Biochem. J. (1990) 265, 219–225 (Printed in Great Britain)

Replacement of isoleucine-397 by threonine in the clotting proteinase Factor IXa (Los Angeles and Long Beach variants) affects macromolecular catalysis but not L-tosylarginine methyl ester hydrolysis

Lack of correlation between the ox brain prothrombin time and the mutation site in the variant proteins

Silvia G. SPITZER,* Bonnie J. WARN-CRAMER,*‡ Carol K. KASPER† and S. Paul BAJAJ*§

*Departments of Medicine, Pathology and Biochemistry, St. Louis University School of Medicine, St. Louis, MO 63104, and †Orthopaedic Hospital, University of Southern California, Los Angeles, CA 90007, U.S.A.

Previously, from the plasma of unrelated haemophilia-B patients, we isolated two non-functional Factor IX variants, namely Los Angeles (IX_{LA}) and Long Beach (IX_{LB}). Both variants could be cleaved to yield Factor IXa-like molecules, but were defective in catalysing the cleavage of Factor X (macromolecular substrate) and in binding to antithrombin III (macromolecular inhibitor). In the present study we have identified the mutation of IX_{LA} by amplifying the exons (including flanking regions) as well as the 5′ end of the gene by polymerase-chain-reaction (PCR) method and sequencing the amplified DNA by the dideoxy chain-termination method. Comparison of the normal IX and IX_{LA} sequences revealed only one base substitution (T → C) in exon VIII of IX_{LA}, with a predicted replacement of Ile-397 to Thr in the mature protein. This mutation is the same as found recently for IX_{LB}. The observation that IX_{LB} and IX_{LA} have the same mutation is an unexpected finding, since, on the basis of their ox brain prothrombin time (PT, a test that measures the ability of the variant Factor IX molecules to inhibit the activation of Factor X by Factor VIIa–tissue factor complex), these variants have been classified into two different groups and were thought to be genetically different. Our observation thus suggests that the ox brain PT does not reflect the locus of mutation in the coding region of the variant molecules. However, our analysis suggests that the ox brain PT is related to Factor IX antigen concentration in the patient's plasma. Importantly, although the mutation in IX_{LA} or IX_{LB} protein is in the catalytic domain, purified IXa_{LA} and IXa_{LB} hydrolyse *L*-tosylarginine methyl ester at rates very similar to that of normal IXa. These data, in conjunction with our recent data on Factor $IX_{Bm\,Lake\,Elsinore}$ (Ala-390 → Val mutant), strengthen a conclusion that the peptide region containing residues 390–397 of normal Factor IXa plays an essential role in macromolecular substrate catalysis and inhibitor binding. However, the two mutations noted thus far in this region do not distort S_1 binding site in the Factor IXa enzyme.

INTRODUCTION

Factor IX is a vitamin K-dependent protein involved in haemostasis. It can be activated by Factor XIa, requiring Ca^{2+}, and by Factor VIIa, requiring tissue factor (TF) and Ca^{2+} [1–3]. The Factor IXa formed then activates Factor X in a reaction requiring Ca^{2+}, phospholipid (PL) and Factor VIIIa [4]. Further, TF–VIIa complex also activates Factor X [4]. Thus, during physiological clotting, Factors IX and X are thought to compete for TF–VIIa sites [5–7]. Additionally, Factor IXa binds antithrombin III, hydrolyses arginine methyl ester (Tos-Arg-OMe, 'TAME'), and activates factor VII [1,8,9].

Human Factor IX is synthesized as a precursor molecule of 461 residues [10–12]. The first 46 residues contain the hydrophobic signal and the hydrophilic propeptide sequence. After removal of the signal peptide the protein undergoes several post-translational modifications, including glycosylation, γ-carboxylation (of the first 12 glutamic acid residues), β-hydroxylation (of Asp-64) and propeptide liberation [13]. Upon activation of Factor IX (415 residues) two bonds are cleaved, which results in the formation of a disulphide-linked two-chain Factor IXa and removal of a 35-residue activation peptide [1,3,12]. The light chain (residues 1–145) contains the 4-carboxyglutamic acid (Gla) domain, which promotes Ca^{2+} and PL binding, and the heavy chain (residues 181–415) contains the serine-proteinase domain which promotes substrate

Abbreviations used: IX_{BmLE}, IX_{ER}, IX_{LA} and IX_{LB}, Factor IX variants Bm Lake Elsinore, Eagle Rock, Los Angeles and Long Beach respectively; PCR, polymerase chain reaction; PT, prothrombin time; TF, tissue factor; Tos-Arg-OMe, L-tosylarginine methyl ester ('TAME'); PL, phospholipid; Gla, 4-carboxyglutamic acid.

‡ Present address: Department of Medicine, University of California at San Diego, H-811K, San Diego, CA 92103, U.S.A.

§ To whom correspondence should be sent, at the following address: Division of Hematology, St. Louis University Medical Center, 3635 Vista Avenue, P.O. Box 15250, St. Louis, MO 63110-0250, U.S.A.

binding and catalysis [4,14]. Portions of both light and heavy chains of factor IXa may be involved in binding to Factor VIIIa [15,16].

Most haemophilia-B patients contain undetectable Factor IX antigen in their blood; however, 10–30% of the patients contain abnormal variants of Factor IX with significantly reduced biological acitivity [17–19]. The patients with abnormal molecules are classified into three groups on the basis of their plasma ox brain prothrombin time (PT). Group I patients have markedly prolonged ox brain PT (as compared with that obtained with normal plasma), Group II patients have moderately prolonged PT, and Group III patients have normal PT. On the basis of the PT test (see the Discussion section), it has been proposed that the abnormal variants in each group are structurally different [18,19].

In our efforts to understand structure–function relationships in Factor IX, we initially described three abnormal variants defective in the catalytic domain [20]: one with a markedly prolonged PT, Factor $IX_{Bm\ Lake\ Elsinore}$ (IX_{BmLE}); one with a moderately prolonged PT, Factor $IX_{Long\ Beach}$ (IX_{LB}); and one with a normal PT, Factor $IX_{Los\ Angeles}$ (IX_{LA}). Recently, by using standard DNA cloning protocols, it was found that the mutation in IX_{BmLE} is at residue 390 (Ala → Val) [9] and in IX_{LB} is at residue 397 (Ile → Thr) [21]. In the present study we have employed the polymerase-chain-reaction (PCR) method [22] to identify the mutation in IX_{LA} (we have also verified the mutation in IX_{LB} by this method). Interestingly, the mutation in IX_{LA} was found to be the same as in IX_{LB}. This was somewhat unexpected, since, on the basis of the ox brain PT, these variant proteins had been thought to be structurally different [18,19]. Thus other factors must be considered in interpreting the PT-test results. In the present paper we provide evidence that the ox brain PT is directly related to the concentration of Factor IX in the patient's plasma.

More importantly, although the mutation in IX_{LA} or IX_{LB} is in the catalytic domain, our kinetic studies reveal that purified IXa_{LA} and IXa_{LB} hydrolyse Tos-Arg-OMe at rates similar to that obtained with normal IXa. These results, coupled with out previous results [9,20] strongly indicate that the peptide region of normal IXa, containing residues 390–397 (which is linearly distant from the active-site serine residue at position 365), plays an essential role in macromolecular substrate catalysis and inhibitor binding.

METHODS

Coagulation studies

The ox brain PT was determined at 37 °C as follows: to 0.1 ml of test plasma, 0.1 ml of ox brain TF and 0.1 ml of 0.02 M-$CaCl_2$ was added, and the clot time recorded from the point of addition of $CaCl_2$. The mean ox brain PT obtained in 50 normal males was 41 s, with an S.D. of 4.3 s. The TF was prepared from bovine brain as described by Owren [23]. Assays for Factor VII, IX and X activity were performed as described previously [19]. Factor IX antigen levels were determined by an antibody neutralization assay and also by an electro-immunoassay performed using a rabbit antibody prepared against Factor IX [19]. Use of volunteer blood donors was approved by the Human Subjects Committee of St. Louis University and of the University of Southern California.

Proteins

Normal human Factor IX, IX_{LA} and IX_{LB} were purified as described previously [20,24]. Human Factor XI was purified by the method of Kurachi & Davie [25], and Factor XIa was prepared as described previously [26]. Protein concentrations were determined by using an $A_{280}^{1\%}$ value of 13.4 for Factor XI [25] and 13.2 for normal Factor IX [1]. An $A_{280}^{1\%}$ value of 13.2 was also used for IX_{LA} and IX_{LB}, since they contain the same number of tyrosine, tryptophan and phenylalanine residues.

Esterase assays for Factor IXa

Esterase assays for normal Factor IXa, IXa_{LA} and IXa_{LB} were performed as described by Roffman *et al.*

Table 1. Oligodeoxynucleotide amplification primers used in PCR

Primer 1a contains the restriction-endonuclease-*Bam*HI site, primer 1b contains a *Taq*I site, primers 2, 4, 6 and 7 contain *Eco*RI sites, primer 3 contains *Pst*I sites, primer 5 contains an *Hpa*II site, and primer 8 contains an *Xba*I site. The restriction site sequences are underlined. The primers used for amplifying exons VI, VII and VIII were listed previously [9]. The sequences in parentheses represent sequences complementary to different regions of Factor IX gene [12]. primers 1a and 2 were used to amplify and sequence the 5′-end of the gene containing the putative promoter region [33], and primers 1b and 2 were used to amplify and sequence exon I segment of the gene [12].

	Primer	
Exon	No.	Sequence
I	1a	5′-ATGGATCCTC(GATGAACTGTGCTGCCACAG)
	1b	5′-(GGTACAACTAATCGACCTTACCACT)
	2	5′-GGACGAATTCGT(AAGGCAAGCATACTCAATGT)
II–III	3	5′-GCCTGCAGC(GATGTAAAATTTTCATGATGT)
	4	5′-AGGAATTCGC(ATGTTTCATATATTAGCTAG)
IV	5	5′-(CAGGGGAGGACCGGGCATTCTAAGC)
	6	5′-AGGAATTCGC(TTTCAACTTGTTTCAGAGGG)
V	7	5′-CGGAATTCGC(TCAGTAGTTCCATGTACTTT)
	8	5′-(ATGATGCAATCTAGAAAATTAATTGG)

[27], using [^{3}H] Tos-Arg-OMe obtained from Amersham International. Factor IXa preparations were made as outlined [20] and evaluated by SDS/polyacrylamide-gel-electrophoretic analysis [28]. Results revealed complete activation of Factor IX to Factor IXa. Details of the esterase activity assays are given in the legend to Fig. 2.

Primer-directed target DNA amplification

Genomic DNA was extracted from blood as outlined by Kan *et al.* [29]. The primers used to amplify Factor IX exons I–V (and their flanking regions) are listed in Table 1. The primers used for amplification of exons VI, VII and VIII were given previously [9]. The target sequences were amplified essentially as described by Saiki *et al.* [22]. Our particular procedure for DNA denaturation, primer annealing and DNA synthesis, using the *Taq* polymerase enzyme (Cetus Corporation, Emeryville, CA, U.S.A.), is outlined in a previous paper [9]. After amplification, DNA was recovered by precipitation with ethanol and electrophoresed on 1.5% low-melting-temperature agarose gel [30]. The amplified segment of the DNA was extracted from the gel as outlined in [30], digested with the specific restriction endonucleases, and cloned into M13 vector for sequencing.

DNA sequence analysis

Restriction fragments containing exons and flanking intron regions were cloned into M13mp18 or mp19 vectors [31], and sequences were determined by the dideoxy chain-termination method [32]. In addition to the 17-mer universal primer, several oligonucleotide (20-mer) primers were designed and used to sequence several of the exons.

RESULTS

All coding and the flanking regions (including the putative promoter region) of normal Factor IX and IX_{LA} gene were amplified *in vitro* by using the primers given in Table 1 for exons I–V and provided in a previous paper [9] for exons VI–VIII. The amplified DNA was then cloned in M13 vector and sequenced by the chain-termination method [32]. Sequence information was obtained on both strands of DNA, and at least two independent clones were sequenced for each strand. Perfect agreement was obtained between our restricted sequence data for the normal factor IX gene and the one reported by Yoshitake *et al.* [12]. However, two differences were noted in the sequence of exon regions of the IX_{LA} gene. One difference was in exon VI. Here, guanine at position 20416 [12] was replaced by adenine. This substitution changes the codon (GCT → ACT) for the amino acid alanine-148 to threonine. Since this change represents a natural variation in the population and is not deleterious [34], it was considered not to be the cause for failure of IX_{LA} to function in haemostasis. The second difference was noted in the sequence for exon VIII. Here, thymine at position 31311 [12] was replaced by cytosine. This substitution changes the codon (ATA → ACA) for the amino acid isoleucine-397 to threonine (Fig. 1). No other changes in the exon sequences of IX_{LB} gene were found. We conclude that factor IX_{LA} fails to function normally because of replacement of isoleucine 397 by threonine in an otherwise normal protein.

The mutation responsible for the molecular defect in IX_{LA} is the same as noted recently for IX_{LB} [21] by the

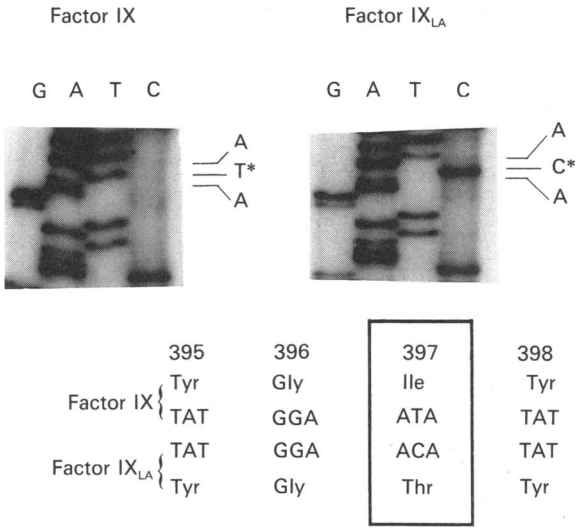


Fig. 1. Identification of the mutation in factor IX_{LA}

Partial nucleotide sequence of the coding strand of exon VIII of the normal gene is shown on the left and that of the LA gene is shown on the right. The mutation (T → C) is highlighted by asterisks. As a result of this substitution (T → C) in the second nucleotide of the codon for amino acid 397, isoleucine in normal Factor IX will be replaced by threonine in Factor IX_{LA}. This is identified by the box at the bottom of the Figure.

conventional cloning and sequencing protocols [30]. By using the PCR amplification technique, we also verified the mutation in IX_{LB}. The sequence data for the coding regions of IX_{LA} and IX_{LB} gene suggest that the catalytic triad (Asp^{269}-His^{221}-Ser^{365}) and the S_1 binding site (aspartic acid-359) may be functional in these molecules. This was tested by studying the rates of hydrolysis of a small synthetic substrate, Tos-Arg-OMe, by purified normal IXa, IXa_{LA} and IXa_{LB}. {We are aware that Tos-Arg-OMe is a poor substrate for Factor IXa. However, since Tos-Arg-OMe will only interact through its positively charged arginine side chain with the negatively charged side chain aspartic acid-359 (S_1 binding site) of Factor IXa, we reasoned that this is a suitable substrate to evaluate whether or not the catalytic triad and S_1 binding site in a variant IXa molecule is functional. Moreover, the availability of [^{3}H]Tos-Arg-OMe and use of a sensitive assay [27] allow one to reduce the background counts essentially to zero.} These data are presented in Fig. 2. At Factor IXa concentrations of 124 μg/ml, the specific rate (Factor IXa minus Factor IX) of hydrolysis of [^{3}H]Tos-Arg-OMe as calculated by the technique of Roffman *et al.* [27] was 3.5 μM/h for normal factor IXa, 4.2 μM/h for Factor IXa_{LB} and 3.6 μM/h for Factor IXa_{LA}. At Factor IXa concentrations of 57 μg/ml, it was 2.2 μM/h for normal Factor IXa, 2.0 μM/h for Factor IXa_{LB} and 1.7 μM/h for Factor IXa_{LA}. Furthermore, using several concentrations of normal factor IXa ranging from 20 to 100 μg/ml, we found that the rates obtained by this method contain a maximum error of $\pm$15%, which we believe is an acceptable error for purposes of these experiments. Moreover, neither unactivated Factor IX nor di-isopropyl fluorophosphate-inhibited XIa hydrolysed Tos-Arg-OMe to an appreciable extent (Fig. 2). Thus the data presented in Fig. 2 indicate that factor IXa_{LA} and factor IXa_{LB} hydrolyse

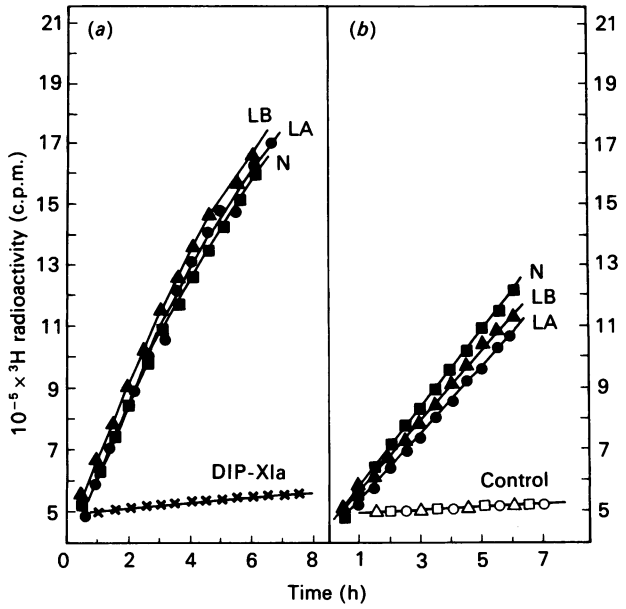


Fig. 2. Esterase activity of normal Factor IXa, IXa_{LA} and IXa_{LB} using [^{3}H]Tos-Arg-OMe substrate

Purified Factor IX preparations [normal IX (N), IX_{LA} (LA) or IX_{LB} (LB)] at concentrations ranging from 600 to 700 μg/ml were incubated for 1 h at room temperature with Factor XIa at an enzyme/substrate ratio of 1:50 (w/w) in 0.05 M-Tris/0.15 M-NaCl buffer, pH 7.5, containing 5 mM-Ca^{2+}. Factor IX was completely activated under these conditions, as evaluated by SDS/polyacrylamide-gel electrophoretic analysis. After incubation for 1 h at room temperature, each sample was made 6 mM in EDTA and 5 mM in di-isopropyl fluorophosphate and further incubated for 1 h at 4 °C. This treatment inactivates Factor XIa without affecting Factor IXa [1,20]. Samples were diluted in the above-mentioned Tris/NaCl buffer and assayed for [^{3}H]Tos-Arg-OMe esterase activity as described by Roffman *et al.* [27]. Specifically, 100 μl of each sample containing 180 μM-[^{3}H]Tos-Arg-OMe (specific radioactivity 0.5 Ci/mol) was added to 10 ml of Omnifluor/toluene scintillation mixture in a glass scintillation vial and counted for ^{3}H radioactivity after every 30 min as described in [27]. The increase in ^{3}H radioactivity, a measure of release of [^{3}H]-methanol, is presented as a function of time. In (*a*), the concentration of Factor IXa in the assay mixture was 124 μg/ml and in (*b*) it was 57 μg/ml. For both (*a*) and (*b*), closed squares (■) represent normal IXa, closed triangles (▲) represent IXa_{LB}, and closed circles (●) represent IXa_{LA}. In (*a*), crosses (×) represent hydrolysis by di-isopropyl fluorophosphate-inhibited Factor XIa (DIP-XIa) at 2.5 μg/ml (this is the concentration of inactive Factor XIa present in experiments containing 124 μg of Factor IXa/ml). In (*b*) open squares (□) represent (corrected for buffer control) unactivated normal IX, open triangles (△) represent unactivated IX_{LB}, and open circles (○) represent unactivated IX_{LA}, each at 57 μg/ml.

Tos-Arg-OMe at rates very similar (77 and 103 % in the case of IXa_{LA}, and 90 and 120 % in the case of IXa_{LB}) to those observed with normal Factor IXa. Thus the catalytic triad and the S_1 binding site appear to be normal in these molecules. Furthermore, these data in conjunction with our previous data [9,20] indicate that the COOH segment of normal factor IXa containing residues 390–397 plays an important role in macromolecular substrate binding/catalysis.

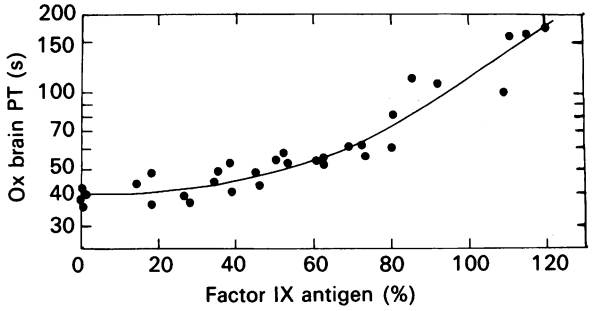


Fig. 3. Dependence of ox brain PT on Factor IX antigen in the patient's plasma

The PT (in s) was plotted on a log scale as a function of Factor IX concentration (expressed as a percentage of that present in normal pooled plasma of 20 normal donors) in the patient's plasma. See the Methods section for determination of the ox brain PT and Factor IX antigen levels.

Since the finding that factors IX_{LA} and IX_{LB} have the same molecular defect in the coding region of the gene is contrary to what had been thought previously (see the Introduction as well as Discussion section), we investigated other factors (in addition to the variant Factor IX molecules) which could contribute to the prolongation of ox brain PT. We measured levels of Factor IX antigen and activities of Factors X and VII in the plasma samples of four haemophilia-B patients studied thus far in our laboratory. {Levels of Factors IX, X and VII were measured because of their participation in the TF pathway of coagulation and because blood clotting is initiated by this pathway during the ox brain PT test (see the Discussion). Moreover, low activities of Factors X and VII observed in patients with haemophilia-B (Table 2) are consistent with previous reports [19,35].} These data along with those for the point mutation and the ox brain PT are provided in Table 2. From the data presented in Table 2, it is clear that there is no correlation between the ox brain PT and the mutation site in the variant molecules. In contrast, it appears that the ox brain PT may be related to the levels of Factor IX in the patient's plasma.

In order to examine further whether or not the antigen levels of Factor IX in the patient's plasma influence the ox brain PT, we determined ox brain PT and Factor IX antigen in several patients with less than 1 % Factor IX activity. These data are presented in Fig. 3. From these data it is apparent that there is a reasonable correlation between the ox brain PT and the levels of factor IX antigen in the test plasma. This observation may be readily understood in terms of competition between the two substrates, Factors IX and X, for the same enzymic complex, TF–VIIa (see the Discussion and Scheme 1).

DISCUSSION

Recent studies of a number of variant molecules present in haemophilia-B patients have enhanced our understanding of the structure–function relationships in Factor IX. A point mutation at residue 145 (Arg → His) precludes cleavage of the Arg^{145}–Ala bond and release of

Table 2. Lack of correlation between the ox brain PT and the mutation site in Factor IX variants

Ox brain PT, Factor IX antigen and activity, and Factor X and VII activity levels were determined as described in the Methods section. Factor levels are expressed as percentages of that present in normal pooled plasma of 20 healthy individuals.

Mutant*	Ox brain PT† (s)	Factor IX‡		Factor levels in the variant plasma samples	
		Activity	Antigen	X	VII
1. IX_{LA} ($Ile^{397} \rightarrow Thr$)	44	< 1	32	60	63
2. IX_{LB} ($Ile^{397} \rightarrow Thr$)	60	< 1	79	37	49
3. IX_{ER} ($Gly^{363} \rightarrow Val$)	60	< 1	68	46	54
4. IX_{BmLE} ($Ala^{390} \rightarrow Val$)	161	< 1	135	48	64

* Mutant 1 (the present study); mutant 2 (the present study; [21]); mutant 3 (S. G. Spitzer & S. P. Bajaj, unpublished work); mutant 4 [9].

† The ox brain PT of normal plasma is 41 s (see the Methods section).

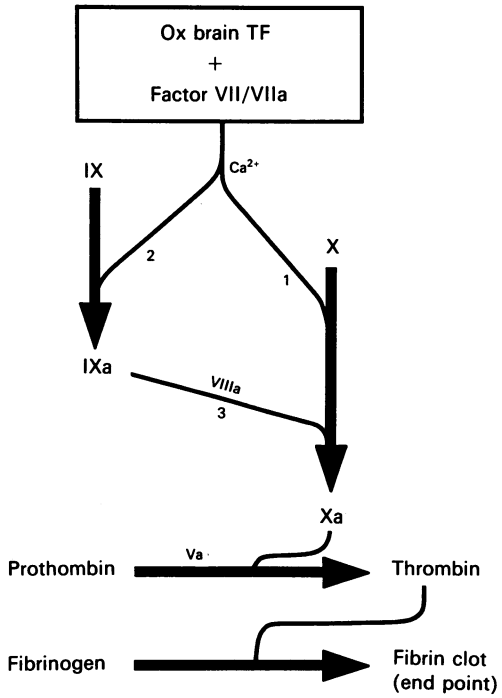
‡ Antigen levels for Factor IX, and activity levels for Factors X and VII, are means of triplicate determinations.

the activation peptide [36]. Since this variant molecule has substantially decreased coagulant activity, it demonstrates that both Arg^{145}–Ala and Arg^{180}–Val bonds must be cleaved in the normal Factor IX zymogen for full expression of biological activity. A point mutation at residue 47 (Asp → Gly) also results in a defective molecule [37]. Although it has not been fully investigated, this variant lacks biological activity, probably because it cannot bind to membrane surfaces [37]. A point mutation either at residue −1 (Arg → Ser) or at residue −4 (Arg → Gln) precludes cleavage of the hydrophilic propeptide and apparently interferes with γ-carboxylation [38,39]. The above mutations are in the non-catalytic domain of Factor IX and illustrate the importance of regions involved in activation of zymogen, in IX–IXa interaction with membrane surfaces, and in post-translational carboxylation.

Our studies have been focused upon the mutations in the catalytic domain of Factor IXa. Towards this end we recently described a variant (BmLE) in which alanine-390 is replaced by valine [9]. In the present paper we describe another variant molecule (LA) in which isoleucine-397 is replaced by threonine. We have previously shown that activated $Ala^{390} \rightarrow Val$ variant (BmLE) has virtually no activity (less than 1 % of that of normal IXa), and the activated $Ile^{397} \rightarrow Thr$ variant (LA or LB) has substantially reduced activity (approx. 5 % of that of normal IXa) in binding to antithrombin III (plus heparin) and in activating Factor VII (plus Ca^{2+} and PL), or Factor X (plus Ca^{2+} and PL) ± Factor VIIIa [20]. Our previous studies also indicate that these variants have normal Ca^{2+}-, PL- and Factor VIIIa-binding characteristics [20]. Moreover, in a recent study [9] we demonstrated that the cleaved $Ala^{390} \rightarrow Val$ variant hydrolyses Tos-Arg-OMe at a rate very similar to that observed for activated normal Factor IX. In the present study we have found that the cleaved $Ile^{397} \rightarrow Thr$ variant (LA or LB) also hydrolyses Tos-Arg-OMe at rates indistinguishable from that of normal Factor IXa. Thus, taken together, these data strongly support a conclusion that both the charge-relay system and the S_1 binding site are normal in the $Ala^{390} \rightarrow Val$ variant [9] and the $Ile^{397} \rightarrow Thr$ variant (LA or LB; the present study). Our conclusion is further supported by a very recent report that the primary defect in the $Ile^{397} \rightarrow Thr$ variant is in the S_3 binding site [40].

The observation that IX_{LA} and IX_{LB} proteins have the same molecular defect prompted us to examine other variables influencing the ox brain PT test. In the PT test, coagulation is initiated by the TF pathway (Scheme 1). In normal plasma the concentration of Factor X ($\simeq 8$ μg/ml or $\simeq 130$ μM) is roughly 2-fold that of Factor IX ($\simeq 4$ μg/ml or $\simeq 70$ μM). Moreover, the K_m values for the activation of Factor X and Factor IX are essentially the same [5–7]. Thus, in normal plasma, two-thirds of TF–VIIa complex may engage in activating Factor X and one-third in activating Factor IX [7]. {These results were obtained using human brain TF. Studies using ox brain TF also suggest that Factor IX will compete with Factor X for TF–VIIa sites [41,42]. However, detailed kinetic studies using ox brain TF and human Factors VII, IX and X are not available.} Further, when a weak source of TF is used, such as in the ox brain PT test, enough Factor IXa may be generated by the TF pathway such that a significant amount of Factor X is activated by IXa–VIIIa–Ca^{2+}–PL complex [43]. This so-called 'alternative mechanism' (reactions 2 and 3 in Scheme 1) for the activation of Factor X has been shown to shorten the clot time of normal plasma in the PT test [43]. However, in a haemophilia-B plasma, the activation of Factor X by this alternative mechanism would be prevented. Moreover, Factor IX variants which are defective in their interaction with TF–VIIa complex would permit all (instead of approx. 66 %) of the TF–VIIa to activate Factor X. The plasma from these variants may have PT value close to that of normal plasma. Factor IX variants which are partially defective in their interaction with TF–VIIa should have a PT moderately prolonged, and the variants which are normal in their interaction with TF/VIIa should have PT value markedly prolonged (since these variants would consume part of the TF–VIIa complex in a non-productive fashion). On the basis of the above reasoning, it appeared logical and reasonable to propose that the variant proteins with different ox brain PT values are genetically heterogeneous and are defective to various extents in their interaction with TF–VIIa complex [18,19,44].

Our data presented in Table 2 and Fig. 3, however, are



Scheme 1. Biochemical basis for the ox brain PT test

In this test a weak source of TF (ox brain) was added to the test plasma, which provides Factor VII/VIIa. The clot formation was initiated by the addition of Ca^{2+} and the time (s) needed to form a clot at 37 °C was recorded (see the Methods section). In this test, the Factor VIIa–TF complex initiates clot formation through the activation of Factor X (reaction 1) directly and via the Factor IXa pathway (reactions 2 and 3) indirectly. In cases such as those where haemophilia-B plasma contains a non-functional factor IX molecule which binds to the VIIa–TF complex, but either does not get converted into an IXa-like molecule or the IXa-like molecule generated does not convert X into Xa, a prolongation in the time needed to form the fibrin clot is observed. A proposed explanation for this prolongation in clot-formation time is that part of the Factor VIIa–TF complex is engaged in a dead-end inhibitory pathway. Thus a higher concentration of variant Factor IX in the haemophilia-B plasma and an unfavourable ratio of IX to X will result in a markedly prolonged time needed to form the clot (see the parenthetic note in paragraph 4 of the Discussion and Table 2).

not consistent with the above hypothesis. Our studies indicate that all four variants listed in Table 2 are normal in their interaction with TF–VIIa complex, despite having different ox brain PT values [20; B. J. Warn-Cramer & S. P. Bajaj, unpublished work]. The three variants (BmLE, LA or LB) are defective in their interaction with the macromolecular-substrate interactions, and the fourth variant (Factor IX_{ER}) (S. G. Spitzer & S. P. Bajaj, unpublished work) is defective in the serine-proteinase common-sequence motif [45]. Our data are consistent with a hypothesis that, when the concentration of Factor IX antigen in plasma is disproportionately lower than that for Factor X (such as in IX_{LA} variant), the PT value would be close to that for normal plasma. This is consistent with several observations that all variant plasmas containing no Factor IX antigen have normal ox brain PT values [17–19]. Furthermore, when the concentration of Factor IX antigen is not disproportionately lower than that for Factor X (such as in IX_{LB} and IX_{ER} variants), the PT would be moderately prolonged. And when the concentration of Factor IX antigen is disproportionately higher than that for Factor X (such as in IX_{BmLE}), the PT would be markedly prolonged. {The following observation is also consistent with this hypothesis. The ox brain PT of a haemophilia-B plasma containing 40% of Factor X (3 μg/ml) and no detectable Factor IX antigen was 45 ± 2 s. When it was supplemented with 25% (1 μg/ml) LA or LB antigen, the PT was 54 ± 2 s. Similarly, supplementation with 50 (2 μg/ml), 75 (3 μg/ml) or 100% (4 μg/ml) LA or LB antigen prolonged the PT of this plasma to 65 ± 2, 83 ± 3 and 112 ± 3 s respectively. In contrast. adding 100% (4 μg/ml) normal Factor IX antigen to the above haemophilia-B plasma gave ox brain PT values of 47 ± 2 s. These observations support the previously reported observations by Bertina & van der Linden [46], who also found that adding Factor $IX_{Deventer}$ (a non-functional molecule) to a Factor-deficient plasma prolongs its ox brain PT in a dose-dependent manner. Those authors also observed that adding normal Factor IX to Factor IX-deficient plasma did not appreciably change the ox brain PT.} An exception to the above proposal might be a situation where the variant molecule is defective in its interaction with TF–VIIa, a situation in which the ox brain PT should be normal. We further propose that the levels of Factor VII (unless extremely low) would not influence the PT test, since the limiting reagent in this assay is TF.

From the foregoing it is obvious that the variant-Factor IX concentration rather than the mutation site in the coding region of the protein is responsible for the ox brain PT results. Thus factors which control the regulation of expression of Factor IX, such as those described in preliminary reports by Kurachi *et al.* [47,48] and by Reitsma *et al.* [33], may indirectly influence the ox brain PT of a variant plasma sample. We have sequenced the putative promoter region of Factor IX [33,48] in both IX_{LA} and IX_{LB} variants and have found no difference from the sequence of normal Factor IX gene in this region (up to -265 base number [12]). Therefore, regulatory elements located in the 5′-end further upstream [47] in the factor IX_{LA} gene may be responsible for its low concentration in plasma. Since these regulatory elements are not as yet fully defined, we did not examine them in the present study. However, it is clear from the observations reported here that Factor IX variant concentration rather than the locus of mutation in the coding region influences the ox brain PT. Our concept is further supported by studies of Huang *et al.* [49], who described a Factor IX mutant ($Arg^{180} \rightarrow Gln$) isolated from a patient with a normal plasma Factor IX antigen level. As expected (Fig. 3), the patient's plasma has markedly prolonged ox brain PT [49].

In conclusion, using PCR methodology, we have shown the molecular defect in Factor IX_{LA} to be the substitution of $Ile^{397} \rightarrow Thr$. Since this substitution and $Ala^{390} \rightarrow Val$ substitution [9] do not impair the charge-relay system and the S_1 binding site but seriously impair the binding of antithrombin III and catalysis of Factors VII and X [20], we propose that residues 390–397 in Factor IXa are involved in the macromolecular-extended substrate/inhibitor binding site. Finally, we propose that the prolonged ox brain PT is related

primarily to the antigen levels rather than the locus of mutation in Factor IX molecules.

We thank Dr. Paul Wollenzein for synthesizing the oligonucleotide probes and Beth Haase for preparation of the manuscript. This work was supported by grants from the National Institutes of Health (HL36365 and HL30572) and the American Heart Association (86–1182). S.P.B. was supported in part by an established investigator award (AHA 83-176) and S.G.S. by a Multidisciplinary Lung Disease Training Program (HL07050) during the performance of this work. B.J.W.-C. was supported by a postdoctoral training grant (HL07107).

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Received 19 April 1989/21 August 1989; accepted 7 September 1989