Supplemental Data for Meeks et al.

A major determinant of the immunogenicity of factor VIII in a murine model is independent of its procoagulant function

Materials

DMEM/F12 (11330-032), fetal bovine serum (FBS), penicillin/streptomycin, and AIM V medium were purchased from Invitrogen (Carlsbad, CA). Alcian Blue was purchased from Sigma-Aldrich (St Louis, MO). Immobilized Protein A, Sulfo-NHS-LC-Biotin, Tween-80 were purchased from Pierce Biotechnology (Rockford, IL). Immulon-1B ELISA plates and high binding half area ELISA plates (Costar) were purchased from Thermo Fisher Scientific (Waltham, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Alkaline phosphatase-conjugated streptavidin was purchased from Jackson ImmunoResearch (West Grove, PA). Monoclonal antibody (MAb) ESH-4 was purchased from American Diagnostica (Stamford, CT). Clotting times were measured using a STart coagulation instrument (Diagnostica Stago, Asnieres, France). Activated partial thromboplastin reagent was purchased from Trinity Biotech (Bray, Ireland). Pooled citrated normal human plasma and fVIII-deficient plasma were obtained from George King Biomedical (Overland Park, KS). Reagents for thrombin generation assays including thrombin fluorogenic substrate, TGA SUB (Z-Gly-Gly-Arg-7-amino -4-methylcoumarin, Z-Gly-Gly-Arg-AMC), tissue factor/phospholipid solution, TGA RC low), and thrombin calibrator (TGA CAL) were purchased from DiaPharma (West Chester, OH). Domain-specific murine anti-human fVIII MAbs 2-116 (A1), 1D4 (A2), 2-54 (A2), 2-93 (A2), 4A4 (A2), 2-113 (A3), 5G12 (A3), G38 (A3), 2A9 (C1), I-109 (C2) and I14 (C2) were prepared as described previously [1]. Human VWF was purified from plasma as previously described [2]. All other materials were reagent grade or are described in the cited literature.

Construction of a cDNA encoding B domain – deleted R372A/R1689A fVIII and V634M fVIII

The primer sets used to construct V634M fVIII and R372A/R1689A fVIII cDNAs by SOE mutagenesis [3] as well as the restriction sites used for cloning into the mammalian expression vector ReNeo are listed below. The template used for the PCR reactions was wt fVIII with unique *MluI*, *SacII*, *AvrII*, and *BsiWI* sites in the ReNeo vector.

Primers for making R372A fVIII:

Forward primer – 5'-CATGTGGACCTGGTAAAAGACTTGAA -3'

Reverse primer – 5'-CTTGCTTGATTCTTAAATATAATCAA-3'

Internal primers – 5'-CCTTCCTTTATCCAAATTGCCTCAGTTGCCAAGAAGCAT -3

5'-ATGCTTCTTGGCAACTGAGGCAATTTGGATAAAGGAAGG -3'

Restriction enzymes for ligation to ReNeo: *SpeI*/*MluI*

Primers for adding R1689A to R372A fVIII:

Forward primer – 5'-CATTGGAGCACAGACTGACTTC-3'

Reverse primer – 5'-GCCATTGATTGCATGGAAGCGATA-3'

Internal primers - 5'-GATGAAAATCAGAGCCCCGCCAGCTTTCAAAAGAAAACA-3'

5'-TGTTTTCTTTTGAAAGCTGGCGGGGCTCTGATTTTCATC-3'

Restriction enzymes for ligation to ReNeo: *AvrII*/*HincII*

Primers for making V634M fVIII:

Forward primer - 5'-TCAGATCCGCGGTGCCTGACCCGCTATTACT-3'

Reverse primer - 5'-AAGAGTCGTACGAGTTATTTCCCGTTG-3'

Internal primers -5'- TCAGTTTGTTTGCATGAGATGGCATACTGGTACATTCTA-3'

5'-TAGAATGTACCAGTATGCCATCTCATGCAAACAAACTGA-3'

Restriction enzymes for ligation to ReNeo: *SacII*/*BsiWI*

Expression of wt-fVIII, R372A/R1689A fVIII and V634M fVIII from BHK-M cells

A recombinant B domain-deleted (BDD) human fVIII construct, wt fVIII, was expressed from a baby hamster kidney-derived cell line, designated BHK-M, and purified as described previously [4]. Transfection of BHK-M cells was performed using Lipofectamine 2000 according to the manufacturer's instructions. Transfected cells were diluted onto 10 cm plates and cultured for 10 days in the presence of DMEM/F-12 containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 500 µg/ml geneticin. Geneticin-resistant clones were picked and cultured in 96-well plates with the same media until confluent, and screened for fVIII production using an ELISA assay. Because R372A/R1689A fVIII and V634M fVIII have little or no activity by bioassay, their expression was measured by ELISA using anti-fVIII C2 MAb I14 and biotinylated anti-fVIII A1 domain MAb 2-116 as capture and detection antibodies, respectively, followed by addition of alkaline-phosphatase conjugated streptavidin, and p-nitrophenylphosphate as described previously [1].

The best expressing clones of R372A/R1689A fVIII and V634M fVIII were grown to near confluence before switching to serum-free AIM-V medium. Media was harvested and purification of recombinant fVIII constructs was performed as described previously for wt BDD fVIII [5]. Concentrations of purified wt fVIII, R372A/R1689A fVIII and V634M fVIII were calculated based on absorbance at 280 nm and an estimated of molar extinction coefficient of 256,300 $M^{-1}cm^{-1}$ based on their tyrosine, tryptophan and cysteine contents [6]. Concentrations were converted to mass units using a molecular weight of 165,300 g/mol based on the polypeptide chain weight. Purified wt fVIII, R372A/R1689A fVIII and V634M fVIII were analyzed by 4-15% gradient SDS-PAGE (3 µg per lane) with and without prior activation by thrombin and stained using Thermo Pierce Gel Code Blue. Molecular weight markers were Bio-Rad Precision Plus Protein Standards. A previously described sandwich ELISA [7] was used to assess the structural integrity of wt fVIII, R372A/R1689A fVIII and V634M fVIII by their ability to bind immobilized domain-

specific murine anti-human fVIII MAbs. Purification tables for wt fVIII, R372A/R1689A fVIII and V634M fVIII preparations are presented in “Supplemental Data”. wt fVIII, R372A/R1689A fVIII, and V634M fVIII were diluted into 0.4 M NaCl, 20 mM HEPES, 5 mM $CaCl_2$, 0.01% Tween-80 pH 7.4 and stored in small aliquots at -80°C.

Purification Tables for wt fVIII, R372A/1689A fVIII and V634M fVIII

	Volume (mL)	A280	Total A280	Units/mL	Total Units	Units/A280	% Yield	Fold Purification
wt fVIII								
AIM-V Medium	5000	2.73	13700	8	41500	3.0	100%	1
SP-Sepharose	40	0.134	5.36	960	38400	7170	93%	2360
Source Q	8	0.330	2.64	2370	19000	7190	46%	2370
R372A/1689A fVIII								
AIM-V Medium	4000	1.490	5960	0.038	152	0.026	100%	1
SP-Sepharose	80	0.043	3.44	0.877	70	20.3	46%	795
Source Q	12.8	0.108	1.38	3.05	39	28.3	26%	1110
V634M fVIII								
AIM-V Medium	4000	2.690	10800	0.011	44	0.004	100%	1
SP-Sepharose and MonoS	76	0.020	1.550	0.307	23	15.1	53%	3700
Source Q	4	0.170	0.678	6.20	25	36.6	56%	8950

Genotyping FVIII$^{-/-}$ and fVIII$^{-/-}$/VWF$^{-/-}$ mice

PCR was performed on mouse DNA obtained by ear punch using primers suggested by the JAX Mice Database Genotyping Protocols.

k/o	Primer	5' – 3' sequence	Primer Type
fVIII	oIMR1754	GAG CAA ATT CCT GTA CTG AC	Common
fVIII	oIMR1753	TGC AAG GCC TGG GCT TAT TT	Wild type
fVIII	oIMR1752	TGT GTC CCG CCC CTT CCT TT	Mutant
VWF	oIMR2084	AGT GAG ACC TTT GGC TTT GC	Common
VWF	oIMR3006	CCC AAC TTT TGC CAA CAA ATA	Wild type
VWF	oIMR6218	CCT TCT ATC GCC TTC TTG ACG	Mutant

Clearance of wt fVIII, R372A/R1689A fVIII, and V634M fVIII in fVIII$^{-/-}$ mice

wt fVIII, R372A/R1689A fVIII, and V634M samples were diluted to 1.0 µg in 100 µl of sterile saline and then injected. Blood was collected by terminal cardiac puncture 0.25. 0.5, 1, 2 and 4 h after the final injection, with 3 mice at each time point. Samples were held on ice prior to centrifugation at 3000 x g for 15 minutes at 4°C to collect plasma for analysis by ELISA.

FVIII antigen levels were measured using an ELISA. Corning 96 well 1/2 area high binding ELISA plates were coated with 6 ug/ml MAb 2-116 in Dulbecco's phosphate-buffered saline with 0.05% sodium azide, and were incubated at 4°C overnight. Plates then were blocked for 2 h at room temperature with blocking buffer (20 mM HEPES, 0.15M NaCl, 2 mM CaCl2, 0.05% Tween-20, 0.05% sodium azide, 2% (w/v) bovine serum albumin, pH 7.4). In order to create a standard curve of each fVIII construct, constructs were diluted separately to 50 ng/ml in fVIII deficient plasma and incubated for 30 min at 37°C. After incubation, 100 mM of 2-mercaptoethanol (BME) was added to each fVIII construct and incubated for 30 min at room temperature in order to disassociate fVIII from vWF. FVIII constructs then were serially diluted 2-fold in fresh dilution buffer (20 mM HEPES, 0.15M NaCl, 2 mM CaCl2, 0.01% Tween-80, 100 mM BME, pH 7.4). The dilutions were transferred to the blocked ELISA plates that had been washed 3 times with wash buffer (20 mM HEPES, 0.15M NaCl, 2 mM CaCl2, 0.05% Tween-20, 0.05% sodium azide, pH 7.4).

Mouse plasma samples to be tested were also incubated for 30 minutes at room temperature with 100 mM BME, serially diluted to 1/32, 1/64, 1/128, and 1/256 in dilution buffer, and placed on the blocked ELISA plate with their corresponding fVIII construct standard curve. Plates were incubated for 1 h at room temperature and then washed 3 times with wash buffer. Next, biotinylated 1B5 (1 µg/ml) was diluted 1:500 in blocking buffer and added to the plates as the

detection antibody. After incubation for 1 hour at room temperature, the plates were washed 3 times with wash buffer. Binding was then detected using alkaline-phosphatase conjugated streptavidin and *p*-nitrophenyl-phosphate. FVIII levels for each mouse plasma sample were interpolated off the standard curves.

Anti-fVIII IgG ELISA

ELISA titers plates were coated with 1.5 μg/ml fVIII in 20 mM Bicine, 2 mM $CaCl_2$, pH 9 buffer and blocked with 20 mM HEPES, 0.15M NaCl, 2 mM $CaCl_2$, 0.05% Tween-20, 0.05% Na azide, 2.0% bovine serum albumin, pH 7.4. Murine plasma was serially diluted into blocking buffer starting at a 1/20 dilution. Ab binding was detected with streptavidin-conjugated goat anti-mouse IgG and *p*-nitrophenyl-phosphate. ELISA titration curves of bound biotinylated MAb binding were fitted to the four-parameter logistic equation. The dilution that produced an A_{405} of 0.3 at 20 minutes was defined as the ELISA titer.

References

(1) Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The humoral response to human factor VIII in hemophilia A mice. *J Thromb Haemost* 2007;5(3):512-517.

(2) Lollar P, Hill-Eubanks DC, Parker CG. Association of the factor VIII light chain with von Willebrand factor. *J Biol Chem* 1988;263(21):10451-10455.

(3) Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR. Gene splicing by overlap extension. *Methods Enzymol* 1993;217:270-279.

(4) Doering CB, Parker ET, Healey JF, Craddock HN, Barrow RT, Lollar P. Expression and characterization of recombinant murine factor VIII. *Thromb Haemost* 2002;88(3):450-458.

(5) Doering CB, Healey JF, Parker ET, Barrow RT, Lollar P. High-level expression of recombinant porcine coagulation factor VIII. *J Biol Chem* 2002;277(41):38345-38349.

(6) Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci* 1995;4(11):2411-2423.

(7) Summers RJ, Meeks SL, Healey JF et al. Factor VIII A3 domain substitution N1922S results in hemophilia A due to domain-specific misfolding and hyposecretion of functional protein. *Blood* 2011;117(11):3190-3198.