Modification of the N-terminus of human factor IX by defective propeptide cleavage or acetylation results in a destabilized calcium-induced conformation: effects on phospholipid binding and activation by factor XIa

Emiel G. C. WOJCIK*, Marieke VAN DEN BERG, Swibertus R. POORT and Rogier M. BERTINA

Hemostasis and Thrombosis Research Centre, Department of Hematology, University Hospital, Building 1: C2-R, P.O. Box 9600, 2300 RC Leiden, The Netherlands

The propeptide of human coagulation factor IX (FIX) directs the γ-carboxylation of the first 12 glutamic acid residues of the mature protein into γ-carboxyglutamic acid (Gla) residues. The propeptide is normally removed before secretion of FIX into the blood. However, mutation of Arg^{-4} in the propeptide abolishes propeptide cleavage and results in circulating profactor IX in the blood. We studied three such genetic variants, factor IX Boxtel ($Arg^{-4} \rightarrow Trp$), factor IX Bendorf ($Arg^{-4} \rightarrow Leu$) and factor IX Seattle C ($Arg^{-4} \rightarrow Gln$). These variant profactor IX molecules bind normally to anti-FIX:Mg(II) antibodies, which indicates that the mutations do not seriously affect γ-carboxylation. Metal ion titration of the binding of variant profactor IX to conformation-specific antibodies demonstrates that the calcium-induced conformation is destabilized in the variant molecules. Also the binding of FIX Boxtel to phospholipids and its activation by factor XIa requires a high (>5 mM) calcium concentration. The three-dimensional structure of the Gla domain of FIX in the presence of calcium indicates that the acylation of the amino-terminus, rather than the presence of the propeptide, was responsible for the destabilization of the calcium-induced conformation. In order to confirm this, the α-amino group of Tyr^1 of FIX was acetylated. This chemically modified FIX showed a similar destabilization of the calcium-induced conformation to variant profactor IX. Our data imply that the amino-terminus of FIX plays an important role in stabilizing the calcium-induced conformation of the Gla domain of FIX. This conformation is important for the binding to phospholipids as well as for the activation by factor XIa. Our results indicate that mutations in FIX that interfere with propeptide cleavage affect the function of the protein mainly by destabilizing the calcium-induced conformation.

INTRODUCTION

Factor IX (FIX) is the zymogen of a serine protease that plays a key role in the blood coagulation cascade [1]. It can be activated to factor IXa by factor XIa and by the factor VIIa–tissue factor complex. In the presence of factor VIIIa, negatively charged phospholipids and Ca^{2+}, factor IXa activates factor X by limited proteolysis [2,3]. FIX is synthesized in hepatocytes, where it undergoes intracellular processing before it is secreted into the blood [4]. These modifications include signal peptide cleavage, N- and O-linked glycosylation, (partial) β-hydroxylation of Asp^{64}, γ-carboxylation and cleavage of the propeptide. The propeptide sequence, which is highly conserved in γ-carboxyglutamic acid (Gla)-containing proteins [5], directs the vitamin K-dependent carboxylase to the γ-carboxylation of the first 12 glutamic acid residues of the mature FIX [6]. The propeptide is removed by an endoprotease located in the trans Golgi apparatus of the secretory pathway [7]. The most likely candidate for this enzyme is PACE (Paired basic Amino acid Cleaving Enzyme [8]). Analysis of genetic propeptide variants [9,10] and *in vitro* expression of recombinant propeptide variants [6,11–13] suggested that residues Leu^{-6}, Arg^{-4}, Lys^{-2} and Arg^{-1} are important for propeptide cleavage and that residues Phe^{-16}, Glu^{-12}, Ala^{-10}, Leu^{-6}, Arg^{-4} and Arg^{-1} are important for γ-carboxylation. On the other hand, analysis of other genetic propeptide variants [14,15] and *in vitro* γ-carboxylation experiments of synthetic and recombinant peptides [16,17] indicated that $Arg^{-4} \rightarrow Gln$ and $Arg^{-1} \rightarrow Ser$ mutations have no effect on the γ-carboxylation efficiency.

Gla residues confer metal ion binding properties to the amino-terminal Gla domain. The binding of metal ions induces a conformational change in FIX, which can be monitored with conformation-specific antibodies [18,19]. Anti-FIX:Mg(II) antibodies recognize a conformational epitope (FIX′) that is non-selective for metal ions, such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Ba^{2+}. Anti-FIX:Ca(II) antibodies recognize a conformational epitope (FIX*) that is only supported by Ca^{2+} and Sr^{2+} ions, and that is involved in phospholipid binding and in activation by factor XIa [19]. The binding sites for both anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies have recently been shown to be located in the Gla domain [20].

Mutations affecting the Arg^{-4} residue in the propeptide are the most frequently reported mutations within the FIX gene. In this study we confirm that the substitution of this residue by Gln (FIX Seattle C), Leu (FIX Bendorf) or Trp (FIX Boxtel) results in defective propeptide cleavage. We also provide support for the notion that these mutations do not affect γ-carboxylation and that the presence of the propeptide destabilizes the calcium-

Abbreviations used: DAB, 3,3′-diaminobenzidine; FIX, factor IX; FIX^{Ci}, factor IX citraconylated in the absence of calcium; $FIX:Ca^{Ci}$, factor IX citraconylated in the presence of calcium; FIX^{Ci}_{Ac}, acetylated FIX^{Ci}; $FIX:Ca^{Ci}_{Ac}$, acetylated $FIX:Ca^{Ci}$; d-FIX^{Ci}, deprotected FIX^{Ci}; d-FIX^{Ci}_{Ac}, deprotected FIX^{Ci}_{Ac}; d-$FIX:Ca^{Ci}_{Ac}$, deprotected $FIX:Ca^{Ci}_{Ac}$; Gla, γ-carboxyglutamic acid; HRP, horseradish peroxidase; TEA, triethanolamine; TNBS, trinitrobenzenesulphonic acid.

* To whom correspondence should be addressed.

induced conformation. Therefore higher calcium concentrations are needed to enable binding to anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies and phospholipids and to enable activation by factor XIa. To determine whether the presence of the propeptide or the acylation of the amino-terminus of FIX was responsible for the destabilization, the α-amino group of Tyr^1 of FIX was acetylated. Our data imply that the amino-terminus of FIX is involved in an intramolecular interaction which is important for the stabilization of the calcium-induced conformation of FIX.

EXPERIMENTAL

Materials

Protein A–Sepharose, CNBr-activated Sepharose 4B, Fast Flow Q Sepharose and PD-10 gel filtration columns were from Pharmacia (Uppsala, Sweden). Goat anti-(rabbit IgG)–horseradish peroxidase (HRP) conjugate and pre-stained molecular mass standards were purchased from Bio-Rad (Richmond, CA, U.S.A.). Human factor XIa was from Enzyme Research Laboratories (Swansea, U.K.). PVDF membrane (Immobilon P) was from Millipore (Bedford, MA, U.S.A.). Non-Ca(II)-dependent antibodies, anti-FIX:Ca(II) and anti-FIX:Mg(II) antibodies against human FIX were isolated from rabbit anti-FIX serum by affinity chromatography on factor IX–Sepharose as previously described [18,19]. Synthetic propeptide (residues -18 to -1 of human FIX) was synthesized on a solid-phase peptide synthesizer, using standard [(9-fluorenylmethyl)-oxy]-carbonyl chemistry (Department of Immuno-Haematology and Blood Bank, University Hospital Leiden, the Netherlands). The peptide was conjugated to rabbit serum albumin by glutaraldehyde and used to immunize a New Zealand White rabbit following standard protocols [21].

Subjects

Blood for the preparation of plasma and white blood cells from patients with haemophilia B and healthy volunteers was obtained with informed consent of the donors. Plasma with FIX Boxtel was obtained from two related patients with moderately severe haemophilia B, who carried the same mutation. Plasma with FIX Bendorf was kindly provided by Dr. M. Ludwig from the Institute of Clinical Biochemistry, University of Bonn, Germany. Plasma with FIX Seattle C was provided by Dr. A. R. Thompson of the Department of Medicine, University of Washington, Seattle, U.S.A.

Identification of the mutation in FIX Boxtel

All of the coding and their flanking regions, the promoter region and the 3′-untranslated region of the FIX gene of the patient with FIX Boxtel were analysed, using PCR and direct sequencing of the amplified regions essentially as described previously [22]. The only modification was an internal 3′-primer (^{6463}CTCGTGCTTCTTCAAAAC^{6446}, numbering according to Yoshitake et al. [23]), that was used for the sequence determination of the fragment containing exons 2 and 3.

Isolation of FIX

FIX Boxtel was isolated from plasma (200 ml) by barium citrate precipitation, ammonium sulphate precipitation, immunoaffinity chromatography and ion exchange chromatography. The procedures for the barium citrate and ammonium sulphate precipitation have been described previously [24]. The 70% ammonium sulphate precipitate was dissolved in a small volume of 50 mM triethanolamine (TEA; pH 7.5), 150 mM NaCl, 10 mM benzamidine and 10 mM $MgCl_2$ and dialysed against the same buffer. The preparation was applied to an anti-FIX:Mg(II) IgG–Sepharose 4B column (1 mg of IgG/ml of gel). Bound FIX was eluted from the column by a buffer containing 50 mM TEA (pH 7.5), 150 mM NaCl, 10 mM benzamidine and 10 mM EDTA. FIX was concentrated by application on Fast Flow Q Sepharose (100 μl) and elution with 1 M NaCl. The protein was dialysed against 50 mM TEA (pH 7.5), 150 mM NaCl. Normal FIX was isolated using the same procedure for small quantities (up to 200 μg) or by previously reported procedures [24] for larger quantities (up to 10 mg). Isolated FIX was more than 95% pure, as judged by SDS/PAGE. Molar concentrations of normal, variant or modified FIX were calculated from the antigen concentration determined in an ELISA using non-Ca(II)-dependent antibodies and a calibrated standard [24].

Profactor IX ELISA

Normal or patient plasma, diluted in 50 mM TEA (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween-20 (ELISA buffer) was incubated for 2 h in microtitre plates, coated with monoclonal antibody 1B5 [25]. Profactor IX was detected with rabbit anti-propeptide antiserum (1:10000 in ELISA buffer). Anti-propeptide antibodies were detected with goat anti-(rabbit IgG)–HRP. The HRP activity was detected with H_2O_2 and 3,3′,4,4′-tetramethylbenzidine as described [26]. All incubations were performed at room temperature. Plates were washed five times with ELISA buffer between incubations.

Binding of FIX to anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies and phospholipids

The ability of normal, variant and modified FIX to bind to anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies or phospholipids was studied in a sandwich ELISA, using anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies or human brain phospholipids [27] respectively to coat microtitre plates. Bound FIX was detected with non-Ca(II)-dependent anti-(factor IX IgG)–HRP conjugate as described [24] and expressed as the A_{450}. The assays were performed in the presence of 20 mM $CaCl_2$. Further procedures were as for the profactor IX ELISA.

Metal ion dependence of binding of FIX to anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies and phospholipids

The metal ion dependence of binding of FIX to anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies and phospholipids was determined as described previously [24]. In short, immobilized anti-FIX:Mg(II) antibodies, anti-FIX:Ca(II) antibodies or human brain phospholipids were incubated with a fixed concentration of FIX in the presence of the indicated metal ion concentrations. Bound FIX was detected with non-Ca(II)-dependent anti-FIX IgG–HRP conjugate. The relative amount of FIX able to bind to the antibodies or the phospholipids was then derived from the A_{450} by interpolation of a dose–response curve for the same FIX preparation at 20 mM $CaCl_2$ or $MgCl_2$.

Activation of FIX by factor XIa

FIX (50 nM) was activated by factor XIa (1.8 nM) in 50 mM TEA (pH 8.0), 150 mM NaCl, 20 mM $CaCl_2$, 100 μg/ml chicken egg ovalbumin, 1 mg/ml polyethylene glycol 8000. At indicated time intervals 25 μl samples were taken and added to an equal volume of SDS/PAGE sample buffer, containing 40 mM EDTA and 50 mM dithiothreitol, and analysed by SDS/PAGE and Western blotting.

SDS/PAGE and Western blotting

The preparation of plasma samples for SDS/PAGE was performed as described previously [28]. SDS/PAGE was performed according to Laemmli [29]. Protein bands were visualized by silver staining according to Morrissey [30] or transferred to PVDF membranes using a semi-dry blotting system (Pharmacia). FIX was then detected either directly with non-Ca(II)-dependent anti-human FIX antibodies, conjugated to HRP or with antiserum raised against the synthetic propeptide of FIX, and goat-anti-(rabbit IgG)–HRP. HRP activity was visualized with 3,3′-diaminobenzidine (DAB) or using a chemiluminescence kit (Boehringer Mannheim, Germany).

Chemical modification of FIX

Amino groups of FIX were reversibly blocked with citraconic anhydride, according to the procedure of Habeeb and Atassi [31], with minor modifications. To 1 ml of isolated normal FIX (0.98 mg/ml) in 0.5 M TEA (pH 8.5) (FIX) or in 0.5 M TEA (pH 8.5)/50 mM $CaCl_2$ (FIX:Ca), citraconic anhydride was added in eight aliquots of 2.5 μl. The pH was adjusted with 1.0 M NaOH after each addition. The solutions were stirred for 30 min. The completion of the reaction was verified by determination of the free amino groups with trinitrobenzenesulphonic acid (TNBS), according to Haynes et al. [32]. The reaction could also be monitored by SDS/PAGE, since the modification resulted in the increase in the apparent molecular mass from 63000 to 80000 Da. For both citraconylated FIX (FIX^{Ci}) and citraconylated FIX:Ca ($FIX:Ca^{Ci}$) the buffer was changed to 0.5 M TEA (pH 8.5) by gel filtration on a PD10 column. Protein was acetylated by the addition of two aliquots of 5 μl of acetic anhydride/ml of protein solution. The pH was adjusted to pH 8.5 after each addition. Deprotection of citraconylated amino groups was accomplished by dialysis against 0.1 M sodium acetate (pH 3.0)/150 mM NaCl. The completion of the deprotection was verified by determination of the free amino groups with TNBS. The deprotection could also be monitored by SDS/PAGE, since the deprotection resulted in a decrease in the apparent molecular mass to 63000 Da. The modified proteins were dialysed against 50 mM TEA (pH 7.5)/150 mM NaCl, and concentrated by ion exchange chromatography over Fast Flow Q Sepharose (see above). For N-terminal sequence analysis, the buffer was exchanged for 0.1 M NH_4HCO_3 by gel filtration over a PD10 column, before freeze-drying.

N-terminal sequence analysis

N-terminal sequence analysis was performed at the gas phase sequenator facility (Department of Medical Biochemistry, State University of Leiden) which is supported by N. W. O. through the Netherlands Foundation for Chemical Research (S. O. N.).

RESULTS

Identification of the point mutation

The only mutation that was observed in the FIX gene of the patient with FIX Boxtel was a C → T transition at residue 6364 (numbering according to Yoshitake et al. [23]). This mutation predicts the change of Arg^{-4} in the propeptide to Trp. The mutations in FIX Bendorf (Arg^{-4} → Leu) and FIX Seattle C (Arg^{-4} → Gln) have been reported previously [33,34].

Isolation of FIX Boxtel

FIX Boxtel was isolated from patient plasma using anti-FIX:Mg(II) antibodies. These antibodies were previously used by Ware et al. [10] to remove normal FIX from a variant FIX (FIX San Dimas) which also had a mutation in the −4 position (Arg^{-4} → Gln). In contrast with the San Dimas variant, FIX Boxtel bound normally to the anti-FIX:Mg(II) antibodies (see below), which could therefore be used for its isolation. SDS/PAGE analysis (Figure 1A) revealed that FIX Boxtel has an increased apparent molecular mass (80000 Da) compared with normal FIX (65000 Da). This indicated that, like in other variants with a point mutation at residue −4 [10,13,15], FIX Boxtel still contained the propeptide. This was confirmed by Western blotting, using an antiserum raised against the synthetic propeptide of FIX (Figure 1B). Only FIX Boxtel did react with anti-propeptide antiserum (lane 5). The presence of the propeptide in FIX Bendorf and FIX Seattle C was confirmed in a sandwich ELISA (Figure 2).

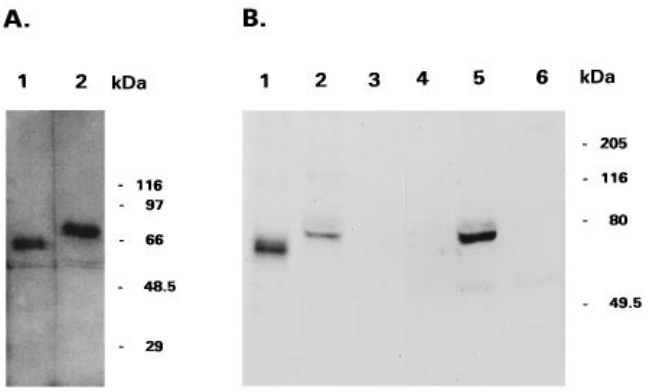


Figure 1 SDS/PAGE analysis of FIX Boxtel

(**A**) 0.1 μg of isolated normal FIX (lane 1) and FIX Boxtel (lane 2) were separated by 5–15% gradient SDS/PAGE and silver stained for protein. (**B**) Barium citrate eluates of normal plasma (lanes 1 and 4), FIX Boxtel plasma (lanes 2 and 5) and congenital FIX-deficient plasma (lanes 3 and 6) were separated by 5–15% gradient SDS/PAGE and blotted to PVDF membranes, which were subsequently incubated with non-Ca(II)-dependent anti-(human FIX)–HRP (lanes 1–3) or anti-propeptide antiserum and goat-anti-(rabbit IgG)–HRP (lanes 4–6). HRP activity was detected by staining with DAB.

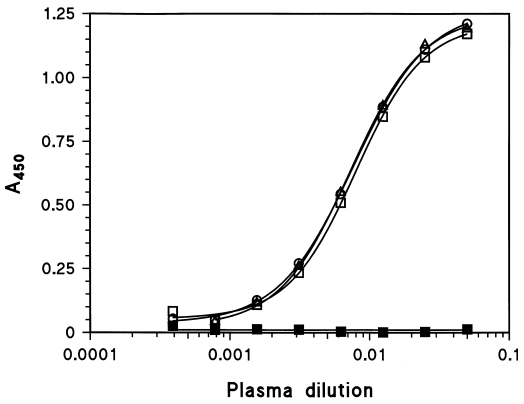
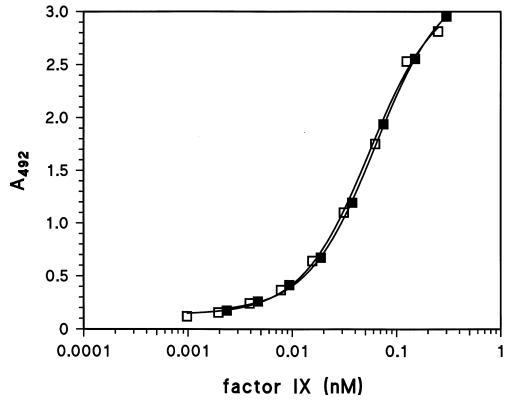


Figure 2 Sandwich ELISA for profactor IX

Normal plasma (■), FIX Boxtel plasma (□), FIX Seattle C plasma (○), or FIX Bendorf plasma (Δ) were incubated at the indicated dilutions with immobilized monoclonal antibody (1B5), directed against human FIX. Bound profactor IX was detected with anti-propeptide antiserum as described in the Experimental section.



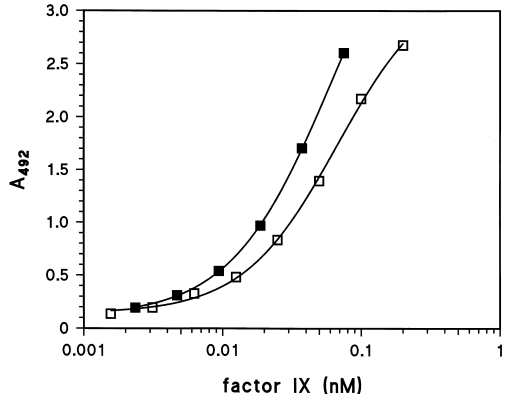
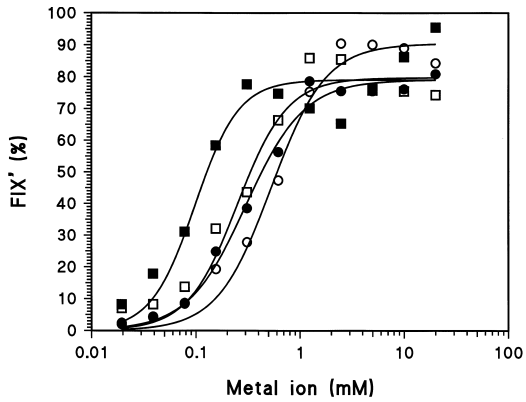


Figure 3 Binding of normal FIX and FIX Boxtel to anti-FIX:Mg(II) (upper panel) and anti-FIX:Ca(II) antibodies (lower panel)

Normal FIX (■) or FIX Boxtel (□) were incubated at the indicated concentrations with immobilized antibodies in the presence of 20 mM $CaCl_2$. Bound FIX was detected as described in the Experimental section.



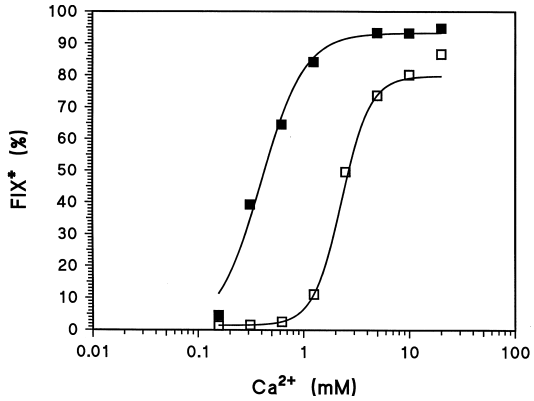


Figure 4 Metal ion dependence of the FIX′ and FIX* conformations of normal FIX and FIX Boxtel

(Upper panel) $CaCl_2$ (■,□) and $MgCl_2$ (●,○) dependence of the binding of normal FIX (310 pM) (■,●) and FIX Boxtel (94 pM) (□,○) to anti-FIX:Mg(II) antibodies. (Lower panel) $CaCl_2$ dependence of the binding of normal FIX (170 pM) (■) and FIX Boxtel (100 pM) (□) to anti-FIX:Ca(II) antibodies. The relative amount of FIX able to bind to anti-FIX:Mg(II) antibodies (i.e. FIX′) or to anti-FIX:Ca(II) antibodies (i.e. FIX*) in the presence of the indicated metal ion concentration was determined as described in the Experimental section.

Binding of FIX Boxtel to anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies

The binding of FIX Boxtel to anti-FIX:Mg(II) antibodies in the presence of excess calcium (20 mM) is identical with that of normal FIX (Figure 3, upper panel). This shows that the $Arg^{-4} \rightarrow Trp$ mutation and the presence of the propeptide do not interfere with the ability of FIX Boxtel to form the FIX′ conformation. However, FIX Boxtel shows a reduced binding to anti-FIX:Ca(II) antibodies (Figure 3, lower panel). It is not evident whether this results from a reduced affinity for the antibodies or that only a fraction of the antibodies (approx. 50 %) is able to bind FIX Boxtel (reduced avidity). The binding of FIX Bendorf and FIX Seattle C to anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies was essentially identical with that of FIX Boxtel (results not shown).

Metal ion dependence of the binding of normal and variant FIX to anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies

In normal FIX, the FIX′ conformation (recognized by anti-FIX:Mg(II) antibodies; Figure 4, upper panel) can be induced by similar concentrations of Ca^{2+} ($EC_{50} = 0.1$ mM) and Mg^{2+} ($EC_{50} = 0.3$ mM). The same is true for FIX Boxtel, although slightly higher metal ion concentrations are required ($EC_{50} =$ 0.3 mM and 0.5 mM for Ca^{2+} and Mg^{2+} respectively). This indicates that the stability of the FIX′ conformation of FIX Boxtel is similar to that of normal FIX. However, the Ca^{2+} dependence of the FIX* conformation [recognized by anti-FIX:Ca(II) antibodies; Figure 4, lower panel] shows that this conformation is much less stable in FIX Boxtel ($EC_{50} = 2.3$ mM) than in normal FIX ($EC_{50} = 0.4$ mM; Figure 4, lower panel).

To determine whether this effect was dependent on the actual amino acid substitution at the -4 position, the same experiment was performed with FIX Bendorf ($Arg^{-4} \rightarrow Leu$) and FIX Seattle C ($Arg^{-4} \rightarrow Gln$). Because of the limited amount of available material, the calcium titration was performed with diluted plasma. For normal FIX, the titration curves of diluted plasma and isolated protein were identical (results not shown), indicating that this procedure was valid and not affected by citrate (50 μM) or calcium (< 6 μM) from the plasma. Figure 5 shows that the Ca^{2+} dependence of the FIX* conformation of both FIX Bendorf and FIX Seattle C is similar to that of FIX Boxtel ($EC_{50} =$ 4.1 mM and 3.7 mM respectively). In FIX Bendorf, the small shoulder in the titration curve is probably due to the presence of a low level of normal FIX, due to the substitution given to the patient 4 days before the blood collection.

Phospholipid binding conformation of normal FIX and FIX Boxtel

At a high (20 mM) Ca^{2+} concentration, the binding of FIX Boxtel to phospholipids is similar to that of normal FIX (Figure

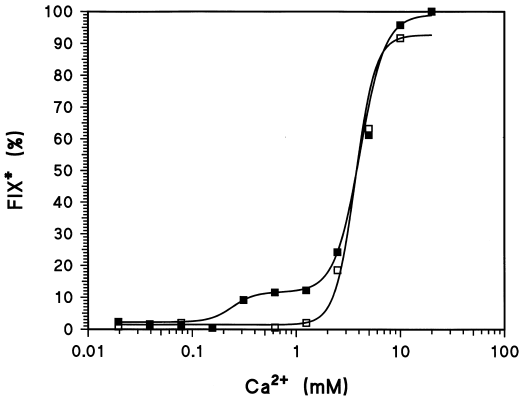


Figure 5 Calcium dependence of the FIX* conformation of FIX Bendorf and FIX Seattle C

The relative amount of FIX Bendorf (■) or FIX Seattle C (□) able to bind to anti-FIX:Ca(II) antibodies (i.e. FIX*) in the presence of the indicated $CaCl_2$ concentration was determined as described in the Experimental section.

6, upper panel). This demonstrates that FIX Boxtel is able to form a phospholipid-binding conformation (FIX^{PL}) and has an apparently similar affinity for phospholipids as normal FIX. However, FIX Boxtel requires a much higher calcium concentration (EC_{50} = 9 mM) to bind to phospholipids than normal FIX (EC_{50} = 3 mM) (Figure 6, lower panel).

Activation of normal FIX and FIX Boxtel by factor XIa

Normal FIX and FIX Boxtel were activated by factor XIa in the presence of 20 mM Ca^{2+} in order to induce the calcium-dependent conformation also in FIX Boxtel. When an enzyme/substrate ratio of 1:30 was used no activation of FIX Boxtel could be observed (Figure 7b), although normal FIX was almost fully activated within 20 min (Figure 7a). At a higher enzyme/substrate ratio (1:10), FIX Boxtel was also activated by factor XIa (Figure 7d). However, activated FIX Boxtel did not react with the anti-propeptide antiserum (results not shown), suggesting that propeptide cleavage had occurred before the activation.

Acetylation of the N-terminal α-amino group of FIX

To acetylate the N-terminal α-amino group of FIX, a two-step modification procedure was used in which only those amino groups were acetylated that were not reactive in the calcium-induced conformation. In the first step, reactive amino groups were modified reversibly with citraconic anhydride in the presence of calcium ($FIX:Ca^{Ci}$). Those amino groups that were not reactive in the presence of calcium were then irreversibly modified with acetic anhydride in the absence of calcium ($FIX:Ca^{Ci}_{Ac}$). For comparison, FIX was also citraconylated in the absence of calcium (FIX^{Ci}) before the modification with acetic anhydride (FIX^{Ci}_{Ac}). The proteins were then deprotected (d-) and subjected to N-terminal sequence analysis (Table 1). FIX that was only citraconylated and then deprotected (d-FIX^{Ci}) was used as a control for the effects of the transient citraconylation The initial yield of the sequence analysis (26 %) of d-FIX^{Ci} was the same as for d-FIX^{Ci}_{Ac} and similar to the initial yield generally obtained for non-modified FIX. This shows that in the absence of calcium the amino-terminus was protected with citraconic acid, which prevented acetylation of the amino-terminus in the next step.

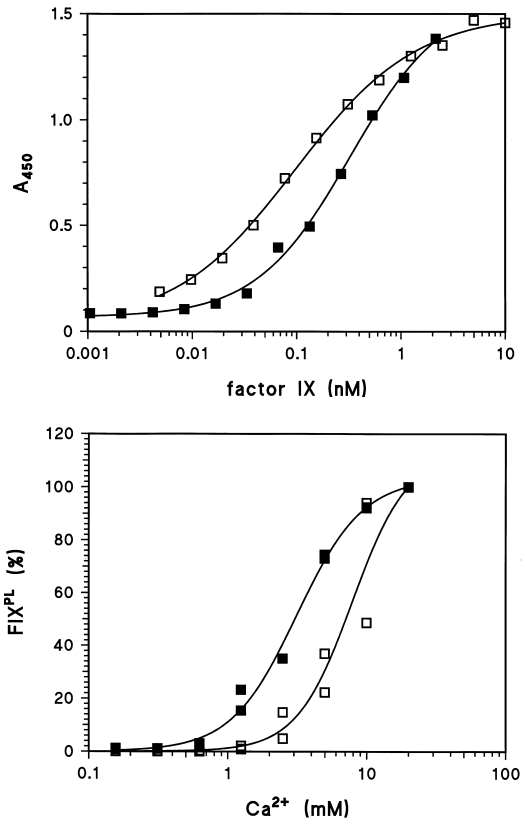


Figure 6 Binding of normal FIX and FIX Boxtel to phospholipids

(Upper panel) Isolated normal FIX (■) and FIX Boxtel (□) were incubated at the indicated concentration with immobilized human brain phospholipids in the presence of 20 mM $CaCl_2$. Bound FIX was detected as described in the Experimental section. (Lower panel) $CaCl_2$ dependence of the binding of normal FIX (2.5 nM) (■) and FIX Boxtel (10 nM) (□) to human brain phospholipids. The relative amount of FIX able to bind to human brain phospholipids (FIX^{PL}) in the presence of the indicated concentration of $CaCl_2$ was determined as described in the Experimental section.

However, the amino-terminus of d-$FIX:Ca^{Ci}_{Ac}$ was blocked, showing that in the presence of calcium the amino-terminus was not modified with citraconic acid, but was acetylated in the next step.

Binding of modified FIX to anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies

d-$FIX:Ca^{Ci}_{Ac}$, d-FIX^{Ci}_{Ac} and d-FIX^{Ci} show similar binding to anti-FIX:Mg(II) antibodies (Figure 8, upper panel). This demonstrates that citraconylation of FIX either in the presence or absence of calcium results in the protection against acetylation of all (if any) amino groups that are involved in the binding to these antibodies. Since in d-$FIX:Ca^{Ci}_{Ac}$ the amino-terminus is acetylated and in d-FIX^{Ci}_{Ac} it is not, the acetylation of the amino-terminus of FIX has no effect on the formation of the FIX′ conformational epitope. Similar to FIX Boxtel, d-$FIX:Ca^{Ci}_{Ac}$ shows a reduced binding to anti-FIX:Ca(II) antibodies, compared with both d-FIX^{Ci}_{Ac} and d-FIX^{Ci} (Figure 8, lower panel). Apparently in d-$FIX:Ca^{Ci}_{Ac}$ one or more amino groups that are involved in the binding to these antibodies are modified. Since this is not true for d-FIX^{Ci}_{Ac}, these residues were protected against modification by citraconic anhydride in the presence of calcium.

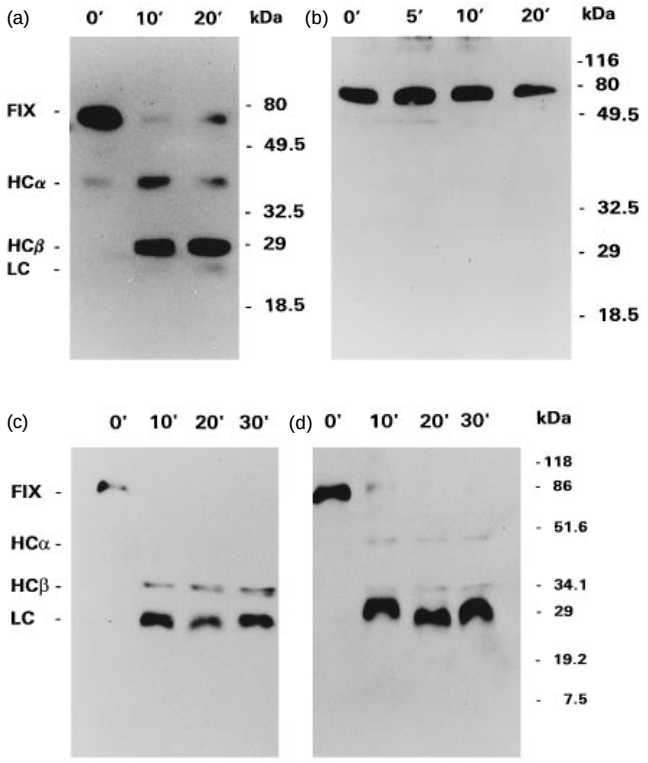


Figure 7 Activation of FIX by factor XIa

50 nM normal FIX (**a**, **c**) and FIX Boxtel (**b**, **d**) were activated by 1.8 nM (**a**, **b**) or 5 nM (**c**, **d**) factor XIa in the presence of 20 mM $CaCl_2$. At the indicated time intervals (min), 10 ng samples were taken, reduced, separated on SDS/12% PAGE gels and transferred to PVDF membranes. FIX was detected with non-Ca(II)-dependent anti-(human FIX) (**a**, **c**, **d**) or anti-propeptide antiserum (**b**). FIX, uncleaved FIX; HCα, heavy chain after cleavage of FIX at Arg^{145}; HCβ, heavy chain after cleavage of FIX at Arg^{180}; LC, light chain of factor IXa.

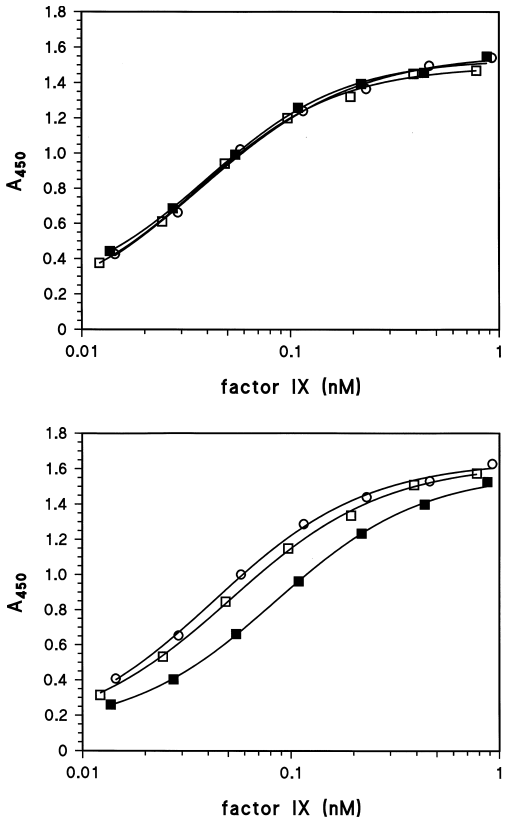


Figure 8 Binding of modified FIX to anti-FIX:Mg(II) antibodies (upper panel) and anti-FIX:Ca(II) antibodies (lower panel)

d-FIX^{Ci} (○), d-FIX^{Ci}_{Ac} (□) or d-FIX:Ca^{Ci}_{Ac} (■) were incubated at the indicated concentrations with immobilized antibodies in the presence of 20 mM $CaCl_2$. Bound FIX was detected as described in the Experimental section.

Table 1 N-terminal sequence analysis of chemically modified factor IX

Analysis was performed on 50 pmol of protein. The yield of the Edman-degradation reactions was determined from the peak height of the phenylthiourea-amino acids in the chromatogram, after subtraction of the background.

Residue	d-FIX^{Ci} yield (pmol)	d-FIX^{Ci}_{Ac} yield (pmol)	d-FIX:Ca^{Ci}_{Ac} yield (pmol)
1 Tyr	13	13	–
2 Asn	5	6	–
3 Ser	–	–	–
4 Gly	7	7	–
5 Lys	4	7	–
6 Leu	5	7	–

Metal ion dependence of the binding of modified FIX to anti-FIX:Mg(II) and anti-FIX:Ca(II) antibodies

The metal ion dependence of the FIX′ conformation of d-FIX^{Ci}_{Ac} (EC_{50} = 0.2 mM for Mg^{2+} and 0.4 mM for Ca^{2+}; Figure 9, upper panel) is similar to that of normal FIX (Figure 4, upper panel), although d-FIX^{Ci}_{Ac} requires somewhat higher Ca^{2+} concentrations than normal FIX. This indicates that the stability of the FIX′ conformation of d-FIX^{Ci}_{Ac} is similar to that of normal FIX. The same is true for the stability of the FIX* conformation of d-FIX^{Ci}_{Ac} (EC_{50} = 0.5 mM; Figure 9, lower panel). The Mg^{2+} dependence of the FIX′ conformation of d-FIX:Ca^{Ci}_{Ac} (EC_{50} = 0.2 mM) is similar to that of normal FIX. The Ca^{2+} dependence of the FIX′ conformation (Figure 9, upper panel) of d-FIX:Ca^{Ci}_{Ac} (EC_{50} = 1.0 mM) is, however, seriously affected. This is also true for the Ca^{2+} dependence of the FIX* conformation (EC_{50} = 2.5 mM; Figure 9, lower panel), which is similar to that of FIX Boxtel (Figure 4, lower panel).

DISCUSSION

FIX Boxtel is a genetic FIX variant with a low specific activity (0.04 unit/ml activity; 0.30 unit/ml of antigen). The proband and his nephew suffered from moderately severe symptoms of haemophilia B. The genetic defect results in a substitution of residue Arg^{-4} in the propeptide to Trp. This mutation, as well as the mutation of this residue to Gln (FIX Seattle C) or Leu (FIX Bendorf), interferes with propeptide cleavage (Figures 1 and 2).

As mentioned in the Introduction, reports are conflicting on whether or not Arg^{-4} is important for γ-carboxylation. The ability to bind to metal-dependent monoclonal [13,15] and polyclonal antibodies [35] can be used as a test for complete γ-carboxylation. Since FIX Boxtel, FIX Bendorf and FIX Seattle C all bind normally to anti-FIX:Mg(II) antibodies, we conclude that the mutation of Arg^{-4} to either Trp, Leu or Gln does not necessarily interfere with γ-carboxylation. The previously observed γ-carboxylation defects in profactor IX variants (FIX

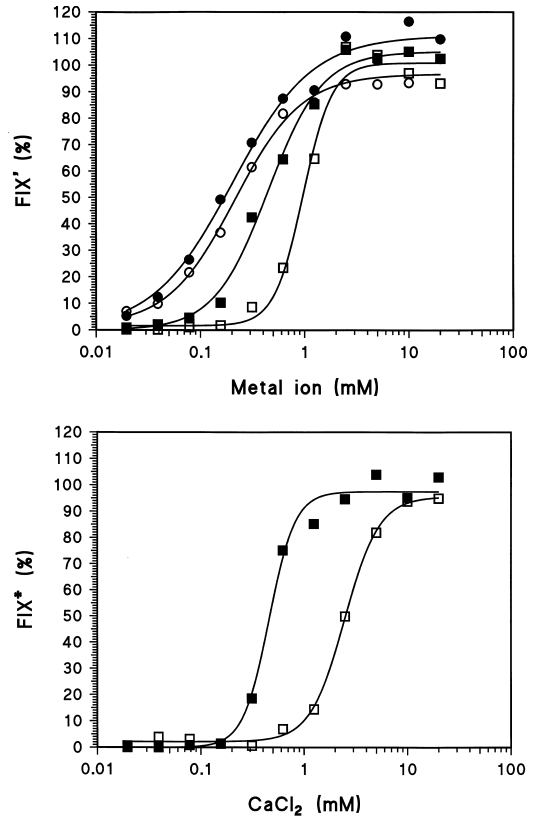


Figure 9 Metal ion dependence of the FIX′ and FIX* conformations of modified FIX

$CaCl_2$ (■,□) and $MgCl_2$ (●,○) dependence of the binding of d-FIX^{Ci}_{Ac} (20 pM) (■,●) and d-$FIX:Ca^{Ci}_{Ac}$ (28 pM) (□,○) to anti-FIX:Mg(II) antibodies (upper panel) or anti-FIX:Ca(II) antibodies (lower panel). The relative amount of FIX able to bind to anti-FIX:Mg(II) antibodies (i.e. FIX′) or to anti-FIX:Ca(II) antibodies (i.e. FIX*) in the presence of the indicated metal ion concentration was determined as described in the Experimental section.

Cambridge [9] and FIX San Dimas [10]) may reflect variations in γ-carboxylation efficiency between individuals or may result from the isolation procedure.

Even when propeptide containing FIX molecules were found to be normally carboxylated, abnormalities relating to calcium binding were observed [14,36,37]. The calcium titration of the binding of variant profactor IX to anti-FIX:Ca(II) antibodies showed that the FIX* conformation is less stable due to the presence of the propeptide [Figures 4 (lower panel) and 5]. Furthermore, the reduced reactivity of FIX Boxtel with anti-FIX:Ca(II) antibodies (Figure 3, lower panel) indicates that the FIX* conformation of FIX Boxtel is non-native-like or that there is steric hindrance from the propeptide.

In profactor IX the phospholipid binding site can still be formed, since we observe apparent normal phospholipid binding of FIX Boxtel. However, FIX Boxtel requires much higher calcium concentrations than normal FIX to bind to phospholipids (Figure 6, lower panel). This explains why Bristol et al. [37] did not observe significant phospholipid binding of normal profactor IX at 2 mM calcium. At physiological calcium concentrations (3 mM), FIX Boxtel will also show no important binding to phospholipids. The similar effect of the presence of the propeptide on the stabilities of the phospholipid binding site and the FIX* conformation supports the notion that these sites are very closely related [19].

Others have previously reported that profactor IX variants could not [37,38] or only partially [36] be activated by factor XIa. However, these studies were performed with either crude FIX and factor XIa preparations, or were performed in the presence of 5 mM calcium, which might not be sufficient to support activation of these variants. We demonstrate that in the presence of a high calcium concentration (20 mM) and a high enzyme/substrate ratio (1:10) FIX Boxtel can be activated by factor XIa, but that the reaction product does not contain the propeptide. At lower enzyme/substrate ratios no activation could be observed. This suggests that factor XIa can not activate (variant) profactor IX directly. However, factor XIa (or a contaminating enzyme in the preparation) can cleave the propeptide of profactor IX, which then is activated normally. This can also explain why some FIX activity (0.04 unit/ml) can still be detected in the patients' plasma, and why the clinical symptoms are only moderately severe.

Activation of FIX by factor XIa occurs via an initial cleavage at Arg^{145}, which is not metal-ion-specific and can be supported by Mg^{2+}. The subsequent cleavage at Arg^{180} is strictly dependent on the presence of calcium. Although the FIX′ conformation that is recognized by anti-FIX:Mg(II) antibodies is fully expressed in FIX Boxtel, we do not observe any cleavage of profactor IX by factor XIa (Figure 7). This indicates that the presence of the FIX′ conformation is not sufficient for cleavage at Arg^{145}. This raises the question of how the conformation of the Gla domain of FIX regulates the activity of factor XIa. It is possible that the formation of the metal-ion-induced conformations of the Gla domain exposes residues Arg^{180} or Arg^{145}. The presence of the propeptide in FIX Boxtel may still obscure these residues by steric hindrance. The removal of the propeptide would then allow the activation of the newly formed normal FIX.

The crystal structure of the calcium-stabilized form of bovine prothrombin fragment 1 shows that the α-amino group of the Ala^1 residue forms hydrogen bonds with Gla^{17}, Gla^{21} and Gla^{27} [39]. The NMR structure of the calcium-bound conformation of the synthetic Gla domain of FIX [40] shows that the α-amino group of Tyr^1 makes similar interactions with Gla^7, Gla^{21} and Gla^{27}. Regarding the high homology between the Gla domains, it is likely that there is also an interaction of the amino-terminus with the homologous Gla residues in other vitamin K-dependent proteins. These interactions would be prevented when the proper amino-terminus was acylated. Indeed, chemical derivatization of the α-amino group of the Ala^1 residue of prothrombin fragment 1 resulted in a decreased affinity for calcium and phospholipids [41–43]. Furthermore, protein C variants in which the amino-terminus is prolonged with only one residue as a result of abnormal propeptide processing, show no immunoreactivity with calcium-dependent monoclonal antibodies [28,44,45]. We demonstrate here that also in FIX in which the amino-terminus is acetylated (d-$FIX:Ca^{Ci}_{Ac}$), the conformations involved in the binding to calcium-dependent antibodies are less stable than in normal FIX. Therefore it is reasonable to assume that also in the propeptide variants the lower stability of the calcium-induced conformation (Figures 4–6) results from the modification of the proper amino-terminus. The similarly reduced reactivity of FIX Boxtel and d-$FIX:Ca^{Ci}_{Ac}$ with anti-FIX:Ca(II) antibodies indicates that the reduced reactivity of FIX Boxtel with anti-FIX:Ca(II) antibodies does not result from steric hindrance by the propeptide. Since in both d-$FIX:Ca^{Ci}_{Ac}$ and FIX Boxtel the N-terminal α-amino group is modified, these data suggest that the N-terminal α-amino group of FIX is involved (directly or indirectly) in the formation of the FIX* conformation.

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REFERENCES

1 Jackson, C. M. and Nemerson, Y. (1980) Annu. Rev. Biochem. **49**, 765–811
2 Furie, B. and Furie, B. C. (1988) Cell **53**, 505–518
3 Mann, K. G., Jenny, R. J. and Krishnaswamy, S. (1988) Annu. Rev. Biochem. **57**, 915–956
4 Furie, B. C. and Furie, B. (1995) Thromb. Haemostasis **74**, 274–277
5 Pan, L. C. and Price, P. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 6109–6113
6 Rabiet, M. J., Jorgensen, M. J., Furie, B. and Furie, B. C. (1987) J. Biol. Chem. **262**, 14895–14898
7 Wallin, R., Stanton, C. and Hutson, S. M. (1993) Biochem. J. **291**, 723–727
8 Wasley, L. C., Rehemtulla, A., Bristol, J. A. and Kaufman, R. J. (1993) J. Biol. Chem. **268**, 8458–8465
9 Diuguid, D. L., Rabiet, M. J., Furie, B. C., Liebman, H. A. and Furie, B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5803–5807
10 Ware, J., Diuguid, D. L., Liebman, H. A., Rabiet, M. J., Kasper, C. K., Furie, B. C., Furie, B. and Stafford, D. W. (1989) J. Biol. Chem. **264**, 11401–11406
11 Galeffi, P. and Brownlee, G. G. (1987) Nucleic Acids Res. **15**, 9505–9513
12 Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B. and Furie, B. (1987) Cell **48**, 185–191
13 Handford, P. A., Winship, P. R. and Brownlee, G. G. (1991) Protein Eng. **4**, 319–323
14 Sugimoto, M., Miyata, T., Kawabata, S., Yoshioka, A., Fukui, H. and Iwanaga, S. (1989) Br. J. Haematol. **72**, 216–221
15 Bentley, A. K., Rees, D. J. G., Rizza, C. and Brownlee, G. G. (1986) Cell **45**, 343–348
16 Hubbard, B. R., Jacobs, M., Ulrich, M. M. W., Walsh, C., Furie, B. and Furie, B. C. (1989) J. Biol. Chem. **264**, 14145–14150
17 Wu, S.-M., Soute, B. A. M., Vermeer, C. and Stafford, D. W. (1990) J. Biol. Chem. **265**, 13124–13129
18 Poort, S. R., van der Linden, I. K., Krommenhoek-van Es, C., Briët, E. and Bertina, R. M. (1986) Thromb. Haemostasis **55**, 122–128
19 Liebman, H. A., Furie, B. C. and Furie, B. (1987) J. Biol. Chem. **262**, 7605–7612
20 Jacobs, M., Freedman, S. J., Furie, B. C. and Furie, B. (1994) J. Biol. Chem. **269**, 25494–25501
21 Harlow, E. and Lane, D. (1988) in Antibodies: A Laboratory Manual (Lane, D., ed.), pp. 53–138, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
22 Poort, S. R., Briët, E., Bertina, R. M. and Reitsma, P. H. (1990) Thromb. Haemostasis **64**, 379–384
23 Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W. and Kurachi, K. (1985) Biochemistry **24**, 3736–3750
24 Wojcik, E. G. C., van den Berg, M., van der Linden, I. K., Poort, S. R., Cupers, R. and Bertina, R. M. (1995) Biochem. J. **311**, 753–759
25 Bertina, R. M., van der Linden, I. K., Muller, H. P., Derks, J. and Klein-Breteler, E. (1981) Thromb. Haemostasis **46**, 165 (Abstract)
26 Gallati, H. and Pracht, I. (1985) J. Clin. Chem. Clin. Biochem. **23**, 453–460
27 Van den Besselaar, A. M. H. P., Ram, I. E., Alderkamp, G. H. J. and Bertina, R. M. (1982) Thromb. Haemostasis **48**, 54–58
28 Wojcik, E. G. C., Simioni, P., Berg, M. V. D., Girolami, A. and Bertina, R. M. (1996) Thromb. Haemostasis **75**, 70–75
29 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
30 Morrissey, J. H. (1981) Anal. Biochem. **117**, 310
31 Habeeb, A. F. S. A. and Atassi, M. Z. (1970) Biochemistry **9**, 4939–4944
32 Haynes, R., Osuga, D. T. and Feeney, R. E. (1967) Biochemistry **6**, 541–547
33 Knobloch, O., Zoll, B., Zerres, K., Brackmann, H.-H., Olek, K. and Ludwig, M. (1993) Hum. Genet. **92**, 40–48
34 Thompson, A. R., Schoof, J. M., Weinmann, A. F. and Chen, S.-H. (1992) Thromb. Res. **65**, 289–295
35 Kotkow, K. J., Roth, D. A., Porter, T. J., Furie, B. C. and Furie, B. (1993) Methods Enzymol. **222**, 435–449
36 Liddell, M. B., Lillicrap, D. P., Peake, I. R. and Bloom, A. L. (1989) Br. J. Haematol. **72**, 208–215
37 Bristol, J. A., Freedman, S. J., Furie, B. C. and Furie, B. (1994) Biochemistry **33**, 14136–14143
38 Pabinger, I. and Schneider, B. (1996) Arterioscler. Thromb. Vasc. Biol. **16**, 742–748
39 Soriano-Garcia, M., Park, C. H., Tulinsky, A., Ravichandran, K. G. and Skrzypczak-Jankun, E. (1989) Biochemistry **28**, 6805–6810
40 Freedman, S. J., Furie, B. C., Furie, B. and Baleja, J. D. (1995) Biochemistry **34**, 12126–12137
41 Welsch, D. J., Pletcher, C. H. and Nelsestuen, G. L. (1988) Biochemistry **27**, 4933–4938
42 Welsch, D. J. and Nelsestuen, G. L. (1988) Biochemistry **27**, 4939–4945
43 Weber, D. J., Berkowitz, P., Panek, M. G., Huh, N.-W., Pedersen, L. G. and Hiskey, R. G. (1992) J. Biol. Chem. **267**, 4564–4569
44 Gaussem, P., Gandrille, S., Duchemin, J., Emmerich, J., Alhenc-Gelas, M., Aillaud, M.-F. and Aiach, M. (1994) Thromb. Haemostasis **71**, 748–754
45 Miyata, T., Zheng, Y. Z., Sakata, T. and Kato, H. (1995) Thromb. Haemostasis **74**, 1003–1008

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