Profactor IX: The Propeptide Inhibits Binding to Membrane Surfaces and Activation by Factor XIa†

J. Andrew Bristol, Steven J. Freedman, Barbara C. Furie, and Bruce Furie*

Departments of Biochemistry and Medicine, Tufts University School of Medicine and the Sackler School of Biomedical Sciences, and the Center for Hemostasis and Thrombosis Research, Division of Hematology/Oncology, New England Medical Center, Boston, Massachusetts 02111

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ABSTRACT: The γ-carboxylase recognition site in the propeptide of profactor IX signals the γ-carboxylation of specific glutamic acid residues in the adjacent Gla domain during factor IX biosynthesis. To study posttranslational processing of the vitamin K-dependent blood coagulation factors and the properties of processing intermediates, we have isolated an incompletely processed factor IX species, profactor IX, from the medium of heterologous mammalian cells expressing the human factor IX cDNA. Profactor IX was purified by sequential immunoaffinity chromatography using antibodies specific for the propeptide and antibodies specific for the well-carboxylated factor IX species. This purified profactor IX preparation was fully γ-carboxylated and contained the N-terminal propeptide, but it exhibited no factor IX procoagulant activity. Profactor IX was not cleaved following incubation with factor XIa. In contrast to mature factor IX, profactor IX did not demonstrate Ca(II)-dependent binding to acidic phospholipid vesicles, nor can the membrane binding surface be expressed, as detected by antibodies specific for this epitope. The propeptide of profactor IX can be removed *in vitro* by a specific endopeptidase, furin/PACE, yielding factor IX, which can be converted to fully active factor IXa by factor XIa and which binds normally to acidic phospholipid vesicles. These results indicate that fully γ-carboxylated profactor IX is biologically inactive due to the presence of the propeptide.

Factor IX is a vitamin K-dependent blood coagulation protein, which is defective in patients with hemophilia B, that undergoes posttranslational modifications during its synthesis and secretion into the blood. These modifications include signal peptide cleavage, N- and O-linked glycosylation, β-hydroxylation of aspartic acid 64, γ-carboxylation of the first 12 glutamic acid residues in the amino terminus, and cleavage of the 18 amino acid propeptide [for a review, see Furie and Furie (1988)]. The propeptides of the vitamin K-dependent proteins, including prothrombin, factor IX, factor X, factor VII, protein C, and protein S, demonstrate marked sequence homology (Pan & Price, 1985). We and others have shown the propeptide to be a necessary and sufficient recognition element to direct the γ-carboxylation of glutamic acid residues adjacent to the propeptide in the Gla[1] domain (Jorgensen et al., 1987; Foster et al., 1987; Ulrich et al., 1988; Huber et al., 1990; Ratcliffe et al., 1991). The γ-carboxylation recognition site is located at the amino terminus of the propeptide, a polypeptide that includes a 10 residue amphipathic α-helix (Sanford et al., 1991), whereas the propeptidase recognition element is located in the carboxy-terminal region of the propeptide, adjacent to the scissile bond (Bentley et al., 1986; Galaeffi & Brownlee, 1987; Handford et al., 1991; Bristol et al., 1993a). The γ-carboxylation event is mediated by a vitamin K-dependent carboxylase that resides in the endoplasmic reticulum (Carlisle & Suttie, 1980), although the enzyme is also present in the Golgi apparatus (Stanton et al., 1991). Propeptide cleavage is thought to be a later event mediated in the Golgi apparatus (Wasley et al., 1993; Stanton et al., 1991) by an endopeptidase. These independent processing events predict an intracellular factor IX intermediate that is fully carboxylated and contains a propeptide.

Factor IX from some hemophilia B patient plasmas has mutations that preclude propeptide cleavage. For example, factor IX Cambridge (Diuguid et al., 1986) contains a mutation from arginine −1 to serine, while factor IX Oxford 3 (Bentley et al., 1986), factor IX San Dimas (Ware et al., 1989), factor IX Troed-y-Rhiw (Liddell et al., 1989), and factor IX Kawachinagano (Sugimoto et al., 1989) contain mutations from arginine −4 to glutamine. In addition to containing a propeptide extension, with the exception of factor IX Kawachinagano (Sugimoto et al., 1989) and possibly factor IX Oxford 3 (Bentley et al., 1986), which are reported to be fully carboxylated, other propeptide-containing mutants have been reported to be only partially carboxylated (Diuguid et al., 1986; Galeffi & Brownlee, 1987; Ware et al., 1989).

The vitamin K-dependent blood coagulation proteins require full or nearly full carboxylation to bind to membrane surfaces in the presence of calcium ions (Furie & Furie, 1988). For example, des-γ-carboxyprothrombin (abnormal) does not interact with phospholipid membranes in the presence of calcium ions, whereas prothrombin does (Esmon

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* Address correspondence to this author: Center for Hemostasis and Thrombosis Research, Division of Hematology/Oncology, New England Medical Center, 750 Washington St., Box 832, Boston, MA 02111. FAX: (617) 956-4833.

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[1] Abbreviations: CHO, Chinese hamster ovary; TBS, Tris-buffered saline [20 mM Tris (pH 7.4)/140 mM NaCl], PACE, paired basic amino acid cleavage enzyme; EDTA, ethylenediaminetetraacetic acid; RIA, radioimmunoassay; Gla, γ-carboxyglutamic acid.

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et al., 1975). Chemical modification of γ-carboxyglutamic acid residues (Jones et al., 1985), partial carboxylation (Borowski et al., 1985), and the substitution of other metal ions for calcium ions (Nelsestuen et al., 1976) prevent the vitamin K-dependent proteins from binding to acidic phospholipids. Furthermore, N-terminal acetylation interferes with the expression of phospholipid membrane binding (Welsch & Nelsestuen, 1988). Recent studies of a factor IX peptide from residues 1−47 that was prepared by chemical synthesis have suggested that the Gla domain and the aromatic amino acid stack domain express the full membrane binding properties of factor IX (Jacobs et al., 1994).

Factor IX binds calcium ions via the Gla domain to assume a unique conformation that is recognized by conformation-specific antibodies (Liebman et al., 1985). The calcium-bound factor IX complexes to membrane surfaces to participate in blood coagulation. The propeptide-containing factor IX mutants do not bind to antibodies that recognize the conformation that is stabilized by divalent metal ions, and they do not bind to phospholipid vesicles (Diuguid et al., 1986; Ware et al., 1989). It is unclear whether the functional defect in these factor IX mutants is due to their undercarboxylation, the propeptide point mutation, or the presence of a propeptide that sterically interferes with the folding of factor IX and expression of the lipid binding site. Recombinant factor IX, isolated from cells amplified to produce very high levels of factor IX, lacks the propeptide but has a γ-carboxyglutamic acid content equivalent to that of factor IX Cambridge or factor IX San Dimas; this preparation exhibits significantly higher procoagulant activity (60%) than do the mutant factor IX species (<1%) (Kaufman et al., 1986). Wild-type profactor IX, which contains the propeptide, the normal sequence of profactor IX, and 12 Gla residues, would allow the evaluation of whether the presence of the propeptide is sufficient to cause the functional defects associated with the naturally occurring mutant.

In this report, we present the purification and characterization of a factor IX species, wild-type profactor IX, which is fully γ-carboxylated and contains the propeptide. We have previously shown that approximately 7% of the secreted factor IX expressed heterologously in Chinese hamster ovary cells escapes propeptide cleavage in the expression system employed (Bristol et al., 1993a). We have now isolated this fraction by sequential immunoaffinity chromatography. This profactor IX species is fully γ-carboxylated, but it has no procoagulant activity, fails to bind to phospholipid vesicles, and cannot be activated by factor XIa.

MATERIALS AND METHODS

Expression and Purification of Profactor IX. The maintenance of Chinese hamster ovary (CHO) cells expressing recombinant human factor IX has been described (Kaufman et al., 1986; Rabiet et al., 1987; Bristol et al., 1993a). Cells were grown in 10-layer cell factories (Nunc), and medium (2 L) was collected after 10 days. The supernatants were made 2 mM in benzamidine hydrochloride (Sigma) and concentrated approximately 10-fold in an Amicon ultrafiltration unit, filtered through a 0.2 mm filter (Millipore), and then applied at 4 °C to an anti-factor IX:total antibody column (3 mg of antibody/mL of Sepharose-4B; 2 × 10 cm) (Liebman et al., 1987). This column was washed with 50 column vol of 10 mM Tris-HCl (pH 7.4)/1.0 M NaCl/0.02% Tween-20, equilibrated with TBS, and then eluted with 4 M guanidine hydrochloride. Protein elution was monitored by the absorbance at 280 nm. The eluted protein was immediately dialyzed against TBS at 4 °C. The total factor IX fraction was next applied to an anti-profactor IX antibody column (2 mg of antibody/mL of Sepharose-4B; 1 × 5 cm) (Bristol et al., 1993a). This column was washed with 10 column vol of the wash buffer described above and eluted with 4 M guanidine hydrochloride. The eluted protein was immediately dialyzed against TBS containing 5 mM $CaCl_2$. Finally, the profactor IX preparation was applied to an anti-factor IX:Mg(II) antibody column (3 mg of antibody/mL of Sepharose-4B; 1 × 5 cm) (Liebman et al., 1987) equilibrated with TBS containing 5 mM $MgCl_2$. This column was washed with 5 column vol of 10 mM Tris (pH 7.4)/1 M NaCl/0.02% Tween-20/5 mM $MgCl_2$, equilibrated with TBS containing 5 mM $MgCl_2$, and then eluted with TBS containing 5 mM EDTA. The profactor IX species that eluted specifically with EDTA was concentrated in a Centriprep-30 filter unit (Amicon) and stored at −80 °C. The purity of the profactor IX was assessed by electrophoresis (Laemmli, 1970) under reducing conditions on SDS/7.5% polyacrylamide gels. Proteins were visualized by staining with silver (Bio-Rad) or with Coomassie Blue.

Analysis of the γ-Carboxylation of Profactor IX. The Gla content of profactor IX was analyzed essentially as described (Kuwada & Katayama, 1983; Kotkow et al., 1993). Briefly, protein samples were purified to homogeneity by reverse phase HPLC using a butyl guard column (Bio-Rad). Proteins (2 μg) were hydrolyzed *in vacuo* for 16 h at 110 °C in 2.5 M KOH. The amino acids were derivatized with the fluorophore *o*-phthalaldehyde (Sigma) and chromatographed to resolve glutamic acid, aspartic acid, and γ-carboxyglutamic acid using a Nucleosil 5SB column (4.6 × 50 mm, Macherey-Nagel, Germany). The integrated areas of the aspartic acid, glutamic acid, and γ-carboxyglutamic acid peaks were used to calculate the γ-carboxyglutamic acid content. Plasma-derived factor IX was used as a standard.

Amino-Terminal Sequence Analysis of Profactor IX. Approximately 200 pmol of purified profactor IX was analyzed by electrophoresis on an SDS/8% polyacrylamide gel. The protein was transferred to a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore) in gel running buffer containing 10% methanol over 1 h at 80 V (constant voltage). The blot was stained with 0.1% Coomassie Blue (Bio-Rad) in 50% methanol/50% water and then destained in 50% methanol/50% water. The profactor IX band was excised and subjected to automated Edman degradation on a Milli-Gen 6600 Prosequencer.

Radioimmunoassay of Profactor IX Using Conformation-Specific Antibodies. Protein samples (10 μg) were labeled with ^{125}I using Enzymobeads as described by the manufacturer (Bio-Rad). The labeled proteins were purified by gel filtration on a Sephadex G-25 column (PD-10, Pharmacia) and subsequently on an anti-factor IX:Mg(II) column as described earlier. Antibodies directed to epitopes that recognize all conformers of factor IX (anti-factor IX:total antibodies), antibodies directed to epitopes of factor IX that are stabilized by most divalent metal ions (anti-factor IX:Mg(II) antibodies), or antibodies directed to calcium-stabilized epitopes at or near the membrane binding site of factor IX (anti-factor IX:Ca(II)-specific antibodies) were

prepared and used in a standard radioimmunoassay (RIA) as described (Liebman et al., 1987). The RIA buffer used contained 20 mM Tris-HCl (pH 7.4)/140 mM NaCl/0.02% Tween-80/0.1% BSA/0.02% NaN_3. Briefly, iodinated protein samples were incubated overnight at 4 °C with increasing concentrations of the purified rabbit polyclonal antibody populations (as indicated) in RIA buffer containing either 5 mM EDTA, 5 mM $MgCl_2$, or 5 mM $CaCl_2$, and 1 mg/mL normal rabbit γ-globulin (Pel-Freez, Inc.). The immunocomplexes were precipitated with goat anti-rabbit immunoglobulin (Pel-Freez, Inc.; 7% in TBS/2.5% PEG-3400), and the precipitates were pelleted by centrifugation for 20 min at 4 °C in a Beckman J-6 centrifuge. The supernatants were aspirated and the pellets were assayed for ^{125}I in a Packard Autogamma 5000 scintillation spectrometer.

Phospholipid Binding Assays. The ability of profactor IX to bind to membrane surfaces was determined by measuring fluorescence energy transfer from tryptophan residues in the protein to dansyl-labeled phospholipid vesicles (Schwalbe et al., 1989). Phospholipid vesicles were composed of phosphatidylserine/phosphatidylcholine/dansylphosphatidylethanolamine (40:50:10, respectively). Increasing amounts of protein were added to 3 mL of 3 mM vesicles/50 mM Tris-HCl (pH 7.4)/50 mM NaCl/5 mM $CaCl_2$ contained in a cuvette. Fluorescence was measured in an SLM 8000C fluorescence spectrophotometer using an excitation wavelength of 280 nm (4 nm slit width) and an emission wavelength of 520 nm (16 nm slit width). Dilution effects were corrected using a buffer control. At saturation of the binding of protein to vesicles (80 mM), EDTA was added to a final concentration of 10 mM to reverse calcium-dependent binding. Factor IX, prepared by furin/PACE digestion of profactor IX (described below), was evaluated for binding at a concentration that corresponds to the highest concentration of profactor IX used in the phospholipid binding experiment (80 mM). The furin/PACE preparation alone was assayed with the phospholipid vesicles to determine its effect on the fluorescence emission. Initial emission intensity (I_0) was subtracted from the total emission intensity ($I-I_0$) and then divided by the initial emission intensity ($(I-I_0)/I_0$) to determine the fraction of the signal due to fluorescence energy transfer.

Activation of Profactor IX with Factor XIa. Factor XIa (Enzyme Research Laboratories, Inc.) was mixed with either plasma-derived factor IX (Enzyme Research Laboratories, Inc.) or profactor IX at a ratio of 1:40 (w/w) in TBS containing 5 mM $CaCl_2$. The reactions were incubated at 37 °C for the indicated times. Aliquots were removed to ice, and EDTA was added to a final concentration of 10 mM to stop the reactions. Cleavage products were monitored by SDS−PAGE on 18% polyacrylamide gels (Laemmli, 1970) and stained with Coomassie Blue (Bio-Rad). For the activation of the recombinant profactor IX, a soluble secreted form of recombinant furin/PACE (kindly provided by Drs. R. Kaufman and A. Rehemtulla) was used to cleave the propeptide to profactor IX; the cleaved protein was then added to factor XIa as described earlier. For the propeptide cleavage, furin/PACE-containing medium from a COS cell transient transfection was dialyzed against 0.1 M HEPES buffer (pH 7.0) containing 5 mM $CaCl_2$ and then concentrated 10-fold in a Centricon-30 (Amicon). This furin/PACE preparation, contaminated with other proteins secreted by the COS cells, was incubated with purified profactor IX overnight at 37 °C in the presence of 5 mM $CaCl_2$.

Analysis of Furin/PACE-Treated Profactor IX. Purified profactor IX was incubated overnight with mock-transfected COS cell supernatant or furin/PACE-transfected COS cell supernatant, as described above. Samples were subjected to gel electrophoresis on 0.1% SDS/7.5% acrylamide gels (Laemmli, 1970). Proteins were visualized by staining with Coomassie Blue or, following transfer to a poly(vinylindene difluoride) membrane, by immunoblotting with either anti-propeptide antibodies (Bristol, 1993a) or immunoaffinity-purified polyclonal anti-factor IX:total antibodies. The blots were incubated for 30 min in 2% bovine serum albumin dissolved in TBS to block nonspecific protein binding sites and then incubated for 2 h at 25 °C with either rabbit anti-propeptide antibodies (5 μg/mL) (Bristol et al., 1993a) or goat anti-factor IX:total antibodies (1 μg/mL) (Liebman et al., 1987) diluted in 2% BSA/TBS. The blots were washed three times with TBS and then incubated for 1 h at 25 °C in either goat anti-rabbit IgG-conjugated or rabbit anti-goat IgG-conjugated alkaline phosphatase. The blots were washed and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (Bio-Rad). The reactions were terminated by the addition of water.

Procoagulant Activity Assay. Profactor IX and profactor IX pretreated with furin/PACE were assayed for procoagulant activity in a one-stage clotting assay using factor IX-deficient plasma (Proctor & Rapaport, 1961). The procoagulant activity was evaluated using an ST4 clot detection instrument (Diagnostica Stago). Normal plasma and plasma-derived factor IX were used as standards.

RESULTS

At expression levels of about 0.5−1.5 μg/mL, approximately 7% of the factor IX secreted from CHO cells transfected with the factor IX cDNA contains propeptide due to incomplete intracellular processing (Bristol et al., 1993a). Sequential immunoaffinity chromatography was employed to isolate the fully γ-carboxylated profactor IX species secreted from these cells. The CHO cell medium containing factor IX was applied to an anti-factor IX:total antibody column to bind all of the factor IX species in the medium, regardless of the presence or absence of propeptide or the state of γ-carboxylation of the factor IX. The factor IX species isolated from this column were applied to an anti-profactor IX antibody column to isolate the propeptide-containing factor IX species. These factor IX species were further fractionated to isolate the well-carboxylated profactor IX species by immunoaffinity chromatography on an anti-factor IX:Mg(II) antibody column equilibrated in 5 mM $MgCl_2$. The bound protein was eluted with EDTA. The progress of this three-step purification is shown in Figure 1. We isolated approximately 4 mg of factor IX-related species from 6 L of CHO cell supernatant. Fully carboxylated profactor IX represented approximately 220 μg of the total factor IX species isolated. This material was nearly homogeneous (Figure 1, lane 3). Laser densitometric scanning of silver-stained SDS polyacrylamide gels showed this preparation to be approximately 90% profactor IX. Factor IX characteristically migrates as a broad band, as does this profactor IX preparation. Western blot analysis using anti-factor IX:total antibodies indicated that the antibodies were reactive with the profactor IX band at M_r 68 000, but were

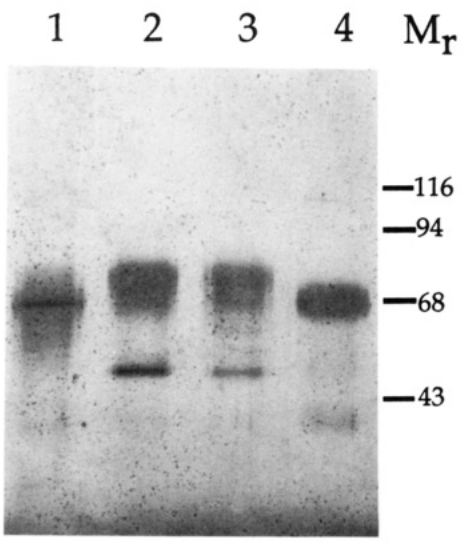

FIGURE 1: Purification of fully carboxylated profactor IX. Profactor IX was purified from CHO cell medium by immunoaffinity chromatography using three antibodies that recognize different epitopes of factor IX: lane 1, 4 M guanidine hydrochloride eluate from the anti-factor IX:total antibody column; lane 2, 4 M guanidine hydrochloride eluate from the anti-profactor IX antibody column; lane 3, 5 mM EDTA eluate from the anti-factor IX:Mg(II) antibody column; lane 4, plasma-derived factor IX. The samples were reduced with β-mercaptoethanol, and separated on a SDS/7.5% polyacrylamide gel, and then visualized by silver staining. Molecular mass standards are noted on the right.

not reactive with the minor M_r 50 000 band (*vide supra*). The profactor IX preparation was subjected to amino-terminal sequence analysis. A single sequence was observed to be that of the propeptide of factor IX. No sequence for mature factor IX was observed.

The Gla content of the purified profactor IX was determined by direct γ-carboxyglutamic acid analysis. Profactor IX was purified to homogeneity by reverse phase HPLC prior to hydrolysis. Profactor IX contained 12.6 mol of Gla/mol of protein compared to 12 mol of Gla/mol of the plasma-derived factor IX standard (Table 1). Thus, the profactor IX that was isolated is fully γ-carboxylated.

The fully carboxylated profactor IX was evaluated for biological activity. When assayed using a one-stage factor IX procoagulant assay, profactor IX possessed less than 2% of the specific coagulant activity of plasma-derived factor IX (Table 1). The effect of the propeptide on the activation of profactor IX by factor XIa was evaluated in a purified system. Profactor IX was incubated with factor XIa in the presence of 2 mM $CaCl_2$. Even after 8 h of incubation with factor XIa, profactor IX was not cleaved (Figure 2, lanes 4−8), whereas plasma-derived factor IX (Figure 2, lanes 1−3) was nearly fully cleaved after 2 h under the conditions employed. The factor IX heavy chain (M_r 28 000) and light chain (M_r 20 000) were observed as the cleavage products that accompany activation. The activation peptide (M_r 11 000 is not detectable in this gel system. Recombinant factor IX synthesized in Chinese hamster ovary cells and isolated in a parallel manner behaves like plasma-derived factor IX in these assays. As the profactor IX species is fully carboxylated, these results indicate that the lack of functional activity in profactor IX is due to its inability to undergo proteolytic cleavage mediated by factor XIa.

The purified profactor IX was characterized by its ability to express certain metal-stabilized antigenic determinants.

Table 1: Analysis of Gla Content and Procoagulant Activity of Profactor IX

protein	γ-carboxyglutamic acid content (mol of Gla/mol of protein)	factor IX procoagulant activity[a] (%)
plasma-derived factor IX	12	100
profactor IX	12.6 ± 1.1 ($n = 5$)[b]	<2
profactor IX pretreated with COS cell supernatant from PACE-transfected cells		78
profactor IX pretreated with COS cell supernatant from mock-transfected cells		<2

[a] Procoagulant activity was determined in a one-stage clotting assay in factor IX-deficient plasma. [b] n represents the total of two different preparations of profactor IX, the first of which was hydrolyzed three separate times and the second of which was hydrolyzed two separate times.

Anti-factor IX:Mg(II) antibodies, which are directed against a conformation stabilized by any divalent metal ion, react similarly with plasma-derived factor IX and profactor IX (Figure 3A). This reactivity is dependent on the presence of Mg(II) since binding is abolished by the addition of EDTA. This demonstrates that fully γ-carboxylated profactor IX can express antigenic determinants shared by factor IX when it undergoes the first conformational transition that is induced by divalent metal ions (Liebman et al., 1987). The experiment depicted in Figure 3B shows that profactor IX does not bind tightly to anti-factor IX:Ca(II)-specific antibodies, in contrast to the high-affinity interaction with plasma-derived factor IX. These antibodies recognize a conformation-specific antigenic determinant that is expressed on factor IX only in the presence of Ca(II). This calcium-induced transition exposes a membrane binding site on factor IX, and these antibodies recognize epitopes at, or near, the membrane binding site of factor IX (Liebman et al., 1987). The interaction of this antibody with plasma-derived factor IX is also abolished by the addition of EDTA. As a control, the experiment shown in Figure 3C demonstrates that anti-factor IX:total antibodies interact similarly with both plasma-derived factor IX and profactor IX.

To show that profactor IX has the potential to be converted to factor IXa upon removal of the propeptide, we demonstrated that treatment of profactor IX with furin/PACE yields functional factor IX. Unlike profactor IX, factor IX derived *in vitro* from profactor IX is cleaved to near completion by factor XIa after 2 h of incubation, yielding bands characteristic of factor IXa (Figure 4, lanes 2−5). Within experimental error, the procoagulant activity of the factor IX derived from the profactor IX was similar to that of plasma-derived factor IX (Table 1). The contaminating bands in the lanes containing furin/PACE represent other proteins in the COS cell supernatant from furin/PACE-expressing cells (Figure 5A).

To prove that furin/PACE expressed into the COS cell supernatant and not another contaminating protease in the COS cell supernatant was responsible for converting profactor IX to factor IX, we analyzed the conversion of profactor IX to factor IX using both furin/PACE-transfected COS cell supernatant and mock-transfected COS cell supernatant. A protein stain of furin/PACE-transfected COS cell supernatant is shown in Figure 5A, lane 1, and profactor IX is shown in Figure 5A, lane 2. As shown in panel B,

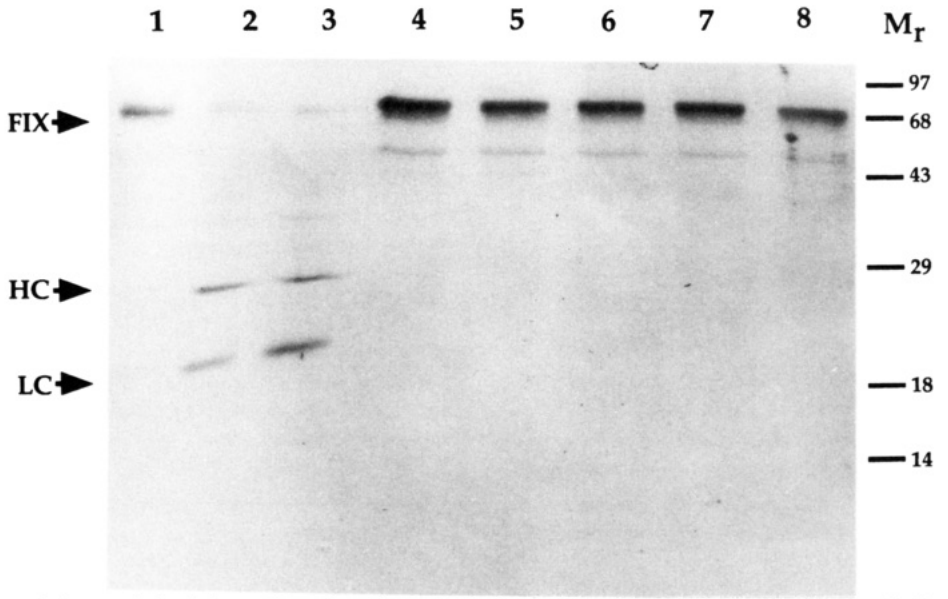

FIGURE 2: Cleavage of plasma-derived factor IX and profactor IX by factor XIa. Factor XIa was incubated with factor IX or profactor IX at a ratio of 1:40 (w/w) at 37 °C for the indicated times. The reaction was stopped by placing aliquots on ice and adding EDTA. The products were reduced with β-mercaptoethanol, separated on an SDS/18% polyacrylamide gel, and then stained with Coomassie Blue. Cleavage products of plasma-derived factor IX (lanes 1−3) and profactor IX (lanes 4−8): lanes 1 and 4, 0 min incubation with factor XIa; lanes 2 and 5, 30 min; lanes 3 and 6, 2 h; lane 7, 4 h; lane 8, 8 h. Molecular mass standards are noted on the right. Arrows point to intact factor IX (FIX), the heavy chain of factor IXa (HC), and the light chain of factor IXa (LC).

profactor IX was reactive with anti-propeptide antibodies (lane 2). When profactor IX was incubated with mock-transfected COS cell supernatant (lane 3), there was no discernable cleavage of the propeptide, as determined by an immunoblot with anti-propeptide antibodies (panel B) or a change in the electrophoretic migration of profactor IX (panels A−C). In contrast, profactor IX was converted to factor IX when profactor IX was incubated with furin/PACE-transfected COS cell supernatant (lane 4). The resultant factor IX was not reactive with the anti-propeptide antibodies (panel B), and there was a detectable shift in molecular size between profactor IX and factor IX, consistent with the removal of the propeptide (panel A).

Since profactor IX failed to bind antibodies that recognize epitopes at or near the membrane binding site on factor IX, we verified that profactor IX does not bind phospholipid vesicles, even in the presence of calcium ions. The interaction of profactor IX and factor IX with phospholipid vesicles was studied by fluorescence energy transfer using PS:PC vesicles containing dansylphosphatidylethanolamine. With increasing protein concentration, plasma-derived factor IX binds to fluorescently labeled phospholipid vesicles in the presence of calcium ions (Figure 6). This interaction is reversible upon the addition of EDTA. However, profactor IX does not bind to the phospholipid vesicles in the presence of calcium ions, a result anticipated by the inability of profactor IX to interact with the anti-factor IX:Ca(II)-specific antibodies. When profactor IX was incubated with the endopeptidase furin/PACE to remove the propeptide, the product of this digestion, factor IX, bound normally to the vesicles. This interaction was also reversible by the addition of EDTA. The furin/PACE preparation did not perturb the fluorescent signal in the binding assay system (data not shown), demonstrating that the observed signal was due to the interaction of phospholipid vesicles with factor IX and not due to the presence of the furin/PACE preparation. These results demonstrate that the presence of the propeptide attached to the N-terminus of factor IX is responsible for inhibiting normal factor IX binding to phospholipid membranes, and removal of the propeptide from profactor IX restores phospholipid binding.

DISCUSSION

The propeptides of the vitamin K-dependent blood coagulation proteins are necessary and sufficient to direct the γ-carboxylation of adjacent glutamic acid residues (Jorgensen et al., 1987; Foster et al., 1987; Ulrich et al., 1988; Huber et al., 1990; Ratcliffe et al., 1991). The propeptide contains a recognition element, termed the γ-carboxylase recognition site (Jorgensen et al., 1987), that binds to the γ-carboxylase (Hubbard et al., 1989). The γ-carboxylase resides in the rough endoplasmic reticulum (Carlisle & Suttie, 1980), as well as in the Golgi membranes (Wallin et al., 1993; Bristol et al., 1993b). It has been proposed that the cleavage of these propeptides is an event mediated by an endopeptidase residing in the Golgi apparatus (Wasley et al., 1993; Stanton et al., 1991). This propeptidase activity may be furin/PACE or a furin/PACE-like enzyme with specificity for the consensus propeptide cleavage site R-X-K/R-R (Wise et al., 1990; Bresnahan et al., 1990; Barr, 1992). On the basis of this presumed order of events, for vitamin K-dependent proteins, intracellular processing intermediates that are fully γ-carboxylated and have an N-terminal propeptide extension should exist. Although never isolated, such an intermediate has been indirectly observed for prothrombin (Stanton et al., 1991).

We report here the isolation of a factor IX species that contains the propeptide and is fully γ-carboxylated. The purification and characterization of this species are novel because this profactor IX represents the wild-type amino acid sequence, is fully γ-carboxylated, and can be cleaved to generate factor IX. These points contrast the hemophiliac profactor IX molecules that contain a point mutation in the propeptide, thus confusing whether their lack of activity is

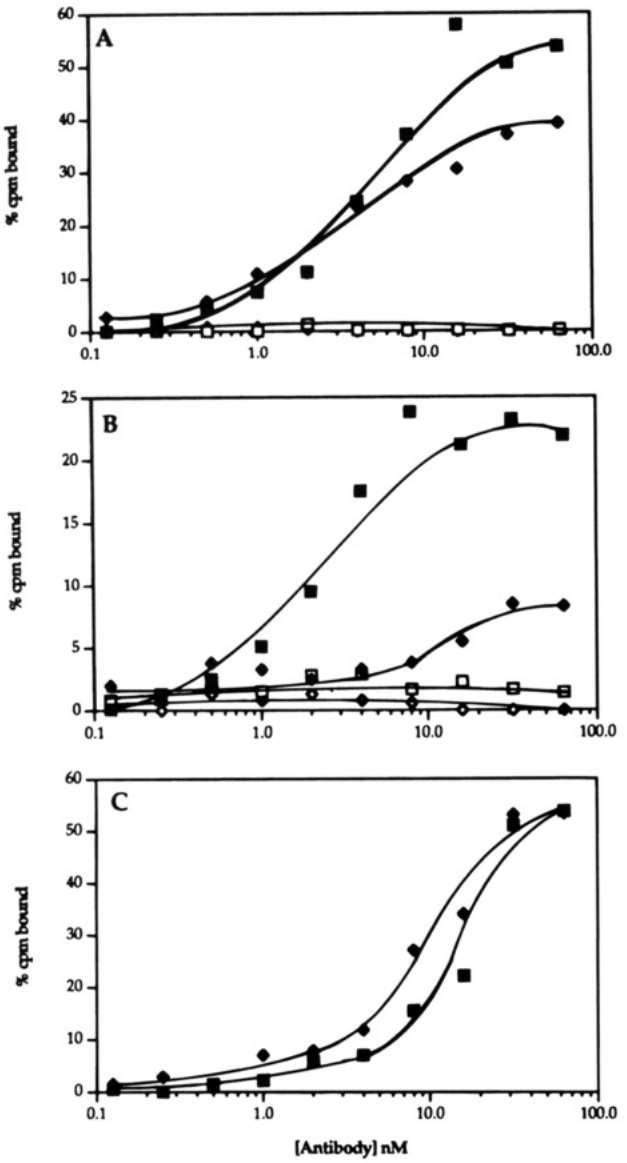

FIGURE 3: Radioimmunoassay of plasma-derived factor IX and profactor IX binding to anti-factor IX antibodies. Iodinated proteins were incubated with increasing concentrations of antibodies that recognize different epitopes of factor IX. The immunocomplexes were precipitated with goat anti-rabbit antiserum, and the pellets were assayed in a gamma counter: (A) anti-factor IX:Mg(II) antibodies; (B) anti-factor IX:Ca(II)-specific antibodies; (C) anti-factor IX:total antibodies; squares, plasma-derived factor IX; diamonds, profactor IX. Filled symbols are assays performed in RIA buffer containing either 5 mM $MgCl_2$ (panel A) or 5 mM $CaCl_2$ (panel B); open symbols are assays performed in RIA buffer containing 5 mM EDTA. Background values were subtracted, and the data were plotted as the percent of cpm bound to antibodies versus the antibody concentration.

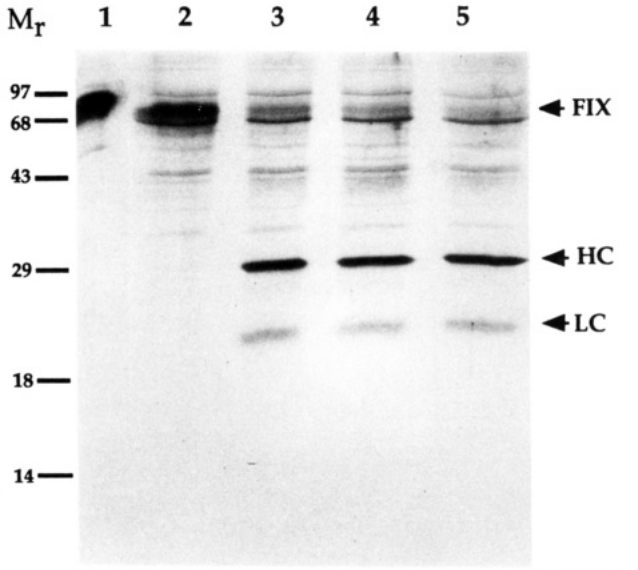

FIGURE 4: Propeptide cleavage of profactor IX with furin/PACE and cleavage by factor XIa. Profactor IX was incubated with furin/PACE overnight at 37 °C to cleave the propeptide from profactor IX. The mature factor IX was incubated with factor XIa for the indicated times and analyzed as described in the legend to Figure 2: lane 1, profactor IX; lane 2, 0 min of incubation with factor XIa; lane 3, 1 h of incubation; lane 4, 2 h of incubation; lane 5, 8 h of incubation. Molecular mass standards are noted on the left. Arrows point to intact factor IX (FIX), the heavy chain of factor IXa (HC), and the light chain of factor IXa (LC).

due to the propeptide extension, the point mutation, or the partial carboxylation that characterizes some of these naturally occurring mutants. The fully carboxylated profactor IX that we have isolated does not express factor IX procoagulant activity, and factor XIa is unable to cleave profactor IX at either the arginine 145−alanine 146 bond or the arginine 180−valine 181 bond, both of which are cleaved during factor IX activation. Thus, the presence of the covalently bound propeptide alone can generate an inactive species in a fully carboxylated profactor IX. The inability of factor XIa to activate propeptide-containing factor IX has been reported for a naturally occurring mutant, factor IX Troed-y-Rhiw (Liddell et al., 1989). In contrast to our results with the wild-type profactor IX, factor XIa is reported to cleave factor IX Troed-y-Rhiw at arginine 180−valine 181 to yield profactor IXaα. The activation of factor IX Troed-y-Rhiw was carried out with partially purified mutant factor IX and partially purified factor XIa, while our experiments used highly purified profactor IX and factor XIa. The basis for these differing results remains unexplained. Nevertheless, both experiments indicate that the presence of the propeptide on factor IX is sufficient to prevent the generation of factor IXa, i.e., cleavage of the scissile bonds adjacent to both arginine 145 and arginine 180.

In contrast to the wild-type profactor IX that we have isolated, factor IX San Dimas and factor IX Cambridge are only 60% carboxylated and do not bind to anti-factor IX:Mg(II) antibodies (Diuguid et al., 1986; Ware et al., 1989). These results suggest that the binding of anti-factor IX:Mg-(II) antibodies requires full or nearly full carboxylation but is not inhibited by the presence of the propeptide. In contrast, anti-factor IX:Ca(II)-specific antibodies will only bind to the fully carboxylated factor IX that lacks the propeptide. The anti-factor IX:Ca(II)-specific antibodies recognize epitopes at or near the membrane binding region exposed upon calcium binding since they inhibit binding of factor IX to phospholipid vesicles and cleavage to factor IXa by factor XIa (Liebman et al., 1987). Based upon this demonstration that anti-factor IX:Mg(II) antibodies bind to fully carboxylated profactor IX while the anti-factor IX:Ca(II)-specific antibodies bind only to fully carboxylated factor IX lacking the propeptide, we have shown by immunofluorescent microscopy studies that carboxylated profactor IX is localized to the rough endoplasmic reticulum and that mature carboxylated factor IX is localized to a late Golgi compartment (Bristol et al., 1993b).

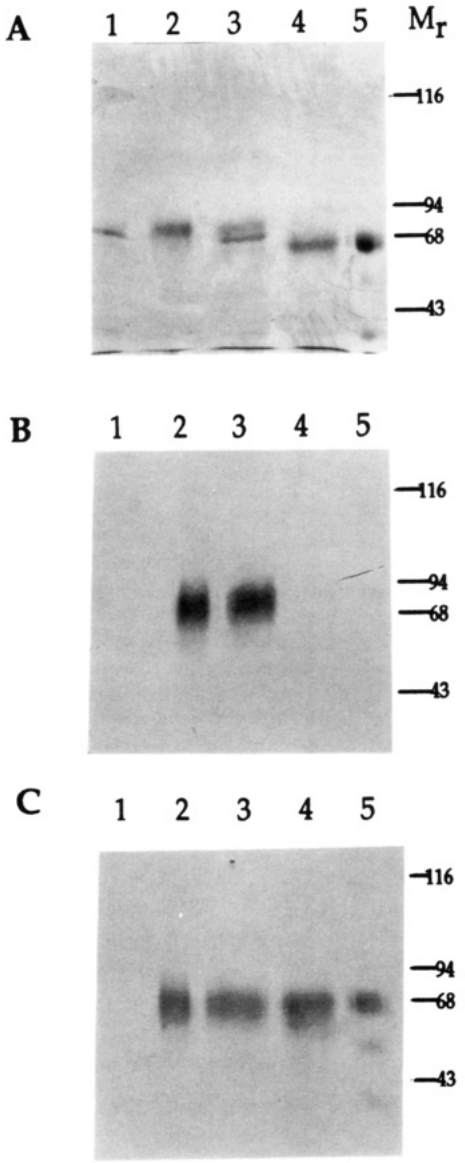

FIGURE 5: Analysis of the conversion of profactor IX to factor IX by furin/PACE. Profactor IX was incubated with either furin/PACE-transfected COS cell supernatant or mock-transfected COS cell supernatant and then separated by SDS gel electrophoresis in the presence of β-mercaptoethanol. For immunoblotting experiments, proteins were transferred from the gel to membrane for subsequent analysis for antibody reactivity: (A) protein stain with Coomassie Blue: (B) immunoblot using anti-propeptide antibodies; (C) immunoblot using anti-factor IX:total antibodies; lane 1, furin/PACE-transfected COS cell supernatant; lane 2, profactor IX; lane 3, profactor IX treated with mock-transfected COS cell supernatant; lane 4, profactor IX treated with furin/PACE-transfected COS cell supernatant; lane 5, plasma-derived factor IX. Molecular mass standards are indicated to the right of each panel.

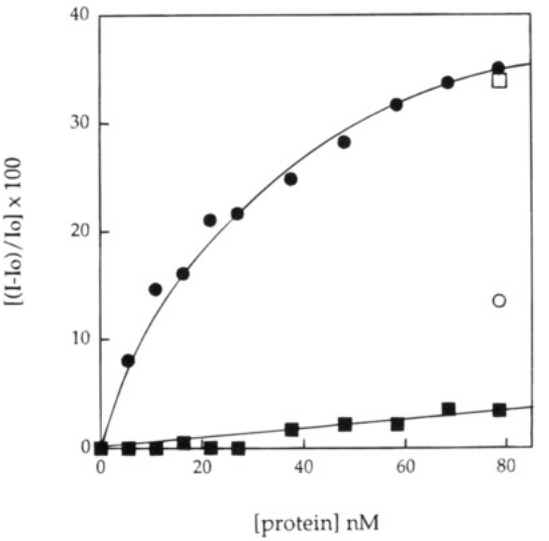

FIGURE 6: Phospholipid binding assay of plasma-derived factor IX and profactor IX. Increments of plasma-derived factor IX (●) or profactor IX (■) were added to a solution containing dansyl-labeled phospholipid vesicles (40% PS/50% PC/10% PE-dansyl) and 2 mM $CaCl_2$. At the saturating concentration of factor IX (80 mM), EDTA was added to a final concentration of 10 mM. The highest concentration of profactor IX was preincubated with furin/PACE to cleave the propeptide as described in Materials and Methods and then assayed for binding to the phospholipid vesicles (□). Binding was reversed by the addition of EDTA (○). Background values of a buffer blank were subtracted, and the data were plotted as the fraction of the fluorescence energy transferred versus the amount of protein added to the cuvette.

Profactor IX does not bind to phospholipid vesicles in the presence of calcium ions, in contrast to plasma-derived factor IX. However, excision of the propeptide from profactor IX with furin/PACE yields factor IX that interacts with vesicles similarly to plasma-derived factor IX. The crystal structure of bovine prothrombin fragment 1 indicates that the amino-terminal alanine is buried in the calcium-stabilized form of the Gla domain; this amino acid forms salt bridges with Gla 17, Gla 21, and Gla 27 (Soriano-Garcia et al., 1992). Furthermore, chemical derivatization of the amino group of alanine 1 of prothrombin interferes with folding of the Gla domain and expression of the lipid binding site (Welsch & Nelsestuen, 1988). Given the similarity of the primary structures of the Gla domains of prothrombin and factor IX, it is reasonable to assume homology of their tertiary structure. The presence of the propeptide would disrupt the formation of an ion pair between the mature amino terminus and the homologous Gla residues in factor IX. Thus, the propeptide extension may preclude the expression of lipid binding properties because it interferes with folding of the amino terminus. This region likely is critical for phospholipid binding. The propeptide may also prevent the intracellular expression of the membrane binding site in fully carboxylated profactor IX. The endoplasmic reticulum and compartments of the Golgi apparatus contain sufficient calcium ion concentrations (>2 mM) (Sambrook, 1991; Ravazzola, 1976) to support calcium ion-dependent membrane binding. The concept that the propeptide plays a protective role early in the intracellular biosynthetic pathway is consistent with studies that show that propeptide cleavage of prothrombin is a trans-Golgi-mediated event (Wallin et al., 1993).

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REFERENCES

Barr, P. J. (1992) *Cell 76*, 1−5.

Bentley, A. K., Rees, D. J. G., Rizza, C., & Brownlee, G. G. (1986) *Cell 45*, 343−348.

Borowski, M., Furie, B., Goldsmith, G. H., & Furie, B. C. (1985) *J. Biol. Chem. 260*, 9258−9264.
Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J., & Thomas, G. (1990) *J. Cell Biol. 111*, 2851−2859.
Bristol, J. A., Furie, B. C., & Furie, B. (1993a) *J. Biol. Chem. 268*, 7577−7584.
Bristol, J. A., Roth, D. A., Furie, B. C., & Furie, B. (1993b) *Blood 82*, 207a.
Carlisle, T. L., & Suttie, J. W. (1980) *Biochemistry 19*, 1161−1167.
Diuguid, D. L., Rabiet, M. J., Furie, B. C., Liebman, H. A., & Furie, B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5803−5807.
Esmon, C. T., Suttie, J. W., & Jackson, C. M. (1975) *J. Biol. Chem. 250*, 4095.
Foster, D. C., Rudinski, M. S., Schach, B. G., Berkner, K. L., Kumar, A. A., Hagen, F. S., Sprecher, C., Insley, M., & Davie, E. W. (1987) *Biochemistry 26*, 7003−7011.
Furie, B., & Furie, B. C. (1988) *Cell 53*, 505−518.
Galeffi, P., & Brownlee, G. G. (1987) *Nucleic Acids Res. 15*, 9505−9513.
Handford, P. A., Winship, P. R., & Brownlee, G. G. (1991) *Protein Eng. 4*, 319−323.
Hubbard, B. R., Jacobs, M., Ulrich, M. M. W., Walsh, C., Furie, B., & Furie, B. C. (1989) *J. Biol. Chem. 264*, 14145−14150.
Huber, P., Schmitz, T., Griffin, J., Jacobs, M., Walsh, C., Furie, B., & Furie, B. C. (1990) *J. Biol. Chem. 265*, 12467−12473.
Jacobs, M., Freedman, S. J., Furie, B. C., & Furie, B. (1994) *J. Biol. Chem. 269*, 25494−25501.
Jones, M. E., Griffin, M. J., Monroe, M. D., Roberts, H. R., & Lentz, B. R. (1985) *Biochemistry 24*, 8064−8069.
Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., & Furie, B. (1987) *Cell 48*, 185−191.
Kaufman, R. J., Wasley, L. C., Furie, B. C., Furie, B., & Shoemaker, C. B. (1986) *J. Biol. Chem. 261*, 9622−9628.
Kotkow, K., Roth, D., Porter, T., Furie, B., & Furie, B. C. (1993) *Methods Enzymol. 25*, 435−449.
Kuwada, M., & Katayama, K. (1983) *Anal. Biochem. 131*, 173−179.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Liddell, M. B., Lillicrap, D. P., Peake, I. R., & Bloom, A. L. (1989) *Brit. J. Haematol. 72*, 208−215.
Liebman, H. A., Limentani, S. A., Furie, B. C., & Furie, B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3879−3883.
Liebman, H. A., Furie, B. C., & Furie, B. (1987) *J. Biol. Chem. 262*, 7605−7612.
Nelsestuen, G. L., Broderius, M., & Martin, G. (1976) *J. Biol. Chem. 251*, 6886.
Pan, L. C., & Price, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6109−6113.
Proctor, R. R., & Rapaport, S. I. (1961) *Am. J. Clin. Pathol. 36*, 212−219.
Ratcliffe, J. V., DiMichelle, D., Jorgensen, M. J., Furie, B., & Furie, B. C. (1991) *Blood 78* (Suppl. 1), 180a.
Ravazzola, M. (1976) *Endocrinology 98*, 950−953.
Sambrook, J. F. (1991) *Cell 61*, 197−199.
Sanford, D. G., Kanagy, C., Sudmeier, J. L., Furie, B. C., Furie, B., & Bachovchin, W. W. (1991) *Biochemistry 30*, 9835−9841.
Schwalbe, R. A., Ryan, J., Stern, D. M., Kisiel, W., Dahlback, B., & Nelsestuen, G. L. (1989) *J. Biol. Chem. 264*, 20288.
Soriano-Garcia, M., Padmanabhan, K., de Vos, A. M., & Tulinsky, A. (1992) *Biochemistry 31*, 2554−2566.
Stanton, C., Taylor, R., & Wallin, R. (1991) *Biochem. J. 277*, 59−65.
Sugimoto, M., Miyata, T., Karabata, S., Yoshioka, A., Fukui, H., & Iwanaga, S. (1989) *Brit. J. Heamatol. 72*, 216−221.
Ulrich, M. M. W., Furie, B., Jacobs, M., Vermeer, C., & Furie, B. C. (1988) *J. Biol. Chem. 263*, 9697−9702.
Wallin, R., Stanton, C., & Hutson, S. M. (1993) *Biochem. J. 291*, 723−727.
Ware, J., Diuguid, D. L., Liebman, H. A., Rabiet, M. J., Kasper, C. K., Furie, B. C., Furie, B., & Stafford, D. W. (1989) *J. Biol. Chem. 264*, 11401−11406.
Wasley, L. C., Rehemtulla, A., Bristol, J. A., & Kaufman, R. J. (1993) *J. Biol. Chem. 268*, 8458−8465.
Welsch, D. J., & Nelsestuen, G. L. (1988) *Biochemistry 27*, 4939−4945.
Wise, R. J., Barr, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J., & Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A 87*, 9378−9382.