It must also be noted that the use of CsA in treatment of idiopathic TTP has for the most part been used as an additional measure associated with plasma exchange (65–67). In this scenario the correctly folded ADAMTS13 has already been synthesized and acute exposure to CsA would have no effect on the activity, as demonstrated in Fig. 3.
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Recently, CypB was shown to play a critically important role in the proper post-translational processing of collagen. Knock-out mice, as well as humans completely lacking functional CypB, have a form of osteogenesis imperfecta that results from altered collagen production. Because complete loss of CypB in mice models is compatible with life as reported by Bram and co-workers (38), it is possible that variations in the endogenous level of this chaperone in humans that may exist in different populations could perhaps explain some of the variability in the incidence of TTP following treatment with CsA. However, a drastic difference between low viability of human cells undergoing CypB siRNA treatment and normal viability of CypB-deficient mice is still not quite clear and hard to explain. Here we provided evidence for the direct association of CypB with ADAMTS13; however, as CsA affects other cellular processes, it cannot be completely excluded that other mechanisms may be involved in controlling ADAMTS13 maturation in conjunction or in parallel with CypB action.

However, taken into consideration all of the above, we believe that our findings may provide a mechanistic explanation for the occurrence of TTP in transplant patients undergoing CsA treatment. We suggest that there may be a direct link between the use of CsA and the occurrence of TTP, a relatively rare but clinically devastating hemostatic derangement in transplant patients.

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