Increased Production of Functional Recombinant Human Clotting Factor IX by Baby Hamster Kidney Cells Engineered to Overexpress VKORC1, the Vitamin K 2,3-Epoxide-reducing Enzyme of the Vitamin K Cycle*

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Some recombinant vitamin K-dependent blood coagulation factors (factors VII, IX, and protein C) have become valuable pharmaceuticals in the treatment of bleeding complications and sepsis. Because of their vitamin K-dependent post-translational modification, their synthesis by eukaryotic cells is essential. The eukaryotic cell harbors a vitamin K-dependent γ-carboxylation system that converts the proteins to γ-carboxyglutamic acid-containing proteins. However, the system in eukaryotic cells has limited capacity, and cell lines overexpressing vitamin K-dependent clotting factors produce only a fraction of the recombinant proteins as fully γ-carboxylated, physiologically competent proteins. In this work we have used recombinant human factor IX (r-hFIX)-producing baby hamster kidney (BHK) cells, engineered to stably overexpress various components of the γ-carboxylation system of the cell, to determine whether increased production of functional r-hFIX can be accomplished. All BHK cell lines secreted r-hFIX into serum-free medium. Overexpression of γ-carboxylase is shown to inhibit production of functional r-hFIX. On the other hand, cells overexpressing VKORC1, the reduced vitamin K cofactor-producing enzyme of the vitamin K-dependent γ-carboxylation system, produced 2.9-fold more functional r-hFIX than control BHK cells. The data are consistent with the notion that VKORC1 is the rate-limiting step in the system and is a key regulatory protein in synthesis of active vitamin K-dependent proteins. The data suggest that overexpression of VKORC1 can be utilized for increased cellular production of recombinant vitamin K-dependent proteins.

Recycnamnt factors VII, VIIa, VIII, IX, and protein C have become important pharmaceuticals in treatment of traumatic bleeding complications (1), hemophilia (2), and sepsis (3). These proteins are parts of the coagulation system (4), a system of plasma and membrane proteins that are responsible for arrest of blood loss at sites of injury.

Factors VII, IX, and protein C belong to a family of vitamin K-dependent proteins that are modified post-translationally to contain γ-carboxyglutamic acid (Gla),1 Ca2+ binding amino acid residues (5). The modification is carried out by the vitamin K-dependent γ-carboxylation system located in the endoplasmic reticulum (ER) (5). Two essential enzymes of the system are 1) the vitamin K-dependent γ-carboxylase, an integral membrane protein of 92 kDa that requires reduced vitamin K (vit.K1H2) as cofactor, and 2) the warfarin-sensitive enzyme vitamin K 2,3-epoxide reductase (VKOR), which produces the cofactor (5).

A major problem with production of recombinant vitamin K-dependent coagulation factors for use as pharmaceuticals has been poor recovery of functional proteins from the cell medium (6, 7). It has been shown that poor recovery results from incomplete γ-carboxylation of the secreted proteins (7) and incomplete removal of the propeptide by the protease PACE/furin in the Golgi apparatus (8). Incomplete γ-carboxylation is a significant problem as <10% of the secreted recombinant vitamin K-dependent proteins have been reported to be fully γ-carboxylated and functional (7). It is believed that incomplete γ-carboxylation occurs when an excess of newly synthesized precursors of vitamin K-dependent proteins appears in the ER and exceeds the capacity of the cell’s γ-carboxylation system to fully modify all of the precursors (6, 7).

Following cloning of γ-carboxylase (9), it was shown, surprisingly, that overexpression of γ-carboxylase in cells stably expressing recombinant human factor IX (r-hFIX) inhibited synthesis of functional r-hFIX (10). Because of our limited knowledge of the vitamin K-dependent γ-carboxylation system, the mechanism of inhibition is not fully understood (11). However, the recent identification of VKORC1 (12), an 18-kDa ER membrane protein believed to be a subunit of VKOR (13), has provided new opportunities to understand the system. When expressed in various cell lines, VKORC1 increases reduced vitamin K cofactor production (14, 15). We have shown that overexpression of VKORC1 in baby hamster kidney (BHK) cells increases the capacity of the cell’s γ-carboxylation system (14). Furthermore, and importantly, we have shown that VKOR is the rate-limiting step in the system (14, 16). This finding is consistent with results obtained by Hallgren et al. (11). In a previous article we measured increased γ-carboxylation capacity by VKORC1-transfected cells as γ-carboxylation of the synthetic γ-carboxyglutamic acid peptide substrate FLEEL. In this report, we have shown that r-hFIX-producing BHK cells, stably trans-
fected with a VKORC1 cDNA construct, produce 2.9-fold more fully γ-carboxylated functional r-hFIX than r-hFIX-producing BHK cells using the endogenous vitamin K-dependent system of the cell to γ-carboxylate the protein. This finding provides new opportunities for increased production of fully γ-carboxylated and functional recombinant vitamin K-dependent proteins.

EXPERIMENTAL PROCEDURES

Cell Lines

Engineering of BHK 21 cell lines stably overexpressing γ-carboxylase, VKORC1, and VKORC1 + γ-carboxylase is described in detail in Ref. 14.

Cell Protein Extraction

BHK cells were washed with ice-cold phosphate-buffered saline, and cell protein was extracted with 50 mM Tris, 1% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, pH 7.2, containing 10 μg/ml of the protease inhibitor mixture for use with mammalian cell and tissue extracts (Sigma) as described (14).

SDS-PAGE and Western Blotting

SDS-PAGE was carried out on 8–16% Criterion gradient gels (Bio-Rad). Western blotting was carried out as described (14). Furin Convertase antibodies (PA1–062) were from Affinity BioReagents, Golden, CO.

Cloning of Human Factor IX into the pLXIN Retroviral Vector

Plasmid pCMV5FIX containing the full-length human factor IX (hFIX) cDNA was purchased from American Type Culture Collection (catalogue number 79871). BamHI restriction sites were generated at the 5’- and 3’-ends of hFIX cDNA using PCR to clone the cDNA into the pLXIN retroviral vector (Clontech) for expression in mammalian cell lines. Kozak sequence was also generated at the 5’-end of the hFIX cDNA. The oligonucleotides used for hFIX PCR were sense primer 5’-GGAGCCGCCGACCATGCGCGTGATCT-3’ and antisense primer 5’-GGATGCGCTTATGAAGCTT-3’. Thirty-five cycles of PCR were performed under the following conditions: Denature at 94 °C for 30 s, anneal for 1 min at 55 °C, followed by an extension at 72 °C for 7 min. The modified cDNA for human factor IX was then cloned into the BamHI site of the pLXIN vector under control of the cytomegalovirus promoter. Recombinant plasmid with cDNA for hFIX was sequenced on both strands to eliminate any PCR errors.

Retroviral Packing

PG 13 cells (a kind gift from Dr. Charles Morrow, Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC) were transfected with the pLXIN retroviral vector containing the hFIX insert for packing. Retroviral particles were harvested from the PG 13 cell medium 72 h post-transfection. Harvested medium was centrifuged at 3000 × g to remove cell debris. The supernatant was collected and used to infect BHK cells and BHK cells engineered to overexpress γ-carboxylase, VKORC1, and VKORC1 + γ-carboxylase. Stable r-hFIX-producing cell lines were selected with G418 pressure.

Cell Culture

Cells were seeded and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 500 μg/ml G418, and 400 μg/ml Zeocin. At confluence, the attached cells were washed twice with PBS and continued growing in Dulbecco’s modified Eagle’s medium without serum but with the addition of 5 μg/ml vitamin K3 (AquaMEPHYTON; Merck & Co. Inc.). After 24 h the serum-free medium was collected and used for purification of r-hFIX.

Preparation of hFIX Antibodies

Purified human factor IX (hFIX 1009; 1.31 mg/ml) was purchased from Enzyme Research Laboratories, South Bend, IN and used for antibody production as described (17). The presence of antibodies in the rabbit sera was confirmed by Ouchterlony immunodiffusion (18). All sera showed the presence of precipitating anti-hFIX antibodies.

Preparation of Affinity-purified Conformational-specific and Nonconformational-specific Anti-hFIX Antibodies

The hFIX antiserum was made 50% saturated with ammonium sulfate, and precipitated proteins were collected by centrifugation at 10,000 × g for 10 min in a fixed angle rotor. The protein pellet was surface washed with Tris-buffered saline (TBS) containing 5 mM CaCl2 (TBS/ CaCl2) and dissolved in half the antiserum volume with TBS/EDTA. The solubilized serum proteins were passed through a 0.2-μm syringe nylon filter before being loaded onto an affinity column with purified hFIX as immobilized ligands. The column was equilibrated in TBS/CaCl2. The column was prepared by attaching purified hFIX to 6-aminohexanoic acid N-hydroxysuccinimide ester-Sepharose 4B as recommended by the supplier (Sigma). Conformational-specific antibodies that recognize the Ca2+-induced secondary structure of the Gla region in hFIX were released from the column with 50 mM EDTA in TBS (TBS/EDTA). Following release of the conformational-specific antibodies, the column was washed extensively with TBS/EDTA before non-conformational-specific anti-hFIX antibodies were eluted with 0.1 M sodium acetate buffer, pH 4.0, containing 4 M urea and 0.5 M NaCl (urea, pH 4) as described by our laboratory (17). Both fractions of affinity-purified antibodies were dialyzed at 4 °C against 50% glycerol in water and stored as aliquots at −85 °C.

Characterization of r-hFIX

Blood Clotting Activity—Clotting activities of purified r-hFIX and purified plasma hFIX were determined with the Activated Partial Thromboplastin Time kit (APTT-SP) (liquid) and the APTT-SP from Instrumentation Laboratory, Lexington, MA. To determine the specific activity of r-hFIX, a standard curve of dilutions of purified plasma hFIX was made. One unit of hFIX activity was defined as the activity of hFIX in 1 ml of pooled human plasma.

N-terminal Edman Degradation—This analysis was carried out by the Protein Core Laboratory at Wake Forest University School of Medicine.

Gla Analysis—This analysis of r-hFIX was carried after alkaline hydrolysis of the purified protein in 4 mM KOH under vacuum for 16 h at 120 °C. Amino acids were separated by HPLC according to a modification of the method described by Wu and Knabe (19), which provides excellent separation of the o-phthaldialdehyde derivatives of Gla, Asp, and Glu. Separation of the number of Gla residues in r-hFIX was based on the known numbers of Asp and Glu residues in purified hFIX (20). Separation was made by isocratic elution from a Supelcosil™ LC-18 column (15 cm × 4.6 mm, 3 μm) (Sigma) with a mixture of 86% of solvent A (0.1 M sodium acetate, pH 7.2, containing 9% methanol and 0.5% tetrahydrofuran) and 14% of solvent B (100% methanol). Prior to amino acid analysis, 0.1 ml of amino acid standards and protein hydrolysates was mixed with 0.1 ml of a solution of benzoic acid with 175 ml of saturated K2B4O7 in water and bringing the total volume to 1.6 ml with HPLC water (Solution C). Equal volumes of Solution C and o-phthaldialdehyde (OPA) reagent (made by dissolving 50 mg of o-phthaldialdehyde in 1.25 ml of methanol to which 11.2 ml of 0.04 M sodium borate buffer, pH 9.5, was added) were mixed and incubated for 2 min in the WA- TERS 2695 HPLC system before separation of the o-phthaldialdehyde derivatives. Detection of fluorescence was made with a JASCO FP-152 detector with excitation and emission wavelengths of 340 and 450 nm, respectively.

Denaturation—Digitized images of immunoreactive protein bands on Fuji Medical X-Ray Film SuperRX (Fisher Scientific, Pittsburgh, PA) were analyzed with Kodak 1D software (Eastman Kodak) to determine the integrated areas representing the protein bands. For determination of r-hFIX protein content, standard curves of Western blots with known purified human FIX were established and compared with unknown samples. All measurements were adjusted to be in the linear range of the standard curve.

RESULTS

Stable Cell Lines Expressing r-hFIX and Recombinant Proteins of the Vitamin K-dependent γ-Carboxylation System—Retroviral particles containing an hFIX cDNA construct were used to infect BHK cells and BHK cells stably overexpressing 1) VKORC1, 2) γ-carboxylase, and 3) VKORC1 + γ-carboxylase. Engineering of cell lines 1), 2), and 3) is described in Ref. 14. Retroviral-infected BHK cells and the BHK cell lines 1, 2, and 3 produced 16 ± 3 (n = 3), 16 ± 2 (n = 3), 15 ± 2 (n = 3), and 16 ± 2 (n = 3) μg of r-hFIX/day/106 cells, respectively, where n represents S.D. between three parallel cell incubations. Thus, total r-hFIX production by each cell line was found to be unaf-
Single band with an apparent molecular mass of 68 kDa. Plasma FIX (IX) is shown in Fig. 2. All proteins migrated as a 2 and Fraction EDTA. Fr FIX outlined in Fig. 1 quantitatively removed all r-hFIX from the column 2 did not contain any hFIX. This finding confirmed that the tandem chromatography procedure from column 2 with urea, pH 4, which resulted in eluted from the column with 50 mM EDTA (Fraction 1). Subsequently, the column was eluted with urea, pH 4, to release any proteins remaining on the column. Such proteins would appear in Fraction 2. Fig. 3A shows the resulting chromatogram. Purified plasma FIX was retained by and eluted from the column with urea, pH 4. The urea, pH 4 Fraction 2 obtained from medium harvested from BHK cells expressing r-hFIX and VKORC1 were used the urea, pH 4 Fraction 2 obtained from medium harvested from BHK cells expressing r-hFIX and VKORC1 were partially γ-carboxylated, none of the proteins in the urea, pH 4 fraction showed an average of 4 mol Gla/mol of r-hFIX. When chromatographed on column 1 (Fig. 1) in the presence of TBS-Ca²⁺, none of the proteins in the urea, pH 4 fraction bound to the conformational-specific column. Together these experiments strongly suggested that the tandem chromatography procedure outlined in Fig. 1 provided 1) the “tool” we needed to purify functional, fully γ-carboxylated r-hFIX and 2) an in situ assay to measure the capacity of our cell lines to produce functional, fully γ-carboxylated variants of r-hFIX among total r-hFIX produced by the cells.

**Characterization of r-hFIX Isolated by Conformational-specific Affinity Chromatography**—To assure that conformational-specific immunoaffinity chromatography on column 1 (Fig. 1) resulted in purification of functional r-hFIX, the purified protein obtained from each cell medium was tested for clotting activity and Gla content. N-terminal sequence analysis was carried out on the protein isolated from the medium that gave the highest yield of functional r-hFIX. Table I lists the data. Total r-hFIX represents the total amount of r-hFIX isolated. The numbers listed under the heading “Functional r-hFIX” represent data obtained with r-hFIX isolated by conformational-specific immunoaffinity chromatography on column 1 (Fig. 1). When tested with the commercial APTT-SP test kit, r-hFIX was released from column 2 with 4M urea, 0.5 M NaCl, 0.1 M sodium acetate, pH 4.0, and appear in Fraction 2.

Figure 1: Flow scheme of immunoaffinity chromatography purification of functional and nonfunctional r-hFIX from serum-free medium obtained from engineered BHK cells. Individual medium from BHK cells stably expressing r-hFIX and the various protein components of the vitamin K-dependent γ-carboxylation were chromatographed on columns 1 and 2 as described under “Results.” Column 1 has conformational-specific anti-hFIX antibodies attached, which in the presence of Ca²⁺ retain functional, fully γ-carboxylated r-hFIX. These variants of r-hFIX are released from column 1 in TBS containing 5 mM CaCl₂ and eluted from column 1 with 50 mM EDTA in TBS and appear in Fraction 1. Column 2 has nonconformational-specific anti-hFIX antibodies attached, which retain the remaining nonfunctional r-hFIX present in the void volume fraction (Void) from column 1. These variants of r-hFIX are released from column 2 with 4 M urea, 0.5 M NaCl, 0.1 M sodium acetate, pH 4.0, and appear in Fraction 2.
from all cell lines exhibited the same specific FIX activity, 145–151 units/mg as purified human plasma hFIX (150 units/mg) within the range of S.D. The Gla content averaged the expected Gla content of plasma hFIX, which is 12 residues/mol of protein (20). Four cycles of N-terminal Edman degradation were carried out on r-hFIX isolated from BHK cells expressing VKORC1, which gave the highest yield of functional r-hFIX. The sequence was Tyr-Asn-Ser-Gly, consistent with the N-terminal sequence of human plasma FIX (20). No underlying sequence indicating the presence of the hFIX propeptide in unprocessed precursors of hFIX could be detected. These findings indicated that the Golgi apparatus-located PACEd/furin convertase, responsible for proteolytic removal of the propeptide (8), was active in the BHK cell lines. Indeed, PACE/furin was easily detected by Western blotting in radioimmunoprecipitation assay buffer extracts of the BHK cell extract as a 66-kDa protein consistent with the molecular mass reported by Preininger et al. (21) for the active furin protease responsible for van Willebrand factor propeptide processing. The new and most important data resulting from this work are listed in Table I and show the increase in production of functional r-hFIX by cell lines engineered to overexpress proteins of the γ-carboxylation system. Consistent with findings by others (10), BHK cells overexpressing γ-carboxylase showed the lowest production of functional r-hFIX. BHK cell lines engineered to overexpress VKORC1 and VKORC1 + γ-carboxylase produced more active r-hFIX than BHK cells expressing r-hFIX. The VKORC1 overexpressing cell line was the greatest producer of functional r-hFIX and produced 2.9-fold more functional r-hFIX than BHK cells stably expressing r-hFIX (Control). These data are consistent with the notion that VKOR is the rate-limiting step in the vitamin K-dependent γ-carboxylation system responsible for post-translational modification of vitamin K-dependent proteins.

**DISCUSSION**

This report is the first to describe production of a recombinant vitamin K-dependent protein by cell lines engineered to harbor a vitamin K-dependent γ-carboxylation system with enhanced capacity for post-translational Gla modification (14). Because of the extensive work on cellular production of r-hFIX carried out by others (6, 7, 10), we focused on r-hFIX as we could compare our data to those previously published. In a recent article (14), we document successful engineering of BHK cells stably overexpressing 1) VKORC1, the reduced vitamin K cofactor-producing enzyme of the vitamin K-dependent γ-carboxylation system, 2) γ-carboxylase, the Gla-modifying enzyme in the system, and 3) a combination of VKORC1 + γ-carboxylase introduced with a bicistronic vector. BHK cells were chosen based on published reports (6) and our own data showing that BHK cells have an endogenous γ-carboxylation system with activity that is above the average of this activity measured in a variety of other cell lines. In the previous report (14), γ-carboxylation activity was measured in vitro as 14CO2 incorporation into the synthetic γ-carboxylase peptide substrate FLEEL. Here we have investigated whether our BHK cells with engineered γ-carboxylation systems would increase production of recombinant functional, vitamin K-dependent proteins. Using a retroviral vector carrying the cDNA for hFIX, we infected BHK cells and our engineered BHK cell lines and selected for cell lines stably expressing r-hFIX.

The rate of r-hFIX secretion has been shown to vary significantly among cell lines expressing r-hFIX (6, 7). By increased selection pressure, Kaufman et al. (7) were able to produce cell lines with significantly higher production rates than our BHK cell lines. However, their higher rate r-hFIX-producing cell lines did not show increased production of active r-hFIX (7). It was also shown (10) that propeptide processing was incomplete, which could partially be corrected for by introducing the cDNA for the propeptide-releasing protease PACE/furin in the Golgi

**FIG. 3.** Conformational-specific immunoaffinity chromatography of purified human plasma FIX. Purified human FIX (200 μg) and 5 mM CaCl2 were added to the serum-free medium used for culturing engineered BHK cells. Chromatography was carried out on the conformational-specific immunoaffinity column 1 shown in Fig. 1. Column 1 was equilibrated in TBS with 5 mM CaCl2. Retained hFIX was eluted from column 1 with 50 mM EDTA in TBS (Fraction 1) followed by 4 M urea, 0.5 mM NaCl, 0.1 M sodium acetate, pH 4.0 (Fraction 2). The elution diagram is shown in panel A. B. Western blots are shown of proteins in Fractions 1 and 2 with conformational-specific anti-hFIX antibodies (Conf. Spec. FIX Abs.) and nonconformational-specific antibodies (Non Conf. Spec. FIX Abs.). A Western blot of purified human plasma FIX (Plasma hFIX) with the conformational-specific antibodies is also shown.

**TABLE I**

Characterization and production yield of BHK cell-produced r-hFIX

Total r-hFIX and total purified functional r-hFIX obtained from medium from BHK cells stably expressing r-hFIX (BHK) and r-hFIX-expressing BHK cells engineered to overexpress γ-carboxylase (BHK + γ-carboxylase), VKORC1 (BHK + VKORC1), and γ-carboxylase + VKORC1 (BHK + γ-carboxylase + VKORC1) were quantified as described under “Experimental Procedures.” Functional r-hFIX was subjected to Gla analysis (n = 3) and measurement of hFIX-specific activity (n = 3). Production yield of purified functional r-hFIX is calculated as % of total r-hFIX isolated. All media were from cell cultures containing 90–100 × 106 cells.

| Cell lines                  | Total r-hFIX mg | r-hFIX mg | Gla mol/mol | Specific activity units/mg | Functional r-hFIX produced % |
|----------------------------|-----------------|-----------|-------------|---------------------------|-------------------------------|
| BHK                        | 1.40            | 0.25      | 12.0 ± 0.2  | 150 ± 4                   | 18                           |
| BHK + γ-carboxylase        | 1.36            | 0.11      | 12.2 ± 0.3  | 147 ± 6                   | 8                            |
| BHK + VKORC1               | 1.28            | 0.64      | 11.8 ± 0.4  | 145 ± 3                   | 50                           |
| BHK + γ-carboxylase + VKORC1| 1.24            | 0.43      | 11.7 ± 0.4  | 151 ± 5                   | 34                           |
apparatus. It became clear that the vitamin K-dependent γ-carboxylation system is a significant rate-limiting step in eukaryotic cell production of functional r-hFIX (7). We show in this work and in previously published articles (14, 16) that VKOR, the reduced vitamin K cofactor-producing enzyme of the γ-carboxylation system, is the rate-limiting step.

N-terminal sequence analysis of active r-hFIX in Fraction 1 (Figs. 1 and 2) showed that the purified functional clotting factor was free of contaminating precursors that had not been proteolytically processed by furin. This finding was important, as it has been shown (22) that fully γ-carboxylated precursors of r-hFIX will undergo the Ca\(^{2+}\)-induced conformational change and form the epitope recognized by our conformational-specific antibodies used to isolated functional r-hFIX. The absence of unprocessed precursors indicates that PACE/furin in the BHK cells used for our studies had the capacity to process r-hFIX secreted at an average rate of 16 μg/day/10\(^6\) cells. However, unprocessed nonfunctional r-hFIX precursors could have been present in Fraction 2 from column 1 (Fig. 1). Proteins appearing in this fraction were not subjected to N-terminal protein sequencing.

The new and important finding in this work is increased production of functional r-hFIX by BHK cells engineered to overexpress VKORC1. A 2.9-fold increase in production yield is significantly above what has been reported previously (7). Thus, the technology described in this report could potentially be used to improve production of r-hFIX for use as a pharmaceutical in hemophilia B treatment (2). Our engineered cell lines could potentially also be used for production of recombinant factor VII and protein C (1, 3).

As demonstrated in this work and by others (10), cells overexpressing recombinant γ-carboxylase produce less functional r-hFIX. Hallgren et al. (11) have suggested that an excess of γ-carboxylase in the ER inhibits release of γ-carboxylated proteins by forming intracellular complexes with the vitamin K-dependent protein precursors. It appears from our data that overexpression of γ-carboxylase is not needed for increased production of recombinant vitamin K-dependent proteins. The endogenous γ-carboxylase in the ER has a high capacity for γ-carboxylation (14), but this requires sufficient supplies of its cofactor. As shown, increased cofactor supplies can be provided by overexpressing recombinant VKORC1. However, to fully optimize the capacity of the γ-carboxylation system in engineered cells, a detailed understanding of the system at the molecular level will be required.

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