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ORIGINAL ARTICLE

Expression and characterization of a codon-optimized blood coagulation factor VIII

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Essentials

- Recombinant factor VIII (FVIII) is known to be expressed at a low level in cell culture.
- To increase expression, we used codon-optimization of a B-domain deleted FVIII (BDD-FVIII).
- This resulted in 7-fold increase of the expression level in cell culture.
- The biochemical properties of codon-optimized BDD-FVIII were similar to the wild-type protein.

Summary. *Background:* Production of recombinant factor VIII (FVIII) is challenging because of its low expression. It was previously shown that codon-optimization of a B-domain-deleted FVIII (BDD-FVIII) cDNA resulted in increased protein expression. However, it is well recognized that synonymous mutations may affect the protein structure and function. *Objectives:* To compare biochemical properties of a BDD-FVIII variants expressed from codon-optimized and wild-type cDNAs (CO and WT, respectively). *Methods:* Each variant of the BDD-FVIII was expressed in several independent Chinese hamster ovary (CHO) cell lines, generated using a lentiviral platform. The proteins were purified by two-step affinity chromatography and analyzed in parallel by PAGE-western blot, mass spectrometry, circular dichroism, surface plasmon resonance, and chromogenic, clotting and thrombin generation assays. *Results and conclusion:* The average yield of the CO was 7-fold higher than WT, whereas both proteins were identical in the amino acid sequences (99% coverage) and very similar in patterns of the molecular fragments (before and after thrombin cleavage), glycosylation and tyrosine sulfation, secondary structures and binding to von Willebrand factor and to a fragment of the low-density lipoprotein receptor-related protein 1. The CO preparations had on average 1.5-fold higher FVIII specific activity (activity normalized to protein mass) than WT preparations, which was attributed to better preservation of the CO structure as a result of considerably higher protein concentrations during the production. We concluded that the codon-optimization of the BDD-FVIII resulted in significant increase of its expression and did not affect the structure-function properties.

Keywords: coagulation factor VIII; hemophilia A; lentivirus; LRP1 protein, human; von Willebrand factor.

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Introduction

Factor VIII (FVIII) is an important component of hemostasis, as its functional deficiency results in the bleeding disorder hemophilia A. In blood coagulation, activated FVIII serves as a cofactor of activated factor IX, which activates factor X. FVIII is translated as a single-chain polypeptide (SCh) with the domain structure of A1-A2-B-A3-C1-C2, which is subjected to glycosylation, sulfation and variable cleavages [1–3]. The resulting FVIII molecule is a heterodimer composed of a heavy chain (HCh, A1-A2-B domains, 90–210 kDa) and a light chain (LCh, A3-C1-C2 domains, 80 kDa). In plasma, FVIII circulates in a complex with von Willebrand factor (VWF). Upon site-specific cleavage (activation) by thrombin or activated factor X, FVIII converts into a heterotrimer A1/A2/A3-C1-C2, which dissociates from VWF and performs its cofactor function [4]. The clearance of FVIII depends on several receptors, the most prominent of which is the low-density lipoprotein receptor-related protein 1 (LRP) [5–8].

The introduction of recombinant FVIII (rFVIII) for the treatment of hemophilia A reduced the risk of

transmission of blood pathogens by products with plasma-derived FVIII [9]. However, production of rFVIII is challenging because of its low level of expression that correlates with a low FVIII level in plasma (0.1 μg mL^{-1}) [10]. Among factors contributing to that are various inhibitory elements present in both the gene [11–16] and protein [17,18]. A number of approaches to improve the rFVIII expression have been developed. For gene delivery, a classical approach employed transfection of FVIII cDNA followed by its amplification [19,20]. A lentiviral system was found to be more effective for this purpose [21,22], as it provides delivery of the gene into transcriptionally active chromatin [23,24].

Improved production of rFVIII was achieved by deletion of the B-domain, comprising about 40% of the molecule [25–27]. Substitution of the B-domain for a small fragment, preserving the flank sites for the protease cleavages, resulted in improved production of FVIII [27–33]. The efforts to eliminate the use of serum in the culturing media met additional challenges. It was shown that in serum-free conditions, the secreted rFVIII mostly associates with the outer cell membrane, and the B-domain-deleted FVIII (BDD-FVIII) is bound to the cells to an even higher degree [34]. In fact, the primary serum component, which stabilizes FVIII in media, is VWF. Therefore, the stabilization of FVIII in serum-free media can be achieved by co-expression with VWF [35,36]; otherwise, the expressed FVIII can be harvested from the cells at high ionic strength [28].

Most recently, improvement of expression of rFVIII was achieved using synonymous codon changes (codon-optimization) of its gene [37]. In a study by Ward *et al.* [38], 907 of 1457 codons of a BDD-FVIII cDNA were altered, resulting in removal of cryptic splice sites, internal ribosomal entry sites, AT-rich and GC-rich sequences and RNA secondary structures. Several variants of the codon-optimized and respective non-optimized constructs were compared for expression. In cultured cells (293T), the secretion of optimized variants was significantly increased. In hemophilia A mice, these constructs resulted in a significantly higher FVIII activity in plasma and improved hemostasis. However, biochemical properties of the codon-optimized BDD-FVIII variants were not evaluated, which left the following concerns unaddressed.

It is known that synonymous mutations may affect the protein post-translational modifications (PTMs), conformation, function, stability and even the fidelity of the amino acid sequence [39,40]. In particular, codon usage may regulate the protein translation rate, including the pausing at certain mRNA sites. Such pausing may be required for proper self-folding of the nascent polypeptide, occurring step-wise via spatial interactions of its non-sequential segments. A change in codon usage may change the translation rate and result in an alternative protein folding [41,42], which may affect the PTMs and functional properties [39,40]. In clinical use of the protein, these changes may result in increased immunogenicity, altered pharmacokinetics and pharmacodynamics, and decreased bioactivity [43]. These concerns are especially relevant to such complex proteins as FVIII.

Thus, the major aim of this study was to compare properties of a BDD-FVIII expressed from a codon-optimized cDNA (CO) with the protein expressed from the wild-type cDNA (WT). Both cDNAs were designed to encode a B-domain substitute fragment with a site for O-glycosylation [30] to improve solubility of the molecule, and the remaining cDNA of CO was identical to that described by Ward *et al.* [38]. For protein expression, we employed a lentivirus-based platform with use of multiple rounds of transduction to increase the gene copy number [22] and a two-step affinity chromatography for protein purification. To consider possible variability of WT and CO due to clonal selection, each protein was produced from several independent clonal cell lines and these preparations were analyzed in parallel for various biochemical parameters. We found that the codon-optimization of the BDD-FVIII cDNA resulted in a significant increase of yield and did not affect the structural and functional properties of the protein.

Materials and methods

Reagents

Commercial FVIII products, Xyntha (Pfizer Inc., New York, NY, USA) and Novoeight (Novo Nordisk, Bagsvaerd, Denmark), were purchased from the National Institutes of Health (NIH) Pharmacy (Bethesda, MD, USA). 8th International Standard (IS) for FVIII Concentrate was from the National Institute for Biological Standards and Control (South Mimms, UK). VWF (FVIII free) was from Haematologic Technologies Inc. (Essex Junction, VT, USA). Recombinant LRP cluster IV was from R&D Systems (Minneapolis, MN, USA). FVIII congenitally deficient plasma was from HRF Inc. (Raleigh, NC, USA). Anti-FVIII antibodies were sheep polyclonal (CedarLane, Burlington, NC, USA) and ESH8 (American Diagnostica Inc., Stamford, CT, USA).

Generation of vectors for expression of FVIII variants

In the design, we used numbering of FVIII residues from the first residue of the mature protein amino acid sequence (NP_000123). For generation of the constructs, we used FVIII cDNA obtained from Dr Steven Pipe (University of Michigan) and a codon-optimized cDNA described previously [38]. Using the standard DNA cloning technique, in each gene, the B-domain substitute fragment coding sequence was modified to that coding a polypeptide SFSQNSRHPSQNPPVLKRHQR [30] and the C-terminus coding sequence was fused with that coding a Strep/10xHis tag with thrombin and Tobacco Etch

Virus endopeptidase cleavage sites for the tag removal with or without activation of the BDD-FVIII (Figs 1A and S2). The resulting expression cassettes were inserted into pLNT/SFFV-MCS vector with the Spleen Focus Forming Virus promoter obtained from Dr Steven Howe (London, UK). The lentiviruses carrying the WT and CO cassettes were produced as described [38,44].

Generation of cells expressing FVIII variants

The cells, BHK-M (obtained from Dr Christopher Doering, Emory University), SK-Hep-1 and CHO cells (Sigma-Aldrich, St Louis, MO, USA) were cultured in respective media supplemented with 10% fetal bovine serum. For testing expression of FVIII variants, each cell line was transduced with the respective vector. For generation of cell lines stably expressing both proteins, CHO cells were transduced four times with the respective vectors as described [22], and the respective clonal lines were isolated using standard single-cell cloning by limited dilution in 96-well plates and testing each clone for FVIII secretion by enzyme-linked immune sorbent assay (ELISA).

FVIII protein quantitation

Factor VIII was quantitated using an ELISA Protein Detector kit (Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA) and an in-house FVIII standard, with concentration established by the absorbance at 280 nm as previously described [45]. The samples were serially diluted with a 2-fold step and analyzed in duplicates. The polyclonal anti-FVIII antibodies were used for the capture step and ESH8 antibody was used for the detection. Standard curves were obtained from six duplicate dilutions relating log of protein concentration to absorbance. The measurements were performed in three experiments. Quantitation of the purified FVIII variants by ELISA was confirmed by the absorbance at 280 nm.

Analysis of FVIII variants by polyacrylamide gel electrophoresis (PAGE) and western-blot (WB)

Protein samples were resolved in a 4–12% SDS gel followed by staining with a SimplyBlue Safe Stain or SilverQuest Stain (Thermo Scientific, Waltham, MA, USA). By PAGE-WB, the protein bands were detected using the polyclonal anti-FVIII antibodies. For thrombin cleavage analysis, the samples were treated with thrombin (0.001 IU per 0.8 mg of protein) for 10 min at 37 °C; the reaction was terminated by addition of Phe-Pro-Arg-chloromethylketone up to 7 μM followed by PAGE.

Expression and purification of FVIII variants

The CHO cells expressing either WT or CO were cultured in multi-layer 1000-cm^2 flasks (EMD Millipore, Darmstadt, Germany); the medium was collected daily, replaced and kept at −30 °C. The samples were pooled, filtered and loaded onto a HisTrap Excel column (GE Healthcare, Pittsburgh, PA, USA) in 20 mM Bis-Tris, 0.5 M NaCl, 5 mM $CaCl_2$, 0.04% NaN_3, 0.01% polysorbate 80, pH 7.4. The column was washed with the buffer containing 10 mM imidazole, and the bound protein was eluted with the buffer containing 0.5 M imidazole. The eluate was diluted with two volumes of 20 mM Bis-Tris, 5 mM $CaCl_2$, 0.04% NaN_3, 0.01% polysorbate 80, pH 7.0, and loaded onto a column with VIIISelect sorbent (GE Healthcare) [46] and equilibrated with the buffer containing 150 mM NaCl. The column was washed with

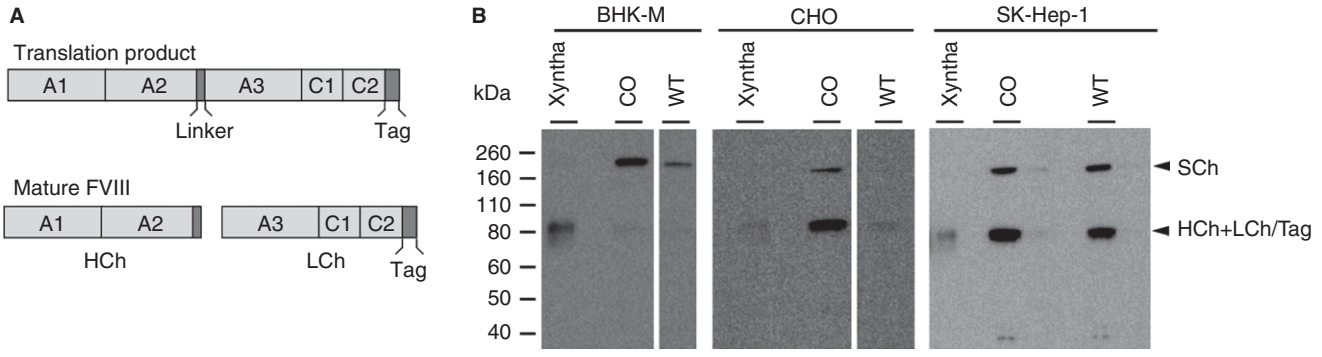


Fig. 1. The structure of the factor VIII (FVIII) variants and their expression in different cell lines. (A) Design of the B-domain-deleted FVIII (BDD-FVIII) encoded by either wild-type or codon-optimized genes. The BDD-FVIII primary translation product is designated as SCh, the protein domains are designated as A1, A2, A3, C1 and C2, fragments of the mature protein (heterodimer) are designated as HCh and LCh and the B-domain substitute fragment is designated as Linker (connecting the A2 and A3 domains). The C-terminal tag (Tag), composed of 40 amino acids (4.6 kDa), includes the Strep/10xHis, thrombin and Tobacco Etch Virus endopeptidase recognition sites. (B) Analysis of expression of the wild-type BDD-FVIII (WT) and codon-optimized BDD-FVIII (CO) in BHK-M, SK-Hep-1 and CHO cells. Each cell line was transduced with either WT or CO construct; after 72 h, the cells' media were analyzed by PAGE-WB (western blot) with anti-FVIII polyclonal antibodies (mostly reacted with the HCh). In contrast to a BDD-FVIII control (Xyntha), the LCh species of the WT and CO, having the C-terminal tags, were not resolved from the respective HCh species, but were detectable by an anti-LCh antibody (data not shown).

20 mM HEPES, 5 mM $CaCl_2$, 1 M NaCl, 0.04% NaN_3, 0.02% polysorbate 80, pH 7.0, followed by elution of the protein with 20 mM HEPES, 5 mM $CaCl_2$, 0.9 M L-Arginine, 45% (v/v) propylene glycol, 0.02% polysorbate 80, 0.04% NaN_3, pH 7.0 [46]. The eluate was dialyzed against 20 mM histidine, 500 mM NaCl, 5 mM $CaCl_2$, 9 g L^{-1} sucrose, 0.005% polysorbate 80, pH 7.2, aliquoted and stored at −80 °C.

Nanospray liquid chromatography tandem mass spectroscopy assay (LC/MS/MS)

The protein (16 μg) samples were processed with and without Deglycosylation Enzyme Mix (New England Biolabs, Ipswich, MA, USA) in duplicates, purified by PAGE, digested with either trypsin or chymotrypsin as previously described [47] and analyzed using a Q-Exactive hybrid Quadrupole-Orbitrap Mass Spectrometer and Dionex UltiMate 3000 RSLCnano System (Thermo Scientific). For peptide identification and protein assembly, the data were analyzed against the FVIII sequence (NP_000123) and the human protein database from the National Center for Biotechnology Information (NCBI) using the SEQUEST and percolator algorithms through the Proteome Discoverer 1.4.1 platform (Thermo Scientific).

Circular dichroism assay (CD)

Far-UV CD spectra of proteins (0.1–0.4 mg mL^{-1} in 10 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$ and 0.005% polysorbate 80, pH 7.4, in 0.5-mm path length quartz cuvettes) were acquired within 180–260 nm at 25 ± 0.2 °C using a Jasco J-815 Spectropolarimeter (Jasco, Oklahoma City, OK, USA). For the secondary structure estimate, each spectrum was converted into residual ellipticity and analyzed using the CDPro/CONTIN program.

FVIII activity measurements

The chromogenic substrate assay was performed using the Coatest SP4 Factor VIII kit (Chromogenix, West Chester, OH, USA). The samples were diluted up to one international unit (IU)/mL in FVIII-deficient plasma, and further dilutions were performed in a buffer supplied with the kit. One-stage clotting assay was performed using a micro-centrifugal analyzer ACL Elite Pro and activated partial thromboplastin time (APTT) reagent SynthAFax (Instrumentation Laboratory, Bedford, Massachusetts, USA). An activated factor XI and tissue factor-activated thrombin generation assay (TGA) was performed as described [48], and the FVIII activity was determined based on thrombin peak height and time-to-peak parameters. In all assays, the calibration curves were prepared using the 8th IS for FVIII Concentrate.

Surface plasmon resonance assay (SPR)

The measurements were performed using Biacore 3000 (GE Healthcare). Either VWF or LRP cluster IV was covalently immobilized on a CM5 chip and tested for binding to FVIII variants as described previously [6]. Between injections, the chip with immobilized VWF was regenerated by 20 mM HEPES, 0.35 M $CaCl_2$, 0.6 M NaCl, pH 7.4, and the chip with immobilized cluster IV was regenerated by 0.1 M phosphoric acid. From the association and dissociation signals, the respective signals from the blank flowpath were subtracted, and the kinetic parameters were derived using the BIAevaluation 4.1.1 program.

Results

Expression of FVIII variants

Upon transduction of the WT and CO constructs into the BHK-M, SK-Hep-1 and CHO cells, we found that all the cells secreted CO at significantly higher levels than WT (Fig. 1B). However, the BHK-M cells secreted both proteins mostly in a single-chain form, whereas two other cell lines secreted the proteins in predominantly processed form (i.e. as heterodimers of the HCh and LCh). Our attempts to produce the clonal isolates using SK-Hep-1 cells were not successful, thus we proceeded with the use of CHO cells. Using these cells, we produced three clones expressing WT and six clones expressing CO. To test stability of proteins' expression, selected clonal lines were cultured for 29 days; the media were replaced daily and assessed for FVIII antigen. We found that secretion of both WT and CO increased during the first 12 days of cultivation and then was stable (Fig. S1).

FVIII variants purification and PAGE analysis

Three preparations each of WT and CO were isolated from different cell lines using two-step affinity chromatography (Fig. 2A). The average yield of CO was 7-fold higher than WT (423 ± 55 and 60 ± 40 μg L^{-1} media, respectively, Fig. 2B). By PAGE, both WT and CO corresponded to a BDD-FVIII control (Fig. 2C). A broad appearance of the HCh and LCh bands was attributed to heterogeneity in truncation and glycosylation, previously described for FVIII [27,30,31]. Upon treatment of selected samples of WT and CO with thrombin (Fig. 2D), the resulting fragments corresponded to the pattern of activated FVIII [30,49,50].

FVIII variants structures by LC/MS/MS

Two preparations each of WT and CO were processed with or without deglycosylation, followed by site-specific proteolysis with either trypsin or chymotrypsin, and the resulting peptides were identified. Both WT and CO amino acid sequences were covered at about 99% of the

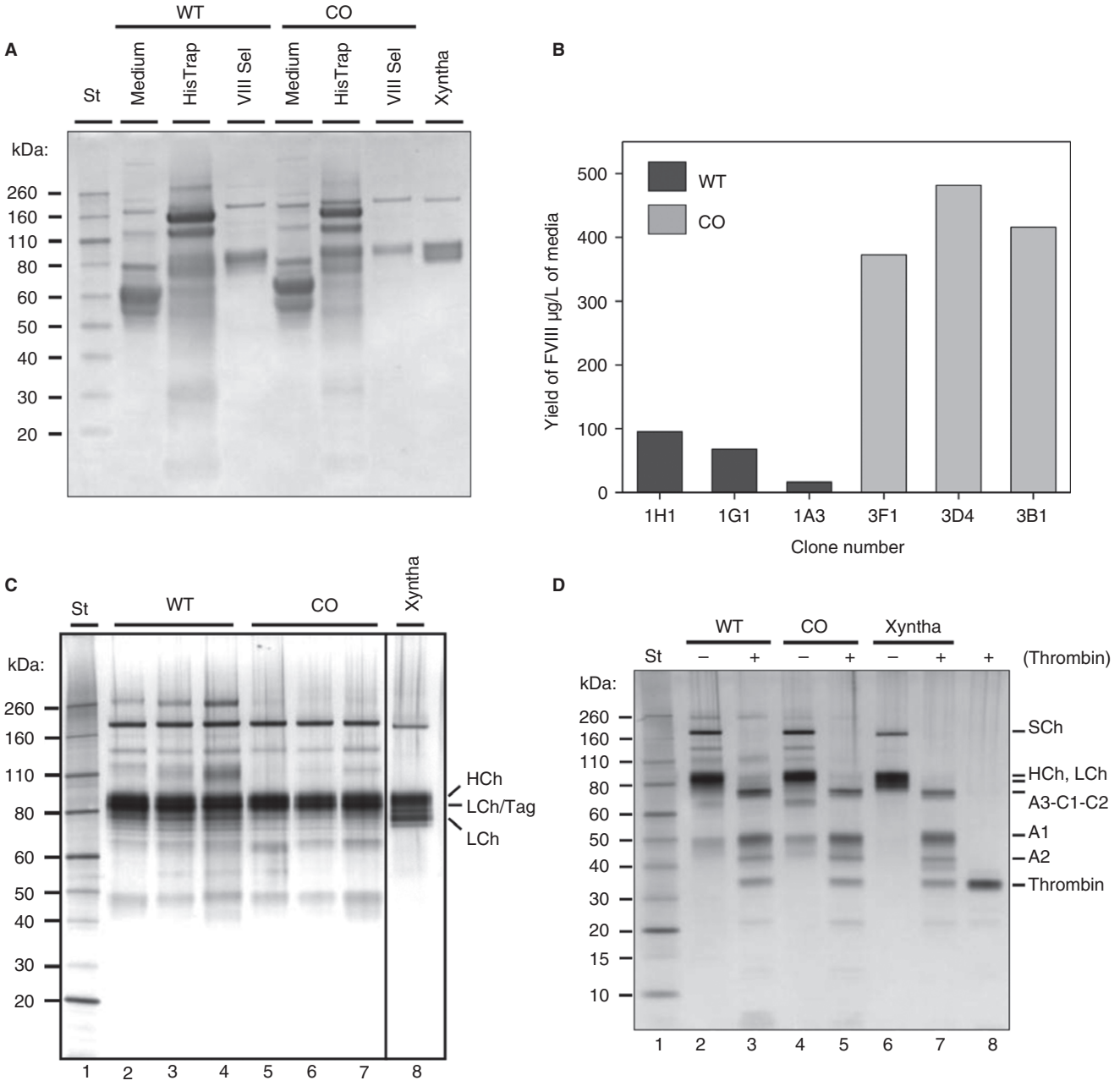


Fig. 2. Purification and analysis of the factor VIII (FVIII) variants by PAGE. (A) Purity of samples from the purification steps: the starting medium (Medium), Ni-column eluate (HisTrap) and VIIISelect-column eluate (VIIISel). The reference lanes were loaded with a B-domain-deleted FVIII (BDD-FVIII) control (Xyntha) and a molecular weight standard (St). (B) Protein yields in preparations of the wild-type BDD-FVIII (WT) (dark grey bars) and codon-optimized BDD-FVIII (CO) (light grey bars) purified from the respective CHO cell clonal lines (n = 1). (C) Final purity of the proteins: lanes 2–4, WT (clones 1H1, 1G1 and 1A3); lanes 5–7, CO (clones 3F1, 3D4 and 3B1). The respective BDD-FVIII fragments are marked as SCh, HCh and LCh. Because of the presence of the C-terminal tags, the LCh bands of WT and CO were not resolved from the HCh bands. (D) Thrombin-cleavage analysis of WT (clone 1H1) and CO (clone 3D4). Before the gel run, the samples were (+) or were not (−) treated with thrombin: WT (lanes 2 and 3), CO (lanes 4 and 5) and Xyntha (lanes 6 and 7). The respective fragments of thrombin-cleaved BDD-FVIII species are marked as A1, A2 and A3-C1-C2. Notably, the mobility of thrombin-cleaved LCh species of both WT and CO became similar to that of the LCh of Xyntha as a result of removal of the respective C-terminal tags. The gels were stained with either a Coomassie-based reagent (A) or a silver-based reagent (C and D).

BDD-FVIII sequence (Fig. S2). The sites of PTMs of both WT and CO were found to be the same and had similar occupancy (Table 1). The identified PTMs were N-glycosylation of asparagines 41, 239, 1810 and 2118, O-glycosylation of Ser-750 and sulfation of tyrosines 346, 718, 719, 723, 1664 and 1680, corresponding to FVIII PTMs described previously [30,32,49,51]. In each of WT and CO, we also identified three new PTMs: N-glycosylation of Asn-235, O-glycosylation of Ser-568 and sulfation of Tyr-729.

Table 1 Post-translational modifications of the factor VIII variants*

Modification	Modified residue	WT† (%) clone 1H1	WT‡ (%) clone 1G1	CO† (%) clone 3D4	CO‡ (%) clone 3F1	Comment
N-linked glycosylation	Asn-41	100.0	100.0	100.0	85.5 ± 0.0	Known [30,32,49,51]
	Asn-235	100.0	95.6 ± 0.3	87.2	74.3 ± 7.3	New
	Asn-239	100.0	100.0	94.4 ± 7.9	80.8 ± 7.4	Known [30,32,49,51]
	Asn-1810	84.5 ± 1.7	75.5 ± 0.2	72.6 ± 14.3	70.1 ± 2.4	Known [30,32,49,51]
	Asn-2118	100.0	86.8 ± 1.0	100.0	84.5 ± 2.3	Known [30,32,49,51]
O-linked glycosylation	Ser-568	12.0	4.4 ± 0.1	14.3	4.5 ± 3.3	New
	Ser-750	5.9	12.4 ± 2.0	23.9	11.3 ± 11.0	Known [30]
Tyrosine sulfation	Tyr-346	ND§	100.0	100.0	100.0	Known [30,32,51]
	Tyr-718	ND	11.0 ± 5.7	21.4	11.4 ± 5.8	Known [32]
	Tyr-719	ND	5.9 ± 4.8	28.6	8.1 ± 1.1	Known [30,32,51]
	Tyr-723	ND	35.2 ± 10.3	ND	23.8 ± 19.8	Known [32]
	Tyr-729	ND	47.9 ± 11.2	28.6	56.7 ± 26.8	New
	Tyr-1664	100.0	100.0	100.0	100.0	Known [30,32,51]
	Tyr-1680	51.5 ± 2.2	71.2 ± 3.4	64.7 ± 6.3	72.2 ± 4.0	Known [30,32,51]

*Results of the liquid chromatography tandem mass spectroscopy assay (LC/MS/MS) analysis of wild-type BDD-FVIII (WT) (clones 1H1 and 1G1) and codon-optimized BDD-FVIII (CO) (clones 3D4 and 3F1) obtained in two experiments. In experiment 1, we compared the results for the clones 1H1 and 3D4, and in experiment 2, we compared the results for the clones 1G1 and 3F1. The values show percentage of peptides containing the respective modified residues ± standard deviation (SD). †In experiment 1, the SD was calculated if two or more different peptides containing the same modification residual were detected. ‡In experiment 2, the standard deviation was calculated based on the results of duplicate measurements. §Not determined.

FVIII variants secondary structures by CD

The folding of selected preparations of the WT and CO was assessed by far-UV CD in comparison with a BDD-FVIII control. Figure 3(A) shows an overlay of the normalized spectra; each of these exhibited a strong negative extremum at 219 nm, typical for the β-structure-rich proteins and reported for FVIII [27,52–54]. The estimated percentage of the β-structures, including β-sheet and β-turn elements, was similar between the samples (Fig. 3B) and consistent with such data reported for other rFVIII variants [27,55]. These results indicated similarity of the WT and CO secondary structures.

FVIII variants activity testing

We assumed that manifestation of FVIII activity would be the most informative for correctness of the overall WT and CO structures. First, we compared the specific activities, calculated as ratios of the activities by a chromogenic assay to protein concentration (IU mg^{-1}). Three preparations each of WT and CO were tested, which showed similarity between the preparations of each FVIII variant. However, the average specific activity of the CO was 1.5-fold higher than WT, although not statistically significant using the two-tailed *t*-test (Table 2). We assumed that such a difference could reflect better structural preservation of the CO because of its consistently higher concentrations during the production.

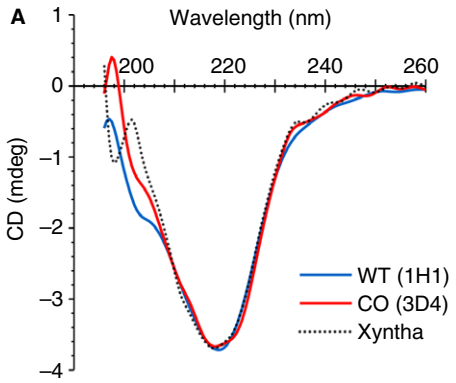
Because the B-domain deletion may be associated with assay-dependent discrepancy in the FVIII activity [48,56], the WT and CO were additionally tested by clotting and thrombin generation assays. To compare results, the values (IU mL^{-1}) were normalized to those determined by the chromogenic assay. As the former experiment showed similarity of specific activities between the preparations of each WT and CO, we limited this analysis by testing two preparations per each FVIII variant. The WT and CO samples had comparable ratios of the activities based on clotting time (Fig. 4A), thrombin peak height (Fig. 4B) and time-to-peak (Fig. 4C). Compared with the BDD-FVIII controls, the WT and CO samples had the higher ratios based on the peak height (Fig. 4B) and time-to-peak (Fig. 4C,D). We attributed these differences to differences in quality between our samples and control samples (protein quality, content of impurities and buffer formulation). Thus, the WT and CO samples behaved similarly in the assays.

FVIII variants interactions with VWF by SPR

Factor VIII has an extended site for binding to VWF [57]. To evaluate the structures of WT and CO, the selected samples of both proteins and a BDD-FVIII control were tested for binding to immobilized VWF (Fig. 5A). All variants of FVIII were found to have similar kinetic parameters for the binding (Fig. 5B), which were in accordance with such data reported previously for other rFVIII variants binding to VWF [31,32,58].

FVIII variants interactions with LRP cluster IV by SPR

Factor VIII has also an extended site for binding to LRP [59], in particular to its cluster IV [60]. We evaluated the



B	H,%	S,%	T,%	U,%
WT (1H1)	5.8	41.3	21.1	31.8
CO (3D4)	5.7	40.2	22.4	31.7
Xyntha	5.9	41.6	20.8	31.1

Fig. 3. Analysis of the factor VIII (FVIII) variants' secondary structures by far-UV circular dichroism (CD). (A) The samples of wild-type BDD-FVIII (WT) (clone 1H1), codon-optimized BDD-FVIII (CO) (clone 3D4) and a BDD-FVIII control (Xyntha) were recorded in triplicates and normalized to the same protein concentration (~0.2 μM). The baseline was subtracted by the running buffer as blank prior to each measurement. Similar spectra were obtained for CO clone 3B1 (data not shown). (B) Estimates of the secondary structure elements: α-helix, total of regular and distorted (H); β-sheet, total of regular and distorted (S); β-turns (T) and unordered (U). [Color figure can be viewed at wileyonlinelibrary.com]

structures of WT and CO by testing their interactions with immobilized recombinant cluster IV in a similar assay set-up to the above experiment. As previously reported, because of the complexity of FVIII and LRP interaction, their binding sensorgrams could not be adequately fitted with any of the standard models [58,61]. This was the case in our experiment (Fig. 5C); thus we limited our analysis to visual evaluation of the sensorgrams. The WT and CO sensorgrams were similar and, in turn, similar to sensorgrams for a BDD-FVIII and LRP reported previously [58].

Discussion

In our study, we investigated expression, purification and biochemical properties of a BDD-FVIII encoded by either a codon-optimized or the wild-type cDNA. Several preparations each of WT and CO were produced from independent CHO cell clones that resulted on average in a 7-fold higher yield of the CO. This increase of expression was similar to that observed upon transduction of a human cell line by the codon-optimized BDD-FVIII (SQ FVIII) cDNA in the study by Ward *et al.* [38]. However, our major aim was to verify preservation of biochemical properties of the protein, based on the concerns outlined in the introduction. In the majority of further assays, several independent preparations each of WT and CO were tested in parallel to minimize any possible effect of genetic variability between the production cell lines on protein structure.

The structural characterizations of WT and CO showed their high similarity to each other and to a BDD-FVIII control. In particular, LC/MS/MS showed the same distribution of the PTM sites with similar occupancy (Table 1). In regard to the PTMs known for FVIII [30,32,49,51], we confirmed four sites with N-glycosylation, one site with O-glycosylation and six sites with tyrosine sulfation. We also found additional PTMs: N-glycosylation of Asn-235 (belonging to a motif not common for N-glycosylation [62]), O-glycosylation of Ser-568 and sulfation of Tyr-729. It is unclear whether the newly found PTMs occur *in vivo* or appeared under our experimental conditions because, in particular, glycosylation can depend on the culturing conditions [63,64]. In this regard, the sulfation of Tyr-1680, critical for the binding to VWF [65], was observed at 50–70%, whereas it was above 90% in other studies [51], and the O-glycosylation of Ser-750 was 6–24%, whereas it was about 65% in another study [30]. Notably, the literature indicates that the PTM sites of FVIII, expressed in the CHO and human cells, are the same [30,32,49,51] and CHO cells are used for manufacturing of some FVIII products

Table 2 Specific activity of the factor VIII (FVIII) variants*

Group	Specific activity (IU mg^{-1})			Average	SD	CI	
						LL	UL
WT Clone	2630 ± 320 1H1	2521 ± 265 1G1	2064 ± 269 1A3	2405	300	1659	3151
CO Clone	4456 ± 258 3F1	3321 ± 237 3D4	3115 ± 146 3B1	3631	722	1837	5424

*The specific activity was determined for each preparation of the wild-type BDD-FVIII (WT) (clones 1H1, 1G1 and 1A3) and codon-optimized BDD-FVIII (CO) (clones 3F1, 3D4 and 3B1). In each sample, the FVIII activity was measured by the chromogenic assay and protein concentration was determined by ELISA (confirmed by the absorbance at 280 nm); the specific activity was calculated as ratio of activity to protein concentration. Shown values are means ± SD of three independent experiments. SD, standard deviation; CI, confidence interval; LL, low limit and UL, upper limit, calculated using the two-tailed *t*-test. This test indicated that the difference between average values of specific activities was not statistically significant ($P = 0.083$).

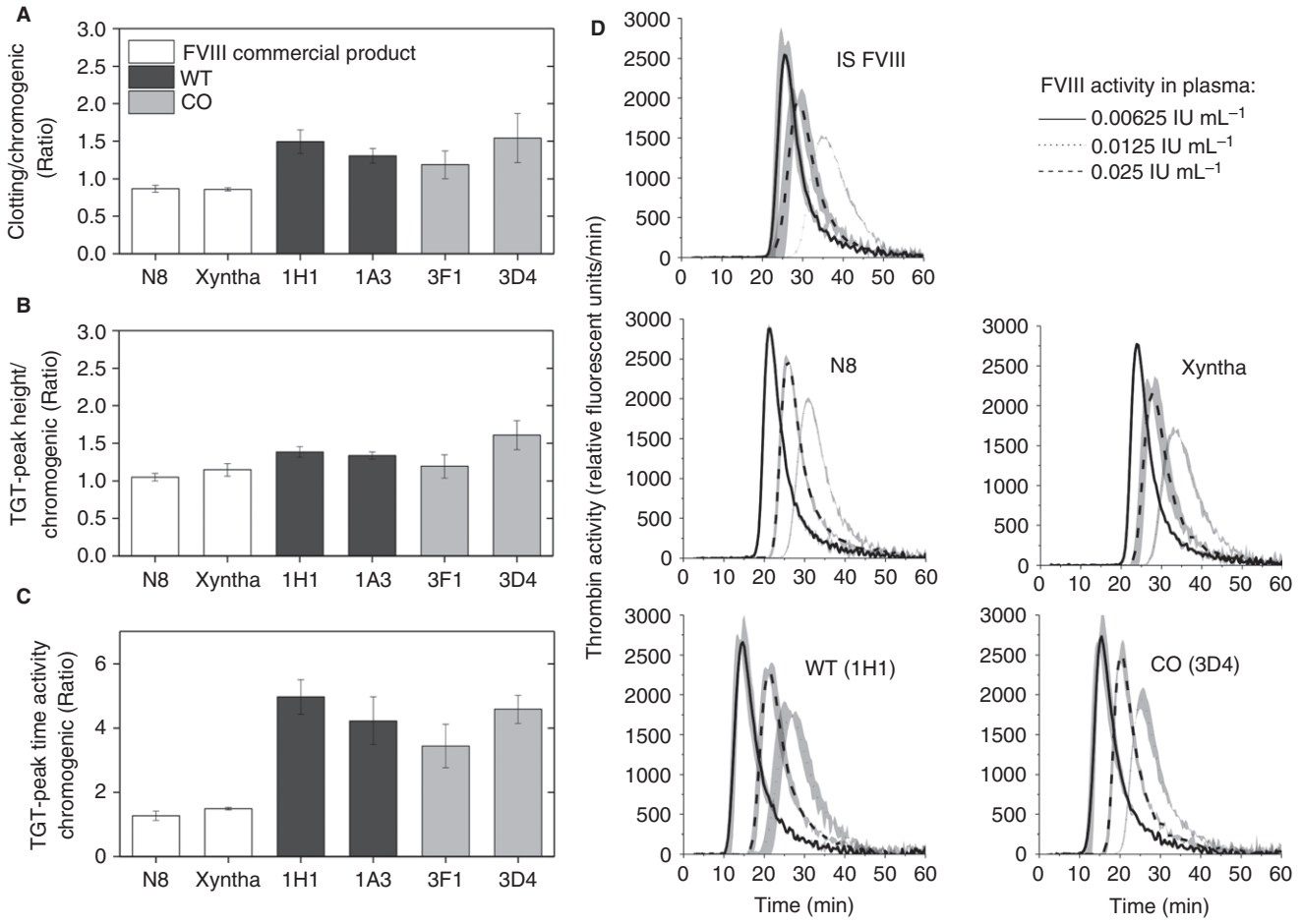
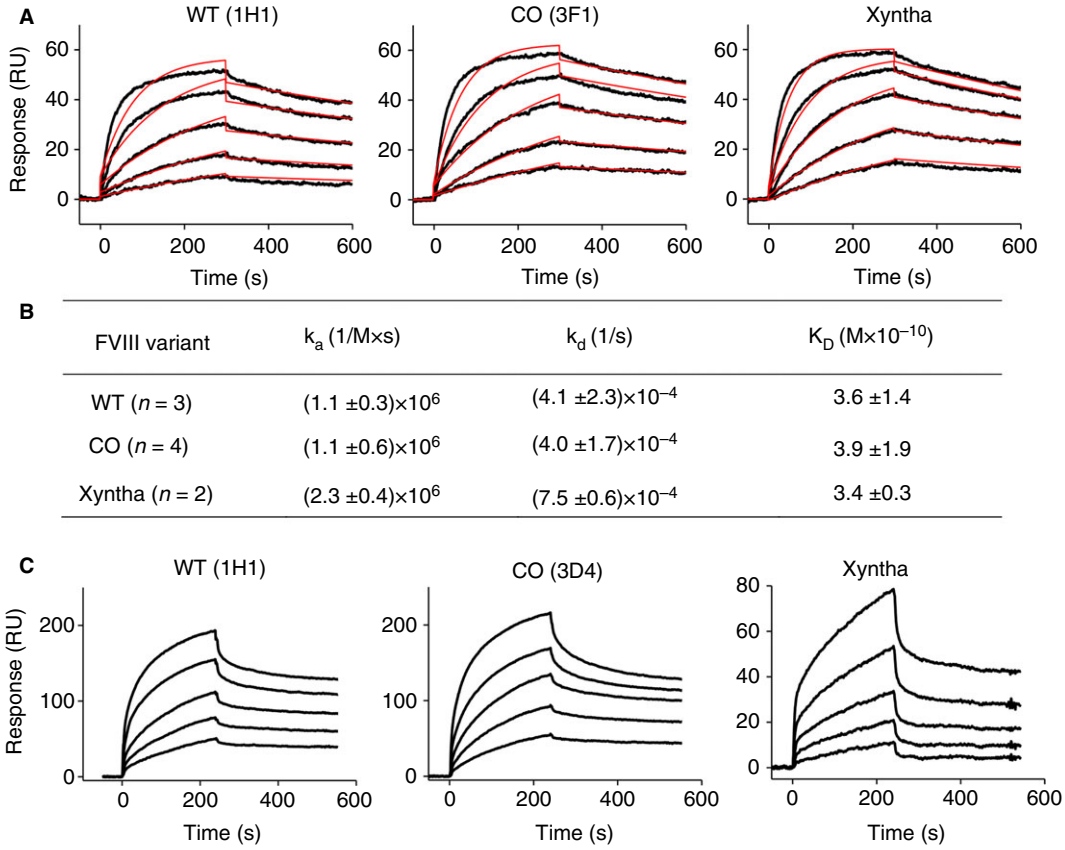


Fig. 4. Characterization of the factor VIII (FVIII) variants activity by the chromogenic, clotting and thrombin generation assay (TGA) assays. In each assay, the activity of wild-type BDD-FVIII (WT), codon-optimized BDD-FVIII (CO) (the respective clone numbers are shown) and the BDD-FVIII controls, Xyntha and Novoeight (N8), was determined using the 8th IS for FVIII (IS FVIII). Bars show relative activities (means ± SD, $n = 3$) determined by the chromogenic assay vs. clotting assay (A), thrombin generation peak height (B) and time-to-peak (C). (D) TGA curves for representative samples of WT (clone 1H1) and CO (clone 3D4); shadows indicate standard deviation of the mean ($n = 2$).

(Advate, Xyntha (Refacto) and Novoeight; information from the package inserts).

The evaluation of folding of WT and CO was based on assessment of the secondary structures, FVIII activity and binding to VWF and to LRP cluster IV. The activity manifestation was assumed to be the most indicative for correctness of the overall structure, as it is dependent on FVIII interactions with several ligands within the tenase complex. Unexpectedly, the average specific activity of the CO was found to be 1.5-fold higher than WT. This difference was not statistically significant because of the wide confidence intervals (Table 2) and was not due to difference in the proteins' secondary structures, assessed by CD. We attributed this difference to better preservation of the CO protein because of its considerably higher levels at all production steps, as high protein concentration is known to stabilize the molecule. In this regard, the specific activities of commercial BDD-FVIII products, having relatively high concentrations during production, are markedly higher (3200–14 000 IU mg^{-1}) [45] than those found for WT and CO. Also, we found differences in manifestation of FVIII activity between both the WT and CO and two BDD-FVIII controls. These were earlier formation of thrombin by our samples in TGA and higher ratios of their activities by clotting or thrombin generation assays to the respective activities by chromogenic assay (Fig. 4). Most likely, these differences were a result of differences in quality of preparations. In particular, our samples contained higher abundance of the single-chain BDD-FVIII, which is known to have altered *in vitro* activity [66], and could have contained pre-activated FVIII, accounting for the earlier thrombin formation. However, our results clearly showed that the codon-optimization did not negatively affect the BDD-FVIII properties, which supports suitability of using this protein as an equivalent of FVIII in relevant applications.

A reasonable question would be whether such protein can be used for treatment of hemophilia A. Currently, a number of variants of codon-optimized FVIII have been patented [38,67,68] and some of them were studied [69] or are under development for gene therapy of hemophilia A [70,71]. In this regard, it would be of interest to



FVIII variant	k_a (1/M×s)	k_d (1/s)	K_D (M×10^{-10})
WT (n = 3)	(1.1 ±0.3)×10^6	(4.1 ±2.3)×10^{-4}	3.6 ±1.4
CO (n = 4)	(1.1 ±0.6)×10^6	(4.0 ±1.7)×10^{-4}	3.9 ±1.9
Xyntha (n = 2)	(2.3 ±0.4)×10^6	(7.5 ±0.6)×10^{-4}	3.4 ±0.3

Fig. 5. Binding of the factor VIII (FVIII) variants to von Willebrand factor (VWF) and low-density lipoprotein receptor-related protein 1 (LRP) cluster IV by surface plasmon resonance assay (SPR). (A) VWF was immobilized to a level of 300 resonance units (RU) and tested for binding to the wild-type BDD-FVIII (WT) (clone 1H1), codon-optimized BDD-FVIII (CO) (clone 3F1) and a BDD-FVIII control (Xyntha) used at concentrations of 0.5, 1, 2, 4 and 8 nM. The dissociation was recorded upon injection of the buffer only. Red lines show fits of the sensorgrams to the 1 : 1 (Langmuir) binding model. (B) The derived kinetic constants for interactions between the FVIII variants and VWF. (C) LRP cluster IV was immobilized at 500 RU and tested for binding to the WT (clone 1H1), CO (clone 3D4) and Xyntha used at concentrations of 6, 13, 25, 50 and 100 nM. [Color figure can be viewed at wileyonlinelibrary.com]

characterize the properties of the CO *in vivo*. Notably, a clinical testing of a codon-optimized factor IX demonstrated its safety and efficacy for treatment of hemophilia B [72,73] and resulted in its approval by the U.S. Food and Drug Administration. These trends indicate that new therapeutic products based on codon-optimized FVIII will appear in the future. During evaluation of such products, the potential risks associated with changing the coding sequence should be thoroughly evaluated. In addition to the known concerns, upon the use of such constructs in gene therapy, there is a possibility of *in vivo* translation of out-of-frame novel polypeptides, which may be immunogenic and interfere with metabolism [39]. The respective preclinical studies should address these concerns on a case-by-case basis.

Addendum

S. Shestopal designed and performed the majority of the experiments and wrote the paper; J-J. Hao performed the mass-spectrometry experiment; E. Karnaukhova performed the CD experiment; Y. Liang and M. Ovanesov performed the clotting and TGA assays; M. Lin performed the statistical evaluation of the results; J. Kurasawa provided experimental assistance; J. McVey contributed to the CO-construct design and the writing of the paper; T. Lee provided the project support; A. Sarafanov designed the research, analyzed the results and wrote the paper. All authors contributed to analysis of data relevant to their experimental discipline and writing respective sections, and approved the final version of the paper.

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Disclosure of Conflict of Interests

This work was supported by funds from the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration and by an appointment to the research program administered by the Oak Ridge Institute for Science and Education through an interagency agreement with the U.S. Department of Energy. These contributions are an informal communication and represent the best judgment of the authors and do not bind or obligate the U.S. Food and Drug Administration. The mentioning of particular commercial FVIII products in this work was not intended to promote or point out any advantage or disadvantage of those over other FVIII products, and was due solely to the experimental convenience. The authors have no financial interest in development of any of the FVIII variant mentioned in this work and no other competing interests. J. McVey reports personal fees from Profactor Pharma Ltd, outside the submitted work, and has patents WO/2011/005968; PCT/US2010/041378 licensed to Biomarin and 1304973.9 pending to Profactor Pharma Ltd.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Time-course of the factor VIII variant expression by CHO cell clonal lines.
Fig. S2. The amino acid sequences of the factor VIII variants by LC/MS/MS.

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Supporting Information

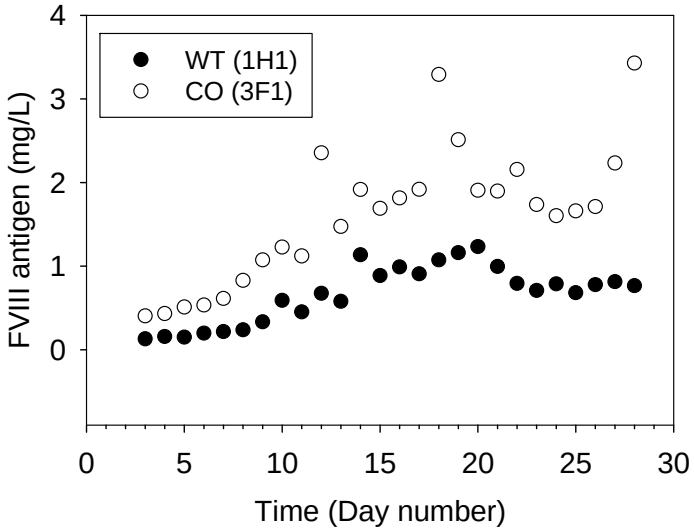

Fig. S1. Time-course of the FVIII variants expression by CHO cells clonal lines. The cells expressing WT (clone 1H1) and CO (clone 3F1), were cultured in multi-layer flasks for 29 days. From the day three, the media were replaced daily and quantitated for the secreted FVIII antigen by ELISA.

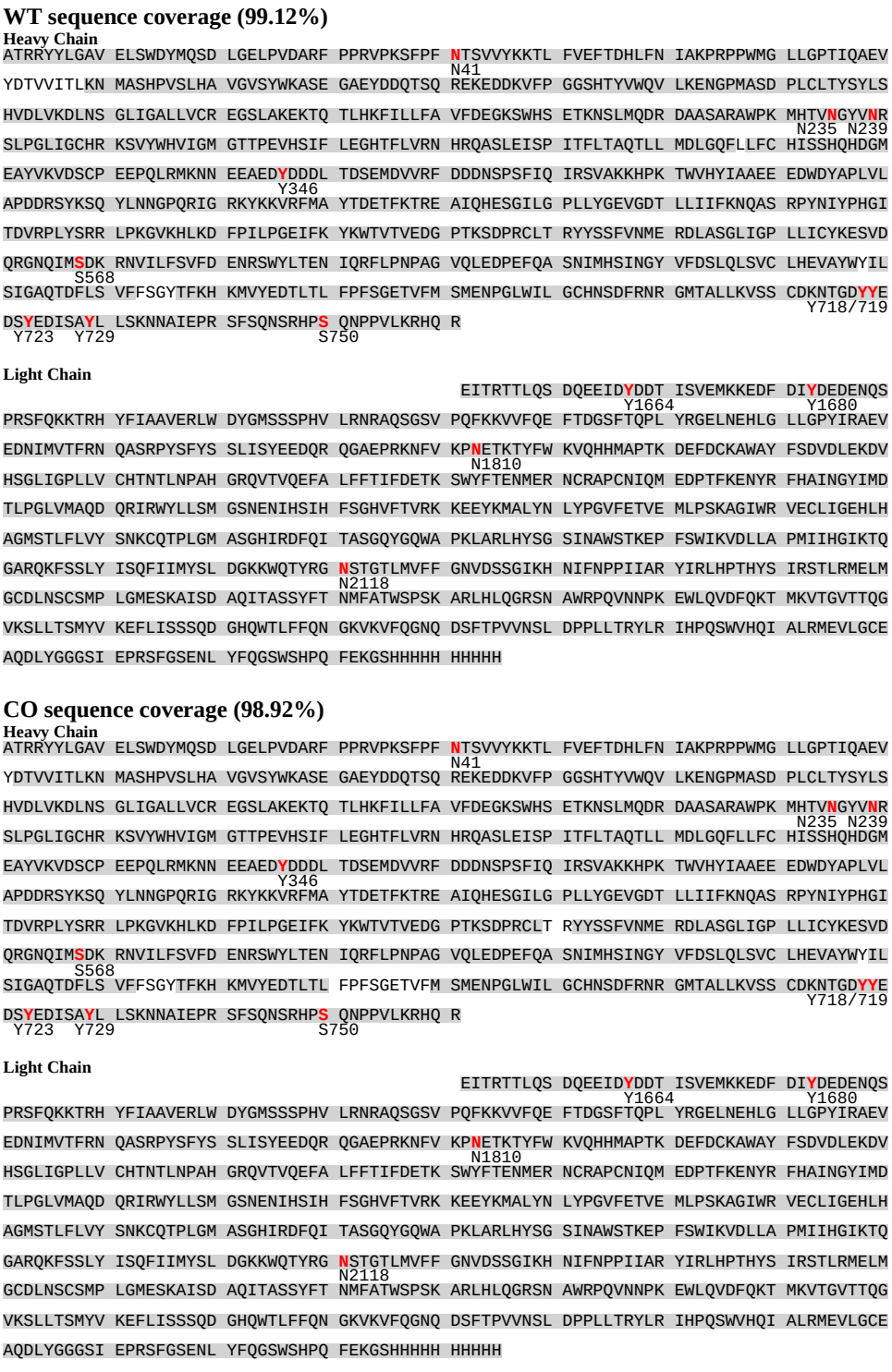

Fig. S2. The amino acid sequences of the FVIII variants by LC/MS/MS. The analysis was performed in two independent experiments using the WT preparations isolated from the clonal lines 1H1 and 1G1, and the CO preparations isolated from the clonal lines 3D4 and 3F1. Amino acid residues detected with the high confidence (1% false discovery rate) are highlighted in grey. Residues with post-translational modifications are shown in red. The sequence was based on human FVIII (NCBI reference NP 000123); the numbering of amino acids corresponds to that of the mature peptide of full-length human factor VIII.