Characterization of the Defect in Activation of Factor $IX_{Chapel\ Hill}$ by Human Factor XIa

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ABSTRACT Factor $IX_{Chapel\ Hill}$ (Factor IX_{CH}), an abnormal Factor IX molecule isolated from the plasma of a patient with mild hemophilia B, has previously been shown to exhibit delayed activation by Factor XIa and calcium. In this study, we have found that Factor IX_{CH} is cleaved upon incubation with human Factor XIa and calcium; however, cleavage of this protein is not observed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis under nonreducing conditions. Under reducing conditions, the rate of disappearance of the zymogen parallels both the appearance of the heavy chain and the generation of clotting activity. In addition, a protein band that migrates with an apparent molecular weight of 45,000 also increases in parallel with clotting activity. Factor IX_{CH} and normal Factor IX (Factor IX_N), after incubation with Factor XIa and calcium, were subjected to amino terminal sequence analysis. Activated Factor IX_N is cleaved at an arginine-alanine (Arg-Ala) bond and an arginine-valine (Arg-Val) bond as demonstrated by formation of the three amino terminal sequences corresponding to the amino terminal of the light chain, heavy chain, and activation peptide. However, activated Factor IX_{CH} has only two amino terminal sequences, corresponding to the original amino terminal sequence and the heavy chain (formed by cleavage at the Arg-Val bond). It is concluded that the major defect in Factor IX_{CH} is the inability of Factor XIa to cleave the Arg-Ala bond at a significant rate. The rate of formation of clotting activity of Factor IX_{CH} is ~60% of the rate of formation of clotting activity of Factor IX_N. The specific clotting activity of activated Factor IX_{CH} is between 20 and 33% of activated Factor IX_N.

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INTRODUCTION

Although it has long been known that Factor IX may be activated by Factor XIa via the intrinsic pathway (1–6), it has only recently been demonstrated that Factor VIIa in the extrinsic pathway may activate Factor IX (7, 8). The relative physiological contribution of both of these factors to Factor IX activation in vivo is unknown. The nomenclature of the cleavage products produced during the activation of normal Factor IX (Factor IX_N)[1] is shown in Fig. 1. In the presence of Factor XIa and calcium, an arginine-alanine (Arg-Ala) bond and an arginine-valine (Arg-Val) bond are cleaved resulting in the release of an 11,000-mol wt activation peptide and the formation of a 45,000-mol wt active enzyme, IXaβ (6). Factor VIIa in the presence of tissue factor and calcium apparently cleaves Factor IX_N at similar sites (8). However, with both enzymes the order of bond cleavage has yet to be confirmed (6, 8). An inactive intermediate, Factor IXα, is formed when only the Arg-Ala bond is cleaved (6). With Russell's viper venom and calcium, cleavage occurs at the Arg-Val bond yielding an active product, IXaα, which has a molecular weight of 57,000 (6). Human Factors IXaα and IXaβ are reported to have similar clotting activity, whereas bovine Factor IXaα has ~50% of the clotting activity of bovine IXaβ (6, 9, 10).

In an earlier report from this laboratory some of the properties of Factor $IX_{Chapel\ Hill}$ (Factor IX_{CH}) isolated from a patient with clinically mild hemophilia B were described (11). The antigenic level of Factor IX in the plasma from this individual was equal to the

[1] *Abbreviations used in this paper*: CRM, cross-reacting material; CRM^+, CRM positive variants; CRM^-, CRM negative variants; CRM^R, CRM reduced variants; Factor IX_{CH}, Factor $IX_{Chapel\ Hill}$; Factor IX_N, Factor IX normal.

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level of Factor IX antigen in pooled normal plasma. However, by routine one-stage Factor IX clotting assay, only 5% Factor IX clotting activity was found. Purified Factor IX_{CH} was similar to Factor IX_N in terms of molecular weight, amino acid composition, content of gamma-carboxyglutamic acid residues, carbohydrate content and composition, and antigenicity. When monitored by SDS polyacrylamide disc gel electrophoresis under nonreduced conditions, Factor IX_{CH} was not converted at a significant rate to a lower molecular weight form by incubation with Factor XIa and Ca^{++}; however, Factor IX_N was completely converted to a 45,000-mol wt species in 30 min. Although a slight decrease in clotting time was detected, the inability to convert Factor IX_{CH} to the expected lower molecular weight form (Factor IXaβ) prevented determination of the specific activity of the activated molecule. In the absence of a rigorously determined specific activity for activated Factor IX_{CH}, the relative effects on clotting activity of a known slow rate of cleavage by Factor XIa and an unknown activity of cleaved Factor IX_{CH} could not be distinguished.

In this study we have extended our investigation to characterize further the defect in Factor IX_{CH}. We have determined the specific clotting activity of Factor IX_{CH} cleaved by human Factor XIa in the presence of calcium, as well as the rate of cleavage of Factor IX_{CH}. Because the activation of Factor IX_{CH} was not associated with a decrease in its apparent molecular weight, we have also characterized the products formed by the incubation of Factor IX_{CH} with Factor XIa. The results of this study suggest that Factor IX_{CH}, in the presence of human Factor XIa and calcium, has an abnormal pattern of activation resulting in a product with decreased specific clotting activity when compared with normal Factor IX.

METHODS

Materials. The patient with Factor IX_{CH} underwent plasmapheresis twice weekly, after informed consent, until sufficient plasma was obtained for the studies outlined below. The blood was processed in a manner similar to that for normal blood as described below. The plasma was stored at −70°C and thawed at 37°C just before use. Normal human plasma was obtained from Cutter Laboratories (provided by Dr. Milton Mozen, Berkeley, Calif.) and the Blood Bank of the North Carolina Memorial Hospital, Chapel Hill, N. C. Human blood was collected in plastic bags that contained standard citrate phosphate dextrose as an anticoagulant and centrifuged at 3,000 *g* for 30 min at 4°C to render the plasma platelet-poor and free of erythrocytes. The plasma was then recentrifuged under the same conditions. Some of the normal human plasma was obtained via an IBM 2997 Blood Cell Separator or a Haemonetics 30-S Blood Processor (IBM Instruments Inc., White Plains, N. Y.). The plasma used for purification of normal Factor IX were either fresh or stored for <2 mo at −70°C.

Barium chloride, polyethylene glycol 20,000, sodium acetate, and sodium azide were purchased from Fisher Scientific Co., Pittsburgh, Pa. Benzamidine hydrochloride and cyanogen bromide were purchased from Eastman Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, N. Y. Tris (hydroxymethyl) amino methane was purchased from Mallinckrodt, Inc., Science Products Div., St. Louis, Mo. Heparin (sodium salt), Russell's viper venom, and ovalbumin were purchased from Sigma Chemical Co., St. Louis, Mo. Sequencer chemicals were purchased from Beckman Instruments, Inc., Fullerton, Calif. Sephadex G-200, Sepharose 4B, and DEAE-Sephadex (A-50) were purchased from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J. Bovine brain cephalin (Thrombofax) was purchased from Ortho Pharmaceutical, Raritan, N. J. All materials used in gel electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif. Celite 545 was pur-

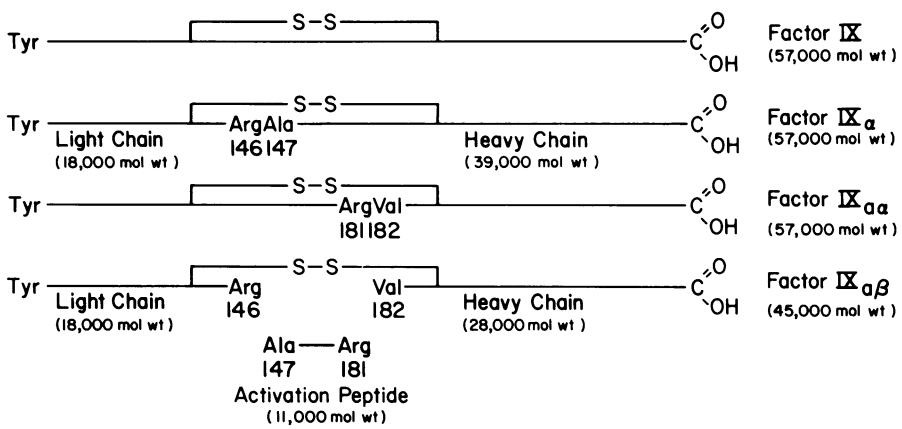

FIGURE 1 Factor IX terminology. Factor IX zymogen is the native form of the protein which has no clotting activity. Factor IXα is the inactive intermediate formed by cleavage of the arginine^{146}-alanine^{147} bond only. Factor IX$\alpha\alpha$ is the active intermediate formed by cleavage of the arginine^{181}-valine^{182} bond only. Factor IXaβ is the fully activated enzyme formed by cleavage of the arginine^{146}-alanine^{147} and arginine^{181}-valine^{182} bonds. Amino acid numbers are from the sequence of bovine Factor IX (10).

chased from Johns-Manville, Denver, Colo. All other chemicals were of at least reagent grade or better.

Human Factor XIa preparation. 19 g of celite was added to 950 ml of normal human pooled plasma and stirred continuously for 10 min at 37°C before centrifugation at 5,600 *g* for 10 min. The precipitate was washed three times with 1 liter of distilled water with a 10-min centrifugation at 5,600 *g* after each wash. The precipitate was then resuspended in 950 ml of 10% NaCl and stirred constantly for 10 min. After centrifugation, the supernatant was dialyzed overnight against three changes of 0.15 M NaOAc, 0.02% NaN_3, pH 5.2. After dialysis the crude Factor XIa was passed over a carboxymethylcellulose (12) column (2.5 × 30 cm) equilibrated with 0.15 M NaOAc (pH 5.2). The column was washed with 250 ml of 0.15 M NaOAc (pH 5.2). Human Factor XIa was eluted with a linear gradient of NaOAc 0.15 M (pH 5.2) to 0.33 M sodium phosphate, 0.5 M NaCl (pH 6.8) (250 ml each). Fractions containing Factor XIa activity, as measured by shortening of a modified nonactivated partial thromboplastin time (13), were pooled and dialyzed overnight against three changes of 4 liters of 0.15 M NaOAc (pH 5.2). The dialyzed material was concentrated with PEG 20,000 to a final volume of 3.5 ml and then placed on a Sephadex G 200 column (3.0 × 60 cm) equilibrated with NaOAc 0.15 M (pH 5.2). Fractions containing human Factor XIa activity were pooled and placed on a heparin Sepharose column (2.0 × 5.0 cm) previously equilibrated with 0.15 M NaOAc (pH 5.2). The column was washed with 100 ml of 0.15 M NaOAc (pH 5.2), 0.2 M NaCl (pH 5.2) and the Factor XIa eluted with 0.15 M NaOAc 2.0 M NaCl (pH 5.2). Factor XIa fractions were again pooled and dialyzed overnight against 0.15 M NaOAc (pH 5.2). The dialyzed material was finally concentrated with PEG 20,000 to a volume of 3.5 ml. Portions of 0.5 ml were stored in polypropylene tubes at −70°C. The final material, diluted 1:10 in 0.05 M Tris HCl (pH 7.4) clotted normal pooled plasma in plastic tubes in 47 s but had no effect on plasma obtained from a patient with hemophilia B with undetectable Factor IX clotting activity and antigen levels nor on fibrinogen after 5 min of incubation at 37°C. The Factor XIa corrected the clotting defect of plasma obtained from a patient severely deficient in Factor XI. The Factor XIa required calcium for complete activation of Factor IX, indicating that the Factor XIa contained an insignificant quantity of kallikrein.

Preparation of Factor IX_N and Factor IX_{CH}. Both proteins were prepared essentially as described (11). A single band was seen in reduced and nonreduced SDS gels. The final product contained only Factor IX clotting and antigenic activity. Other clotting factors were undetectable.

Factor IX activation. The activation of Factor IX_N and Factor IX_{CH} was performed on samples that were desalted by passage over Sephadex G-25 (medium) columns (1.0 × 10 cm) equilibrated with 0.05 M Tris (pH 7.4). Factor IX was diluted with 0.05 M Tris (pH 7.4) to a final concentration of 0.56 μM solution.[2] Human Factor XIa was diluted with 0.05 M Tris (pH 7.4) to a final concentration of 0.46 μM. To 1.95 ml of Factor IX (final concentration 0.54 μM) was added 0.05 ml of human Factor XIa (final concentration 0.014 μM) and 0.010 ml of 1.0 M CaCl (final concentration 5 mM). Duplicate 0.1 ml samples were taken at 0, 5, 10, 15, 20, and 30 min for clotting assay. Factor IXa clotting activity was measured by adding 0.10 ml of plasma with undetectable Factor IX clotting activity and antigen to 0.10 ml of the sample to be tested and incubating at 37°C in a 12 × 75-mm polystyrene tube for 60 s. 0.2 ml of a buffered Thrombofax solution (5 ml of calcium imidazole saline,[3] 4.5 ml of normal saline, and 0.5 ml of Thrombofax) was then added to the mixture and a stopwatch was started. The tubes were shaken 20 times and after 30 s at 37°C, the clotting time was determined. Both Factor IX_N and Factor IX_{CH} were tested at the same time in an identical manner. After determining that Factor IX_N was fully activated after 30-min incubation, a standard curve was made of the normal product by preparing dilutions of the fully activated material in 0.05 M Tris (pH 7.4) of 50, 33, 20, 12.5, and 6.25%. The clotting times were plotted on a log-log graph and a straight line was obtained as described by DiScipio et al. (6). From this standard curve the percent activation was extrapolated for the time points previously measured. After 30 min all of the Factor IX_N zymogen had been cleaved to the activated product as demonstrated by electrophoresis on 7.5% polyacrylamide gels under reducing conditions with β-mercaptoethanol as described by Weber and Osborn (14). The specific activity of Factor IX_{CH} was determined by running the activation as previously described with three different Factor XIa concentrations yielding enzyme substrate ratios of 1:10, 1:20, and 1:40. Determination of the percentage of Factor IX activity of Factor IX_{CH} was continued until all of the Factor IX_{CH} zymogen had been cleaved.

Monitoring Factor IX activation by polyacrylamide gel electrophoresis. Factor XIa was incubated with 5 mM $CaCl_2$ and Factor IX for 30 min. The final concentrations of Factor XIa and Factor IX were 0.09 μM and 1.75 μM, respectively. 10 μg of the activated Factor IX was removed from the reaction mixture at 0, 5, 10, 15, 20, and 30 min and placed in a solution of 1% SDS, dithiothreitol (7 mg/ml), and 5 mM EDTA at 56°C for 1 h. The samples were electrophoresed by the method of Wyckoff et al. (15). The gels were stained with Coomassie Blue and scanned using a Soft Laser Scanning Densitometer, Biomed Instruments, Inc., Chicago, Ill. The areas under the peaks were determined and their relative amounts calculated. Purified Russell's viper venom (16) in an enzyme/substrate ratio of 1:3 was incubated with Factors IX_N and IX_{CH} in 5 mM CaCl. Samples were taken at starting time, 15, and 30 min for electrophoresis on reduced and nonreduced polyacrylamide gels by the method of Weber and Osborn (14).

Sequence analysis. 10 nmol of Factor IX_{CH} and 5 nmol of Factor IX_N were incubated with Factor XIa at an enzyme/substrate ratio of 1:20 and 5 mM $CaCl_2$ in a total volume of 2 ml. Initial samples and 60-min samples were exposed to 0.5 ml of 50% acetic acid to inactivate the Factor XIa. The samples were dialylzed against 4 liters of 5% acetic acid and subjected to amino terminal sequence analysis.

Automated Edman degradations (17) were performed by a Beckman 890C sequencer (Beckman Instruments, Inc.). The 0.1 M Quadrol program of Brauer et al. (18) was used, with modification of the cleavage portion to minimize blowing of heptafluorobutyric acid vapors through the effluent valve. The phenylthiohydantoin amino acids produced by the Edman degradation were identified by high performance liquid chromatography, using a 4.6-mm, i.d. × 250-mm Ultrasphere-ODS column with a Beckman model 324 gradient liquid chromatograph (Beckman Instruments, Inc.) and model

[2] Protein concentrations were determined by absorption at 280 nM. Concentration of Factor IX was based on the extinction coefficients ($E_{280\ nm}^{1\%}$) 11.8 for Factor IX_N and 12.5 for Factor IX_{CH} (11). Concentration of Factor XIa was based on the extinction coefficient 13.4 (6).

[3] Calcium imidazole saline is 560 ml of 0.082 M $CaCl_2$, 480 ml of 0.253 M imidazole buffer pH 7.2, and 400 ml of normal saline (0.15 M).

153 absorbance detector operated at 254 nm. Absorbance at 323 nm (for detection of phenylthiohydantoin-dehydrothreonine) was monitored by an Isco model 1840 variable wavelength detector (Instrumentation Specialties Company, Lincoln, Nebr.). A 2.1-mm, i.d. × 7-cm guard column filled with Co:Pell ODS (Whatman, Inc., Chemical Separation Div., Clifton, N. J.) was connected ahead of the analytical column. Gradient elution was performed at 55°C with an acetate buffer: acetonitrile solvent system.[4]

RESULTS

Factor IX_N and Factor IX_{CH} were incubated at 37°C for 60 min with Factor XIa in the presence of 5 mM Ca^{++}. Samples were removed and subjected to SDS polyacrylamide gel electrophoresis under reducing and nonreducing conditions. The Coomassie Blue-stained gels are shown in Fig. 2. Under nonreducing conditions it was observed that the mol wt of Factor IX_N was reduced from 58,000 for the zymogen to 48,000 for Factor IXaβ; however, the mol wt of Factor IX_{CH} was unchanged. Under reducing conditions, two major bands could be observed for Factor IX_N. One band (the heavy chain of IXaβ) appeared to correspond to a mol wt of ~29,000 whereas the other band (the light chain of IXaβ) had an apparent mol wt of ~22,000. Factor IX_{CH} also appeared as two bands (in addition to the original zymogen) under reducing conditions (Fig. 2). One band migrated with a mobility identical to that of the heavy chain band from Factor IX_N. The other band, however, had an apparent mol wt of 45,000, which corresponded to a cleavage product composed of the light chain and activation peptide found on cleavage of both Factor IX_N and Factor IX_{CH} by Russell's viper venom (not shown).

The relative rates of cleavage of Factor IX_N and Factor IX_{CH} were determined by scanning polyacrylamide gels of reduced samples taken at various times of incubation (Fig. 3A and B). With both Factor IX_N and Factor IX_{CH} the rate of decrease in the zymogen band was nearly identical to the rate of increase in the heavy chain band (Fig. 3B). However, the rate of cleavage of Factor IX_{CH} was 60% of the rate of Factor IX_N cleavage under these experimental conditions. The rate of increase in the intermediate 45,000 mol wt band for Factor IX_{CH} was also nearly identical to the rate of decrease in Factor IX_{CH} zymogen (Fig. 3A). A 45,000 mol wt band was also apparent in gels from Factor IX_N but was present in only small amounts and remained essentially constant during the period of incubation (Fig. 3A). The light chain band from Factor IX_N cleavage did not appear in the first 10 min of incubation but increased significantly after that time (Fig. 3B). There was no evidence of light chain products derived from Factor IX_{CH} by this technique.

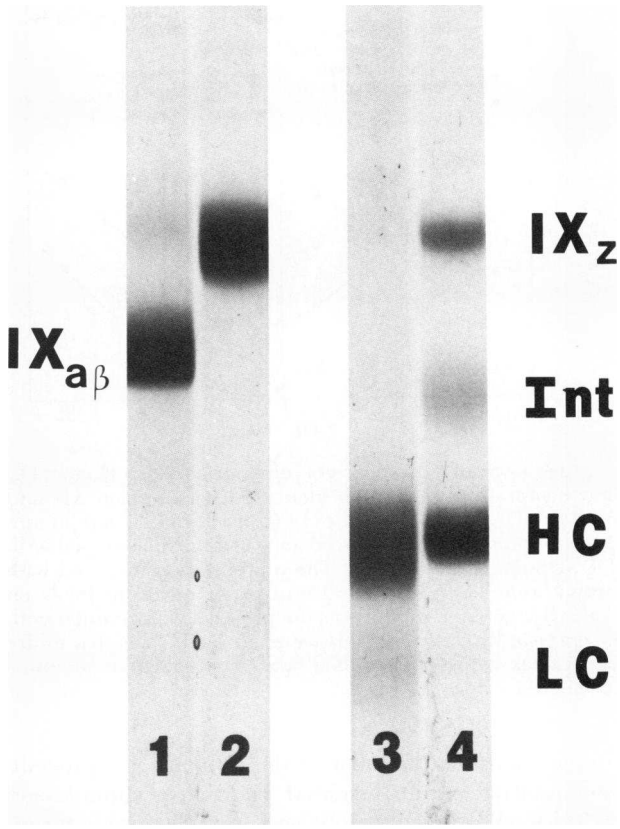

FIGURE 2 Factor IX_N and Factor IX_{CH} after Factor XIa cleavage. 7.5% polyacrylamide SDS disc gel electrophoresis of: (1) nonreduced Factor IX_N; (2) nonreduced Factor IX_{CH}; (3) reduced Factor IX_N, and (4) reduced Factor IX_{CH}. All samples had been incubated with human XIa and 5 mM $CaCl_2$ at 37°C for 60 min, as described in Methods section. Z, zymogen; Int., intermediate band; HC, heavy chain, LC, light chain.

In an attempt to confirm the identity of the cleavage products formed by incubation of Factor IX_{CH} with Factor XIa, samples were prepared for sequence analysis of the amino terminal regions of the resulting activation products. (The samples used were those shown in Fig. 2). Unactivated Factor IX_N and Factor IX_{CH} were identical in amino acid sequence through the first six positions tyrosine-asparagine-serine-glycine-lysine-leucine (Tyr-Asn-Ser-Gly-Lys-Leu). The first sequence cycle of activated Factor IX_N indicated three amino terminals; Tyr, Ala, Val as shown in Table I. The amino acids found in the subsequent cycles likewise corresponded to the reported sequences (6) of the light chain, the activation peptide, and the heavy chain. In contrast, sequence analysis of Factor XIa-activated Factor IX_{CH} revealed only two sequences, corresponding to the original amino terminal of the zymogen (Tyr) and the amino terminal of the heavy chain (Val) (Table I). No detectable

[4] Noyes, C. M. Manuscript in preparation.

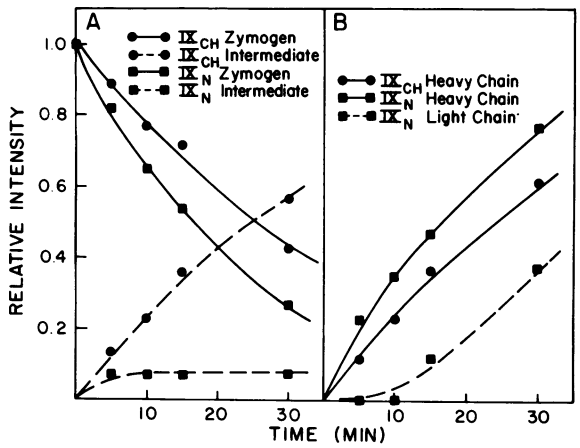

FIGURE 3 Scans of SDS gel electrophoresis. Factor IX_N and Factor IX_{CH} were each incubated with human XIa and 5 mM $CaCl_2$ at 37°C. At times of 0, 5, 10, 15, 20, and 30 min 10 μg of protein were removed and the reaction stopped with 1% SDS and 5 mM EDTA. The proteins were reduced with dithiothreitol at 56°C for 60 min. After electrophoresis on 7.5% disc gel electrophoresis, the proteins were stained with Coomassie Blue and the gels were scanned. The areas under each peak were determined and their relative amounts calculated.

amount of the activation peptide sequence was present. Quantitative amino terminal yields are summarized in Table II, where it can be seen that the amino terminal Ala of the activation peptide for Factor IX_{CH} was present in only a trace amount, which did not exceed the background level found in subsequent cycles and in analysis of unactivated material.

The experiments described above indicate that Factor IX_{CH} was cleaved by Factor XIa at the Arg-Val bond, but do not examine the effect of cleavage on activation as determined by clotting assays. To pursue this question, samples were taken after maximal cleavage of the Factor IX_{CH} zymogen and the clotting activity determined as described in Methods. In comparison with Factor IX_N, Factor IX_{CH} had an average clotting activity of 29%, with a range of 20 to 33% on eight determinations. The generation of clotting activity was followed by assaying samples taken at various times after the addition of Factor XIa to Factor IX_N and Factor IX_{CH}. The results are shown in Fig. 4. From the data obtained in four identical experiments, the rate of Factor IX_{CH} activation was found to be ~60% of the rate of activation of Factor IX_N. SDS polyacrylamide gel electrophoresis of reduced samples taken during incubation indicated that maximum activity for both Factor IX_N and Factor IX_{CH} coincided with complete disappearance of the zymogen band.

DISCUSSION

About 10% of hemophilia B patients have a normal amount of Factor IX antigen (crossreacting material; CRM) present in their plasma (19). Such variants have been termed CRM^+. Some hemophilia B patients have reduced crossreacting material (CRM^R) whereas others have no detectable antigen (CRM^-). Only a few of the CRM^+ variants have been investigated. Hemophilia B_M patients are CRM^+ and are severely affected bleeders. Their defect is characterized by a prolonged plasma ox brain prothrombin time (20). Østerud et al. were unable to detect a defect in either the activation rate or the cleavage products of radiolabeled Factor IX_{BM} when incubated with Factor XIa and calcium or Factor VIIa, tissue factor, and calcium (21). However, Bertina (22) claims to have a Hemophilia B_M patient whose Factor IX is not cleaved at the Arg-Val bond by either Russell's viper venom or Factor XIa. Hemophilia B_{Leyden} has a reduced Factor IX clotting and antigen level that increases with age (23). In addition, Bertina and Veltkamp (24) have identified CRM^+ variants that have altered heparin and calcium binding. Factor IX_{CH} was the first IX variant described with a defect in its activation rate. Østerud et al. (25) have subsequently added three other patients with defective activation rates, but have not described the nature of the defect further. In the present study we have identified the abnormality in the activation of Factor IX_{CH} by human Factor XIa, although we have not yet determined the putative amino acid substitution.

The rate of formation of cleavage products determined by gel scanning revealed unexpected results. Whereas the clotting activity of Factor IX_N coincides with the formation of the heavy chain (Arg-Val cleavage), there is an apparent lag in the appearance of the light chain (Arg-Ala cleavage) relative to the

TABLE I
Amino Terminal Sequences Derived from Factor XIa-activated IX_N and IX_{CH}

	1	2	3	4	5	6
Light chain IX_N	Tyr	Asn	Ser	Gly	Lys	Leu
Light chain-activation peptide IX_{CH}	Tyr	Asn	Ser	Gly	Lys	Leu
Heavy chain IX_N	Val	Val	Gly	Gly	Glu	Asp
Heavy chain IX_{CH}	Val	Val	Gly	Gly	Glu	Asp
Activation peptide IX_N	Ala	Glu	Thr	Val	Phe	Pro

TABLE II

Amino Terminal Amino Acids from Factor XIa-Activated IX_N and IX_{CH} with Quantitation

	IX_N	IX_{CH}*
	(nmol)	*(nmol)*
Tyr	4.1	8.5
Ala	3.7	0.4
Val	4.2	8.9

* The amount of Factor IX_{CH} used in these experiments was approximately twice that of Factor IX_N.

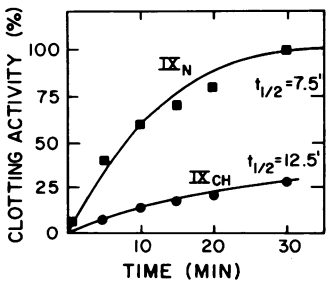

FIGURE 4 Generation of clotting activity vs. time. Factor IX_N and Factor IX_{CH} were each incubated with human XIa and 5 mM CaCl. At times of 0, 5, 10, 15, 20, and 30 min, portions of the reaction mixture were removed and clotting times were determined in duplicate as described in Methods. At 30 min all of the Factor IX_N zymogen had been cleaved and a standard curve was made from dilutions of 50, 33, 20, 12.5, and 6.25%. From this standard curve the percent activation was determined for the time points previously measured.

appearance of the heavy chain. Furthermore, a protein band appears in trace amounts in the reduced gels of partially activated Factor IX_N and corresponds to the peptide band composed of light chain and activation peptide seen in the reduced gels of activated Factor IX_{CH} (or Russell's viper venom activated IX_N). Thus, the activation of Factor IX_{CH} by XIa gives rise to a product similar to IXaα. Because Factor IX_{CH} can be activated by human Factor XIa without the cleavage of the Arg-Ala bond, it is clear that the cleavage of this bond is not an absolute prerequisite for the cleavage of the Arg-Val bond by human Factor XIa. These data, particularly the lag in the appearance of the Factor IX_N light chain, suggest that the cleavage of the Arg-Val bond may precede cleavage of the Arg-Ala bond in human Factor IX. This is different from the activation of bovine Factor IX where it appears that the cleavage of the Arg^{146}-Ala^{147} bond precedes the cleavage of the Arg^{181}-Val^{182} bond by bovine Factor XIa (9, 10).

It is interesting to note that the apparent molecular weight of the light chain and the light chain-activation peptide component are somewhat heavier as judged by SDS polyacrylamide disc gel electrophoresis than expected. Precise explanation for this observation cannot be given; however, it is well-known that glycoproteins may migrate with higher apparent molecular weights than expected in SDS-polyacrylamide gel electrophoresis (26). From the reported carbohydrate composition of human Factor IX (6), it is known that all three peptides (heavy chain, light chain, and activation peptide) contain significant carbohydrate and, thus, could migrate in an unexpected manner on SDS disc gel electrophoresis.

By comparison with Factor IX_N, Factor IX_{CH} is distinguishable by a slower rate of activation (~60%) and a lower final specific activity (~30%). These observations can be contrasted with the 5% Factor IX activity determined for Factor IX_{CH} by a one-stage clotting assay. The relatively low specific activity of the fully activated Factor IX_{CH} may be related to the fact that Factor IX_{CH} is cleaved by Factor XIa to a Factor IXaα form rather than to the Factor IXaβ form. Because normal human Factor IXaα is reportedly as active as Factor IXaβ (6), it is not clear that the lack of cleavage of the Arg-Ala bond in Factor IX_{CH} would necessarily decrease its specific clotting activity. It is not possible at the present time to speculate further concerning the region of Factor IX_{CH} where an amino acid substitution would lead to the observed effect.

Factor IX_{CH} has given us new insights into the mechanism of Factor IX activation. The formation of Factor $IX_{CH}a\alpha$ by human Factor XIa indicates that cleavage of the Arg-Ala bond is not an absolute requirement for human Factor XIa cleavage of the Arg-Val bond. This is further supported by the appearance in Factor IX_N of an intermediate peptide, which corresponds to the light chain-activation peptide band of Factor IX_{CH}. Interestingly, the patient has only a mild bleeding tendency. Whether this represents significant generation of Factor IX activity via the intrinsic Factor XIa system, or whether this is due to the ability of Factor VIIa to overcome the molecular defect in Factor IX_{CH}, at present, remains speculative. However, this variant of hemophilia B may be particularly important in establishing the significance of the interactions of Factor IX with Factor XIa and Factor VIIa.

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