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ORIGINAL ARTICLE

Most factor VIII B domain missense mutations are unlikely to be causative mutations for severe hemophilia A: implications for genotyping

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Summary. *Background & Objective:* The factor VIII (FVIII) B domain shares very little amino acid homology with other known proteins and is not directly necessary for procoagulant activity. Despite this, missense mutations within the B domain have been reported in patients with hemophilia A. Given that the B domain is dispensable for secretion and function of FVIII, we hypothesized that these mutations should not be causative of hemophilia A in these patients. *Methods:* Plasmid vectors containing B domain missense mutations that were reported to be associated with moderate/severe hemophilia A (T751S, D826E, V993L, H1047Y, T1353A, N1441K, L1462P, E1579D, A1591S, P1641L and S1669L) were analyzed for their effect on synthesis and secretion compared with FVIII wild-type (WT) following transient transfection into COS-1 and CHO cells *in vitro*. Further, H1047Y, N1441K and E1579D mutants were expressed *in vivo* in a hemophilia A mouse model by hydrodynamic tail-vein injection. *Results:* FVIII activity and antigen levels for all mutants expressed into the conditioned media of COS-1 and CHO cells were similar to FVIII WT. Also, plasma expression of these mutants was similar to FVIII WT in hemophilia A mice. An *in vivo* tail clip bleeding assay also demonstrated that blood loss from hemophilia A mice expressing FVIII WT, H1047Y, N1441K and E1579D was similar. *Conclusions:* We conclude that most missense mutations within the FVIII B domain would be unlikely to lead to severe hemophilia A and that the majority of such missense mutations represent polymorphisms or non-pathologic mutations.

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Introduction

Factor (F) VIII is a large plasma glycoprotein that functions as an essential cofactor for the proteolytic activation of FX by activated FIX within the intrinsic pathway of blood coagulation [1]. Deficiency or dysfunction of FVIII results in hemophilia A, which is phenotypically stratified as mild, moderate or severe disease based on residual plasma FVIII activity of $> 5\%$, 1%–5% or $< 1\%$, respectively. FVIII is synthesized as a single-chain polypeptide of approximately 280 kDa with the domain structure A1-*a1*-A2-*a2*-B-*ap*-A3-C1-C2, with italics denoting heavy chain acidic regions and a light chain activation peptide [2,3]. This domain structure is also shared by the homologous coagulation cofactor, FV. Following synthesis, cleavage within the B domain results in a variably sized heavy chain (A1-*a1*-A2-*a2*-B, 90–200 kDa) and a light chain (*ap*-A3-C1-C2, 80 kDa) that are associated as a heterodimer through a divalent metal ion linkage between the A1 and A3 domains [4]. The A domains share 35–40% amino acid identity and are homologous to the A domains of ceruloplasmin and FV. The C domains also display 35–40% amino acid identity to each other and to FV and are homologous to proteins that are capable of binding negatively charged phospholipids, suggesting a role in phospholipid interactions [2,3,5].

A database of mutations described as causing hemophilia A is available on the Internet at The Haemophilia A Mutation, Structure, Test and Resource Site (HAMSTeRS: http://hadb.org.uk/; Accessed 12 October 2010). Common gene derangements include gene inversions, insertions and large and small gene deletions, typically associated with a severe phenotype. However, point mutations (missense, nonsense and messenger RNA splice-site mutations) represent approximately 70% of the described molecular defects in hemophilia A. The study of FVIII missense mutations, in particular, has provided insights on: structure and function of FVIII, mechanisms of hemophilia A, and risk of inhibitors developing to FVIII replacement therapy. Over 900 such missense mutations have

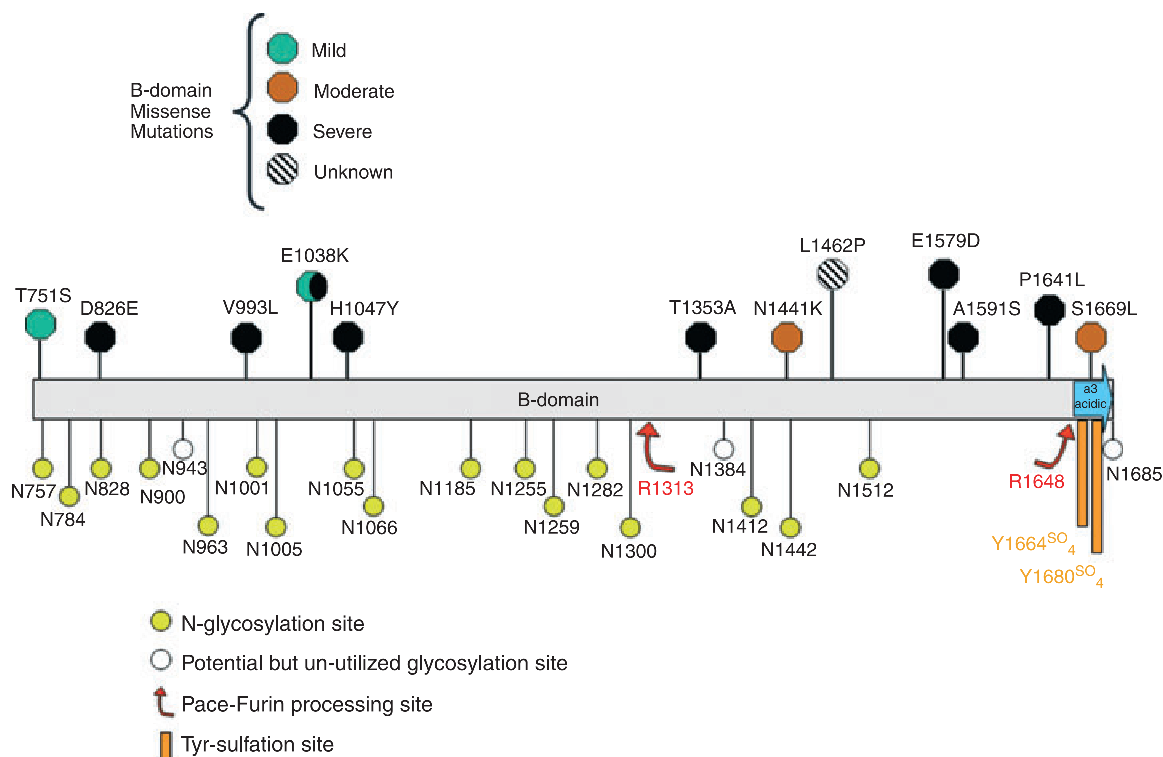

Fig. 1. Missense mutations and sites of key post-translational modifications within the factor (F) VIII B domain. Sites of reported missense mutations within the FVIII B domain and their reported clinical phenotypes are indicated in the upper portion of the figure. Residues that predict sites of asparagine(N)-linked glycosylation, tyrosine(Y)-sulfation and proteolytic modification are indicated in the lower portion of the figure.

now been described and occur throughout the entire coding region for FVIII. Although missense mutations have been shown to interfere with FVIII biosynthesis and secretion, there are a number of hemophilia A missense mutations that affect the functional properties of FVIII. These are clustered in regions known to interact with FIXa, FX, von Willebrand factor (VWF) and the phospholipid surface or affect the stability of FVIII after activation by thrombin [6]. However, those missense mutations characterized to date have all been located within the A and C domains of FVIII.

The large FVIII B domain is encoded by a single large exon (exon 14) and roughly spans from amino acids 741 to 1648. Unlike the other functional domains of the FVIII molecule, the B domain shares very little amino acid homology with the B domain of FV. FVIII does share with FV the distinctive property of having extensive asparagine (N)-linked glycosylation in its B domain. The B domain alone accounts for 19 of the 25 potential asparagine (N)-linked glycosylation attachment sites found on the FVIII molecule. While the B domain is not directly necessary for the central procoagulant activity of FVIII, more recently it has been shown to play a major role in the intracellular processing and trafficking of FVIII. In addition, there is emerging evidence that portions of the B domain may have functional influences throughout the life cycle of FVIII [7]. However, no molecular mechanisms for how missense mutations within the B domain cause hemophilia A have been described.

Figure 1 depicts the reported missense mutations within the FVIII B-domain that have been associated with moderate/severe hemophilia A. None of these reported mutations occurred at sites known to be involved in post-translational modifications within the B domain. In this report we have characterized these FVIII B domain missense mutations, including *in vitro* and *in vivo* expression and functional analysis, to elucidate any plausible mechanism for causation of hemophilia A. The list of B domain missense mutations analyzed in this study is shown in Table 1, along with references to their clinical reports.

Methods

Materials

FVIII-deficient and normal pooled human plasma were obtained from George King Biomedical (Overland Park, KS, USA). Activated partial thromboplastin (automated aPTT reagent) and $CaCl_2$ were purchased from bioMérieux (Durham, NC, USA). Dulbecco modified Eagle medium (DMEM), alpha-modified essential medium (alpha-MEM) and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, USA). COAMATIC was purchased from DiaPharma (West Chester, OH, USA). Matched-Pair Antibody Set for ELISA of human Factor VIII antigen was purchased from Affinity Biologicals (Ancaster, ON, USA). FuGENE-6 transfection

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Table 1 Factor VIII B domain missense mutations analyzed in the present study

Nucleotide change*	Amino acid change†	Reported clinical phenotype	HAMSTeRS	References
c.2309 C > G	T751S (T770S)	Mild	No	[8]
c.2535 C > A	D826E (D845E)	Severe	No	[9]
c.3034 G > C	V993L (V1012L)	Severe	Yes	[10]
c.3196C > T	H1047Y (H1066Y)	Severe	Yes	[11]
c.4114 A > G	T1353A (T1372A)	Severe	No	[12]
c.4380T > A	N1441K (N1460K)	Cross-reacting material-reduced; Mild/moderate	Yes	[13]
c.4442T > C	L1462P (L1481P)	Unknown	Yes	[13]
c.4794 G > T	E1579D (E1598D)	Severe	Yes	Unpublished
c.4828 G > T	A1591S (A1610S)	Severe	Yes	[14]
c.4979 C > T	P1641L (P1660L)	Severe	Yes	[15]
c.5063 C > T	S1669L (S1688L)	Moderate	No	[12]

*DNA mutation numbering is based on cDNA sequence (GENBANK # NM_000132), with nucleotide +1 corresponding to A of the ATG translation initiation codon. †Amino acid numbering is for the mature processed protein, with the first alanine numbered as +1, and is representative of the published literature and the factor VIII mutation reference database. The corresponding Human Gene Variation Sequence (HGVS)–type numbering is shown in parentheses.

reagent was purchased from Roche Applied Science (Indianapolis, IN, USA). QuikChange XL Site-Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). Male C57BL/6 mice were purchased from Jackson Laboratory. *F8*$^{-/-}$ (exon 16 knock-out) hemophilia A mice [16] were housed under pathogen-free conditions at the University of Michigan Laboratory Animal Medicine (ULAM) facility. The University Committee on the Use and Care of Animals (UCUCA) approved the animal protocols.

Plasmid mutagenesis

Mutagenesis was performed within the mammalian expression vector pMT2 [17] containing the full-length human FVIII cDNA (NM_000132). Mutant plasmids were generated through site-directed mutagenesis using the QuikChange XL protocol as described previously [18,19]. The plasmid containing the full-length wild-type human FVIII cDNA sequence was designated FVIII WT. The mutations were confirmed by restriction enzyme digestion and DNA sequencing.

Plasmid transfection and analysis of in vitro *expression*

Plasmid DNA of FVIII WT, B domain mutants and control mutants were transfected into COS-1 monkey kidney cells by the diethylamino ethanol (DEAE)-dextran method as previously described [20] and into Chinese hamster ovary (CHO) cells using FuGENE-6 transfection reagent according to the manufacturer's recommendations. Conditioned medium was harvested at 60–70 h post-transfection.

Factor VIII activity and antigen analysis

FVIII activity was measured by a: (i) one-stage aPTT clotting assay on an MLA Electra 750 fibrinometer (Medical Laboratory Automation, Pleasantville, NY, USA) by reconstitution of human FVIII-deficient plasma or (ii) two-stage assay using the COAMATIC chromogenic assay according to the manufacturer's instructions. The FVIII plasma standard was FACT plasma (normal pooled plasma) from George King Biomedical for the one-stage aPTT clotting assay. The calibration standard included with the COAMATIC chromogenic assay is assayed according to the Fourth International WHO standard. FVIII antigen was quantified by an anti-FVIII light chain sandwich ELISA according to the manufacturer's instructions.

Plasmid expression in vivo *by hydrodynamic tail-vein injection into the hemophilia A (exon 16–/–) mouse model*

A *F8* exon 16 knock-out mouse model of hemophilia A was used to analyze the *in vivo* expression of the FVIII B domain mutants. Plasmid DNA (100 ug) was diluted in 2.0 mL Lactated Ringers and infused over 10 s into the tail vein [21,22]. Peripheral blood was collected from the retro-orbital venous plexus at 24 h and mixed in 10% volume of 3.8% sodium citrate. Plasma was prepared by centrifugation of the blood at 2000 ***g*** for 15 min. FVIII activity and antigen levels were analyzed by COAMATIC chromogenic assay and human FVIII-specific ELISA.

Murine tail clip bleeding assay

A quantitative *in vivo* hemostasis assay was performed at between 24 and 48 h after hydrodynamic tail vein injection. Mice were anesthetized (ketamine 80 mg kg^{-1}; xylazine 5 mg kg^{-1} IP) and the tail was placed on a heat pad at 37 °C for 5 min. Next, the tail was transected with a sterile razor blade over a lateral tail vein at a position where the diameter of the tail was approximately 1.5 mm. After transection, the tail was immediately placed in a 15-mL falcon tube filled with 0.9% NaCl warmed to 37 °C. The weight of lost blood was measured by a micro balance within a 10-min window. Immediately after the conclusion of the bleeding assay, peripheral blood was collected from the mouse retro-orbital venous plexus and

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mixed in 10% volume of 3.8% sodium citrate. Plasma was prepared by centrifugation of the blood at 2000 ***g*** for 15 min. FVIII activity was analyzed by COAMATIC chromogenic assay.

Statistical analysis

Data are expressed as mean values ± SD. Statistical significance was determined by a two-sided Student *t*-test and established at $P < 0.05$.

Results

Analysis of FVIII B domain mutants in silico

To compare amino acid sequence variation between species at sites of FVIII B domain hemophilia-associated missense mutations, we utilized the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid alignments were performed by MacVector (Symantec, Cupertino, CA, USA). Although there was relatively high conservation of the residues between species at the homologous sites for the B domain missense mutation, especially at position 1047, the greatest divergence was at amino acid 1241 (Table 2). Interestingly, some of the reported hemophilia A mutations appear as a native sequence in other species.

We also investigated whether missense mutations within the FVIII B domain associated with hemophilia A were reported in normal population studies by searching the National Center for Biotechnology Information (NCBI) database of SNPs (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp, Accessed 12 October 2010). Out of 10 SNPs also reported to result in missense mutations of FVIII, four were found in the B domain (data not shown).

We utilized three software tools available online, namely, PolyPhen (http://genetics.bwh.harvard.edu/pph/), SIFT (http://sift.jcvi.org/) and Align GVGD (http://agvgd.iarc.fr/) to predict whether these missense substitutions were likely to be damaging. Of all the B domain mutations, Polyphen predicted only H1047Y and N1441K to be 'possibly damaging', while all the others were considered to be 'benign'. Similarly, SIFT analysis indicated that only E1038K and N1441K might affect FVIII function and it is to be noted here that both of these were low confidence predictions. Align GVGD analysis suggested that H1047Y, N1441K, L1462P, A1591S, P1641L and S1669L were most likely to affect function while the rest were benign. The utility of these *in silico* prediction tools was further underscored by the fact that all three of them predicted the A and C domain mutations, namely, R593C and R2150C, included as controls in this study, to be deleterious.

Expression of FVIII B-domain mutants is similar to WT in COS-1 and CHO cells

FVIII WT and B-domain mutants were compared after transient DNA transfection into COS-1 monkey kidney cells *in vitro*. FVIII activity and antigen levels were measured by a one-stage clotting assay and FVIII-specific ELISA, respectively, from the conditioned media 60–70 h after transfection. As controls for our experimental approach, previously well-characterized FVIII A and C domain mutants, namely, A284E, R593C and R2150C, were included in our assays. All B-domain mutants exhibited FVIII activity and antigen

Table 2 Conservation across species and *in silico* prediction of reported factor VIII B domain sequence variations

		In silico prediction		
Amino acid change	Species conservation* H/CM/D/M/P/B/Rb/R	Polyphen	SIFT	Align GVGD (likely to affect function)
T751S	T/T/T/***A***/T/T/T/S	Benign	Tolerated	Likely
D826E	D/D/E/D/E/E/D/***H***	Benign	Tolerated	Less likely
V993L	V/V/V/***D***/V/V/V/-	Benign	Tolerated	Least likely
E1038K	E/E/E/E/-/E/E/E	Benign	Affects function†	Likely
H1047Y	H/H/H/H/-/H/Y/H	Possibly damaging	Tolerated	Most likely
D1241E	D/E/E/***Y***/***G***/E/-/E	Benign	Tolerated	Less likely
T1353A	T/T/T/***K***/-/T/T/***K***	Benign	Tolerated	Likely
N1441K	N/N/N/N/N/N/N/***D***	Possibly damaging	Affects function†	Most likely
L1462P	L/***V***/L/P/L/L/L/P	Benign	Tolerated	Most likely
E1579D	E/E/E/***M***/E/E/E/***Q***	Benign	Tolerated	Less likely
A1591S	A/A/A/S/A/A/***D***/***R***	Benign	Tolerated	Most likely
P1641L	P/P/P/P/P/P/***S***/L	Benign	Tolerated	Most likely
S1669L	S/S/S/***T***/S/S/S/***V***	Benign	Tolerated	Most likely
A284E	A/A/A/A/A/A/A/A	Benign	Tolerated	Most likely
R593C	R/R/R/R/R/R/R/R	Probably damaging	Affects function	Most likely
R2150C	R/R/R/R/R/R/R/R	Probably damaging	Affects function	Most likely

H, human; CM, common marmoset; D, dog; M, mouse; P, pig; B, bat; Rb, rabbit; R, rat. Non-conserved amino acids are in bold; divergent amino acids are italicized; reported mutations appearing as native sequences in other species are underlined. *Factor VIII conservation across species. †Low confidence prediction.

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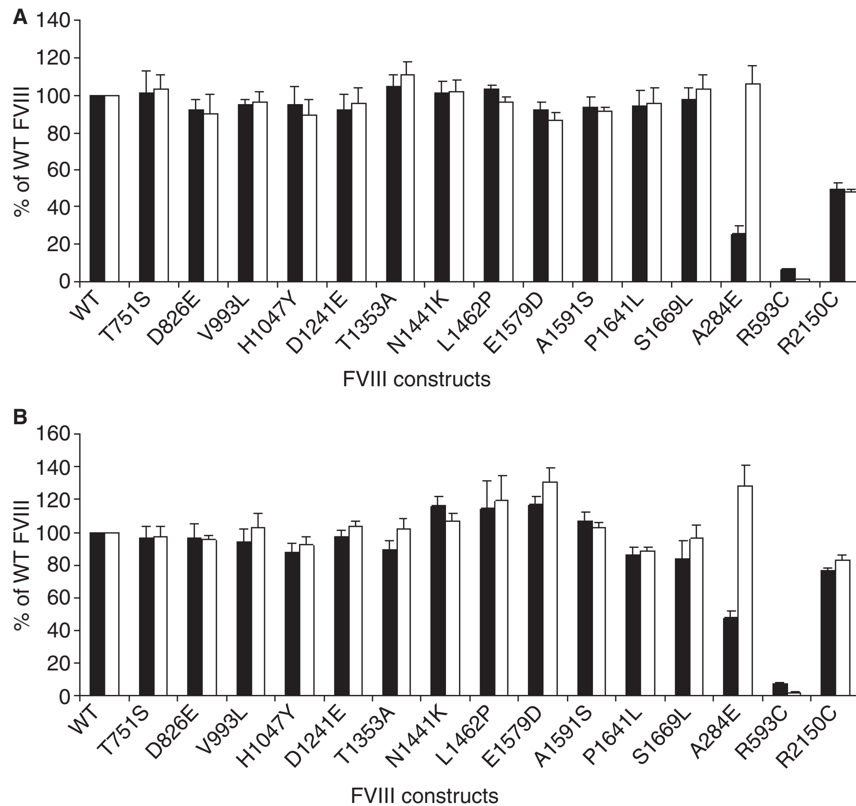

Fig. 2. Expression of factor (F) VIII B domain missense mutants is similar to wild type (WT) in COS-1 and CHO cells. FVIII activity (■) and antigen levels (□) in the conditioned media of COS-1 (A) and CHO (B) cells were measured by APTT and ELISA, respectively, at 60–70 h following transfection with the indicated FVIII constructs. Data are reported as percentages of FVIII WT expression. Data shown are representative of several independent experiments ($n \geq 6$), and the error bars represent the standard deviation (SD).

levels similar to FVIII WT(Fig. 2A). To determine if the observations were cell-line specific, the same experiments were repeated in CHO cells and no difference was observed for the B domain mutants compared with FVIII WT (Fig. 2B). Of the controls, A284E displayed a mild impairment of FVIII activity while FVIII antigen levels were similar to those of FVIII WT. In the case of R593C, a mild to moderate impairment of FVIII activity and a moderate to severe impairment of antigen levels were observed. R2150C on the other hand, displayed a mild phenotype with both FVIII activity and antigen levels.

B domain mutants express efficiently in vivo *in the hemophilia A mouse model*

We next compared the *in vivo* expression for select B domain mutants with FVIII WT in the hemophilia A mouse model. We chose H1047Y and N1441K because they were considered 'possibly damaging' by the different prediction tools. In addition, we also chose the known polymorphism D1241E and E1579D, which is an unpublished entry in HAMSTeRS database with a severe phenotype. We also included A284E and R2150C as control mutants. We induced transient expression in the liver of hemophilia A mice using a hydrodynamic tail vein injection of plasmid DNA. No statistically significant difference in mean plasma levels was found between FVIII WT, H1047Y, D1241E, N1441K and E1579D measured at 265.1 mU mL^{-1} (range, 10.8–734.1), 203.7 mU mL^{-1} (96.1–368.3), 208.4 mU mL^{-1} (25.51–608.1), 335.5 mU mL^{-1} (150–485) and 212 mU mL^{-1} (40.5–720), respectively (Fig. 3A). The mean plasma levels of the controls A284E and R2150C were 12 mU mL^{-1} (9.4–38.3) and 84.1 mU mL^{-1} (18.4–143), respectively, and were significantly lower than FVIII WT ($P < 0.05$).

B domain mutants exhibit effective hemostasis in vivo *in the hemophilia A mouse model*

To investigate the functional effect of select FVIII B domain mutants, we utilized an *in vivo* tail clip bleeding assay in the hemophilia A mouse model ($n \geq 5$). The mean quantitation of blood loss in untreated HA mice was 633.6 mg (range, 395–737; $n = 5$), while in C57BL/6J control mice, it was 115 mg (82–165). The mean blood loss measured for FVIII WT, H1047Y, D1241E, N1441K and E1579D was 216.2 mg (26–453), 260 mg (65–573), 278.14 mg (12–532), 169.4 mg (57–299) and 134.33 mg (25–488), respectively (Fig. 3B). No statistically significant difference was observed between FVIII WT and the B domain mutants ($P > 0.05$).

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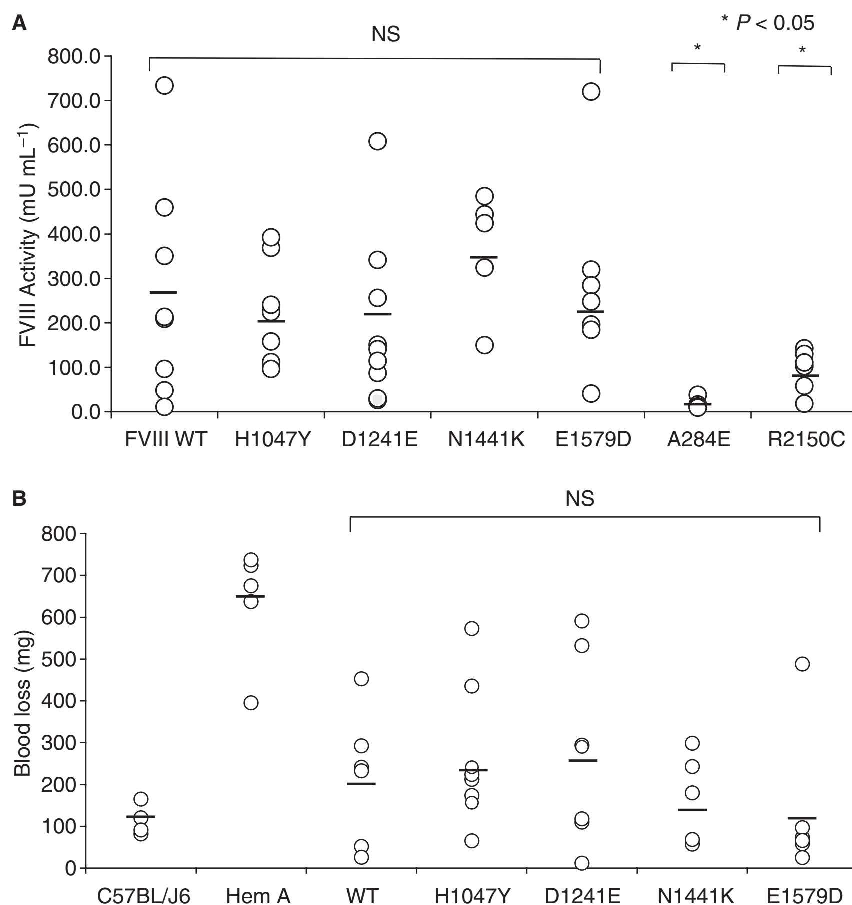

Fig. 3. *In vivo* expression and hemostatic efficacy of factor (F) VIII B domain mutants in a hemophilia A mouse model. (A) FVIII activity in the plasma of hemophilia A mice was measured by a COAMATIC chromogenic assay following transient expression of the indicated FVIII constructs by hydrodynamic tail vein injection as described under Methods. Data presented (○) represent multiple independent expression studies ($n \geq 5$) with the mean indicated by a bar. No statistical difference was observed between FVIII wild type (WT) and the B domain mutants, H1047Y, D1241E, N1441K and E1579D. The FVIII activity of the controls, A284E and R2150C, however, was significantly lower than FVIII WT ($P < 0.05$). (B) Hemophilia A mice were subjected to a tail clip bleeding assay following *in vivo* expression of FVIII WT and B domain mutants and blood loss over 10 min was quantified. C57BL/6J and hemophilia A mice were included as positive and negative controls, respectively. Data presented (○) are from several independent expression studies ($n \geq 5$) with the mean indicated by a bar. No significant difference was observed between FVIII WT and the B domain mutants.

Discussion

Hemophilia A results from varied genetic alterations of the *F8* gene, including gene inversions, deletions and insertions. Intron 22 or intron 1 gene inversions account for nearly half of the families with severe hemophilia A [23]. In mild/moderate hemophilia A, missense mutations within the exon coding for the three A domains or the two C domains account for most of the mutations detected [24]. Missense mutations occur in fewer than 20% of individuals with severe hemophilia A but nearly in all of those with mild or moderate hemophilia [23] due to defects in (i) protein and intracellular trafficking, (ii) VWF interaction, (iii) thrombin activation, (iv) stability of FVIIIa, (v) phospholipid binding, and (vi) interaction with FIXa. Point mutations in the nucleotide sequence resulting in splicing errors, rare small rearrangements and promoter mutations also account for hemophilia with varying phenotypes [6].

Detection and identification of *F8* gene mutations has been demonstrated to predict disease severity, the likelihood of inhibitor development and the success of immune tolerance induction (ITI) therapy for FVIII inhibitor patients [6,25–27]. Since the first sequences of the human *F8* gene were published, various studies have been conducted on the mechanisms of hemophilia A. Although investigation into the structural and functional roles of the FVIII A and C domains has progressed [28], there has not been extensive characterization of the FVIII B-domain [7]. FVIII and FV comprise the same basic domain structure and therefore share structural and functional features. Despite these similarities, there is a marked difference in rates of secretion between the two proteins *in vitro*, with FVIII being less efficiently secreted. Removal of the B domain of FVIII does not impair FVIII activity and results in an increased expression of mRNA [29]. However, this does not result in more efficient secretion due to intracellular retention of the primary translation product. Nevertheless, the net balance is improved expression of B-domain deleted (BDD) FVIII compared with FVIII WT *in vitro* [20]. A significant portion of the FVIII primary translation product is misfolded, resulting

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in its retention within the endoplasmic reticulum (ER). FVIII is co-translationally translocated into the lumen of the ER where it folds and assembles into its tertiary structure. Within the ER, FVIII acquires N-linked oligosaccharide structures. Productive secretion of FVIII requires interaction with and subsequent release from several ER chaperones, including immunoglobulin binding protein, calnexin (CNX) and calreticulin (CRT). CNX and CRT both display substrate specificity for glycoproteins containing partially glycosylated N-linked core oligosaccharides. Interaction of FVIII with CNX and CRT is mediated in part by interaction with N-linked oligosaccharides within the B-domain. Properly folded FVIII is released from these chaperones but requires interaction with the mannose-binding lectin complex LMAN1/MCFD2 for efficient transport from the ER to Golgi apparatus. Recent work has also demonstrated that LMAN1 directly interacts with FVIII and that high mannose-containing oligosaccharides, mostly clustered within the B-domain, provide a significant contribution to this interaction. Thus, the N-linked oligosaccharides within the B-domain can participate in the folding interactions within the ER as well as potentially facilitate ER-Golgi transport[7]. Thus, it could be postulated that missense mutations in the B domain, particularly those that may disrupt post-translational modifications to FVIII, could affect its secretion efficiency.

However, none of the B domain mutants in our study would be predicted to interfere with N-glycosylation. Two mutations, P1641L and S1669L, are close to the intracellular cleavage site at residue R1648 or tyrosine-sulfation sites at residues Y1664 and Y1680, respectively. Nevertheless, we did not recognize any effect on expression or functional activity.

D1241E was previously widely reported as a missense mutation associated with mild or severe hemophilia but recent observations have firmly established it as a non-synonymous single-nucleotide substitution polymorphism [30–32]. The expression results in our study demonstrated that both FVIII antigen levels and activity in conditioned media from cell lines and in plasma within the hemophilia A mouse were similar to FVIII WT. Additionally, in a tail clip bleeding assay, D1241E exhibited hemostatic efficacy similar to FVIII WT. Similar observations were obtained with H1047Y, N1441K and E1579D. Accordingly, these results suggest that none of the FVIII B domain missense mutations we investigated would be likely to be causative of severe hemophilia A.

There are several examples where patients have been identified with FVIII B domain missense mutations coincident with other FVIII gene derangements. E1038K has a reported phenotype ranging from mild to severe [14,33,34]. However, in one of these cases [14], E1038K was found to be associated with the intron 22 inversion, which is a known causal mutation for severe hemophilia A. Therefore, it is quite unlikely that the E1038K B domain missense variant is responsible for the hemophilia A phenotype, but rather represents a polymorphism. Such misattribution of causation to a missense variant highlights the potential hazards when genotyping is adopted broadly, particularly when such information might be used to identify carriers or utilized in prenatal decision-making. A recent paper by Schneppenheim *et al.* [35] clearly emphasizes this point in reporting that a novel rare missense variant was mistakenly attributed to be causative of hemophilia A leading to prenatal diagnosis and peripartal management until the subsequent identification of the actual causative mutation in the family's index case.

In conclusion, most missense mutations within the FVIII B-domain would be unlikely to lead to severe hemophilia A. Possibly additional causative mutations in these patients are outside the targeted sequencing regions. When sequencing patients with hemophilia A, if a B-domain missense mutation is identified, another causative mutation should continue to be sought. It is also important to perform expression studies of these novel sequence variants to prove their causative nature. A search for other potential pathological mutations is recommended within the non-analyzed sequences, such as within introns, in the promoter region or the 3′-untranslated region, or specialized studies to identify duplications.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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