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High Level Expression of Recombinant Porcine Coagulation Factor VIII*

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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)[1] 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). Clotting times were measured using a STart coagulation instrument (Diagnostica 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 TriReagent (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 SFSQNPPVLKRHQR 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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[1] 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 SFAQNSRPPSASAPKPPVLRRHQR (designated rp-fVIII OL). Both constructs contain the RHQR PACE/furin recognition sequence. Expression data for rp-fVIII SQ and rp-fVIII OL were similar; thus, only data for rp-fVIII OL are presented.

fVIII Activity Measurements—For all fVIII-expression experiments, cells were rinsed twice with Dulbecco's phosphate-buffered saline and cultured in serum-free medium for 24 h prior to assaying fVIII activity. Activity was measured using the activated partial thromboplastin reagent-based one-stage coagulation assay. Briefly, 5 μl of sample or standard was added to 50 μl of fVIII-deficient plasma, followed by addition of 50 μl of activated partial thromboplastin reagent reagent and incubation for 3 min at 37 °C. Fifty microliters of 20 mM $CaCl_2$ was added to initiate the reaction, and the time required to develop a fibrin clot was measured viscometrically. Standard curves were generated using several dilutions of FACT and analyzed by linear regression analysis of the clotting time *versus* the logarithm of the reciprocal plasma dilution. For determination of fVIII activity, samples were diluted in HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4) to a concentration within the range of the standard curve. Dilutions of purified rh-fVIII SQ and rp-fVIII OL within the range used to generate the standard curves produced lines parallel to that obtained for normal pooled human plasma on semilogarithmic plots (data not shown). Determination of the activation quotient (13) for rp-fVIII OL was done using a two-stage coagulation assay. Fifty microliters each of fVIII-deficient plasma and activated partial thromboplastin reagent were dispensed into a pre-warmed cuvette and incubated at 37 °C for 180 s. At 140 s, 4 units/ml porcine thrombin was added to the conditioned medium to activate rp-fVIII OL. At 160 s, the activated rp-fVIII OL was diluted from 40- to 80-fold and added to the cuvette containing fVIII-deficient plasma and activated partial thromboplastin reagent. To initiate the clotting time, 50 μl of 20 mM $CaCl_2$ was added to the cuvette. Clotting times were compared with a standard curve as described above. The activation quotient is defined as the ratio of fVIII activity measured by the two-stage assay divided by the activity measured by the one-stage coagulation assay. fVIII activity is expressed as units·10^6 $cells^{-1}$·24 h^{-1}. An estimate of 100,000 cells/cm^2 was used to calculate the fVIII expression values presented. The number of BHK-M cells/cm^2 was determined experimentally to be 99,300 ± 8,900 cells/cm^2 in five independent measurements.

The specific activity of individual preparations of rp-fVIII OL and rh-fVIII SQ was determined by taking the weighted number average of the specific activities measured from the peak fractions collected. Fractions demonstrating absorbance at 280 nm below 0.08 or an activation quotient of less than 20 were excluded. The mean values ± S.D. of the specific activities for all rp-fVIII OL and rh-fVIII SQ preparations are presented.

Transient Expression of Recombinant fVIII—COS-7 cells were grown to 70–80% confluence in 2-cm^2 wells containing 1 ml of DMEM:F-12 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with a 2000:1 mass ratio of fVIII (rp-fVIII OL or rh-fVIII SQ) plasmid:luciferase plasmid DNA. Twenty-four hours after transfection, the cells were rinsed twice with 1 ml of phosphate-buffered saline and 0.5 ml of serum-free medium was added to each well. Cells were cultured 24 h before the conditioned medium was harvested and fVIII activity was measured.

Stable Expression of Recombinant fVIII—BHK-M cells (14) were transfected with either rp-fVIII OL or rh-fVIII SQ and cultured 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 for 10 days. Between 72 and 84 geneticin-resistant clones were initially screened for fVIII production. The 24 clones exhibiting the highest level of fVIII activity were divided into individual 2-cm^2 wells and grown to greater than 80% confluence prior to being switched to 0.5 ml of serum-free medium. Twenty-four hours later, fVIII activity in the conditioned medium was determined by one-stage coagulation assay. The top three fVIII-expressing clones were then divided into 75-cm^2 flasks and grown to 90–95% confluence before being switched to 25 ml of serum-free medium. After 24 h, the conditioned medium was replaced with 25 ml of fresh serum-free medium and cultured for an additional 24 h. This process was repeated a third time to measure fVIII production between 48 and 72 h. Harvested medium from each time point was assayed for fVIII activity as described above.

Quantitation of fVIII mRNA—Total RNA was extracted from fVIII-expressing cell lines using TriReagent following the manufacturer's instructions. RNA concentrations were determined by absorbance at 260 nm (A_{260}) in H_2O. RNA standards, used for absolute quantitation of fVIII transcripts by real time RT-PCR, were generated using T7 polymerase-mediated *in vitro* transcription of rh-fVIII SQ and rp-fVIII OL cDNAs cloned into pBluescript II KS− (Stratagene, La Jolla, CA). *In vitro*-transcribed RNA was treated with DNase I for removal of plasmid DNA. Purified RNA standards were quantitated spectrophotometrically by A_{260} and stored at −70 °C in individual aliquots at a concentration of 10^{10} transcripts/μl. Amplification kinetics of human and porcine fVIII transcripts were found to be similar by performing RT-PCR reactions using serial dilutions of rh-fVIII SQ and rp-fVIII OL (10^2-10^7 transcripts/reaction) as template (data not shown). All data presented were calculated using *in vitro*-transcribed rh-fVIII SQ RNA standards.

Oligonucleotide primers used for RT-PCR assays were located in the fVIII sequence encoding the A2 domain. The oligonucleotide sequences are as follows: forward primer, 5′-ATGCACAGCATCAATGGCTAT-3′ and reverse primer, 5′-GTGAGTGTGTCTTCATAGAC-3′, and are located at positions 2047–2067 and 2194–2213 of the human fVIII cDNA sequence (15). Within these primer regions, the nucleotide sequences for human and porcine fVIII are identical. Therefore, the same primer pair can be used to amplify from both human and porcine fVIII templates. Reactions were done in 25 μl of total volume containing 1× SYBR Green PCR master mix, 300 μM forward and reverse primers, 6.25 units of Multi-Scribe reverse transcriptase and 5 ng of sample RNA. One-step RT-PCR was performed by incubation at 48 °C for 30 min followed by a single incubation at 95 °C for 10 min and 40 amplification cycles of 95 °C for 15 s then 60 °C for 1 min. Post-reaction dissociation analysis was used to confirm single-product amplification. The calculation of the number of transcripts per cell was derived using a value of 35 μg of total RNA per 10^6 BHK-M cells.

For Northern blot analysis, 5 μg of total RNA was separated by 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane as described previously (16). Rp-fVIII OL and rh-fVIII SQ cDNAs were labeled using the BrightStar psoralen-biotin nonisotopic labeling kit (Ambion) following the manufacturer's instructions. Biotin-labeled probes were denatured at 95 °C for 10 min and immediately added to ULTRAhyb (Ambion) hybridization buffer. Hybridization was performed overnight at 48 °C, followed by two 5-min washes in 2× SSC at room temperature and two 15-min washes in 0.1× SSC at 48 °C. (1× SSC is 150 mM NaCl, 15 mM Na_3 citrate-$2H_2O$, pH 7.0.) Probe detection was performed using the BrightStar BioDetect Nonisotopic Detection Kit (Ambion) following the manufacturer's instructions. Cross-hybridization between the human and porcine fVIII 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 $CaCl_2$, 0.01% Tween 80, pH 7.4. Rp-fVIII OL was eluted with a linear 0.18–0.65 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_{280} and SDS-9% polyacrylamide gel electrophoresis.

fVIII Activity Decay Measurements—Activated fVIII (fVIIIa) 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 nM desulfatohirudin, and fVIIIa activity was measured at several time points.

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. fVIII activity in the conditioned medium was measured by one-stage coagulation assay and normalized to luciferase activity in the corresponding cell lysate. Media collected from the cells transfected with rp-fVIII OL displayed 9-fold greater fVIII activity than media obtained from cells transfected with rh-fVIII SQ in three independent experiments (data not shown).

Stable expression of rh-fVIII SQ and rp-fVIII OL was gener-

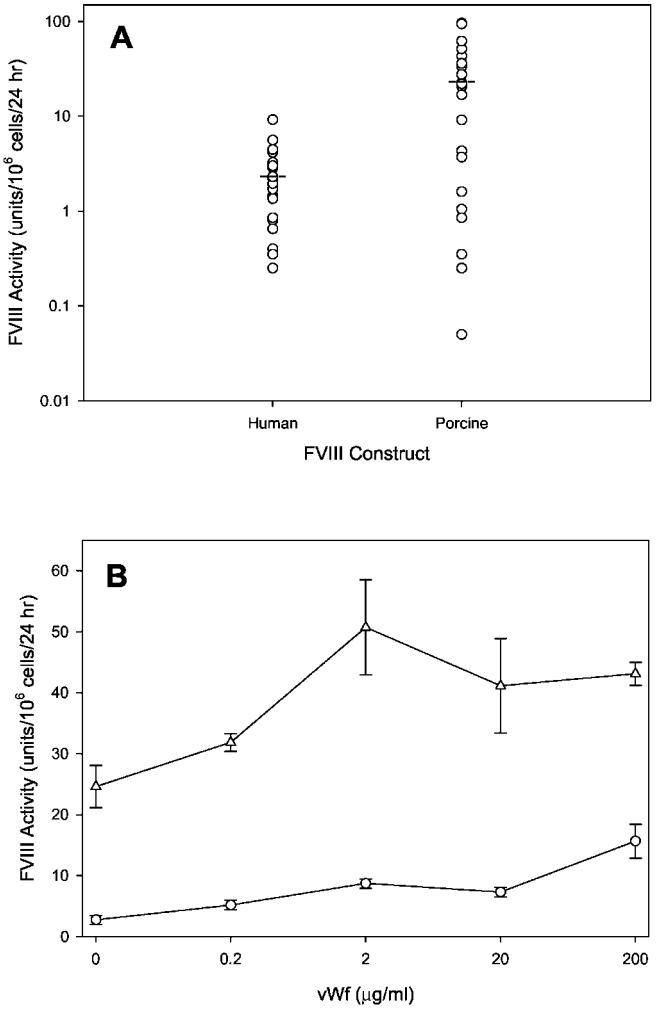

FIG. 1. **Heterologous expression of rp-fVIII OL and rh-fVIII SQ.** *A*, BHK-M cells were transfected with rp-fVIII OL or rh-fVIII SQ and selected for stable transgene integration. Individual clones expressing fVIII were apportioned into 24-well plates, grown to greater than 80% confluence, rinsed twice with phosphate-buffered saline, and cultured for 24 h in serum-free medium. After 24 h, the medium was harvested and assayed for fVIII activity. Each *open circle* represents the value obtained from an individual rp-fVIII OL- or rh-fVIII SQ-expressing clone (n = 22 and 24, respectively). The mean value of all clones in each group is represented by a *horizontal bar*. *B*, the highest expressing clones for rp-fVIII OL (*open triangles*) and rh-fVIII SQ (*open circles*) were cultured in serum-free medium for 24 h in the absence or presence of purified human vWf. vWf was added at concentrations of 0, 0.2, 2, 20, and 200 μg/ml. After 24 h of incubation, the conditioned medium was assayed for fVIII activity by one-stage coagulation assay. fVIII activity data shown represent the mean ± S.D. of four independent replicates.

ated by transfection of BHK-M cells with the respective expression vectors followed by antibiotic-resistance selection. Between 72 and 84 geneticin-resistant clones were selected and screened for fVIII expression. Because ~50% of the geneticin-resistant clones from both sets of transfections do not express levels of fVIII above background, only the top 24 fVIII-expressing rp-fVIII OL and rh-fVIII SQ clones were selected for further analysis. Conditioned serum-free medium from these clones was analyzed for fVIII activity after a 24-h culture period (Fig. 1*A*). fVIII activity measured from the rh-fVIII SQ and rp-fVIII OL populations ranged from 0.25 to 9.2 and 0.05 to 96 units·10^6 cells^{-1}·24 h^{-1}, respectively. The fVIII expression values measured for the two groups are significantly different (p = 0.014, Mann-Whitney U test). The rate of fVIII production from the top three rh-fVIII SQ- and rp-fVIII OL-expressing clones was monitored over a 72-h collection period. 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}.

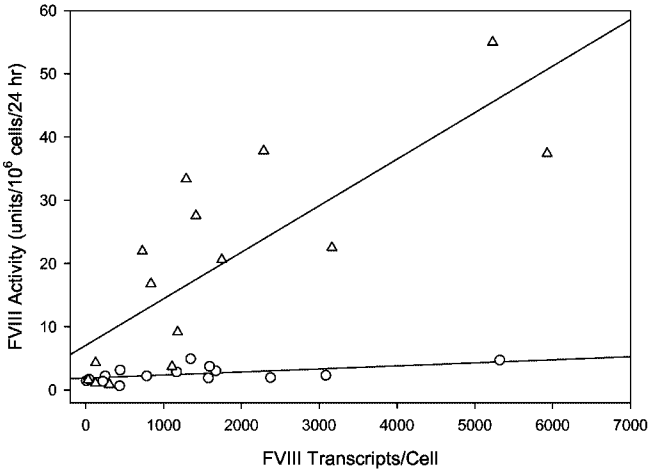

FIG. 2. **Real time RT-PCR quantitation of rp-fVIII OL and rh-fVIII SQ mRNA from individual fVIII-expressing clones.** Total RNA was harvested from rp-fVIII OL-expressing (*open triangles*) and rh-fVIII SQ-expressing (*open circles*) clones (n = 15 each) and assayed for fVIII mRNA by one-step real time 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.

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. 1*B*). 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

TABLE I
Purification of rp-fVIII OL

Sample	Volume	A_{280}	Total A_{280}	Activity	Units	Units/A_{280}	AQ[a]	Yield	Purification
	ml			*units/ml*			*-fold increase*	*%*	*-fold increase*
Medium	10,000	2.70	27,000	5.7	57,000	2.11	20	100	1
SP-Sepharose pool	235	0.05	11	160	37,400	3,460	106	66	1,640
Mono Q pool	4.8	1.00	4.8	11,200	53,700	11,100	70	94	5,290

[a] Activation quotient (see "Experimental Procedures").

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.

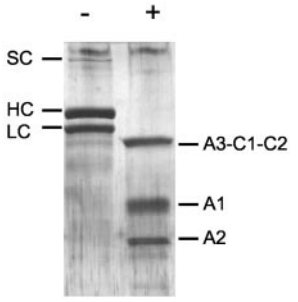

FIG. 3. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of rp-fVIII OL.** Purified rp-fVIII OL was treated (+) or not treated (−) with thrombin, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and visualized by silver stain. Single-chain (*SC*), heterodimeric heavy-chain (*HC*), light-chain (*LC*), thrombin-cleaved light-chain (*A3-C1-C2*), and thrombin-cleaved *A1* and *A2* fragments are identified.

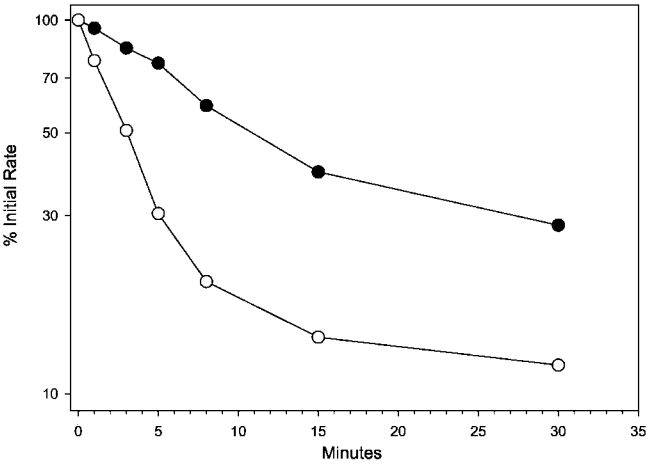

FIG. 4. **Decay of rp-fVIII OL following thrombin activation.** Purified rp-fVIII OL (*closed circles*) and rh-fVIII SQ (*open circles*), 20 nM each, were activated by addition of 100 nM porcine thrombin. After 30 s of incubation, thrombin activity was inhibited by addition of desulfatohirudin, and fVIIIa activity was determined as described under "Experimental Procedures." Data are expressed as percent initial activity and are representative of two independent experiments.

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 PACE/furin recognition sequence. This linker functions 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 developmental 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 two-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 sequences that are necessary and/or sufficient for high level expression. Previous studies have demonstrated that functional fVIII protein can be produced from hybrid human/porcine molecules (10, 37–39). This type of analysis could enable the identification of a novel type of genetic or biochemical regulation for fVIII that may or may not be shared by other proteins. 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 casued 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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