Carboxylase Overexpression Effects Full Carboxylation but Poor Release and Secretion of Factor IX: Implications for the Release of Vitamin K-Dependent Proteins†

Kevin W. Hallgren, Eric L. Hommema, Beth A. McNally, and Kathleen L. Berkner*

Department of Molecular Cardiology, Lerner Research Institute, and Jacobs Center for Thrombosis and Vascular Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, NB50, Cleveland, Ohio 44195

Received April 24, 2002; Revised Manuscript Received June 19, 2002

ABSTRACT: Vitamin K-dependent (VKD) proteins are modified by the VKD carboxylase as they transit through the endoplasmic reticulum. In a reaction required for their activity, clusters of Glu's are converted to Gla's, and fully carboxylated VKD proteins are normally secreted. In mammalian cell lines expressing high levels of r-VKD proteins, however, under- and uncarboxylated VKD forms are observed. Overexpression of r-carboxylase does not improve carboxylation, but the lack of effect is not understood, and the intracellular events that occur during VKD protein carboxylation have not been investigated. We analyzed carboxylation in 293- and BHK cell lines expressing r-factor IX (fIX) and endogenous carboxylase or overexpressed r-carboxylase. The fIX secreted from the four cell lines was highly carboxylated, indicating fIX−carboxylase engagement during intracellular trafficking. The r-carboxylase was functional for carboxylation: overexpression resulted in a proportional increase in fIX−carboxylase complexes that yielded full fIX carboxylation. Interestingly, the carboxylated fIX product was not efficiently released from the carboxylase in r-fIX/r-carboxylase cells, resulting in decreased fIX secretion. r-Carboxylase overexpression changed the ratios of intracellular fIX to carboxylase, and we therefore developed an in vitro assay to test whether fIX levels affect release. FIX−carboxylase complexes were in vitro carboxylated with or without excess VKD substrate or propeptide. These analyses are the first to dissect the rates of release versus carboxylation and showed that release was much slower than carboxylation. In the absence of excess VKD substrate/propeptide, fIX in the fIX−carboxylase complex was fully carboxylated by 10 min, but 95% was still complexed with carboxylase after 30 min. The presence of excess VKD substrate/propeptide, however, led to a significant increase in VKD product release, possibly through a second propeptide binding site in the carboxylase. The intracellular analyses also showed that the fIX carboxylation rate was slow in vivo and was similar in r-fIX versus r-fIX/r-carboxylase cells, despite the large differences in carboxylase levels. The results suggest that the vitamin K cofactor may be limiting for carboxylation in the cell lines.

Vitamin K-dependent (VKD)[1] carboxylation is required for the generation of biologically active VKD proteins with functions in hemostasis, growth control, calcium homeostasis, and signal transduction (*1*, *2*). The enzyme responsible for this modification, the VKD- or γ-carboxylase, converts glutamyl residues to γ-carboxylated glutamyl residues, or Gla's, using the energy of vitamin K hydroquinone (KH_2) oxygenation. The carboxylase converts KH_2 to vitamin K epoxide, and this product is subsequently recycled to KH_2 by a reductase that has not yet been identified. Cys99 and Cys450 in the carboxylase active site are required for KH_2 oxygenation and may form a coordinated complex with the KH_2 cofactor and the Glu substrate (*3*). Multiple Glu's are modified within the Gla domain of VKD proteins, and carboxylation confers the ability to bind phospholipid bilayers where many of these proteins exert their effects. The VKD proteins are targeted for carboxylation by an approximately 18 amino acid sequence, which in most cases is an amino-terminal propeptide immediately adjacent to the Gla domain. The propeptide confers high-affinity binding to, and may also activate, the carboxylase (*2*, *4*). It is cleaved subsequent to carboxylation in what is thought to be a late Golgi event (*2*).

The carboxylase is an integral membrane enzyme that resides in the endoplasmic reticulum (ER), and VKD proteins are carboxylated during their secretion to the cell surface or from the cell. The process is complex because full carboxylation of the multiple Glu's is required for activity and because one carboxylase is engaged by several different VKD proteins, e.g., with at least 10 in liver (*1*). In addition, recent inhibition studies indicate that the affinities of VKD propeptides for the carboxylase vary widely (*5*). Under normal physiological conditions, cells secrete fully carboxylated VKD proteins. This ability implicates a mechanism that achieves a high fidelity of carboxylation. One possible mechanism is cellular regulation to distinguish fully from partially carboxylated forms, and studies with protein C and

† Supported by NIH RO1 HL55666.

* Corresponding author. Phone: (216) 445-9760. Fax: (216) 444-9263. E-mail: berknek@ccf.org.

[1] Abbreviations: VKD, vitamin K-dependent; KH_2, vitamin K hydroquinone; Gla, γ-carboxyglutamic acid; fIX or FIX, factor IX; r-fIX, recombinant fIX; r-carb or r-carboxylase, recombinant carboxylase; endo H, endoglycosidase H; ER, endoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

10.1021/bi026016e CCC: $22.00
Published on Web 11/15/2002

prothrombin have shown that un- or undercarboxylated forms are preferentially degraded (*6*−*8*).

A second mechanism for accomplishing full carboxylation takes advantage of the ability of the carboxylase to effect processive carboxylation (*9*, *10*). We developed an in vitro assay that mimics the in vivo environment, i.e., carboxylation of a carboxylase−factor IX (fIX) complex in the presence of competitor VKD substrate, and found that comprehensive carboxylation of fIX was achieved and that the rate and extent of fIX carboxylation were unaffected by the competitor protein (*10*). Thus, the fIX remains bound to the carboxylase throughout all of the multiple (i.e., 12) Glu-to-Gla conversions. An interesting question that arises from these observations is how release is effected. Since the VKD protein is tethered to the carboxylase by its propeptide throughout the entire reaction, it is not clear why fully carboxylated product is released but partially carboxylated intermediates are not.

There are two instances where poor VKD protein carboxylation is observed in vivo. Warfarin treatment blocks carboxylation by inhibiting vitamin K reduction and therefore the supply of KH_2 cofactor required by the carboxylase. This block leads to the secretion of un- and undercarboxylated VKD proteins, as shown for prothrombin (*11*, *12*). Poor carboxylation has also been observed in mammalian cell lines expressing recombinant VKD proteins. While low-level expression results in full carboxylation, high-level expression often leads to the secretion of poorly carboxylated VKD proteins (*13*−*15*). The mechanism by which carboxylation becomes saturated in these cell lines is not known. In the only reported effort to improve VKD protein carboxylation by increasing carboxylase levels, the poor clotting activity of secreted fIX expressed at high levels was shown to be unaffected by r-carboxylase overexpression (*16*). However, the reason for this failure, including whether the r-carboxylase was functional in vivo or whether the secretory machinery had been saturated, was not investigated.

The secretion of carboxylated VKD proteins is the endpoint of a complex process that is poorly understood. We therefore took a direct approach to biochemically examine the intracellular events that occur during the course of fIX carboxylation in cell lines expressing fIX and endogenous carboxylase or high levels of r-carboxylase. These studies showed that the r-carboxylase was functional for fIX carboxylation, but that carboxylase overexpression resulted in the accumulation of intracellular, carboxylated fIX that remained bound to the carboxylase, causing a substantial decrease in fIX secretion. To test whether the impaired release of carboxylated fIX was due to the low intracellular ratio of fIX to carboxylase caused by carboxylase overexpression, release of fIX from a fIX−carboxylase complex was examined in vitro. Interestingly, in vitro carboxylation of fIX−carboxylase complexes led to full carboxylation of fIX but not to release of fIX from the complex unless excess VKD substrate was also present. Thus, VKD product release requires binding of a second VKD protein.

EXPERIMENTAL PROCEDURES

In Vivo Labeling and Immunoprecipitation. 293 cells stably expressing r-fIX and r-carboxylase (r-carb) were generated by cotransfection with fIX/ZEM228, which contains the full-length fIX cDNA in an expression cassette also encoding a phosphotransferase gene conferring resistance to G418 (*17*), and carboxylase/ZEM228, which contains a full-length carboxylase cDNA in the same vector. Lysates from clonal isolates were screened for fIX expression by western analysis and for carboxylase expression by peptide activity (*18*). The generation of both 293 cells stably expressing r-fIX and BHK cells stably expressing r-fIX or r-fIX and r-carb has been described (*18*−*20*). All cell lines were passaged in media containing charcoal-treated, dialyzed fetal calf serum (GibcoBRL) that lacked vitamin K. For pulse experiments in the presence of vitamin K, cells were fluid changed into media containing vitamin K (Phytonadione, Merck, to 5 μg/mL) 1 day prior to labeling. Cells were rinsed with prewarmed PBS and then incubated with 2 mL of Dulbecco's Cys^-, Met^- MEM (GibcoBRL) supplemented with 1% charcoal-treated, dialyzed fetal calf serum, 1 mM pyruvate (Sigma), 2 mM glutamine (Sigma), 1 μg/mL vitamin K, and [^{35}S]Cys/Met (ICN, final concentration of 20 μC_i/ml). After 4 h, medium was collected and centrifuged (14 000 rpm, 5 min, 4 °C) to remove cellular debris. The cells were rinsed with cold PBS (2 mL), followed by lysis with 2 mL of 1% Triton X-100 (Pierce), 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 μg/mL aprotinin, 40 μg/mL bestatin, 10 μg/mL leupeptin, 0.8 μg/mL pepstatin, 2 mM PMSF (all inhibitors were from Roche Diagnostics), and 10 mM iodoacetamide. After 20 min on ice, the lysates were harvested and centrifuged at 14 000 rpm for 10 min at 4 °C, and the supernatant was saved. Pulse-chase experiments were performed similarly, except that the cells were not changed into vitamin K-containing media the day before and the pulse was performed in media lacking vitamin K. The cells were in vivo labeled for 1 h and then rinsed with prewarmed PBS (2 mL), followed by a chase for 0−22 h in regular media (DMEM/F12, GibcoBRL) containing 1% dialyzed, charcoal-treated fetal calf serum, 1 mM pyruvate, 2 mM glutamine, and either 0 or 1 μg/mL vitamin K. Media and cell pellets were harvested as above.

Samples (400 μL media or cell lysate) were immunoprecipitated using affinity-purified polyclonal anti-fIX Ab [5 μg (*19*)] or affinity-purified anti-fIX propeptide Ab [5 μg (*10*)] and protein A agarose (50 μL, sufficient for binding 1 mg of Ab, Sigma). Preliminary experiments showed that these amounts were saturating for fIX immunocapture. After overnight incubation at 4 °C, the resins were washed four times in 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.05% Tween 20 (Pierce), and 0.1% SDS, with centrifugation at 1000 rpm for 1 min between each wash step. All manipulations were done at 4 °C. After removal of the final wash buffer, the resins were incubated in 100 μL of SDS loading buffer, boiled for 5 min, and then centrifuged at 5000 rpm for 1 min at room temperature. Supernatants (75 μL) were then subjected to SDS−PAGE followed by autoradiography or PhosphorImager analysis, as indicated in the figure legends.

Quantitation of FIX Protein and Activity. To quantitate secreted fIX, 50% confluent cells were rinsed twice in prewarmed PBS (2 mL) and then cultured in DMEM/F12 media containing 1% charcoal-treated, dialyzed fetal calf serum, 1 mM pyruvate, 2 mM glutamine, and 5 μg/mL vitamin K for 24 h. Medium was harvested, and lysates were

prepared (*3*) from the cells to normalize secreted fIX from different cell lines to a fixed amount of cellular material. Secreted fIX was then quantitated by an ELISA using two monoclonal Ab's (ESN1 and ESN2, American Diagnostica) as capturing Ab's, affinity-purified polyclonal anti-fIX Ab for detection and pure, plasma fIX (Enzyme Research Laboratories) for the standard curve. The intracellular fIX levels were measured using a quantitative western because much of the intracellular fIX was membrane-bound and required solubilization with detergents such as SDS, which interfered with the ELISA. Cells [$(1-2) \times 10^7$] were harvested and lysates (5 mL) prepared as previously described (*3*). Varying amounts of lysate (25−100 μg of total protein as determined by BCA analysis, Pierce) were subjected to SDS−PAGE and western analysis (ECF, Clontech) using an affinity-purified polyclonal anti-fIX Ab and pure plasma fIX (20−100 ng, Enzyme Research Laboratories) as a standard.

The activity of the secreted fIX was quantitated in a clotting assay using fIX-deficient serum, as before (*13*). Standard curves were constructed using both pure plasma IX and normal plasma (George King) containing a fIX concentration of 3 μg/mL. The two standard curves were essentially identical.

Isolation of FIX−Carboxylase Complexes. Microsomes were prepared, as previously described (*3*), from r-fIX BHK cells, r-fIX/r-carb BHK cells, r-fIX/r-carb 293 cells, and r-carb BHK cells. The r-carb BHK cells express r-carboxylase ~50-fold over endogenous carboxylase but do not contain r-VKD proteins and were used as a control. The microsomes were prepared from cells [$(4-8) \times 10^8$] cultured in the absence of vitamin K, and microsomes were also prepared from r-fIX/r-carb BHK cells fluid changed into vitamin K (5 μg/mL) 1 day before the cells were harvested. Microsomes were solubilized in 7.5 mL of 50 mM Tris-HCl pH 7.4, 200 mM NaCl, 0.5% CHAPS (Pierce), and 1 mM PMSF and then centrifuged at 100000*g* for 1 h. All manipulations were done at 4 °C. Solubilized microsomes (250 μL, 600−700 μg of total protein as determined by BCA) were incubated with 100 μL of resin containing an affinity-purified polyclonal anti-fIX Ab (500 μg, coupled to CNBr-activated Sepharose) which recognized both carboxylated and uncarboxylated fIX. The slurry was nutated for 16 h, aliquots (3×10 μl) were reserved, and the samples were centrifuged at 1000 rpm for 1 min, saving aliquots of the unbound material (3×10 μL). The resin was then washed four times with 1 mL of 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.25% CHAPS, 0.25% phosphatidyl choline, and 5 mM DTT (TNCPD), with centrifugation at 1000 rpm for 1 min between each wash step. On the final wash, aliquots (3×10 μL) of the 1.1 mL slurry of resin and TNCPD were removed. Washed resin, starting slurry, and unbound sample were then assayed for carboxylase activity using a peptide assay (*18*).

An Assay for Dissociation of Carboxylase from FIX. Solubilized microsomes (1 mL) from r-fIX/r-carb BHK cells cultured in the absence of vitamin K were adsorbed to a purified anti-heavy chain fIX Ab immobilized to Sepharose (250 μL of resin, 500 μg of Ab; the Ab was generously provided by Dr. William Church) by overnight nutation at 4 °C. The resin was washed with TNCPD as described above, and after the final wash, TNCPD (250 μL) was added to give a slurry containing 50% resin/50% TNCPD (v/v). Aliquots of the slurry (100 μL) were then mixed with 400 μL of a protein carboxylation reaction cocktail (*10*). Duplicate reactions were performed that had either 0 or 1.2 μM fIX[180]. This protein is a truncated fIX variant that contains the fIX light chain (*10*) and was used to determine the effect of VKD protein upon release of carboxylase from fIX. The samples were incubated at room temperature for 5 min, and the reactions were then initiated by the addition of KH_2 (to 150 μM). At timed intervals, aliquots (20 μL) were removed, and $^{14}CO_2$ incorporation into fIX was monitored by SDS−PAGE and PhosphorImager as previously described (*10*), except that the protein from the gels was transferred to nitrocellulose before exposure to increase detectability. Samples from the same reaction were also monitored for fIX−carboxylase dissociation by separating free carboxylase from carboxylase bound to fIX−anti-fIX Ab resin. Aliquots (80 μL) removed at timed intervals were centrifuged (1000 rpm, 1 min, room temperature), and all but 10 μL of the supernatant was transferred to a new tube. The small amount of supernatant was left to avoid disturbing the resin, and the amount was corrected for in the final calculations. Both supernatants and resins were then assayed for the amount of carboxylase by a peptide activity assay (*18*).

The effect of propeptide and Gla domain-derived sequences on carboxylase-fIX release was also monitored. The fIX−carboxylase complex was isolated on anti-heavy chain fIX Ab resin as above and incubated in a protein carboxylation reaction cocktail (400 μL) either alone or with factor X (fX) propeptide (20 μM final concentration) or with *N*-Boc-Glu-Glu-Leu-OMe (EEL, Bachem, 2 mM final concentration). After 5 min of incubation at room temperature, carboxylation of the fIX−carboxylase complex was initiated by the addition of vitamin K (to 140 μM), and release was monitored as above. In this experiment, the reaction cocktail contained cold $NaHCO_3$ instead of [^{14}C]$NaHCO_3$ so that $^{14}CO_2$ incorporation into EEL during the release experiment did not interfere with subsequent quantitation of carboxylase in the peptide activity assay.

An Assay To Determine the Activity of Carboxylase-FIX Complexes. Carboxylation of fIX in the fIX−carboxylase complex was monitored to determine whether most of the isolated fIX−carboxylase complexes were productive, i.e., able to undergo in vitro carboxylation. Solubilized microsomes (250 μL, 600 μg of total protein) from r-fIX/r-carb BHK cells cultured without vitamin K were incubated with affinity-purified anti-C-terminal carboxylase Ab resin [50 μL, 150 μg, coupled to CNBr-activated Sepharose (*18*)] to separate fIX bound to carboxylase from free fIX. The resin was washed with TNCPD as above and then incubated in 350 μL of the protein carboxylation reaction cocktail (*10*) for 5 min at room temperature. Carboxylation was then initiated by the addition of vitamin K (to 140 μM). At timed intervals, duplicate aliquots (40 μL each) were withdrawn and incubated with SDS−PAGE loading buffer (40 μL) for 20 min, followed by centrifugation at 5000 rpm for 1 min at room temperature. Supernatants (60 μL) were then subjected to SDS−PAGE. One set was processed for PhosphorImager analysis to monitor $^{14}CO_2$ incorporation into fIX. The other set was analyzed by a western using affinity-purified polyclonal anti-fIX Ab to monitor the shift in migration on SDS−PAGE that occurs when fIX is carboxylated (*10*). The entire experiment was performed in duplicate.

FIX Purification and Gla Quantitation. r-FIX BHK, r-fIX/r-carb BHK, r-fIX 293, and r-fIX/r-carb 293 cells [(3−5) × 10^8] were cultured in DMEM/F12 media containing 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM pyruvate, 2 mM glutamine, 20 mM Hepes pH 7.2, 10 μg/mL insulin (GibcoBRL), 7 μg/mL selenium (GibcoBRL), 6 mg/mL transferrin (GibcoBRL), 10 μg/mL fetuin (Sigma), and 1 μg/mL vitamin K. r-FIX BHK cells were also cultured in the identical media but without vitamin K. After 2−3 days, cell-spent medium was harvested, centrifuged (5000 rpm, 5 min, 4 °C) to remove cellular debris, and stored at −80 °C. Factor IX was then affinity purified using an immobilized Ab against the heavy chain of fIX (ESN1, American Diagnostica, coupled at 1 mg/mL), which recognizes both carboxylated and uncarboxylated fIX. Medium (250 mL) was passaged over the column (1 mL), followed by 10 column volumn washes with 20 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, and 0.05% NP40 (Pierce) and then with PBS. The fIX was eluted with triethylamine (50 mM), and fractions (1.1 mL) were neutralized by collection into tubes containing 400 μL of 0.5 M Tris-HCl pH 8.8. All purification steps were performed at 4 °C. Samples were analyzed by SDS−PAGE and silver staining to show that the proteins were homogeneous and then quantitated for Gla content as before (*13*).

Intracellular fIX in complex with carboxylase was also purified and quantitated for Gla's. Solubilized microsomes (7.5 mL, 19 mg of total protein) were prepared as before (*3*) from r-fIX/r-carb BHK cells cultured in vitamin K-containing media. FIX bound to carboxylase was then separated from free fIX by adsorption to immobilized, affinity-purified anti-C-terminal carboxylase Ab (1 mL of resin, 3 mg of Ab/mL). After overnight nutation at 4 °C, the slurry was poured into a column, and the resin was washed with 50 mL of 50 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.25% CHAPS, and 0.25% phosphatidylcholine, followed by elution with the same buffer containing 100 μM fX propeptide. To remove the propeptide, as well as trace amounts of contaminating bands, the purified fIX was concentrated (Centricon, Amicon), gel-electrophoresed, and transferred to PVDF (Immobilon, Millipore). The membrane was stained with Coomassie R250 (Sigma, 0.2% in 20% methanol) and then destained in 20% methanol, and the Coomassie-staining material in the 50−65 kDa region of the gel was excised. The membrane was then washed four times with water (1 mL) and incubated for 4 h in 50 mM Tris-HCl pH 8.8, 1% Triton X-100 (Pierce), and 1% SDS (200 μL) at room temperature. The membrane was removed, and the eluted material was then precipitated by the addition of 400 μL of acetone and incubation overnight at −20 °C. Following centrifugation (14 000 rpm, 30 min, 4 °C), the precipitate was washed with 1 mL of 67% acetone and then recentrifuged. The pellet was brought up in 5 M KOH (100 μL) and then processed for Gla quantitation as before (*13*).

RESULTS

Overexpression of the Carboxylase Impairs Factor IX Secretion. To analyze the intracellular events that occur during fIX carboxylation and secretion, we used cell lines that expressed fIX at levels that result in the secretion of highly carboxylated, active fIX. The advantage of this approach is that all components of the secretory pathway that allow the appropriate folding and posttranslational modification of fIX to produce biologically active protein are functional. In cell lines expressing higher levels of fIX where carboxylation is saturated, the impaired carboxylation could be due to either carboxylase-dependent or secretion-dependent mechanisms. A secretion-dependent defect would complicate the analysis of r-carboxylase function. Thus, the approach used isolates the question of carboxylase function from questions related to saturation of components of the secretory pathway.

Table 1: Carboxylase and FIX Expression in BHK and 293 Cells Expressing r-FIX and Endogenous or r-Carboxylase[a]

recombinant	carboxylase activity (pmol)	factor IX (pmol)
BHK cells		
r-fIX	0.21	51.5
r-fIX/r-carb	9.28	42.6
untransfected	0.13	0
293 cells		
r-fIX	0.50	5.9
r-fIX/r-carb	9.80	13.2
untransfected	0.24	0

[a] Cell lysates were prepared from cells cultured without vitamin K. Carboxylase levels were quantitated using a peptide activity assay and specific activity (*3*) of 6.7×10^4 cpm h^{-1} $pmol^{-1}$, and fIX levels were measured using a quantitative western. The total protein from the lysates (from ∼2×10^7 cells) was ∼1 mg for the BHK cells and ∼2 mg for the 293 cells, and the values shown have been normalized to the equivalent of 1 mg of lysate.

Sets of cell lines derived from both 293 and BHK cells were analyzed because these two lines have different efficiencies of secreting carboxylated VKD proteins (*13*, *14*) and so had the potential to yield different results. Each set of 293 or BHK cell lines containing r-fIX or r-fIX/r-carboxylase expressed similar levels of intracellular fIX (Table 1). The endogenous carboxylase levels were similar for the r-fIX 293 cells and r-fIX BHK cells, and the r-carboxylase was expressed at 41-fold (293 cells) or 71-fold (BHK cells) higher levels than endogenous carboxylase in untransfected cells (Table 1). Overexpression of activity was proportional to the amount of carboxylase protein, as determined using quantitative western analysis (data not shown).

Most of the carboxylase was shown to form a complex with fIX in all four cell lines. In tissue and mammalian cells, VKD proteins and carboxylase have been shown to exist in a tight complex when vitamin K is not present (*21*, *22*). To test for the extent of complex formation, then, the amount of carboxylase bound to fIX, and therefore to immobilized anti-fIX Ab, was determined for cells cultured in the absence of vitamin K. In both r-fIX BHK and r-fIX/r-carb BHK cell lines, most (∼90%) of the carboxylase was bound to the fIX−anti-fIX Ab resin and was stable to repeated washing of the resin (Table 2). These results were similar to those previously obtained for the r-fIX 293 cell line (*19*), and nearly identical results were also obtained with the r-fIX/r-carb 293 cell line (data not shown). Thus, the overexpressed r-carboxylase was present in the appropriate intracellular compartment and was functional in complex formation with fIX.

In vivo labeling experiments showed that carboxylase overexpression impaired fIX secretion (Figure 1). These

Table 2: Carboxylase Overexpressed in r-fIX/r-carb BHK Cells Lacking Vitamin K Is Complexed with FIX[a]

	fraction	carboxylase activity (cpm h^{-1})	activity (%)
BHK cells containing r-fIX	start	5.0×10^4	100
	flow-through	0.6×10^4	12
	anti-fIX resin	4.5×10^4	90
BHK cells containing r-fIX/r-carb	start	1.4×10^6	100
	flow-through	0.2×10^6	14
	anti-fIX resin	1.2×10^6	86

[a] Solubilized microsomes (250 μL, 0.65 mg of total protein) from BHK cells cultured in the absence of vitamin K were fractionated on polyclonal anti-fIX Ab resin (100 μL, 5 μg/μL Ab), and aliquots of each fraction were assayed for peptide activity.

Table 3: Levels of Secreted FIX from r-fIX and r-fIX/r-carb Cell Lines[a]

cell line	fIX level (pmol)	fIX activity (pmol)	percent active
BHK	0	0	0
r-fIX BHK	75.2	71.4	95
r-fIX/r-carb BHK	7.4	7.7	104
293	0	0	0
r-fIX 293	72.5	70.3	97
r-fIX/r-carb 293	12.6	12.9	102

[a] Cells cultured in the absence of vitamin K were fluid changed into media with vitamin K (5 μg/mL), and 24 h later the spent media were assayed for fIX levels by ELISA and for activity in a clotting assay.

Table 4: Gla Quantitation of (A) Secreted and (B) Intracellular FIX[a]

sample	mol of Gla/mol of fIX
(A) Secreted FIX	
r-fIX BHK	9.0 ± 0.2
r-fIX BHK (−K)	0.3 ± 0.2
r-fIX/r-carb BHK	9.6 ± 0.2
r-fIX 293	11.6 ± 0.3
r-fIX/r-carb 293	12.0 ± 0.2
plasma fIX	11.5 ± 0.2
(B) Intracellular FIX	
carboxylase-bound fIX	11.3 ± 0.2

[a] (A) FIX secreted from the indicated cell lines was purified to homogeneity and quantitated for Gla's as described in the Experimental Procedures. The r-fIX BHK (−K) sample is fIX purified from cells cultured in the absence of vitamin K. (B) FIX in a fIX−carboxylase complex was isolated using an anti-carboxylase Ab and subsequent displacement of fIX from carboxylase by propeptide, as described in the Experimental Procedures.

experiments were performed in media containing vitamin K, and similar levels of newly synthesized intracellular fIX were detected for all four lines (Figure 1A). However, the amount of secreted fIX in the cell media was greatly reduced in the r-fIX/r-carb cell lines (Figure 1B,C). Identical results were obtained with both BHK and 293 cell lines, indicating that the inhibition of fIX secretion was not cell-specific but rather a general property of r-carboxylase overexpression.

Quantitative analysis of the r-fIX and r-fIX/r-carb cell lines confirmed that the amount of secreted fIX from cells coexpressing r-carboxylase was reduced (Table 3). FIX from all four cell lines showed high biological activity. This result indicated that the fIX's were extensively carboxylated and therefore must have engaged the carboxylase during their secretion. Efficient carboxylation was unequivocally demonstrated by purifying the secreted fIX's and performing Gla quantitation (Table 4A). These data therefore indicated that

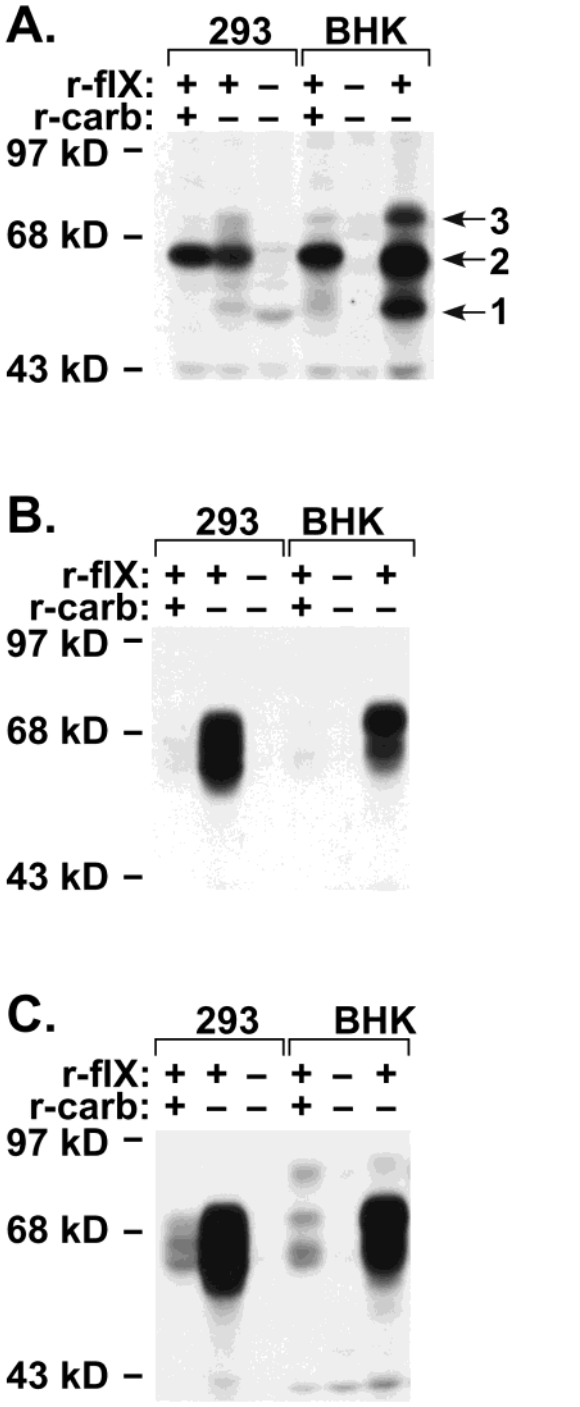

FIGURE 1: Carboxylase overexpression decreases secretion of fIX. BHK or 293 cells stably transfected with r-fIX or r-fIX and r-carb were in vivo labeled with medium containing [^{35}S]Cys/Met and vitamin K, as detailed in the Experimental Procedures. Equivalent amounts of intracellular (A) or secreted (B, C) material were then quantitatively immunoprecipitated with polyclonal anti-fIX Ab, followed by SDS−PAGE and autoradiography. Panel C shows a longer exposure of the gel in panel B. The arrows indicate different fIX intracellular forms.

fIX carboxylation was still efficient when the carboxylase was overexpressed; however, carboxylase overexpression somehow impaired fIX secretion.

Three different intracellular forms were observed in the r-fIX BHK cell line (indicated by arrows in Figure 1A), and band 2 was the most prominent form in all of the cell lines. FIX undergoes several posttranslational modifications which have the potential to account for the different forms,

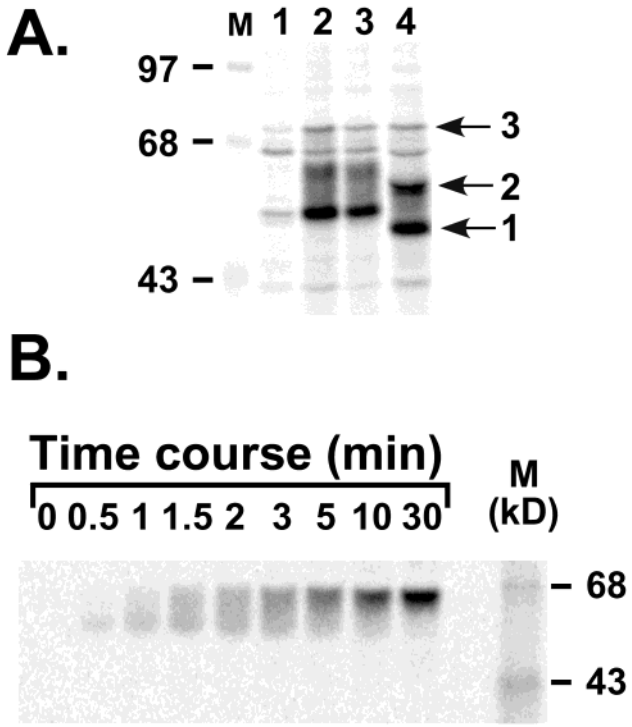

FIGURE 2: Intracellular fIX forms differ in glycosylation and carboxylation. (A) BHK (lane 1) and r-fIX BHK cells (lanes 2−4) were labeled in vivo for 1 h, and lysates were prepared and immunoprecipitated with polyclonal anti-fIX Ab (lanes 1, 2, and 4) or anti-fIX propeptide Ab (lane 3). The immunoprecipitate in lane 4 was digested with endoglycosidase H (2000 units, New England Biolabs). Samples were then processed by SDS−PAGE and PhosphorImager analysis. The arrows indicate the three fIX intracellular forms in the endoglycosidase H-treated sample. The background bands from the untransfected BHK cells (lane 1) are not hamster fIX, as shown by lack of incorporation of $^{14}CO_2$ into protein after in vitro carboxylation of BHK lysates (data not shown). (B) Aliquots of fIX−carboxylase complex in vitro carboxylated for the indicated times were subjected to SDS−PAGE and PhosphorImager to monitor $^{14}CO_2$ incorporation into fIX and show that carboxylation of the 12 Glu's to Gla's in fIX retards fIX migration.

including propeptide processing, N-glycosylation, O-glycosylation, aspartyl β-hydroxylation, and carboxylation. Our analyses indicated that all three forms had the propeptide, that bands 1 and 2 differed in the extent of carboxylation, and that band 3 differed from bands 1 and 2 in glycosylation. Thus, identical immunoprecipitation profiles were obtained using a polyclonal anti-fIX Ab or an Ab against the fIX propeptide (Figure 2A). The immunoprecipitation was performed using r-fIX BHK cells labeled for a shorter time than in Figure 1, and revealed a smear of fIX forms between bands 1 and 2. The smear is similar to the partially carboxylated fIX intermediates that are observed during in vitro carboxylation (Figure 2B). Bands 1 and 2 were shifted in molecular mass in SDS−PAGE when treated with endoglycosidase H (endo H), from 55 and 65 kDa to 50 and 60 kDa, respectively (Figure 2A). Endo H sensitivity indicated localization of these forms to the ER or cis-Golgi, and the molecular mass shift was consistent with the molecular mass of ER/cis-Golgi N-glycans (~2 kDa) and the presence of two N-linked oligosaccharide chains on fIX (*23*). However, the endo H-digested bands 1 and 2 still showed a large difference in migration on SDS−PAGE, ruling out N-glycosylation differences. The endo H-sensitivity also ruled out O-glycosylation because this modification does not occur in the ER/cis-Golgi. Aspartyl β-hydroxylation is also unlikely to explain retarded migration since it does not cause a net charge difference in fIX and causes only a small change in mass. Carboxylation introduces a cluster of negative charges in fIX, and such clusters are known to retard migration of other proteins (e.g., phosphoproteins) on SDS−PAGE. In vitro carboxylation of isolated fIX−carboxylase complexes showed a shift in migration upon fIX carboxylation identical to the migration difference between bands 1 and 2 (Figure 2B). Thus, carboxylation is the only posttranslational modification that can account for the difference between bands 1 and 2.

Band 3 was endo H resistant (Figure 2A). This observation indicated a medial-, trans-, or post-Golgi localization, and the difference between band 3 and bands 1 and 2 is therefore attributable to differences in N-glycosylation and possibly in O-glycosylation.

r-Carboxylase Fully Carboxylates FIX in Vivo but Inefficiently Releases the Carboxylated FIX Product. To determine why overexpression of carboxylase caused a decrease in fIX secretion, the fate of newly synthesized fIX was analyzed by pulse-chase experiments. The BHK and 293 cell lines expressing r-fIX and endogenous or r-carboxylase were in vivo labeled in the absence of vitamin K and then chased in media either lacking or containing vitamin K. Intracellular and secreted fIX forms were then detected by SDS−PAGE and PhosphorImager following immunoprecipitation of samples harvested at various times in the chase. The results for the BHK cell lines are shown in Figure 3.

The predominant intracellular fIX form observed at the zero time point in the chase for both the r-fIX and r-fIX/r-carb cell lines was band 1 (Figure 3B,D), the uncarboxylated fIX precursor which is endo H sensitive and therefore ER- or cis-Golgi localized. When the r-fIX BHK cell line was chased in vitamin K-containing media, a transient amount of band 2, the carboxylated, endo H-sensitive fIX form, was observed (Figure 3B). Band 2 was observed until 6 h but by 22 h was gone, as were bands 1 and 3. All of the uncarboxylated precursor fIX (band 1) was ultimately secreted from the r-fIX BHK cells, and a steady increase in the appearance of fIX in the media was observed between the first chase time point (1 h) and the last (22 h) (Figures 3A,B and 4A).

Carboxylation of fIX was not obligatory for secretion, as shown by the analysis of in vivo-labeled r-fIX BHK cells chased in the absence of vitamin K. Thus, a continual decline in the uncarboxylated, intracellular band 1 was observed over time, concomitant with an increase in the appearance of secreted fIX (Figure 3A,B). Band 2 was not detectable in cells grown without vitamin K (Figure 3B), consistent with its identification as a carboxylated protein, and the secreted fIX was uncarboxylated (Table 4A). The fIX population secreted from r-fIX BHK cells chased without vitamin K was more heterogeneous than for the BHK cells chased with vitamin K (Figure 3A). This heterogeneity was due to differences in propeptide processing and glycosylation: western analysis with anti-fIX propeptide Ab detected propeptide-fIX in r-fIX BHK cells cultured without vitamin K but not when cultured in vitamin K, and endoglycosidase F digestion showed differences in N-glycosylation (data not shown). Impaired propeptide processing and N-glycosylation

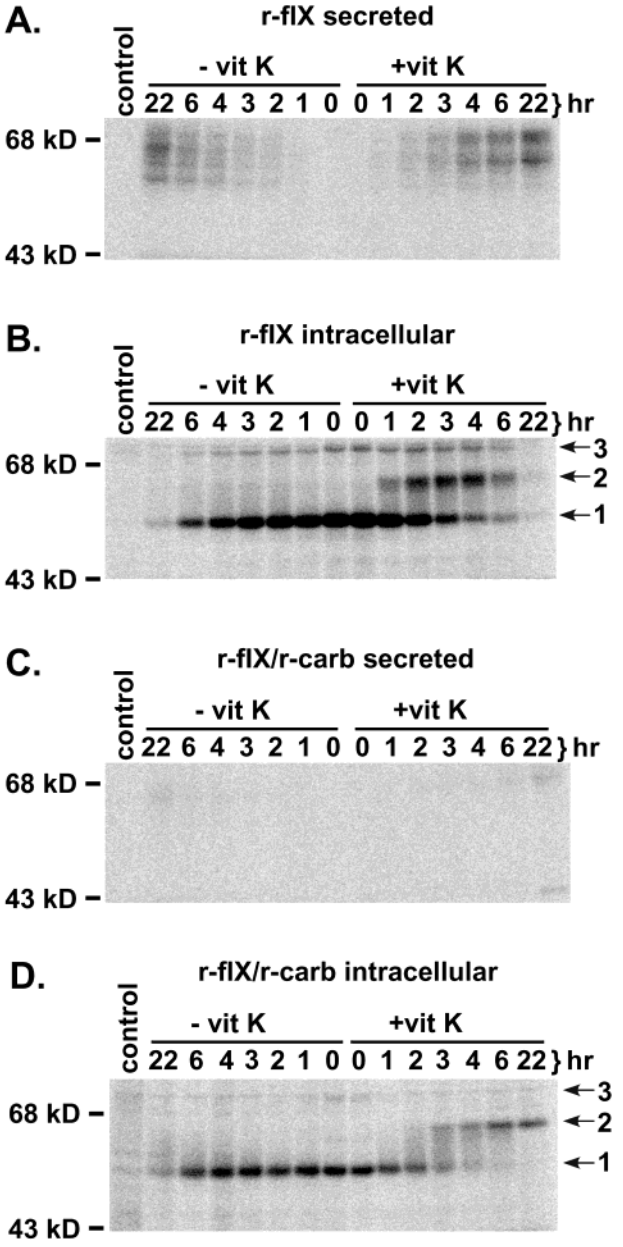

FIGURE 3: Carboxylase overexpression inhibits fIX secretion: pulse-chase analysis. BHK cells containing r-fIX (A, B) or r-fIX/r-carb (C, D) were in vivo labeled for 1 h with media containing [^{35}S]Cys/Met and no vitamin K and then chased for the indicated times in either the absence (−vitK) or presence (+vitK) of vitamin K. Media (A, C) and cell lysates (B, D) were then immunoprecipitated with polyclonal anti-fIX Ab, followed by SDS−PAGE and PhosphorImager analysis, as detailed in the Experimental Procedures. The control is an untransfected BHK cell line in vivo labeled for 1 h in [^{35}S]Cys/Met containing medium.

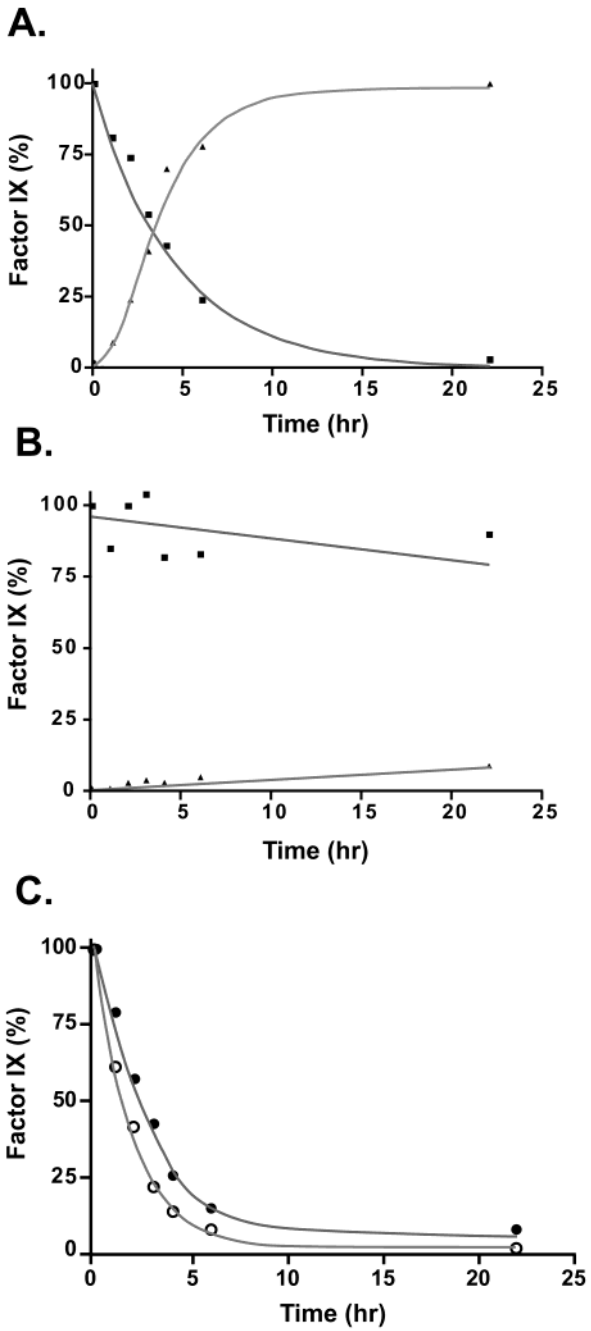

FIGURE 4: Carboxylase overexpression inhibits fIX secretion: gel quantitation. The results of the chase experiments (Figure 3) performed in the presence of vitamin K were quantitated. Secreted fIX (▲) and intracellular fIX (■) from r-fIX BHK cells (A) or r-fIX/r-carb BHK cells (B) correspond to panels A and C or B and D of Figure 3, respectively. Part C shows the disappearance of band 1 in the +vit K chase in r-fIX BHK cells (○) or r-fIX/r-carb BHK cells (●).

could be a consequence of conformational differences between carboxylated and uncarboxylated fIX.

The r-fIX/r-carb BHK cells, like the r-fIX BHK cells, showed a decrease in band 1 and an increase in band 2 over time when chased in vitamin K-containing media (Figure 3D). However, band 2 was not temporally transient in r-fIX/r-carb BHK cells, as observed in r-fIX BHK cells (Figure 3B,D). While very little intracellular fIX was observed at 22 h in the r-fIX BHK cells, a substantial amount of band 2 remained at this time point in r-fIX/r-carb BHK cells. Quantitation of the pulse-chase experiments also showed the striking difference in pattern of secretion for these two cell lines (Figure 4A versus B). The uncarboxylated fIX precursor (band 1, Figure 3) disappeared at the same rate in both r-fIX BHK and r-fIX/r-carb BHK cells (Figure 4C). However, at the end of the chase, all of the fIX in r-fIX BHK cells was secreted (Figure 4A), while most (~80%) of the fIX in the r-fIX/r-carb BHK cells was intracellular (Figure 4B). These data indicate that the intracellular fIX was stable in the r-fIX/r-carb BHK cell line. Therefore, the impairment of secretion caused by overexpression of the carboxylase (Figure 1) was not due to degradation of fIX; instead, high-level carboxylase expression trapped fIX inside the cell.

Table 5: Carboxylase and FIX Are Complexed in r-fIX/r-carb BHK Cells Containing Vitamin K[a]

	fraction	carboxylase activity ($\times 10^{-6}$ cpm h^{-1})	activity (%)
BHK cells containing r-fIX/r-carb	start	1.40	100
	flow-through	0.30	21
	anti-fIX resin	1.01	72
BHK cells containing r-carb	start	1.08	100
	flow-through	1.03	95
	anti-fIX resin	0.02	2

[a] Solubilized microsomes (250 μL, 0.7 mg of total protein) from r-fIX/r-carb BHK cells cultured in vitamin K or from r-carb BHK cells cultured without vitamin K were fractionated on polyclonal anti-fIX Ab resin (100 μL, 5 μg/μL Ab), and aliquots of each fraction were then assayed for peptide activity.

One possible explanation for the impaired fIX secretion caused by carboxylase overexpression is that the fIX was poorly released from the carboxylase in r-fIX/r-carb cells. To test for this possibility, the assay previously used to demonstrate fIX−carboxylase association in r-fIX/r-carb cells grown without vitamin K (Table 2) was applied to the same cells grown in the presence of vitamin K (Table 5). Comparison of the amount of carboxylase in the start and flow-through fractions revealed that 72% of the carboxylase was bound to fIX−anti-fIX Ab resin and was stable to repeated washings of the resin (Table 5). These values were similar to those observed with cells grown in the absence of vitamin K (Table 2), showing that even in cells containing vitamin K, most of the carboxylase is in a complex with fIX. Carboxylase binding to the anti-fIX resin was specifically due to its association with fIX since carboxylase from a BHK cell line that does not express fIX was not retained on anti-fIX Ab resin (Table 5).

Most of the intracellular fIX in the r-fIX/r-carb BHK cells chased in the presence of vitamin K appeared to be carboxylated, as indicated by the shift over time from band 1 to band 2 (Figure 3D). To unequivocally test for fIX carboxylation, the fIX in the fIX−carboxylase complex from r-fIX/r-carb BHK cells cultured in vitamin K was purified and Gla quantitation performed. This analysis showed that the fIX was highly carboxylated (Table 4B). Thus, most of the carboxylase in the r-fIX/r-carb BHK cells cultured with vitamin K was in a complex with fIX that resulted in full carboxylation but not efficient release of the carboxylated fIX product.

VKD Protein Facilitates Release of the VKD Product. r-Carboxylase overexpression resulted in efficient carboxylation but poor release, indicating that at least one factor is limiting in the r-fIX/r-carb cell lines that was required for efficient release. This limiting factor could be a cellular protein that is titrated out when the carboxylase is overexpressed. One protein that is titrated out is fIX: the ratio of carboxylase to intracellular fIX was increased by high-level carboxylase expression (from 1:245 in r-fIX BHK cells to 1:5 in r-fIX/r-carb BHK cells, Table 1). Therefore, one potential explanation for the impaired release of fIX from fIX−carboxylase complexes in the r-fIX/r-carb cells is that an excess of VKD protein is required for release of carboxylated fIX. We directly tested this possibility in an in vitro assay.

Uncarboxylated fIX−carboxylase complex was isolated on an anti-fIX heavy-chain Ab resin, and the amount of carboxylase released from fIX−anti-fIX Ab resin was quantitated following in vitro carboxylation in the presence or absence of excess VKD substrate. The substrate, propeptide-containing fIX[180], comprised amino acids −18 to 180 of fIX. This substrate does not react with the anti-fIX heavy-chain Ab and was shown to be kinetically indistinguishable from full-length propeptide-containing fIX in a carboxylation reaction (*10*). When fIX−carboxylase complex was in vitro carboxylated in the absence of fIX[180], fIX in the complex was carboxylated (Figure 5A), and the amount of $^{14}CO_2$ incorporation observed at the end of the reaction indicated comprehensive carboxylation, as previously reported (*10*). Western analysis of the in vitro-carboxylated sample showed that almost all of the fIX shifted in migration to the carboxylated fIX form (Figure 5B), indicating that most of the complex in the reaction resulted in productive carboxylation. However, in the absence of excess VKD substrate, most (95%) of the carboxylase remained bound to the anti-fIX Ab resin (Table 6), showing that carboxylase and fIX were still associated following fIX carboxylation. This experiment was repeated in the presence or absence of $CaCl_2$, since carboxylated VKD proteins undergo a calcium-

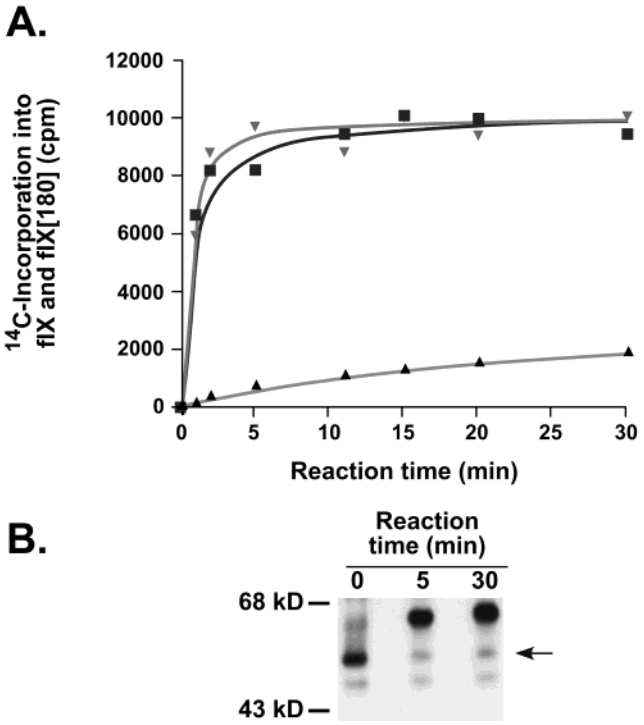

FIGURE 5: In vitro carboxylation of fIX. (A) FIX−carboxylase complexes were in vitro carboxylated in the presence or absence of fIX[180] and quantitated for $^{14}CO_2$ incorporation by SDS−PAGE and PhosphorImager analysis as detailed in the Experimental Procedures and in (*10*). Time courses are shown for $^{14}CO_2$ incorporation into fIX[180] (▲) and fIX from the fIX−carboxylase complex in vitro carboxylated in the absence (■) or presence (▼) of fIX[180]. The results are the average of duplicate gels, and the variation was less than 10%. (B) FIX−carboxylase complex from solubilized microsomes from r-fIX/r-carb BHK cells cultured without vitamin K was adsorbed to anti-carboxylase Ab, and the resin was washed and in vitro carboxylated as described in the Experimental Procedures. At the indicated times, aliquots were withdrawn and analyzed in a western using polyclonal anti-fIX Ab to show that most of the uncarboxylated fIX in the complex (indicated by the arrow) becomes carboxylated to the slower migrating form. The smear in the zero time point is presumably due to trace amounts of vitamin K in the serum the cells are cultured in that gives rise to some in vivo-carboxylated fIX.

Table 6: VKD Protein Promotes VKD Product Release in Vitro[a]

time (min)	fraction	−fIX[180] carboxylase activity ($\times 10^{-3}$ cpm h^{-1})	−fIX[180] free carboxylase (%)	+fIX[180] carboxylase activity ($\times 10^{-3}$ cpm h^{-1})	+fIX[180] free carboxylase (%)
0	bound	710.0		738.8	
	unbound	14.4	2	23.1	3
6	bound	648.1		665.6	
	unbound	28.8	4	76.3	10
31	bound	632.5		597.5	
	unbound	34.4	5	110.0	16

[a] Uncarboxylated fIX−carboxylase complex from r-fIX/r-carb BHK-solubilized microsomes was adsorbed to anti-heavy-chain fIX Ab resin, and the resin was then in vitro carboxylated in the absence or presence of fIX[180] substrate. At the indicated times, aliquots were withdrawn and centrifuged, and the amount of unbound carboxylase or carboxylase bound to fIX−anti-fIX resin was quantitated by assaying the supernatants and resins for activity. The percent free carboxylase is expressed as amount of unbound versus the sum of bound + unbound activity. Aliquots were also withdrawn for analysis of fIX and fIX[180] carboxylation by SDS−PAGE and PhosphorImager (Figure 5). The experiment was performed in duplicate, and the duplicate values varied by less than 10%. The entire experiment was performed three times, all giving similar results.

Table 7: VKD Propeptide, but Not a Gla Domain-Derived Peptide, Promotes VKD Product Release in Vitro[a]

time (min)	fraction	−EEL, −propeptide carboxylase activity ($\times 10^{-3}$ cpm h^{-1})	−EEL, −propeptide free carboxylase (%)	+EEL carboxylase activity ($\times 10^{-3}$ cpm h^{-1})	+EEL free carboxylase (%)	+propeptide carboxylase activity ($\times 10^{-3}$ cpm h^{-1})	+propeptide free carboxylase (%)
0	bound	1007.3		916.0		1003.3	
	unbound	22.2	2	21.5	2	23.8	2
5	bound	1034.3		1095.3		913.8	
	unbound	49.6	5	41.9	4	86.0	9
30	bound	933.7		1078.2		863.6	
	unbound	42.1	4	42.8	4	126.1	13

[a] The experiment was performed as described in the legend to Table 6, except that carboxylation of the fIX−carboxylase complex was performed in the presence or absence of EEL or fX propeptide and cold $NaHCO_3$ was used in the protein carboxylation reaction cocktail to prevent $^{14}CO_2$ incorporation into EEL. The experiment was performed in duplicate, and the duplicate values varied by less than 10%. The test for release in the presence or absence of propeptide was performed four times, giving results similar to those shown above.

dependent conformational change that had the potential to affect release of fIX from the carboxylase. Results identical to those shown in Table 6 were obtained, however, showing that full carboxylation of fIX did not result in release from the carboxylase (data not shown). In contrast, when fIX−carboxylase complex carboxylation was performed in the presence of fIX[180], a significant increase in the amount of unbound carboxylase was observed (Table 6), indicating that carboxylase was released from the fIX in the fIX−carboxylase complex. FIX[180] carboxylation was observed (Figure 5A), consistent with the release of fIX and consequent binding of fIX[180].

To determine if the effect of fIX[180] was due to propeptide or Gla domain sequences, the experiment was repeated in the presence or absence of a propeptide sequence or a peptide derived from the Gla domain (Table 7). These analyses showed that the propeptide effected a 3-fold increase in release of the carboxylated fIX product. When the assay was performed for a longer period of time, the difference in release of carboxylated fIX in the presence versus the absence of propeptide was even greater (9-fold after 2 h, Figure 6). The combined in vitro data, then, indicate that VKD protein, specifically the propeptide, facilitates carboxylated VKD product release.

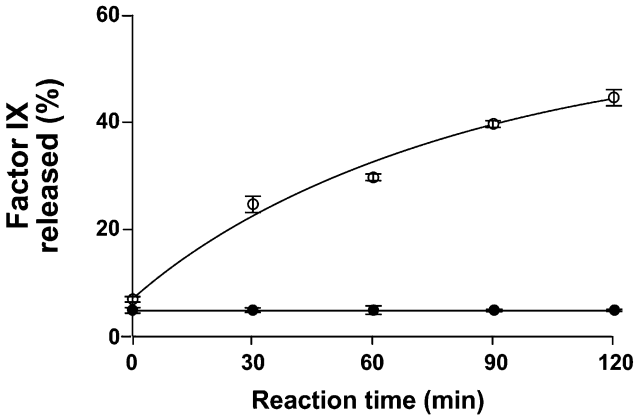

FIGURE 6: VKD propeptide promotes VKD product release in vitro. The experiment was performed in the presence (○) or absence (●) of propeptide, as described in the legends to Tables 6 and 7.

DISCUSSION

The intracellular events in VKD protein carboxylation are only poorly understood, and we therefore undertook an approach to directly analyze these processes. We found that overexpressed r-carboxylase is functional for full fIX carboxylation but caused decreased secretion of fIX due to inefficient release of the carboxylated fIX product. Thus, r-carboxylase overexpression titrated out some factor(s) required for efficient release of the fIX product. In vitro carboxylation studies showed that at least one of the factors could be excess VKD protein. FIX in a fIX−carboxylase complex was fully carboxylated (Figure 5), but most (95%) of the carboxylated fIX product remained associated with the carboxylase (Table 6), showing that full carboxylation is not a sufficient signal for rapid release. However, when in vitro carboxylation of the fIX−carboxylase complex was carried out in the presence of VKD substrate or propeptide, a significant increase in release of fIX from the carboxylase was observed (Tables 6 and 7; Figure 6). Thus, at least one explanation for the in vivo observation that r-carboxylase

overexpression causes inefficient release of carboxylated fIX product is that the r-carboxylase titrates out excess fIX (Table 1), which decreases facilitation of release by excess VKD protein. Whether r-carboxylase overexpression also titrates out other cellular factors that contribute to fIX secretion will require further study.

The effect of excess VKD protein/propeptide on release is of interest with regard to previous studies on carboxylase processivity. We showed that the carboxylase is a highly processive enzyme such that competition between free VKD substrate and carboxylase-bound VKD protein undergoing carboxylation does not occur. Thus, all 12 Glu-to-Gla conversions in fIX result from a single binding event whose kinetics are not perturbed by the presence of excess VKD substrate (*10*). Excess VKD protein/propeptide therefore has a differential effect upon release after, but not during, carboxylation. One possible explanation for this difference could be the individual rates of these two steps of the reaction. Our analyses using preformed carboxylase−fIX complexes allow the first dissection of these two steps and show that release is substantially slower than carboxylation (Figures 5 and 6). Consequently, under normal in vivo conditions, processive carboxylation may be accomplished simply because during the carboxylation step there is less time for release to occur. However, it is plausible that other factors also affect release. For example, some change could occur upon full carboxylation that differentially affects release of fully carboxylated product but not VKD intermediates. One change that occurs is the loss of Glu binding to the carboxylase active site because all of the Glu substrates are converted to Gla's, and recent studies suggest that the propeptide off-rate is affected by the Glu substrate (*24*). A second change could be due to carboxylase carboxylation. We showed, using uncarboxylated fIX−carboxylase complexes, that the carboxylase is carboxylated subsequent to fIX (*10*, *18*). Either change, then, has the potential to weaken propeptide binding, facilitating the release of the carboxylated product.

The increased release of VKD product in the presence of excess VKD substrate/propeptide could be due either to competitive binding at the same site or to binding at a second site in the carboxylase. Thus, the carboxylated VKD product may dissociate from the carboxylase but does not rebind due to the presence of a precursor pool of uncarboxylated VKD substrate that outcompetes the product. Alternatively, release may be facilitated by binding at a second site in the carboxylase. This possibility is supported by studies showing propeptide activation of carboxylase activity toward peptide substrates, which suggest carboxylase allostery (*4*), and by cross-linking studies that map propeptide binding to two different regions of the carboxylase (*25*, *26*). Our studies showed that release of VKD product is specifically due to the propeptide (Table 7; Figure 6), consistent with this alternative explanation.

The pulse-chase analyses provide insight into the rate of in vivo carboxylation and suggest that availability of the reduced vitamin K cofactor is limiting for carboxylation in mammalian cells. These studies showed that the rate of carboxylation is slow. The precursor pool of uncarboxylated fIX in both r-fIX BHK cells and r-fIX/r-carb BHK cells was detected until 6 h (band 1 in Figure 3B,D), indicating a rate of secretion much slower than that typically observed with other proteins (*27*). During the first turnover of fIX, the rate of disappearance of the uncarboxylated fIX precursor is a function of fIX−carboxylase association and the carboxylation reaction, but not release. In r-fIX/r-carb BHK cells, the first turnover took at least 3 h, as evidenced by the lack of appearance of fully carboxylated fIX before this time point (Figure 3D). During this time, the rate of disappearance of uncarboxylated fIX precursor was the same in both r-fIX/r-carb BHK cells and r-fIX BHK cells (Figure 4C), even though fIX−carboxylase binding occurs ∼50 times more frequently in the r-fIX BHK cells (Table 1). These data therefore suggest that the carboxylation reaction is rate-limiting for production of fully carboxylated fIX in r-fIX BHK cells, rather than VKD protein−carboxylase binding. This limitation could be due to low intracellular concentrations of vitamin K, poor reduction of the vitamin K to the hydroquinone cofactor form, or poor delivery of the vitamin K to the carboxylase.

The different pattern of carboxylation observed with r-fIX BHK cells versus r-fIX/r-carb BHK cells is consistent with a slow rate of carboxylation due to limiting amounts of vitamin K. Fully carboxylated fIX was observed at a much earlier time in the chase of the r-fIX BHK cells (1 h, Figure 3B) than with the r-fIX/r-carb BHK cells (3 h, Figure 3D) which have ∼50-fold more complexes (Table 1). In addition, a higher fraction of partially carboxylated intermediates was observed in the r-fIX/r-carb BHK cells (e.g., compare the 3 h time points, Figure 3B versus D). Thus, reduced vitamin K appears to be partitioned among all of the intracellular fIX−carboxylase complexes, and the limiting amount of vitamin K hydroquinone slows the carboxylation rate of individual fIX−carboxylase complexes in the r-fIX/r-carb BHK cells.

Low amounts of reduced vitamin K in cells could explain the large difference in VKD protein carboxylation efficiencies that were observed in vivo versus in vitro. The fIX−carboxylase complex that was analyzed in vitro was isolated from the r-fIX/r-carb BHK cells and so should be the same complex as that analyzed in vivo, yet carboxylation in vitro resulted in the appearance of carboxylated fIX after 5 min (Figure 2B) versus 3 h in vivo (Figure 3D). Saturating amounts of KH_2 were used in vitro, while the concentration of vitamin K in vivo and efficiency of reduction to the active KH_2 cofactor form are unknown.

Low vitamin K levels may then explain why overexpression of r-carboxylase did not improve carboxylation of fIX expressed at high levels (*16*). Overexpressed r-carboxylase is functional because most of the r-carboxylase binds fIX (Tables 2 and 5) and results in full fIX carboxylation (Table 4B). However, if vitamin K is limiting, then the total rate of Glu-to-Gla conversion would not increase with increased r-carboxylase expression. Our results predict that even in cell lines expressing r-VKD protein and r-carboxylase in the appropriate ratios to promote efficient release of carboxylated product, the VKD protein may still be poorly carboxylated because carboxylation is limited by the low in vivo amounts of KH_2. The analysis of cell lines expressing high levels of fIX and endogenous or r-carboxylase, using an approach similar to that described here, should be valuable in testing whether vitamin K is actually limiting. Such studies will also be important for determining how cellular factors affect VKD protein carboxylation and secretion which, at present, is

unknown. The results presented here will provide an important foundation for interpreting such future work.

ACKNOWLEDGMENT

The authors thank Dr. Kurt Runge for critical reading of the manuscript and for helpful comments throughout this work. We also thank Dr. William Church for providing the anti-fIX heavy chain antibody.

REFERENCES

1. Berkner, K. L. (2000) *J. Nutr. 130*, 1877−1880.
2. Furie, B., Bouchard, B. A., and Furie, B. C. (1999) *Blood 93*, 1798−1808.
3. Pudota, B. N., Miyagi, M., Hallgren, K. W., West, K. A., Crabb, J. W., Misono, K. S., and Berkner, K. L. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 13033−13038.
4. Knobloch, J. E., and Suttie, J. W. (1987) *J. Biol. Chem. 262*, 15334−15337.
5. Stanley, T. B., Jin, D. Y., Lin, P. J., and Stafford, D. W. (1999) *J. Biol. Chem. 274*, 16940−16944.
6. Wu, W., Bancroft, J. D., and Suttie, J. W. (1996) *Thromb. Haemost. 76*, 46−52.
7. Wu, W., Bancroft, J. D., and Suttie, J. W. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 13654−13660.
8. Tokunaga, F., Wakabayashi, S., and Koide, T. (1995) *Biochemistry 34*, 1163−1170.
9. Morris, D. P., Stevens, R. D., Wright, D. J., and Stafford, D. W. (1995) *J. Biol. Chem. 270*, 30491−30498.
10. Stenina, O., Pudota, B. N., McNally, B. A., Hommema, E. L., and Berkner, K. L. (2001) *Biochemistry 40*, 10301−10309.
11. Malhotra, O. P., and Carter, J. R. (1971) *J. Biol. Chem. 246*, 2665−2671.
12. Malhotra, O. P. (1972) *Nat. New Biol. 239*, 59−60.
13. Berkner, K. L. (1993) *Methods Enzymol. 222*, 450−477.
14. Yan, S. C., Razzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinnell, B. W. (1990) *Biotechnology (N.Y.) 8*, 655−661.
15. Kaufman, R. J., Wasley, L. C., Furie, B. C., Furie, B., and Shoemaker, C. B. (1986) *J. Biol. Chem. 261*, 9622−9628.
16. Rehemtulla, A., Roth, D. A., Wasley, L. C., Kuliopulos, A., Walsh, C. T., Furie, B., Furie, B. C., and Kaufman, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4611−4615.
17. Busby, S. J., Mulvihill, E., Rao, D., Kumar, A. A., Lioubin, P., Heipel, M., Sprecher, C., Halfpap, L., Prunkard, D., Gambee, J., and Foster, D. C. (1991) *J. Biol. Chem. 266*, 15286−15292.
18. Berkner, K. L., and Pudota, B. N. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 466−471.
19. Lingenfelter, S. E., and Berkner, K. L. (1996) *Biochemistry 35*, 8234−8243.
20. Busby, S., Kumar, A., Joseph, M., Halfpap, L., Insley, M., Berkner, K., Kurachi, K., and Woodbury, R. (1985) *Nature 316*, 271−273.
21. DeMetz, M., Vermeer, C., Soute, B. A. M., Van Scharrenburg, G. J. M., Slotboom, A. J., and Hemker, H. C. (1981) *FEBS Lett. 123*, 215−218.
22. Harbeck, M. C., Cheung, A. Y., and Suttie, J. W. (1989) *Thromb. Res. 56*, 317−323.
23. Kurachi, K., and Davie, E. W. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6461−6464.
24. Presnell, S. R., Tripathy, A., Lentz, B. R., Jin, D. Y., and Stafford, D. W. (2001) *Biochemistry 40*, 11723−11733.
25. Yamada, M., Kuliopulos, A., Nelson, N. P., Roth, D. A., Furie, B., Furie, B. C., and Walsh, C. T. (1995) *Biochemistry 34*, 481−489.
26. Wu, S. M., Mutucumarana, V. P., Geromanos, S., and Stafford, D. W. (1997) *J. Biol. Chem. 272*, 11718−11722.
27. Lodish, H. F., Kong, N., Snider, M., and Strous, G. J. (1983) *Nature 304*, 80−83.

BI026016E