High Level Expression of Recombinant Porcine Coagulation Factor VIII*

Recombinant human factor VIII expression levels, in vitro and in vivo, are significantly lower than levels obtained for other recombinant coagulation proteins. Here we describe the generation, high level expression and characterization of a recombinant B-domain-deleted porcine factor VIII molecule. Recombinant B-domain-deleted porcine factor VIII expression levels are 10- to 14-fold greater than recombinant B-domain-deleted human factor VIII levels by transient and stable expression in multiple cell lines. Peak expression of 140 units 10^6 cells^{-1}·24 h^{-1} was observed from a baby hamster kidney-derived cell line stably expressing recombinant porcine factor VIII. Factor VIII expression was performed in serum-free culture medium and in the absence of exogenous von Willebrand factor, thus greatly simplifying protein purification. Real time reverse transcription–PCR analysis demonstrated that the differences in protein production were not caused by differences in steady-state factor VIII mRNA levels. The identification of sequence(s) in porcine factor VIII responsible for high level expression may lead to a better understanding of the mechanisms that limit factor VIII expression.

Factor VIII (FVIII) is a large (~300 kDa) glycoprotein that functions as an integral component of the intrinsic pathway of blood coagulation. Mutations in the FVIII gene that result in decreased or defective FVIII protein give rise to the genetic disease, hemophilia A, which is phenotypically characterized by recurrent bleeding episodes. Treatment of hemophilia A entails intravenous infusion of either human plasma-derived or recombinant FVIII material. Approximately 25% of all hemophilia A patients treated with FVIII products develop antibodies that inhibit FVIII activity and limit treatment efficacy (1). Patients with FVIII-inhibitory antibodies can be treated using porcine plasma-derived FVIII products, which generally display low cross-reactivity with the human FVIII antibodies (2, 3). Currently there is not a recombinant porcine FVIII product available for clinical use.

Since the introduction of recombinant FVIII for the treatment of hemophilia A, commercial suppliers have struggled to keep up with high patient demand (4). The shortage of recombinant FVIII material has precluded prophylactic treatment of severely affected patients, limited the implementation of immune-tolerance regimens, and kept treatment costs high. Unfortunately, FVIII is expressed and recovered at low levels in the heterologous mammalian cell culture systems used for commercial manufacture. The importance of this problem has fueled significant research efforts to overcome the low FVIII expression barrier, and several basic mechanisms have been identified that limit FVIII expression (for review, see Kaufman et al. (5)). Despite these findings, FVIII expression levels remain low, and a product shortage persists.

The porcine FVIII cDNA sequence has been reported and shown to encode the homology-defined internal protein domain structure, A1-A2-B-ap-A3-C1-C2 (6, 7). Porcine FVIII shares 83% amino acid identity with human FVIII outside of the 2.7-kb B-domain, which has no ascribed function. Additionally, B-domain deletion has been shown to increase FVIII protein production in heterologous systems (7, 8). In this study, we compared recombinant B-domain-deleted porcine FVIII (rp-FVIII OL) to recombinant B-domain-deleted human FVIII (rh-FVIII SQ) in terms of protein expression, purification, and activity.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s phosphate-buffered saline, fetal bovine serum, penicillin, streptomycin, DMEM-F-12 medium, and AIM V culture medium were purchased from Invitrogen (Carlsbad, CA). Cell transfections were performed using Lipofectin (for baby hamster kidney-derived (BHK-M) cells) or LipofectAMINE (for COS-7 cells) (Invitrogen). Antibiotic selection was done using geneticin (Invitrogen). Transient transfections were controlled for transfection efficiency using the RL-CMV vector and Dual-Luciferase assay kit (Promega, Madison, WI). Cloning times were measured using a Start coagulation instrument (Diagnostic Stago, Asnieres, France). Citrated FVIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KS). Activated partial thromboplastin reagent was purchased from Organon Teknika (Durham, NC). Human vWF was isolated as described previously (9). RNA was purified from cultured cells using TrizReagent (Sigma). In vitro-transcribed FVIII RNA standards were generated using the mMessage mMachine Kit (Ambion, Austin, TX). FVIII RNA quantitation was performed using the ABI 7000 Sequence Detection System and the ABI SYBR Green RT-PCR Kit (Applied Biosystems, Foster City, CA). Oligonucleotides were synthesized by Invitrogen. BrightStar nylon membrane was purchased from Ambion.

Construction of Human and Porcine FVIII Expression Vectors—Rh-FVIII SQ was created by cloning the human FVIII cDNA into the mammalian expression vector ReNeo (10) and using splicing-by-overlap extension mutagenesis (11) to modify the nucleotide sequence between the A2 and A3 domains to encode a SFPSQNPVPLRHQR linker. This amino acid sequence includes the RHQR recognition sequence for PACE/furin processing (12).

The cloning and sequencing of the porcine FVIII cDNA has been described previously (6). Two B-domain-deleted FVIII expression constructs were created by ligation of the porcine FVIII cDNA into the ReNeo vector and using splicing-by-overlap extension mutagenesis to modify the nucleotide sequence between the A2 and A3 domains. One

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‡ The abbreviations used are: FVIII, factor VIII; rp-FVIII OL, recombinant B-domain-deleted porcine FVIII; rh-FVIII SQ, recombinant B-domain-deleted human FVIII; BHK-M, baby hamster kidney-derived; RT-PCR, reverse transcription-PCR; DMEM, Dulbecco’s modified Eagle medium; vWF, von Willebrand factor.
vector contains the identical human SQ linker amino acid sequence described above (designated rp-fVIII SQ), and the second vector contains a similar porcine-derived sequence SFAQNSRPPSASPKPVLRHHQR (designated rp-fVIII OL). Both constructs contain the RRQR FACE/furin recognition sequence. Expression data for rp-fVIII SQ and rp-fVIII OL, cultured in serum-free medium and stored under these conditions.

**Results**

Transient and Stable Expression of Human and Porcine Recombinant fVIII—Expression of rp-fVIII OL was compared with rh-fVIII SQ in the context of the same mammalian expression vector. COS-7 cells were transiently transfected with either rh-fVIII SQ or rp-fVIII OL as well as a luciferase control vector and cultured in serum-free medium for 24 h. Luciferase activity was measured using the BrightStar BioDetect Nonisotopic Detection Kit (Ambion) following the manufacturer’s instructions. Cross-hybridization between the human and porcine VIIIA sequences was not observed under these conditions.

**Purification of Rp-fVIII OL**—A two-step ion-exchange chromatography procedure was used to isolate rp-fVIII OL from conditioned serum-free medium. Briefly, rp-fVIII OL-containing medium was loaded onto a 5 × 20-cm SP-Sepharose Fast Flow column equilibrated in 0.18 M NaCl, 20 mM HEPES, 5 mM CaCl2, 0.01% Tween 80, pH 7.4. Rp-fVIII OL was eluted with a linear 0.2 to 0.8 M NaCl gradient in the same buffer. Fractions containing fVIII were pooled, diluted to 0.2 M NaCl in the same buffer, applied to a Mono Q fast protein liquid chromatography column, and eluted with a linear 0.2–1.0 M NaCl gradient. Fractions were analyzed by one-stage coagulation assay at A<sub>540</sub> and SDS-9% polyacrylamide gel electrophoresis. 

**VIIIA Activity Decay Measurements—Activated VIIIA (VIIIA) was measured by chromogenic assay using purified human fIXa, human fX, and synthetic phospholipid vesicles as described previously (17). Briefly, 20 nM rp-fVIII OL or rh-fVIII SQ was activated by 30-s incubation with 100 nM human thrombin at room temperature. The reaction was stopped by the addition of 150 mM desulfotinhirudin, and VIIIA activity was measured at several time points.

**RESULTS**

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Stable expression of rh-fVIII SQ and rp-fVIII OL was gener-
Adding 100 mg of purified human vWf. vWf was added at concentrations of 0, 0.2, 2, 20, 200 μg/ml. After 24 h of incubation, the conditioned medium was assayed for fVIII activity by one-stage coagulation assay. The conditioned serum-free medium was collected every 24 h, assayed for fVIII activity and replaced with an equal volume of fresh serum-free medium. fVIII production from both sets of clones increased with time (data not shown). Rp-fVIII OL expression from the highest expressing clone peaked at 140 units 10^6 cells^-1 24 h^-1, whereas the maximum rh-fVIII SQ expression observed was 10 units 10^6 cells^-1 24 h^-1.

Addition of purified vWf or co-expression of vWf and fVIII transgenes has been shown to increase fVIII production in heterologous expression systems (8, 18, 19). Purified human vWf was added to the culture medium of the highest expressing rh-fVIII SQ and rp-fVIII OL clones at several concentrations, and fVIII production was measured (Fig. 1B). At the highest concentration of vWf tested (200 μg/ml), rh-fVIII SQ expression levels reached 15.7 ± 2.8 units 10^6 cells^-1 24 h^-1 (mean ± S.D.). In contrast, rp-fVIII OL production appeared to peak and level off at 2 μg/ml vWf, with rp-fVIII OL expression levels of 50.7 ± 7.8 units 10^6 cells^-1 24 h^-1.

Quantitation of fVIII mRNA—High level expression of porcine fVIII could result from disproportionately high steady-state mRNA levels. Real time quantitative fVIII RT-PCR was performed on total RNA from BHK-M cell clones stably expressing rh-fVIII SQ and rp-fVIII OL (Fig. 2). The numbers of fVIII transcripts/cell were not significantly different between rh-fVIII SQ- and rp-fVIII OL-expressing clones (Mann-Whitney U test, p = 0.74), despite significant differences in fVIII protein production. Linear regression analysis revealed a statistically significant correlation between fVIII activity and fVIII transcripts for both rh-fVIII SQ and rp-fVIII OL (p < 0.05). Northern blot analysis of total RNA from the three highest expressing rp-fVIII OL and rh-fVIII SQ clones, identified a band at nucleotide 6,000 whose relative intensity correlated directly with the number of fVIII transcripts/cell as determined by real time RT-PCR (data not shown).

Purification and Characterization of Rp-fVIII—Milligram quantities of rp-fVIII OL were purified 5,300-fold from 10 liters of conditioned medium using a two-step ion-exchange chromatography procedure (Table I). Approximately 54,000 units of

![Diagram](http://www.jbc.org/...)

**Fig. 2.** Real time RT-PCR quantitation of rp-fVIII OL and rh-fVIII SQ mRNA from individual fVIII-expressing clones. Total RNA was harvested from rp-fVIII OL-expressing (open triangles) and rh-fVIII SQ-expressing (open circles) clones (n = 15 each) and assayed for fVIII mRNA by one-step real time RT-PCR. Absolute quantitation was achieved by generating a standard curve using known amounts of in vitro-transcribed rh-fVIII SQ RNA. RT-PCR reactions were performed in triplicate, and the data shown are the mean values. Prior to RNA harvest, the cells were cultured for 24 h in serum-free medium and assayed for fVIII activity by one-stage coagulation assay. Data from individual clones are presented as fVIII activity versus fVIII transcripts.
purified rp-fVIII OL were obtained at a yield of 94%. The specific activity of rp-fVIII OL was calculated using an estimation of the molar extinction coefficient obtained by absorbance at 280 nm and the known tyrosine, tryptophan, and cysteine content (20). The mean (± S.D.) specific activity of the peak fractions obtained from three independent preparations was 2,050 ± 770 units/nmol (12,400 ± 4,640 units/mg). This value is slightly higher, but not significantly different (Student's t test, p = 0.4), than that obtained for rh-fVIII SQ from four separate preparations that were purified using the same method (1,630 ± 530 units/nmol (9,870 ± 3,180 units/mg)).

The purity of isolated rp-fVIII OL was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). The majority of the purified protein was present in the heterodimeric (heavy chain/light chain) form characteristic of PACE/furin intracellular processing (21). A small amount of unprocessed, single-chain material also was present. After incubation with thrombin, A1, A2, and A3-C1-C2 bands appeared, representative of heterotrimeric thrombin-activated fVIII (fVIIIa) (22, 23).

Dissociation of the A2 subunit from the heterotrimeric form of fVIII results in loss of fVIIIa cofactor activity. Following thrombin activation, decay of rp-fVIIIa OL and rh-fVIIIa SQ was monitored over a 30-min time course (Fig. 4). Decay of rp-fVIIIa OL cofactor activity was substantially slower than rh-fVIIIa SQ. Rp-fVIIIa OL and rh-fVIIIa SQ demonstrated half-lives from 7 to 10 and from 2 to 3 min, respectively.

**DISCUSSION**

The study of fVIII biosynthesis primarily has been limited to in vitro heterologous expression systems because there are no known cell lines that express endogenous fVIII (5). Using heterologous expression, several factors that limit expression have been identified, including low mRNA levels (24–26), interaction with protein chaperones and inefficient secretion (27–29), and rapid decay in the absence of vWF (18, 19). Despite these insights into fVIII regulation, expression continues to be significantly lower than other recombinant proteins in the heterologous systems used in commercial manufacturing (5) as well as ex vivo (30) and in vivo gene-therapy applications (31).

In this study, we demonstrate that rp-fVIII OL is expressed at levels up to 14-fold greater than rh fVIII SQ. These levels are substantially greater than in previously published reports of fVIII expression (8, 18, 19, 32). No specific manipulations were made to either the cDNAs or the expression vector designed to enhance fVIII expression other than the incorporation of a linker sequence between the A2 and A3 domains that includes a PAC/E-furin recognition sequence. This linker function to facilitate intracellular processing of the single-chain fVIII protein into the heterodimeric (A1-A2/A3-C1-C2) form (21). Rp-fVIII OL and rh-fVIII SQ expression cassettes do not contain endogenous fVIII 5′-untranslated region sequence, whereas both possess the first 749 nucleotides (of 1805 nucleotides) of the human fVIII 3′-untranslated region (33).

Interestingly, the increased production of rp-fVIII OL does not equate with higher steady-state levels of fVIII mRNA (Fig. 2). This finding precludes increased transcription rates, enhanced nuclear export, or greater mRNA stability as possible mechanisms for the high level expression of rp-fVIII. There is likely an increased translational and/or post-translational efficiency of rp-fVIII OL expression. Further studies are warranted to identify differences in the kinetics of expression between human and porcine fVIII.

The expression of rp-fVIII OL from BHK-M cells in serum-free medium is sufficiently high that a two-step procedure using only ion-exchange chromatography is adequate to remove contaminant proteins (Fig. 3). In contrast, commercial manufacture of existing recombinant fVIII products involves the use of immunoaffinity chromatography, which complicates the development validation process. This potential simplification may lead to more economical commercial production of fVIII.

Previously, we compared the specific activity of purified plasma-derived porcine fVIII to plasma-derived human fVIII (17).
The specific activity of porcine FVIII increased as a function of concentration and exceeded the specific activity of human FVIII. In the present study, the concentration of rp-FVIII OL was maintained within the limits of the clotting times obtained for the pooled human plasma standard curve. Under these conditions, the specific activities were not significantly different. In contrast, the decay of thrombin-activated rh-FVIII SQ activity was significantly faster than that of rp-FVIII OL (Fig. 4) and was similar to a previous comparison of plasma-derived human and porcine FVIII (17). The spontaneous loss of activity of thrombin-activated FVIII is caused by dissociation of the A2 subunit (23, 34). This indicates that A2-subunit dissociation does not contribute to FVIII activity under the normal conditions of the one-stage FVIII coagulation assay.

In a two-stage assay, FVIII is activated with thrombin in the first stage. Evidence that A2-subunit dissociation does contribute to the two-stage assay comes from the identification of patients with mild hemophilia A who have low-stage activity relative to one-stage activity (35, 36). These patients have abnormally fast A2 dissociation rates due to mutations in the A1 and/or A2 domains.

It should be possible to identify porcine FVIII cDNA sequence(s) that confer(s) high level expression and to generate a recombinant hybrid human/porcine FVIII that incorporates only the porcine A2 domains. Such a “high-expression” FVIII construct could be extremely valuable for increasing the production capability of commercial recombinant FVIII therapeutics, which remain costly and in limited supply. Additionally, because the high expression phenotype of hybrid porcine/human FVIII is caused by differences at the translational or post-translational level, it should also be expressed at high levels from viral vector systems, thereby functioning to increase the effectiveness of gene-therapy approaches for hemophilia A.

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