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Mvwf, a Dominant Modifier of Murine von Willebrand Factor, Results from Altered Lineage-Specific Expression of a Glycosyltransferase

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Summary

We have identified altered lineage-specific expression of an *N*-acetylgalactosaminyltransferase gene, *Galgt2*, as the gain-of-function mechanism responsible for the action of the *Mvwf* locus, a major modifier of plasma von Willebrand factor (VWF) level in RIIIS/J mice. A switch of *Galgt2* gene expression from intestinal epithelial cell-specific to a pattern restricted to the vascular endothelial cell bed leads to aberrant posttranslational modification and rapid clearance of VWF from plasma. Transgenic expression of *Galgt2* directed to vascular endothelial cells reproduces the low VWF phenotype, confirming this switch in lineage-specific gene expression as the likely molecular mechanism for *Mvwf*. These findings identify alterations in glycosyltransferase function as a potential general mechanism for the genetic modification of plasma protein levels.

Introduction

von Willebrand factor (VWF) is a multimeric plasma glycoprotein that plays an essential role in hemostasis. In plasma, VWF stabilizes coagulation factor VIII and mediates the adhesion and aggregation of platelets to the subendothelium at sites of vascular injury, especially under conditions of high shear (Savage et al., 1996). VWF is synthesized exclusively in megakaryocytes and endothelial cells and is often used as a specific marker for the endothelial cell lineage. Endothelial cell–derived VWF can be secreted by a constitutive pathway or can induce the formation of Weibel-Palade bodies for regulated secretion (Wagner et al., 1991). Extensive posttranslational modification of VWF includes glycosylation, sulfation, multimerization, and propeptide cleavage (Nichols and Ginsburg, 1997).

Qualitative or quantitative abnormalities of VWF function result in von Willebrand disease (VWD), the most common inherited bleeding disorder of humans, with prevalence estimated at up to 1% of the population (Rodeghiero et al., 1987). Seventy percent of VWD is classified as type 1, characterized by quantitative reduction of plasma VWF to 20%–50% of the normal level. Inheritance of type 1 VWD is generally autosomal dominant, though with reduced penetrance and variable expressivity. *VWF* gene mutations have been identified in a subset of type 1 VWD individuals. However, the molecular defects responsible for most cases remain unknown and contributions from other genetic loci have been proposed (Ginsburg and Bowie, 1992; Nichols and Ginsburg, 1997).

The highly variable expressivity and incomplete penetrance of VWD, as well as the wide range of plasma VWF levels among normal individuals, has made it difficult to study potential VWF modifier genes in humans. The mouse has proven to be a powerful model organism for the dissection of genetic factors contributing to complex disorders (Todd et al., 1991) and the identification of modifier genes for variable phenotypes such as cancer predisposition (MacPhee et al., 1995) and infectious disease susceptibility (Vidal et al., 1993; Bellamy et al., 1998). We previously defined a major modifying locus for plasma VWF levels in RIIIS/J mice that we termed *Mvwf*. RIIIS/J plasma VWF levels are reduced by up to 20-fold, when compared to other inbred strains (Sweeney et al., 1990; Nichols et al., 1994). RIIIS/J mice also exhibit other characteristics similar to human type 1 VWD, including prolonged bleeding time and normal VWF multimer structure. Low VWF levels are inherited as an essentially pure autosomal dominant trait (Sweeney et al., 1990), suggesting a gain-of-function mutation mechanism (Mohlke et al., 1996). In an intercross between RIIIS/J and CASA/Rk, an inbred strain of *Mus musculus castaneus*, we mapped the *Mvwf* locus to a ∼0.3 centimorgan (cM) interval on distal mouse chromosome 11 (Mohlke et al., 1998), distinct from the murine *Vwf* gene on chromosome 6 (Mohlke et al., 1996; Nichols et al., 1994).

We now report the identification of *Mvwf* as a gain-of-function regulatory mutation in the gene encoding an *N*-acetylgalactosaminyltransferase, GALGT2. The RIIIS/J *Mvwf* allele switches *Galgt2* gene expression from predominantly gastrointestinal epithelial cell-specific to a pattern largely restricted to the vascular endothelial cell, the latter coincident with the site of plasma VWF synthesis. The resulting aberrant posttranslational modification of VWF by GALGT2 leads to dramatically increased clearance from plasma, accounting for the low VWF phenotype.

Results

To identify the gene responsible for low plasma VWF level in RIIIS/J mice, candidate transcripts previously mapped to the 0.3 cM, ∼300 kb *Mvwf* region on mouse chromosome 11 (Mohlke et al., 1998) were surveyed for mutations. Of the seventeen putative expressed sequences in this region, complete coding sequence was available for an ATP synthase (*Atp6n1*), gastric inhibitory peptide (*Gip*), and a known but previously unmapped gene encoding an *N*-acetylgalactosaminyltransferase that we termed *Galgt2*. The enzyme activity associated

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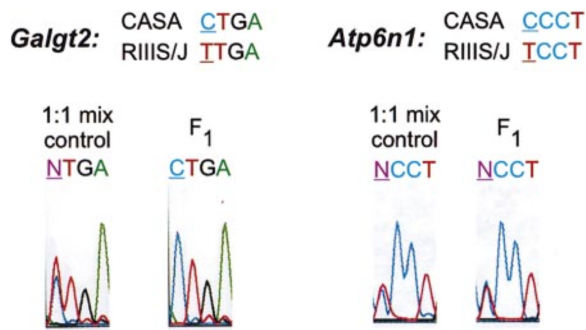

Figure 1. Relative Allelic Expression of *Mvwf* Candidate Genes

Sequencing chromatograms of RNA PCR product amplified from small intestine RNA (*Galgt2*) or liver RNA (*Atp6n1*). The relevant polymorphic nucleotide (underlined) is coincidentally a C in the CASA/Rk allele and a T in the RIIIS/J allele for both genes. Equal quantities of parental RNA PCR products were combined prior to sequencing to form the 1:1 mix controls. One of two (RIIIS/J $\times$ CASA/Rk)F_1 samples analyzed for each gene is shown. The control 1:1 mix samples for both genes and the F_1 PCR product for *Atp6n1* show equal expression from both alleles (N). In contrast, only expression from the CASA/Rk allele is seen for *Galgt2* amplified from the F_1 mRNA.

with the *Galgt2* gene generates the T lymphocyte–specific CT oligosaccharide differentiation antigen, and the human homolog of *Galgt2* is thought to generate the structure recognized as the human blood group Sd[a] oligosaccharide (Smith and Lowe, 1994). As we previously reported, sequence analysis of RNA PCR products spanning the coding regions for these three genes failed to identify any clearly significant amino acid differences (Mohlke et al., 1998).

Altered *Galgt2* Expression in Mice with Low Plasma VWF

To screen for transcriptional regulatory mutations in the *Atp6n1*, *Gip* and *Galgt2* loci, we used an RNA PCR strategy (Nichols et al., 1991) to detect potential allelic differences in expression of the cognate mRNAs. The incidental identification of neutral DNA variation during the initial sequencing facilitated this approach. Analysis of tissue from heterozygote mice allowed a direct comparison of mRNA expression from the mutant and wild-type alleles. In order to determine the relative expression levels of the RIIIS/J and CASA/Rk alleles at these three candidate loci, RNA PCR products spanning polymorphic nucleotides were amplified from total RNA prepared from liver (*Atp6n1*) or small intestine (*Gip* and *Galgt2*) of RIIIS/J, CASA/Rk, and (RIIIS/J $\times$ CASA/Rk)F_1 mice (abbreviated F_1). *Atp6n1* and *Gip* PCR products from F_1 RNA showed equivalent expression from both alleles (Figure 1 and data not shown). In contrast, *Galgt2* mRNA amplification from F_1 samples only detected a signal from the CASA/Rk allele (Figure 1). These results suggested that the CASA/Rk allele of *Galgt2* is preferentially expressed in F_1 mice.

To further characterize the apparently altered *Galgt2* mRNA expression pattern in RIIIS/J mice, a *Galgt2* RNase protection assay was performed on small intestine, kidney, liver, lung, spleen, and brain of adult RIIIS/J and CASA/Rk mice. A protected fragment of the predicted size was observed in the small intestine sample from the CASA/Rk mouse but not in any tissues from the RIIIS/J mouse. Levels of *Galgt2* mRNA detected in small intestine from two additional wild-type strains (C57BL/6J and 129/Sv) were similar to CASA/Rk, and the level from an (RIIIS/J $\times$ CASA/Rk) F_1 mouse appeared to be decreased by approximately 50% (data not shown). These results indicate that the gastrointestinal-specific expression of *Galgt2* observed in most wild-type mouse strains has been lost in RIIIS/J.

Normal Plasma VWF Levels in *Galgt2*-Deficient Mice

The observation of apparently reduced *Galgt2* expression in RIIIS/J mice suggested that maintenance of normal plasma VWF levels might require a threshold level of GALGT2, above that present in heterozygous F_1 mice. To test this hypothesis, we assayed VWF from plasma of mice lacking *Galgt2*. *Galgt2*-deficient mice generated by embryonic stem cell targeting are viable and fertile (P. L. Smith and J. B. Lowe, unpublished data). Plasma VWF level was measured in three female and three male 11-week-old *Galgt2*-deficient mice (produced from an eight-generation backcross to C57BL/6J). The mean VWF level of *Galgt2*-deficient mice (0.10 $\pm$ 0.05 units/ml) was not significantly different from the mean for 13 control C57BL/6J mice (0.13 $\pm$ 0.04 units/ml, $P > 0.24$). These results indicate that *Galgt2* gene expression is not required for the maintenance of normal plasma VWF levels and that *Mvwf* is not a simple loss-of-function mutation in *Galgt2*.

The *Dlb1* Polymorphism Requires Expression of *Galgt2*

Examination of available genetic maps for mouse chromosome 11 (Watkins-Chow et al., 1996) identified a histologic polymorphism termed *Dlb1*, which was functionally mapped by Uiterdijk et al. in 1986 to a region (3.1 $\pm$ 1.4 cM proximal to *Re*; Uiterdijk et al., 1986) potentially overlapping the *Mvwf* candidate interval. *Dlb1* was first detected in mouse small intestine sections with the plant lectin termed *Dolichos biflorus* agglutinin (DBA) by Ponder and colleagues in 1983 (Ponder and Wilkinson, 1983) and was subsequently used as a lineage-specific marker for intestinal epithelial cell migration (Ponder et al., 1985b; Schmidt et al., 1985). The DBA lectin recognizes terminal nonreducing *N*-acetylgalactosamine (GalNAc) residues. The *Dlb1* polymorphism is characterized by DBA binding primarily restricted to the intestinal epithelium of C57BL/6 and most other mouse strains, in contrast to the pattern of DBA binding to the vascular endothelium and absence from the epithelium observed in the RIII strain (an ancestral strain of RIIIS/J). Though the nature of the locus that determined expression of DBA-positive tissues was not known, this lectin's specificity for GalNAc and GalNAc-substituted glycans suggested that the *Dlb1* polymorphism was determined by tissue-specific expression of a GalNAc transferase (Ponder et al., 1985a).

To examine the potential relationship between *Dlb1* and *Galgt2*, we used immunofluorescence to examine

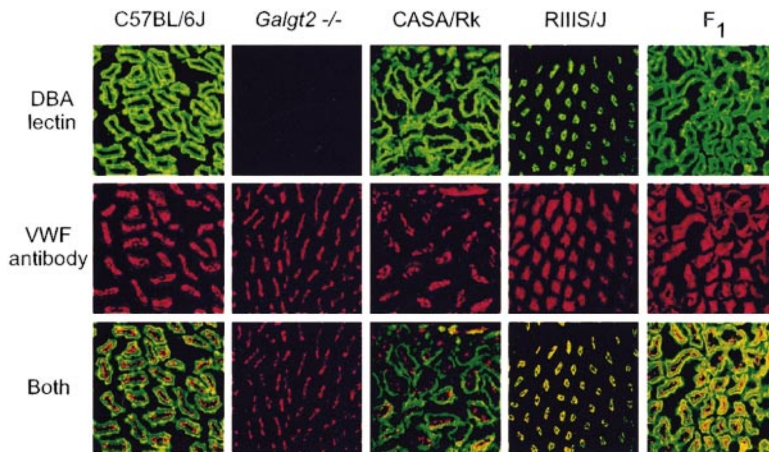

Figure 2. Altered Cell-Type Expression of *Galgt2*

Frozen sections of small intestine from C57BL/6J, *Galgt2*-deficient (*Galgt2*$^{-/-}$), CASA/Rk, RIIIS/J, and (RIIIS/J × CASA/Rk)F_1 mice were visualized with DBA lectin (green) and anti–von Willebrand factor antibody (red). The DBA lectin signal is restricted to intestinal epithelial cells in C57BL/6J and CASA/Rk and to endothelial cells in RIIIS/J. Both types of cells are labeled in (RIIIS/J × CASA/Rk)F_1, whereas no signal is seen in the *Galgt2*$^{-/-}$ section. The VWF antibody detects endothelial cells lining the vessels of the lamina propria located in the center of the intestinal villi and shows a similar pattern in each section. The bottom row shows a composite of the VWF and DBA lectin staining.

the DBA-binding pattern in the small intestine of C57BL/6J and *Galgt2*-deficient mice. Sections of small intestines were simultaneously analyzed with the DBA lectin and a polyclonal rabbit anti-human VWF antibody as a marker for endothelial cells (Figure 2). In the C57BL/6J sample, the DBA lectin bound to the villus epithelial cells, confirming the earlier report (Ponder et al., 1985a), whereas no lectin-binding was observed in *Galgt2*-deficient mice. These results demonstrate that the sugar structure detected by the DBA lectin in small intestine tissue sections requires the presence of the *Galgt2* gene product.

Altered Cell-type Expression of *Galgt2* in RIIIS/J Mice

To determine if altered *Galgt2* expression in the RIIIS/J strain is responsible for the *Dlb1* polymorphism, we performed immunofluorescence on sections from RIIIS/J, CASA/Rk, and F_1 progeny of a cross between the two strains. In the RIIIS/J sample, the DBA lectin signal was absent from the epithelial cells but was present in the central vessels of the villi and colocalized with VWF staining. In the CASA/Rk sample, the DBA lectin bound to the villus epithelial cells, as had been observed in C57BL/6J mice, but the villus microvasculature did not bind the lectin. The F_1 sample showed DBA lectin-binding to both epithelial and endothelial cells, consistent with codominance of the two *Galgt2* expression patterns (Figure 2).

Additional tissues were analyzed for DBA-binding in all of the above strains. In RIIIS/J and F_1 mice, the DBA signal was present in major blood vessels of the spleen and kidney and was coincident with the location of VWF, as observed in the intestinal microvasculature. No DBA signal was detected in these tissues in the other mouse strains, aside from staining in a subset of kidney tubules in C57BL/6J mice (data not shown).

Analysis of *Galgt2* mRNA expression by in situ hybridization detected a strong signal in the colon epithelium of C57BL/6J, with no specific signal evident in the spleen. In contrast, in the RIIIS/J mouse, *Galgt2* expression was clearly evident in the splenic vascular endothelium but was undetectable in the colon (Figure 3). These data demonstrate that the *Dlb1* polymorphism detected by the DBA lectin is the result of a switch in cell-type-specific *Galgt2* gene expression from gastrointestinal epithelial to vascular endothelial cells, rather than variation in the availability of a specific glycoprotein substrate or altered localization of GalNAc-decorated proteins synthesized at other sites. The much greater overall level of *Galgt2* expression in the colon of C57BL/6J compared to that in RIIIS/J, as evident by *in situ* hybridization (Figure 3), also explains the failure to detect a signal from the RIIIS/J allele of the F_1 mouse in the competitive RNA PCR experiment shown in Figure 1.

To explain how the altered *Galgt2* cell-type expression pattern results in reduced plasma VWF levels, we proposed the model shown in Figure 4. In most mouse strains, *Galgt2* is expressed primarily in the intestinal epithelium, but not in the vascular endothelium, the primary site of VWF biosynthesis. In such strains of mice, posttranslational modification of VWF would not include glycan modification catalyzed by GALGT2. However in RIIIS/J mice, a switch of *Galgt2* gene expression to the endothelial cell compartment leads to GALGT2-mediated transfer of GalNAc onto the glycans that decorate endothelial cell-synthesized proteins, including VWF. GalNAc-decorated VWF in this strain is consequently secreted in an inefficient manner, or, alternatively, is more rapidly cleared from plasma, resulting in the observed reduction in VWF level.

GALGT2-Mediated Transfer of GalNAc onto VWF

To determine if VWF can display the proper precursor sugar structure substrate for modification by GALGT2, recombinant VWF was synthesized in the presence or absence of GALGT2 and subsequently assayed for the GalNAc modification. Control CHO cells and CHO cells stably expressing *Galgt2* were transiently transfected with an expression vector containing the full-length human *VWF* cDNA. Conditioned media prepared from both cell types contained similar levels of VWF antigen. Media were also analyzed by a modified sandwich ELISA for the presence of DBA-detectable GalNAc on VWF. A strong signal was observed for VWF synthesized in *Galgt2*-expressing cells ($>12 \times$ background). In contrast, no DBA-specific signal was detected for VWF synthesized in control *Galgt2*-negative cells. These results indicate

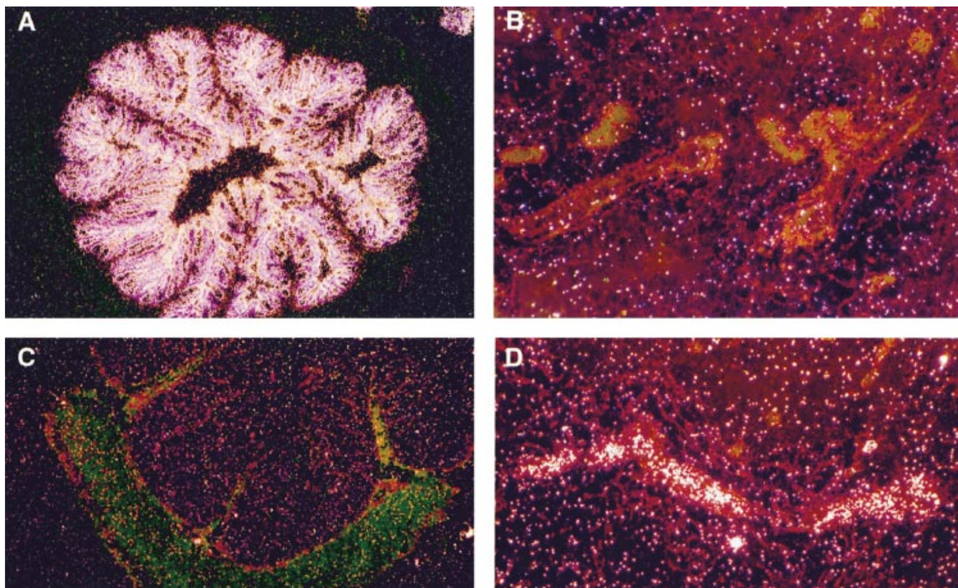

Figure 3. *Galgt2* RNA In Situ Hybridization

RNA in situ hybridization was performed with a *Galgt2* probe using colon (A and C) and spleen (B and D) sections from C57BL/6J (A and B) and RIIIS/J (C and D) mice. A strong signal is seen in the colonic epithelium of C57BL/6J and is absent from the corresponding RIIIS/J section. In contrast, the specific staining seen in the endothelial cells of large vessels in the spleen of RIIIS/J is absent from C57BL/6J.

that GALGT2 can modify the human VWF protein to contain the structure recognized by the DBA lectin and that this reactivity is not detectable in the absence of *Galgt2*.

To determine if a significant fraction of RIIIS/J mouse plasma VWF contains the GALGT2-dependent structure recognized by the DBA lectin, plasma samples were tested in the same DBA-VWF ELISA. No DBA-detectable GalNAc signal was observed in RIIIS/J, CASA/Rk, F_1, C57BL/6J, or *Galgt2*-deficient mice, suggesting that few or no terminal GalNAc residues are present on circulating murine plasma VWF. These results are consistent with the hypothesis that GALGT2-modified VWF is either very rapidly cleared from plasma or inefficiently synthesized or secreted from the endothelial cell compartment (Figure 4).

VWF Modified by GALGT2 Is Rapidly Cleared

To distinguish among these potential mechanisms for the GALGT2-dependent modification of plasma VWF levels, we measured the clearance rate of exogenously administered, epitope-tagged, recombinant human VWF with or without modification by GALGT2. Concentrated conditioned media containing these recombinant forms of VWF were injected into the tail veins of C57BL/6J mice. Blood samples were subsequently collected at times up to 45 min postinjection, and the relative amount of epitope-tagged VWF was determined. VWF modified by GALGT2 was cleared rapidly from plasma, relative to unmodified VWF (Figure 5). The half-life ($t_{1/2}$) of modified VWF was ~25 min, while the $t_{1/2}$ of unmodified VWF was ~300 min. The latter value is comparable to the ~4 hr $t_{1/2}$ of recombinant human VWF in the rat (Stoddart et al., 1996) or dog (Schwarz et al., 1997) though shorter than the 8–16 hr $t_{1/2}$ observed for plasma-derived VWF in humans. These results suggest that the reduced level of VWF observed in RIIIS/J plasma is due to accelerated clearance of GALGT2-modified VWF. The previous report that RIIIS/J mice exhibit normal levels of platelet VWF (Sweeney et al., 1990) is also consistent with this

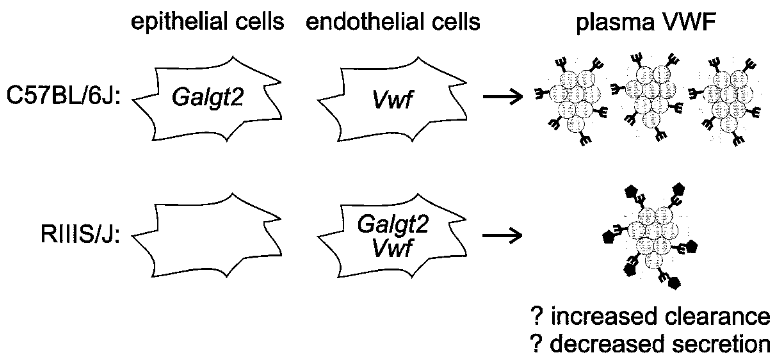

Figure 4. Model for Mechanism of *Mvwf* Action

In most mouse strains, *Galgt2* is expressed primarily in intestinal epithelial cells and *Vwf* is expressed in vascular endothelial cells. VWF secreted from endothelial cells circulates in the plasma at normal steady-state levels. However in RIIIS/J mice, *Galgt2* expression is switched from epithelial to endothelial cells, leading to the transfer of GalNAc (black pentagons) onto oligosaccharides present on VWF. The novel sugar structure on the secreted VWF results in decreased steady-state plasma levels, either through interference with the secretory pathway or more rapid clearance of VWF from plasma.

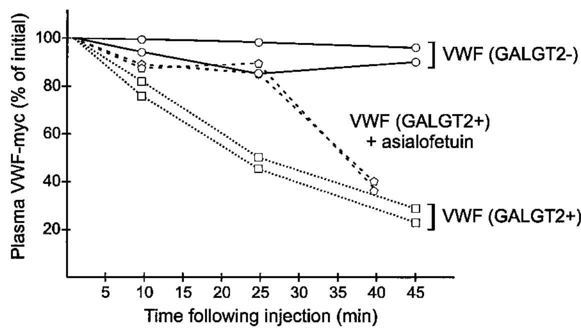

Figure 5. Altered Clearance of VWF Modified by GALGT2

Human recombinant c-myc-epitope-tagged VWF synthesized in the presence (squares on dotted lines) or absence (circles on solid lines) of GALGT2 was injected into C57BL/6J mice. Amounts of c-myc-epitope-tagged VWF in plasma samples collected at 1, 10, 25, and 45 min postinjection were determined and are shown as percent of amount detected at 1 min. In the experiments indicated by pentagons on dashed lines, excess asialofetuin as competitor was injected immediately prior to injection of GALGT2-modified VWF. Each line represents the results obtained from a single mouse.

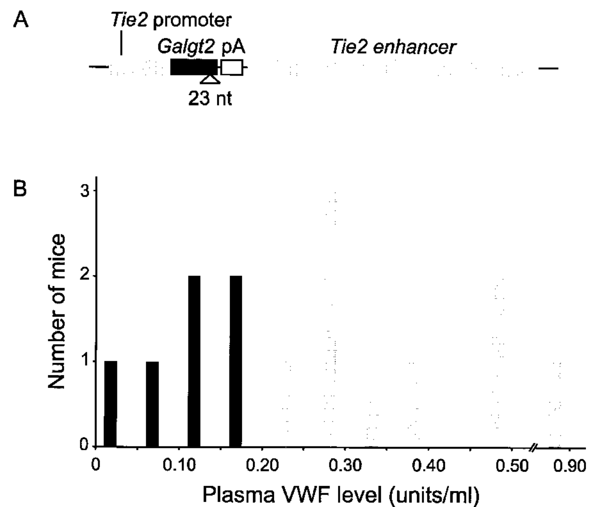

Figure 6. Transgenic Expression of *Galgt2* in Endothelial Cells Results in Decreased Plasma VWF

(A) Structure of the 15 kb transgene construct, showing the murine *Tie2* promoter, the murine *Galgt2* cDNA with a 23 bp deletion engineered as a marker into the 3′UTR, SV40 intron and poly A signals (pA), and the murine *Tie2* enhancer fragment.

(B) Plasma VWF levels of transgenic progeny generated from two transgenic founders mated to CASA/Rk. Six transgenic progeny (black bars) have significantly lower plasma VWF levels than nine non-transgenic littermates (gray bars). The average VWF level in CASA/Rk mice is arbitrarily defined as 1.0 unit/ml.

hypothesis, since VWF synthesized in the megakaryocyte and sequestered within the platelet α granule should be protected from accelerated clearance.

The high affinity of the asialoglycoprotein receptor for structures containing subterminal GalNAc (up to 60-fold greater than for galactose residues exposed following removal of terminal sialic acids; Kolatkar et al., 1998) suggests that GALGT2-modified VWF may be cleared via this receptor. To test this hypothesis, the disappearance of modified VWF was measured following the bolus infusion of excess asialofetuin, a competitive ligand for the asialoglycoprotein receptor. Under these conditions, clearance of GALGT2-modified VWF was temporarily extended to a rate similar to that of unmodified VWF (Figure 5). These results suggest that the accelerated clearance of GALGT2-modified VWF is mediated via the asialoglycoprotein receptor.

Endothelial Cell-Specific *Galgt2* Expression Is Sufficient to Reduce Plasma VWF

To confirm the hypothesis that endothelial cell expression of *Galgt2* is sufficient to lower plasma VWF level, we generated mice carrying a *Galgt2* transgene under the control of the vascular endothelial cell–specific mouse *Tie2* promoter and enhancer (Schlaeger et al., 1997) (Figure 6A). The average plasma VWF level of nine independent founder transgenic mice (0.09 $\pm$ 0.07 U/ml) was significantly lower than the average level of 22 non-transgenic littermates (0.19 $\pm$ 0.11 U/ml, $P < 0.02$). Two transgenic founders with plasma VWF levels of 0.06 and 0.04 U/ml were mated to CASA/Rk mice to generate progeny on a genetic background of high VWF levels. Transgenic progeny showed significantly lower plasma VWF levels (0.11 $\pm$ 0.05 U/ml) than their nontransgenic littermates (0.40 $\pm$ 0.20 U/ml, $P < 0.005$) (Figure 6B). These levels compare to the values of 0.06 U/ml for RIIIS/J mice, 0.14 U/ml for (RIIIS/J $\times$ CASA/Rk)F_1 mice, and 1.0 U/ml for CASA/Rk mice. These results demonstrate that expression of *Galgt2* directed to the endothelial cell compartment is sufficient to reproduce the low plasma VWF phenotype characteristic of the RIIIS/J *Mvwf* allele.

Discussion

Taken together, the data reported here identify a novel mechanism for the inherited deficiency of a specific plasma protein. Reduction of plasma VWF in the RIIIS/J mouse was shown to be due to a gain-of-function regulatory mutation in a previously known glycosyltransferase enzyme, GALGT2, resulting in its switch from a predominantly gastrointestinal epithelial cell to a vascular endothelial cell–specific pattern of expression. This altered expression results in aberrant posttranslational modification leading to rapid clearance of VWF from plasma, as confirmed by the low VWF phenotype observed in mice carrying an endothelial cell–specific *Galgt2* transgene.

Glycosyltransferase Polymorphisms as Genetic Modifiers of Human VWD

The most common form of VWD in humans (type 1) results from quantitative decreases in plasma VWF to 20%–50% of the normal level. Thus, a reduction of plasma VWF in the range produced by *Mvwf* (2- to 20-fold) would be expected to produce clinically significant bleeding. Though at least some cases of human type 1 VWD are associated with haploinsufficiency at the *VWF* locus, the existence of locus heterogeneity has been suggested (Ginsburg and Bowie, 1992; Mohlke and Ginsburg, 1997) including the possibility of mutations resulting in altered posttranslational processing (Stoddart et al., 1996). However, a clear example of human

VWD unlinked to the *VWF* locus has not yet been reported. The human homolog of *Galgt2* is thought to determine the Sd[a] blood group antigen (Smith and Lowe, 1994). Though there is currently no evidence for a human *Galgt2* mutation similar to *Mvwf*, our results demonstrate that genetically determined alterations in glycosylation, and potentially defects at other steps in posttranslational processing, can result in profound reductions in plasma VWF leading to a significant bleeding phenotype. In addition, our findings further illustrate the utility of the mouse as a model system for identifying modifier loci for complex mammalian phenotypes. Genetic modifiers identified in this way may also prove to be directly relevant to humans, as recently demonstrated for the *NRAMP1* gene and susceptibility to tuberculosis (Bellamy et al., 1998).

Prior studies suggest that approximately 60% of the total variance in human plasma VWF level is determined by genetic factors. The most significant of these factors is the effect determined by the ABO blood type (Gill et al., 1987), the latter accounting for 30% of this genetic variation (Orstavik et al., 1985). ABO blood group is determined by polymorphism in the glycosyltransferases encoded by the *ABO* locus, and the resulting structures are known to be present on *N*-linked sugar chains of plasma VWF (Sodetz et al., 1979). Though proposed to alter plasma VWF processing, secretion, or stability (Gill et al., 1987), the precise mechanism for the effect of the *ABO* locus on VWF level is unknown. A clearance mechanism similar to but more subtle than that we have demonstrated for *Mvwf* may also explain the effect of ABO in humans, as well as the known minor effects of other blood group antigens (Orstavik et al., 1989). Our results also suggest that polymorphic variation in glycosyltransferase function or other steps in posttranslational processing may account for the large component of genetic variation in VWF level that remains to be identified. These potential modifier loci could be significant determinants of genetic predisposition to thrombosis and atherosclerosis through their effects on plasma VWF levels (Lip and Blann, 1997).

Alterations in posttranslational processing, including glycosylation, may represent an important general mechanism for genetic modifiers of human disease. Genetically determined variation in glycosylation may be particularly relevant for regulating the steady-state level of plasma proteins such as VWF, as even small changes in carbohydrate structure could have profound effects on either processing through the secretory pathway or clearance mechanisms. In addition, given the large number of genes participating in the assembly of *N*- and *O*-linked glycoprotein structures (Varki, 1993), the opportunity exists for considerable genetic diversity.

Regulatory Changes in Gene Expression as Disease-Causing Mutations

Disease-causing mutations have been previously reported due to alteration at glycosylation sites within a target protein or loss-of-function for specific enzymes within the glycosylation pathway (Varki, 1993; Kornfeld, 1998). In addition, transgenic misdirection of an N-acetylglucoaminyltrasferase to the liver was recently shown to produce aberrant hepatic cellular morphology (Ihara et al., 1998). However, to our knowledge, the current report is the first example of a naturally occurring phenotype resulting from ectopic expression of a component of the posttranslational processing machinery.

The altered tissue-specific pattern of glycosylation described here might be expected to perturb function for a broad class of endothelial cell proteins. It is thus remarkable that the phenotype observed in the RIIIS/J mouse is primarily restricted to a mild bleeding abnormality (Sweeney et al., 1990). This unexpected impact on a specific protein product by a generalized alteration in posttranslational processing would have made it difficult to predict the molecular basis for *Mvwf* without the aid of positional cloning methodologies. Of note, the RIII pattern of staining was also observed in several other mouse strains not known to be related to RIII (Ponder et al., 1985a). This latter observation, taken together with the novel molecular mechanism we have defined, suggests that *Mvwf* may represent an ancient founder allele among laboratory mice and a potential site of variation among wild mouse populations.

The unusual observation of nearly identical phenotypes in homozygotes and heterozygotes for the RIIIS/J allele of *Mvwf* (the presence of one or two gene copies producing a similar effect), originally suggested that the mutant allele results from a gain-of-function mutation. Pure dominant inheritance of this type is unusual among human genetic disorders. In the few clear examples, such as Huntington's disease (Huntington's Disease Collaborative Research Group, 1993), the disease-causing mutation generally results in a coding sequence change leading to a new or altered protein function. In contrast, the *Mvwf* gain-of-function is a switch in the tissue-specific pattern of expression, rather than an alteration in the protein product itself.

There are several well-described examples of inherited human diseases associated with alterations in temporal or developmental gene expression, such as hereditary persistence of fetal hemoglobin (Cunningham and Jane, 1996) and factor IX Leiden (Crossley et al., 1992). However, there are few precedents for a natural mutation producing an altered tissue-specific pattern. Decreased expression in normal tissue, concurrent with aberrant ectopic expression, has been observed in several mouse mutations including the *W-sash* allele of the c-Kit receptor gene (Duttlinger et al., 1993), the *Steel-contrasted* allele of the melanocyte growth factor gene (Bedell et al., 1995), and a number of mutant alleles for the agouti coat color gene (Duhl et al., 1994). However, in all of these examples, gene expression is altered from a tissue-specific to a more general pattern. The unique switch from one cell-specific pattern to another, as observed for the *Mvwf* mutation, suggests a novel molecular mechanism. No obvious regulatory mutation has yet been detected by routine Southern blot analysis of *Galgt2* genomic structure or direct sequence analysis through the first 1.5 kb of the upstream promoter. However, several of the transcriptional mutants described above act at a great distance, with responsible mutations located as far as 400 kb from the relevant promoters (Bedell et al., 1996). Though the *Mvwf* mutation may similarly lie at a great distance from the *Galgt2* gene, a

more proximal location adjacent to the promoter or within an intron of the *Galgt2* gene cannot be excluded. Based on current genetic data (Mohlke et al., 1998), the *Mvwf* mutation must lie somewhere within the 300 kb interval surrounding *Galgt2*.

Heterogeneity among Endothelial Cell Gene Expression Programs

The absence of detectable GALGT2-modified VWF in the plasma of RIIIS/J mice suggests that this large pool of VWF is rapidly cleared. The source of the remaining unmodified VWF in plasma is unclear. The RIIIS/J *Galgt2* allele may direct expression to a subset of endothelial cells only partially overlapping the distribution of cells expressing the *Vwf* gene. The subset of unmodified VWF molecules persisting in RIIIS/J plasma would thus represent the product of a *Vwf*-positive/*Galgt2*-negative endothelial cell subpopulation. Alternatively, the unmodified plasma VWF pool could represent a subset of VWF molecules that have escaped complete modification in *Galgt2*-expressing cells.

It is becoming increasingly clear that the gene expression program is highly variable among endothelial cells of different vascular beds. Though frequently used as a general marker for endothelial cells, VWF is known to be expressed at high levels in capillaries, veins, and venules, with lower expression in most large arterial vessels (Rand et al., 1987). A portion of the human *VWF* promoter (−487 to +246) has been shown to target expression to the yolk sac vasculature and to only a subset of endothelial cells in the adult brain (Aird et al., 1995). Similar age-restricted results were obtained in *Tie2-LacZ* transgenic mice when only the promoter element was included (Schlaeger et al., 1995). However the addition of a 10-kb intron 1 *Tie2* enhancer element expanded expression specifically and uniformly throughout the vasculature of both embryonic and adult mice (Schlaeger et al., 1997). The observation that GALGT2 may modify up to 95% of the total VWF plasma pool suggests that the endothelial cell pattern conferred on *Galgt2* by the *Mvwf* mutation closely matches that of the *Vwf* gene. The transgenic expression of *Galgt2* under control of the *Tie2* promoter/enhancer would also appear to have a very similar endothelial cell pattern. These observations suggest that the *Mvwf* and *Tie2* transcription programs are both very broad, encompassing nearly the full subset of endothelial cells expressing VWF. The future definition of the complex transcriptional elements within the RIIIS/J *Mvwf* allele may provide important insights into the regulation of endothelial cell–specific gene expression. In addition, the shutoff of *Galgt2* expression in the gastrointestinal tract coincident with the endothelial cell–specific induction may shed light on more general processes of tissue-specific gene regulation.

Taken together, our observations illustrate the potential importance of tissue-specific differences in post-translational processing. Though variation in glycosylation across species and different cell types has been well described (Varki, 1993), this variability has generally not presented a problem for the ectopic expression of proteins for therapeutic purposes, such as gene therapy (Verma and Somia, 1997). However, our results suggest that production of a recombinant plasma protein at alternative tissue sites, or in cells with a glycosylation program distinct from wild-type, could have unexpected consequences.

Experimental Procedures

RNA PCR and Sequencing

Small intestine and liver RNA from adult RIIIS/J, CASA/Rk, and (RIIIS/J × CASA/Rk)F_1 mice were isolated using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD). RNA PCR was performed as described (Nichols et al., 1998). To amplify the 5′ portion of the *Galgt2* cDNA, corresponding to nucleotides 6–1031 of the published sequence (Smith and Lowe, 1994), small intestine RNA reverse-transcribed with primer 1 (5′-AGTAGTCACCTGTG-3′) was amplified with primers 2 (5′-GTAGGCAGTCTGCAGAAGTGGCT-3′) and 3 (5′-CAGG TTCCTCCCAGCAAACCAG-3′). Similarly, the 3′ portion of *Galgt2*, nucleotides 973–1600, was reverse-transcribed with primer 4 (5′-GCCGCTAATCTGTT-3′) and amplified with primers 5 (5′-ACTACGT GGAGTACTACACCATG-3′) and 6 (5′-ATTGCGATTCCTGGTCTTG GTACT-3′). For a control in the comparative expression study, equal quantities of *Galgt2* 3′ RNA PCR products amplified from RIIIS/J and CASA/Rk were combined prior to sequencing with primer 7 (5′-CTGGAGAAAACCGAACTGGATGTG-3′). All comparative expression analyses were performed on independent samples from two different (RIIIS/J × CASA/Rk)F_1 mice.

Primers used to sequence and test the relative allele expression of *Atp6n1* were designed from mouse and rat cDNA sequences (GenBank accession numbers L19737 and D13123). The *Atp6n1* cDNA was reverse-transcribed from liver RNA samples using primer 8 (5′-TAATGGTAAAGCTCA-3′), amplified with primers 9 (5′-GGGAG TGGGAGTGCAGATTGAA-3′) and 10 (5′-AGCAGGGACGGCTGGGT GAC-3′), and sequenced with primer 10. Similarly, primers used to analyze *Gip* were designed from mouse, rat, and human sequences (GenBank accession numbers U34295, X66724, and M18185). The *Gip* cDNA was reverse-transcribed from small intestine RNA samples using primer 11 (5′-GTTTGGTTCTCTCAGA-3′), amplified with primers 12 (5′-CTGTTTGGAGCAAGATCCTGAGAAC-3′) and 13 (5′-AAGCAGGAGCCAAGCAAGCTAAG-3′), and sequenced with primer 14 (5′-AGGAAGATGGTGGCTTTGAAGACCT-3′).

RNase Protection

Ribonuclease protection assays were performed using the RPA II kit (Ambion Inc., Austin, TX). A *Galgt2* DNA template containing a T7 RNA polymerase recognition site was generated by PCR using primers 5′-GGCTCTTCCCCAAAAATCAGTGTG-3′ and 5′-GGATCCT AATACGACTCACTATAGGGAGGGCTCTTCACTCTCTCAAG-3′. The protected fragment corresponds to nucleotides 269–406 of the *Galgt2* cDNA (Smith and Lowe, 1994). A pTRI-β-actin-mouse plasmid (Ambion, Inc.) was used as the template for the control actin probe. Riboprobes were labeled with [α-^{32}P]UTP and transcribed with T7 RNA polymerase using 100 ng of *Galgt2* template and 500 ng of actin template. To remove the DNA templates, riboprobes were treated with 20 units of RNase-free DNase (Boehringer Mannheim, Indianapolis, IN) and were extracted using TRIzol Reagent. A total of 10^5 cpm of riboprobe was added to 10 μg of sample RNA.

Immunofluorescence

Small intestine, spleen, and kidney blocks from RIIIS/J, CASA/Rk, (RIIIS/J × CASA/Rk)F_1, C57BL/6J, and *Galgt2*-deficient mice were prepared in O. C. T. compound (Tissue-Tek). Twelve-micrometer sections were cut using a cryostat and air-dried at room temperature for 30 min. Immunofluorescence was performed essentially as described (Nichols et al., 1998). Tissue sections were blocked in 6% goat serum, 0.05% Triton X-100, 0.05% Tween 20 in PBS. Sections were incubated with 5 μg/ml DBA-biotin (EY Laboratories, San Mateo, CA) and 28.5 μg/ml rabbit anti-human VWF (Dako, Carpinteria, CA) overnight at 4°C, followed by incubation with 7.5 μg/ml FITC-avidin (EY Laboratories) and 1.2 μg/ml Texas red goat anti-rabbit IgG (ICN Pharmaceuticals, Aurora, OH) for 1 hr at room temperature.

Slides were mounted with Immuno Fluore Mounting Medium (ICN) and visualized at 100-fold magnification.

RNA In Situ Hybridization

RNA in situ hybridization was performed as described (Smith et al., 1996) using frozen sections of small intestine, kidney, spleen, and colon from 12-week-old RIIIS/J and C57BL/6J mice. The *Galgt2* probe consists of nucleotides 45–361 of the *Galgt2* sequence (Smith and Lowe, 1994). Slides were exposed to emulsion for ten days to four weeks.

Synthesis of VWF Modified by GALGT2

Generation and maintenance of control CHO-Tag cells and Sd^a-CHO-Tag cells, engineered as a stable line expressing *Galgt2*, have been described previously (Smith and Lowe, 1994). The full-length human *VWF* cDNA in pMT2 also has been described previously (Bonthron et al., 1986). The VWF-myc plasmid contains the human VWF cDNA through the penultimate codon of the coding sequence cloned into the XbaI-EcoRI fragment of pcDNA3.1(−)Myc-His A vector (Invitrogen, Carlsbad, CA). The resulting c-myc-epitope-tagged VWF protein contains the following amino acids at the carboxyl end, following the penultimate residue of native VWF (serine 2812): RIPPHWTSGSELGTKLGPEQKLISEEDLNSAVDHHHHHH. CHO-Tag and Sd^a-CHO-Tag cells in 100 mm dishes were transiently transfected with 5 μg plasmid using approximately 35 μl LipofectAMINE (GIBCO-BRL), and recombinant VWF was collected in OptiMEM I Reduced Serum Media (GIBCO-BRL) 48–72 hrs after transfection. Media was collected into a cocktail of protease inhibitors (5 μg/ml aprotinin, 100 μg/ml trypsin inhibitor, 1 mM PMSF, 2.5 mM EDTA, 1 μg/ml pepstatin), clarified by centrifugation at 2100 × g for 10 min, and concentrated 30- to 40-fold using Centriplus concentrators, 100 kDa MW cutoff (Amicon, Beverly, MA).

Clearance Studies

Plasma clearance studies were performed in 9-week-old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) anesthetized with an intraperitoneal injection of sodium pentobarbital (approximately 1 mg/g body weight). Mice remained anesthetized for the duration of the experiment. 1.5–2 μg of VWF in a total volume of 100–250 μl was intravenously injected into the lateral tail vein. Approximately 30 μl of blood was collected at the indicated time points by retro-orbital plexus puncture using heparinized Natelson collecting tubes (Fisher Scientific, Pittsburgh, PA). The first collection was within 1 min of injection and is denoted as 1 min. Plasma was isolated by centrifugation at 2100 × g for 10 min. For clearance competition assays, at least 300 μg of asialofetuin (A1908, Sigma Chemical Co., St. Louis, MO) at 4 μg/μl in PBS was injected 1–4 min prior to VWF injection. $t_{1/2}$ values were calculated from a line fit to data averaged from two mice.

VWF ELISAs

Mouse plasma VWF levels were determined by a sandwich ELISA using unconjugated rabbit anti-human VWF as the coating antibody and horseradish peroxidase-tagged rabbit anti-human VWF (Dako) as the detecting antibody, as previously described (Mohlke et al., 1996). Aliquots of pooled C57BL/6J plasma was used as a standard, and the average plasma VWF level in CASA/Rk was arbitrarily defined as 1.0 unit/ml. A similar ELISA was used to determine the concentration of recombinant human VWF, with normal human plasma (Bio/Data, Horsham, PA) as a standard.

To measure the level of terminal GalNAc present on recombinant and plasma VWF samples, the human plasma VWF ELISA was modified to use a peroxidase-conjugated DBA lectin (0.4 ng/μl) in place of the peroxidase-conjugated anti-VWF antibody. For clearance studies, quantity of c-myc-tagged VWF was determined by sandwich ELISA similar to mouse plasma VWF ELISAs except that detection of the myc epitope was accomplished with mouse monoclonal anti-c-myc peptide antibody (Zymed Laboratories, South San Francisco, CA), biotinylated as described (Lyons et al., 1992). Aliquots of c-myc-tagged VWF synthesized in *Galgt2*-containing cells were used as a standard.

Transgenic Mice

To express *Galgt2* in endothelial cells, *Galgt2* was placed under the control of the previously described murine receptor tyrosine kinase *Tie2* promoter and enhancer (Schlaeger et al., 1997). The partial *Galgt2* cDNA used encompasses the entire coding region of the gene (Smith and Lowe, 1994) but lacks most untranslated sequence. To facilitate detection of the transgene on an endogenous background, 23 nucleotides of the 3′ untranslated region were removed by a PCR technique using the primer 5′-GCTCTAGATGTTGCCCGC CGCTAATCTGTTCTTTTTAAGTGGAGCAGTAGAG-3′ (an underline indicates the nucleotides flanking the deletion). The construct (Figure 6A) consisted of a 2.1 kb HindIII *Tie2* promoter fragment followed by a 1.6 kb XhoI *Galgt2* cDNA fragment followed by an SV40 intron and poly A signal derived from the pcDNA1/Amp vector (Invitrogen). In addition the full 10 kb *Tie2* enhancer, an NgoAIV/NotI fragment transferred through pBluescript II SK^+ to generate a BssHII fragment, is located after the poly A signal. Plasmids containing the *Tie2* promoter and enhancer were kindly provided by T. Sato (University of Texas Southwestern Medical Center). A NotI digest released the 15 kb transgene from the pGEM5z (Promega, Madison, WI) vector.

Transgenic mice were generated in the Transgenic Core facility at the University of Michigan. Transgene DNA was injected into fertilized eggs produced by $(C57BL/6 \times SJL)F_1$ parents. Pups were genotyped using primers that span an intron as well as the 23-nucleotide cDNA deletion: 5′-TCCCATCTTGCAACGAGTGGC-3′ and 5′-TTGTTGCCCGCCGCTAATCTGTT-3′. Selected founder animals were mated to CASA/Rk, a strain previously shown to have plasma VWF levels ∼20-fold higher than RIIIS/J (Mohlke et al., 1996). Blood samples were collected by retro-orbital plexus puncture and plasma VWF level was determined as previously described (Mohlke et al., 1996). While the reported plasma VWF level for founder transgenic mice is based on a single blood sample, levels among progeny are the average of three samples obtained between four and eight weeks of age. Average plasma VWF levels of transgenic and nontransgenic progeny were compared by two-tailed Student's *t* test.

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