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The role of β-hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IX

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β-Hydroxyaspartic acid is a post-translationally modified amino acid found in a number of plasma proteins in a domain homologous to epidermal growth factor. Its presence can be correlated with a high affinity Ca^{2+} binding site, with a dissociation constant of 10−100 μM. We describe a system for the expression of human coagulation factor IX in dog kidney cells in tissue culture, in which the post-translational modifications and the biochemical activity are indistinguishable from factor IX synthesized *in vivo*. This system has been used to express eight different point mutations of human factor IX in the first epidermal growth factor domain in order to study the role of β-hydroxyaspartate at residue 64, and the adjacent carboxylate residues at positions 47, 49 and 78. We conclude that this domain is essential for factor IX function and suggest that Ca^{2+} binds to carboxylate ions in this domain and stabilizes a conformation necessary for the interaction of factor IXa with factor X, factor VIII and phospholipid in the next step of the clotting cascade.

Key words: EGF domains/factor IX/β-hydroxyaspartate/Ca^{2+} binding/factor VIII

Introduction

Factor IX is a plasma glycoprotein (mol. wt 56 000) which plays a central role in the intrinsic phase of blood coagulation (reviewed in Jackson and Nemerson, 1980; Thompson, 1986). It is a serine protease zymogen which undergoes four types of post-translational modification during biosynthesis before secretion. The pre- and pro-sequences are removed by successive proteolytic cleavages, the 12 glutamate residues at the N terminus of the protein are γ-carboxylated in a vitamin K-dependent reaction, the aspartate at residue 64 is β-hydroxylated (Fernlund and Stenflo, 1983; McMullen *et al.*, 1983a) and N-linked carbohydrates are added in the activation peptide domain.

In the coagulation pathway factor IX interacts with a number of proteins, with Ca^{2+} and with phospholipid membranes. The activation of factor IX occurs in solution, in the presence of Ca^{2+}, by proteolytic cleavages catalysed by factor XIa, which is generated by the contact phase of coagulation. This results in the formation of the activation peptide and the generation of the active serine protease, factor IXa. Factor IX can also be activated by factor VIIa in the presence of tissue factor, Ca^{2+} and phospholipid membranes. Factor IXa then interacts with its cofactor, factor VIIIa, to form a Ca^{2+}-dependent complex on the surface of phospholipid membranes which is capable of activating factor X.

β-Hydroxyaspartate (or the related β-hydroxyasparagine) has been characterized as a post-translationally modified residue in a number of plasma proteins, including factor VII, factor IX, factor X, protein C, protein S and protein Z, and complement Clr (Drakenberg *et al.*, 1983; McMullen *et al.*, 1983a,b; Fernlund and Stenflo, 1983; Sugo *et al.*, 1984a; Højrup *et al.*, 1985; Hagen *et al.*, 1986; Stenflo *et al.*, 1987; Arlaud *et al.*, 1987). In each case the β-hydroxyaspartate is found in a domain homologous to epidermal growth factor (EGF). The presence of EGF homologous domains containing β-hydroxyaspartate can be correlated with γ-carboxyglutamate-independent Ca^{2+} binding sites with dissociation constants in the range 10−100 μM, which have been characterized for factor IX (Morita *et al.*, 1984, 1985), factor X (Sugo *et al.*, 1984b), protein C (Esmon *et al.*, 1983), protein S (Sugo *et al.*, 1986) and complement Clr (Villiers *et al.*, 1980). However, it remains to be demonstrated that the Ca^{2+} ion is binding to the β-hydroxyaspartate residue.

A number of reports have described the expression of recombinant human factor IX in heterologous systems (reviewed in Brownlee, 1987; Anson *et al.*, 1987; Choo *et al.*, 1987; Jorgensen *et al.*, 1987). But, with the exception of the expression of factor IX in transgenic mice, none of these systems produces high levels of protein, with completely normal levels of coagulant activity. Biochemical analysis has shown, when high levels of protein are produced, that post-translational cleavage of the propeptide and γ-carboxylation of glutamate residues is inefficient and results in decreased biological activity.

In the current study an expression system is described in which sufficient recombinant factor IX is expressed to allow detailed biochemical analysis. It shows high levels of γ-carboxylation, complete propeptide cleavage, and nearly normal levels of coagulant activity. We then describe the site-directed mutagenesis of four carboxylate residues in the first EGF homologous domain of factor IX, at aspartate 47, aspartate 49, β-hydroxyaspartate 64 and glutamate 78, and the expression and characterization of these mutant proteins. Our results suggest a role for this first EGF-like domain of factor IX in the interaction between factor IXa and the other components, specifically factor X, factor VIII and phospholipid, which are required for the correct activation of factor X. We suggest that Ca^{2+} binding to a site contain-

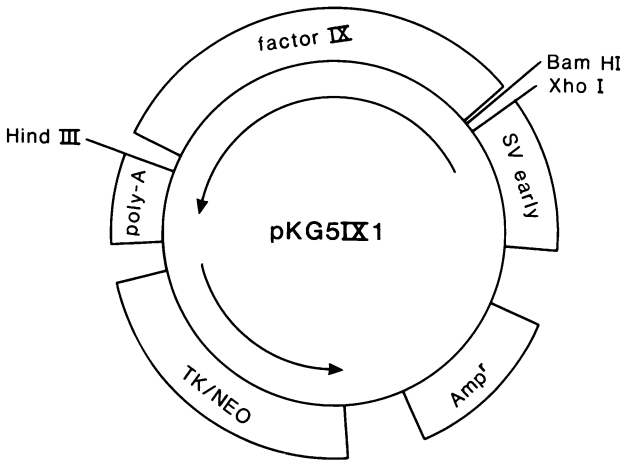

Fig. 1. Structure of the expression plasmid pKG5IX1. See Materials and methods for further details. Arrows show direction of transcription of the factor IX and TK-*neo* genes.

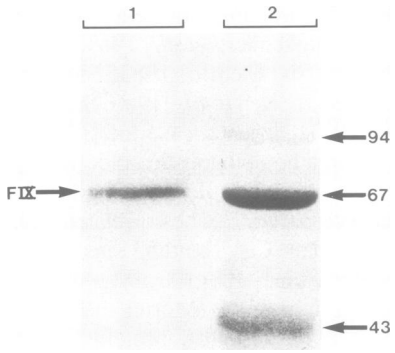

Fig. 2. SDS–PAGE of purified recombinant human factor IX. **Lane 1**: 0.5 μg lyophilized recombinant human factor IX (purified from conditioned medium from the cell line C6 as described in Materials and methods except that BSA was omitted). **Lane 2**: mol. wt markers (Pharmacia): phosphorylase b (94 kd), BSA (67 kd) and ovalbumin (43 kd). Analysis was by SDS–PAGE on a standard Laemmli 10% polyacrylamide gel which was stained with silver.

ing β-hydroxyaspartate results in the correct conformation of factor IXa required for this interaction.

Results

Expression of normal factor IX in MDCK cells

Normal human factor IX was expressed in Madin–Darby canine kidney (MDCK) cells using the mammalian expression plasmid pKG5IX1 (Figure 1 and Materials and methods). MDCK cells transfected with this plasmid were selected for G418 resistance and conditioned medium derived from 90 individually isolated clones were screened for factor IX expression by enzyme-linked immunosorbent assay (ELISA) (see Materials and methods). Of these, 30 clones were positive for the secretion of factor IX antigen at final concentrations varying between 10 and 250 ng factor IX/ml. The clone (C6) producing the highest level of factor IX antigen (~250 ng/ml) was then grown in 1-l culture and factor IX was purified by adsorption onto barium citrate, followed by immunoaffinity chromatography using the monoclonal antibody A7 (Smith and Ono, 1984; Anson *et al.*, 1987) (see Materials and methods). Factor IX antigen was quantitatively adsorbed in both these affinity steps when assayed by ELISA, and the purified material behaved as a single component of apparent mol. wt 68 kd on SDS–PAGE (Figure 2). It was indistinguishable in electrophoretic mobility from factor IX isolated from normal human plasma when analysed by Western blotting (Figure 4). When assayed by one-stage clotting assay (Austen and Rhymes, 1975), the normal recombinant factor IX had a specific coagulant activity of 92% that of factor IX in pooled normal total plasma (Table I). By comparison, normal factor IX, purified by the same protocol from pooled human plasma and stabilized by the addition of bovine serum albumin (BSA), had a specific coagulant activity of 124%. It is likely that this value is greater than the control plasma sample because of some systematic difference in the assay caused by using purified factor IX. We observed that there was 0.18 mol β-hydroxyaspartate (see Materials and methods) per mol of recombinant factor IX. A preparation of plasma-derived factor IX gave 0.12 mol β-hydroxyaspartate per mol of factor IX. These figures do not differ significantly from the low figures of β-hydroxyaspartate found previously (Fernlund and Stenflo, 1983), which suggested that this modification was not essential for function.

Table I. Factor IX mutants constructed and their clotting activities

Mutant	Codon change	Amino acid change	Factor IX antigen	Factor IX clotting activity	Specific activity (%)
217	GAT to AAA	Asp^{47} to Lys	520	4.0	1
220	GAT to GGT	Asp^{47} to Gly	100	4.5	5
223	GAT to GAA	Asp^{49} to Glu	440	37.0	8
19	GAT to AAA	β-OH Asp^{64} to Lys	460	8.5	2
56	GAT to GTT	β-OH Asp^{64} to Val	600	11.0	2
57	GAT to GGT	β-OH Asp^{64} to Gly	675	4.0	1
225	GAA to GAT	Glu^{78} to Asp	100	52.0	52
81	GAA to AAA	Glu^{78} to Lys	170	<0.5	<1
	Normal human factor IX		460	570	124
	Recombinant factor IX		260	240	92

Factor IX antigen and clotting activities are expressed as a percentage of that antigen or activity found in a pool of normal human plasma. Specific clotting activities (activity ÷ antigen) are expressed as a percentage of that in a pool of normal plasma, taken as 100%.

Design and construction of factor IX mutations

Figure 3 shows an alignment of domains of protein sequences related to EGF, derived from a diverse group of proteins, including clotting factor IX. All sequences (whether type A, B or C, see Figure 3) have the arrangement of cysteine residues characteristic of EGF domains. However type B sequences have additional features in common, specifically, a D, D, $\overset{*}{D}$, Y, E consensus at residues 47, 49, 64, 69 and 78. (We use the human factor IX numbering system, where $\overset{*}{D}$ and $\overset{*}{N}$ are β-hydroxyaspartate and β-hydroxyasparagine, respectively.) All type B sequences have at least three and usually four of the residues characteristic of the

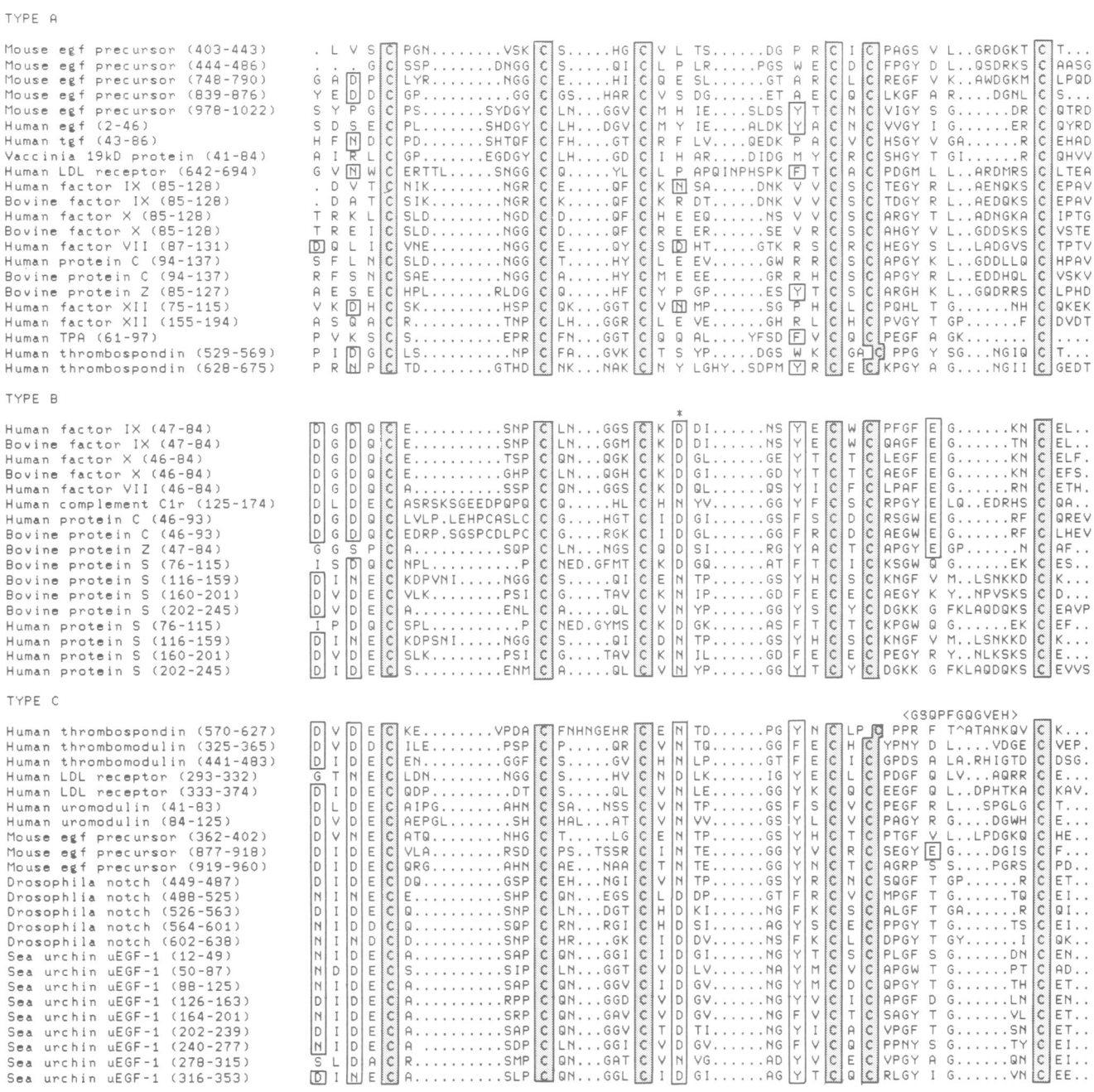

Fig. 3. Alignment of amino acid sequences of domains of various proteins homologous to epidermal growth factor. Conserved cysteine residues are boxed and stippled. Residues homologous to the consensus D,D,$\overset{*}{D}$ Y,E (residues 47, 49, 64, 69 and 78 respectively) are boxed, without stippling.
N $\overset{*}{N}$ F

The aligned sequences (Gribskov *et al.*, 1987) are divided into three sets. Type A lack the consensus (above). Type B contain a minimum of three of the consensus residues including, obligatorily, residue 64 as a β-hydroxy derivative (marked *). Type C are related to type B but are not known to contain a β-hydroxy derivative at the amino acid 64 consensus position (see text). Sequences were taken from National Biomedical Research Foundation Protein sequence data base, except for human factor VII (Hagen *et al.*, 1986), human complement C1r (Arlaud *et al.*, 1987), human protein S (Lundwall *et al.*, 1986), human thrombomodulin (Suzuki *et al.*, 1987), human uromodulin (Pennica *et al.*, 1987), sea urchin uEGF-1 (Hursh *et al.*, 1987).

consensus, whereas those of type A have ≤2. The first EGF domain of factor IX has all five features of the consensus. By contrast, the second EGF domain of factor IX (listed as a type A sequence in Figure 3) has none. This suggested that these consensus residues might be functionally important.

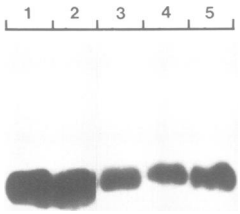

Fig. 4. Analysis of purified factor IX preparations by Western blotting. Approximately 100 ng of each purified factor IX preparation was separated on 10% SDS–PAGE, electroblotted onto nitrocellulose and the filter probed with ^{125}I-labelled monoclonal antibody A7 as described in Materials and methods. The samples are: (**1**) human plasma factor IX purified by heparin–Sepharose chromatography; (**2**) human plasma factor IX, purified as described in Materials and methods; (**3–5**) factor IX mutants 19, 56 and 57 purified similarly.

To investigate whether the conserved carboxylate residues at positions 47, 49, 64 and 78 (specifically D, D, D* and E, respectively) in human factor IX are important (we excluded tyrosine 69 in this initial study), a set of point mutants were constructed to generate single amino acid substitutions at each of these positions (Table I), using site-directed mutagenesis in M13 (see Materials and methods). The entire factor IX coding region of each mutant was completely sequenced (see Materials and methods) to confirm the site-directed mutant and to exclude that other unwanted second mutations had occurred. The mutant expression constructs in pKG5 (see Materials and methods) were transfected into MDCK cells and G418-resistant clones isolated. Mutant factor IX from the highest producing cell lines was then purified exactly as described for recombinant normal factor IX (see above and Materials and methods). All mutants were completely adsorbed onto barium citrate and onto the immunoaffinity column (see Materials and methods) and were identical in mobility to human factor IX, purified from pooled normal plasma on Western blots (Figure 4 shows results on three of the mutants).

Analysis of the specific activities of the mutant factor IXs

The coagulant activities (see Materials and methods) of the purified mutant factor IX molecules are shown in Table I. The data from these assays, normalized to the respective antigen levels of the different mutants (see Table) are given as specific activities. All have specific activities <10% of the normal factor IX, except for mutant 225 (glutamate,78, replaced by aspartate) which has ~50%.

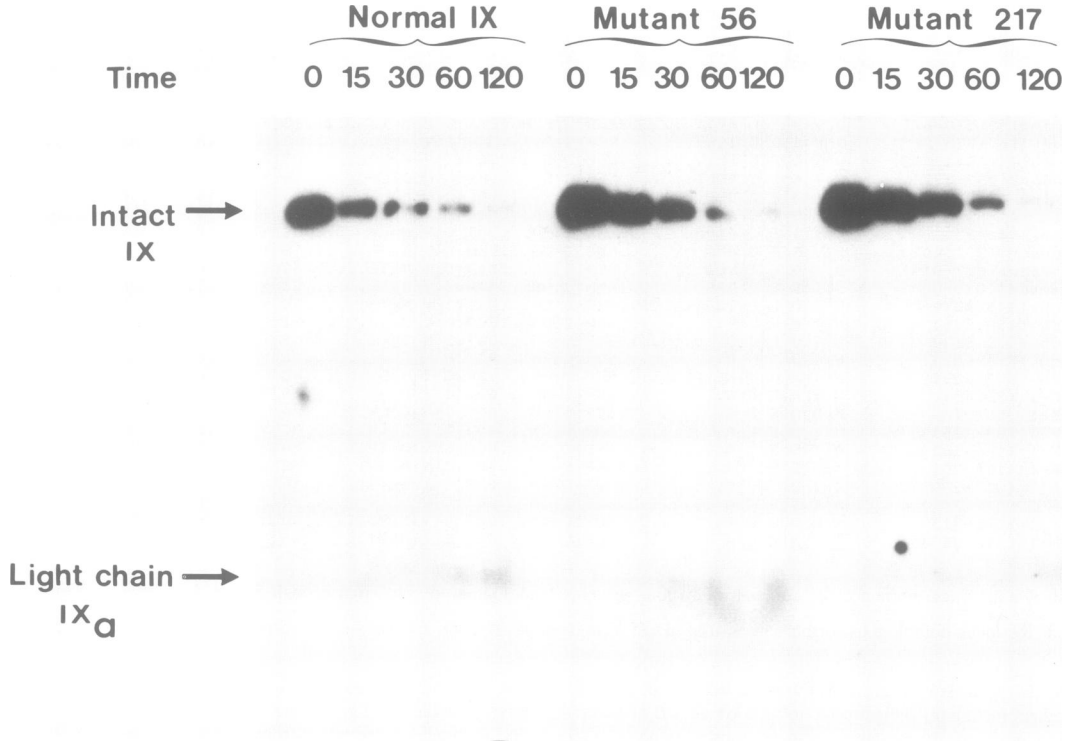

Fig. 5. Time course of the activation of normal and two mutant factor IX molecules by factor XIa. Normal plasma and mutants 56 and 217 factor IX molecules were activated by factor XIa as described in Materials and methods taking time points between 0 and 120 min. The reaction products were resolved in 10% SDS–PAGE and electroblotted, probing with ^{125}I-labelled monoclonal antibody A7. This detects the intact factor IX and only the light chain of factor IXa (both arrowed).

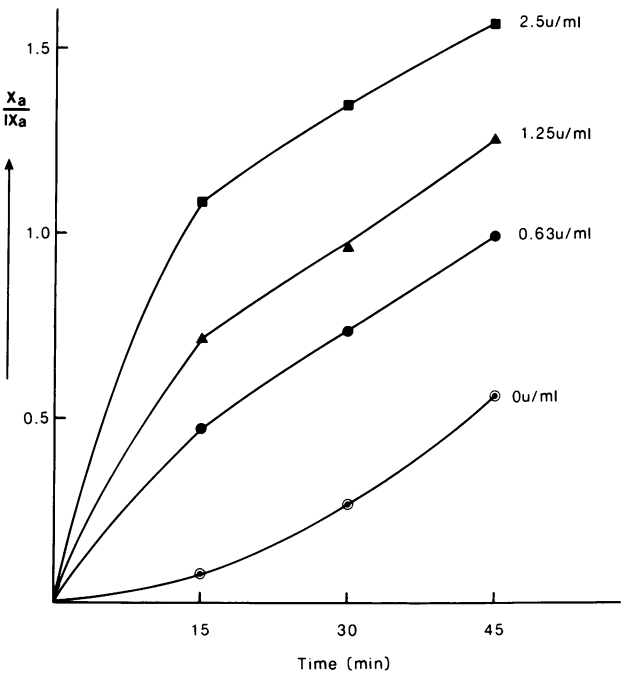

Fig. 6. Time course of activation of factor X by factor IXa assayed at different concentrations of added factor VIII. Release of [^{3}H]peptide from bovine factor X was measured by TCA precipitation and normalized to ng of Xa produced per ng of IXa added. Assays were carried out with 0 (◉), 0.63 (●), 1.25 (▲) and 2.5 (■) units of human factor VIII per ml of incubation.

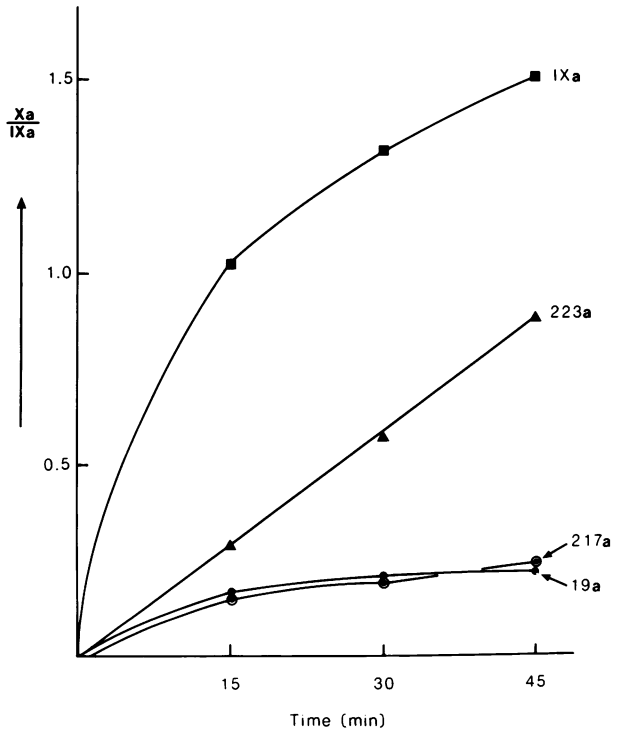

Fig. 7. Time course of activation of factor X by factor IXa from normal plasma and various mutant factor IX preparations in the presence of factor VIII. Release of [^{3}H]peptide from labelled bovine factor X was measured by TCA precipitation and normalized to ng of Xa produced per ng IXa added (see Materials and methods) using (■) activated plasma factor IX or activated mutants 223 (▲), 217 (○) and 19 (●).

Activation by factor XIa

In theory, the low specific activities of the mutant factor IX molecules could be explained either by a decrease in the rate of activation by factor XIa or by inhibition of the subsequent activation of factor X by factor IXa. In order to determine whether the mutant factor IX molecules could be activated, purified factor XIa (contact product) was used to activate each mutant factor IX. Figure 5 shows a comparison of a time course of activation of mutants 56 and 217 compared with normal plasma human factor IX analysed by Western blotting (see Materials and methods). During activation the intact factor IX decreases in intensity with the concomitant production of the factor IXa light chain. Factor IXa heavy chain is also produced but is not detected by the antibody A7 used to probe this blot. We observe that these two mutants undergo proteolytic cleavage indicating normal activation. The other six mutants were also activated at a rate which was indistinguishable from normal plasma factor IX (results not shown). Identical results were obtained when the activation of mutants 19, 56, 220 and 223 was carried out in 0.5 mM Ca^{2+} instead of 15 mM Ca^{2+} (results not shown).

Activation of factor X by factor IXa

In order to analyse factor X activation, the time course of release of the activation peptide of ^{3}H-labelled bovine factor X was analysed in a reaction utilizing factor IXa, Ca^{2+} and phospholipids (see Materials and methods), in the presence or absence of factor VIII. Figure 6 shows that increasing concentrations of factor VIII stimulated the activation of factor X by control plasma-derived factor IXa. When the mutants 19, 81, 217 and 223 were assayed, in the absence of factor VIII, there was a low, but detectable, rate of factor X activation which was indistinguishable from that of wild-type factor IXa (results not shown). However, in the presence of factor VIII at a concentration of 2.5 U/ml, the rate of factor X activation by the mutant factor IXa molecules which were tested was appreciably less than wild-type factor IXa (Figure 7). For the mutants with higher specific activity (e.g. mutant 223, which has 8%, Table I), there was a detectable increase in the rate of reaction on the addition of factor VIII, but this remained significantly lower than the rate for wild-type factor IXa. For the mutants with very low specific activities (e.g. mutants 19 and 217, see Table I), there was a very low rate of factor X activation, which was indistinguishable from that observed in the absence of factor VIII. Mutant 81 gave qualitatively similar results to mutants 19 and 217 (results not shown). Mutants 56, 57, 220 and 225 were not tested.

Discussion

Expression and characterization of normal and mutant factor IXs

In the work described here, the expression of sufficient recombinant normal factor IX has been achieved to allow purification to homogeneity and biochemical characterization. Factor IX biosynthesis seemed normal and showed complete cleavage of the propeptide and apparently normal levels of γ-carboxyglutamate and of β-hydroxyaspartate (Fernlund and Stenflo, 1983). In addition, the mobility of the recombinant factor IX by SDS–PAGE was indistinguishable from factor IX purified from normal human plasma, indicating that oligosaccharides had been added during biosynthesis. The

coagulant activity of the normal recombinant factor IX was high, although slightly less than normal human plasma factor IX. The MDCK expression system described therefore provided a means by which to analyse the effects of point mutation, introduced into the factor IX by site-directed mutagenesis. It has also been used in a parallel study of a factor IX mutant of the propeptide region (Galeffi and Brownlee, 1987).

The initial question approached in the design of these experiments was whether the β-hydroxyaspartate residue had a function in factor IX activity which was commensurate with its extensive conservation in homologous sequences in other proteins. However, the comparison of these related sequences indicated that other residues in the epidermal growth factor-related β-hydroxyaspartate-containing domains were also highly conserved. In addition to the residues of structural significance such as cysteines, a number of other residues appeared important. In the set of data initially available (factor IX, factor X, protein C and Clr) the residues of interest to this work appeared to be aspartate 47, aspartate 49, β-hydroxyaspartate 64 and glutamate 78, and these residues were selected for mutagenesis. The subsequent publication of the sequences for bovine and human protein S showed that the residue equivalent to glutamate 78 was not absolutely conserved. In addition, at three of four positions in protein S the residue β-hydroxyasparagine was found (Stenflo *et al.*, 1987). Finally, it has been suggested that the conserved aromatic residue equivalent to tyrosine 69 in human factor IX may be important in substrate recognition by the enzyme catalysing the β-hydroxylation of aspartate or asparagine in this domain (Stenflo *et al.*, 1987).

When the Ca^{2+} binding properties of the β-hydroxyaspartate- and β-hydroxyasparagine-containing proteins are considered, it is found that, apart from the numerous low affinity sites which can be assigned to the γ-carboxyglutamate (gla)-containing domains, there are also gla-independent Ca^{2+} binding sites with dissociation constants of 10−100 μM in factor IX, factor X, protein C, protein S and complement Clr (Morita *et al.*, 1984, 1985; Sugo *et al.*, 1984b, 1986; Esmon *et al.*, 1983; Villiers *et al.*, 1980). In the cases of protein C and Clr the binding of Ca^{2+} to these sites has been shown to be essential for interactions with other proteins (Johnson *et al.*, 1983; Villiers *et al.*, 1980; Arlaud *et al.*, 1985). The number and location of these sites are consistent with the hypothesis that they involve β-hydroxyaspartate or β-hydroxyasparagine. However, the high affinity of the Ca^{2+} binding suggests that other residues are involved.

The effect of all the mutations described here, with the exception of mutation 225, is to cause a large (>10-fold) reduction in the total coagulant activity of the expressed factor IXs. In the case of 225, the conservative replacement of glu^{78} by aspartate resulted in a mere 2-fold reduction showing that the glu^{78} was satisfactorily substituted by aspartate.

When the activation of the mutant factor IXs by factor XIa was examined, the time courses of activation were found to be essentially identical to that for normal factor IX, either at 15 mM or 0.5 mM Ca^{2+}. However, when the same assay was used to analyse the activation of factor IX from patient haemophilia $B_{Oxford\ 3}$ (Bentley *et al.*, 1986), no activation was detected, showing that this is a valid analysis of factor IX activation and will detect a significant reduction in activation rate if it occurs (D.J.G.Rees, unpublished data).

The assay for factor X activation in the presence of factor VIII, Ca^{2+} and phospholipids relies on the release of the labelled factor X activation peptide after a single proteolytic cleavage (Hultin, 1982). The system used here for normal human factor IX, was shown to be dependent on Ca^{2+}, phospholipids (data not shown) and factor VIII (Figure 6), the rates of activation being very low in the absence of any of these components. For the mutant factor IXs analysed in this system, however, the rates of factor X activation were found to be reduced significantly compared with the rates obtained with equivalent amounts of normal factor IXa. When the assays were performed in the absence of factor VIII however, the normal and mutant factor IXa appeared to have similarly low activities, although we cannot exclude the possibility of small differences in activation, because of the insensitivity of our assay. For the mutants with the lowest clotting activity (e.g. 217), the addition of factor VIII did not increase the initial rate of reaction significantly (see Figure 7). However, for those mutants with significant residual clotting activity (e.g. 223), the rate of activation of factor X was increased in the presence of factor VIII (Figure 7).

The observation that, for mutants with significant residual clotting activity (e.g. 223), the rate of factor X activation is increased by the addition of factor VIII, suggests that the mutation affects the factor IX structure in such a way as to disrupt the interaction between factor IXa and one of the other components, either singly or in combination, −specifically Ca^{2+}, factor X, factor VIII and phospholipid. We prefer the hypothesis that the mutants interfere directly with factor VIII binding, because the effect of the mutation was not apparent when the factor X activation assay was carried out in the absence of factor VIII. However, we cannot exclude that a direct interaction of the first EGF domain of factor IX with Ca^{2+}, factor X or phospholipid could secondarily result in the observed kinetics of activation of factor X, either in the presence or absence of factor VIII. Others (Bajaj *et al.*, 1985) have suggested that factor VIII binds to the heavy chain of factor IX, which conflicts with our hypothesis. However, it is possible that there are multiple binding sites for factor VIII on the factor IX molecule. The EGF domain of factor IX may also be involved in binding to an endothelial receptor which may enhance coagulation *in vivo* (Nawroth *et al.*, 1986).

Relationship to factor $IX_{Alabama}$ and factor $IX_{Eindhoven}$

One of the mutants described here (220) was constructed to provide an *in vitro* equivalent of the previously characterized mutant factor $IX_{Alabama}$. This mutant, aspartate 47 to glycine (Davis *et al.*, 1987), has 8−10% coagulant activity and is normally activated by factor XIa to generate a factor IXa with reduced activity. The interaction of this factor IX with phospholipids was normal, but the activation of factor X either in the presence or absence of factor VIII was found to occur at a reduced rate, relative to the normal protein (Briet *et al.*, 1982; Jones *et al.*, 1985). The level of β-hydroxyaspartate was found to be normal in this variant (McGraw *et al.*, 1985). Thus, the clotting activities of the patient factor IX and the mutant factor IX 220 generated in this work are essentially similar, although we have not compared their properties in factor X activation.

Another factor IX mutant, factor $IX_{Eindhoven}$, has been characterized enzymologically (Mertens *et al.*, 1983) and

found to have 1% coagulent activity, with the defect occurring in the factor VIII dependence of the factor X activation. The amino acid substitution in this mutant is unknown, but could be at one of the carboxylate residues mutated in this study.

Homology to EGF tertiary structure

The solution structures for both human and mouse EGFs have recently been determined (Cooke *et al.*, 1987; Montelione *et al.*, 1987). There is an antiparallel β sheet formed from residues equivalent to residues 60–73 in human factor IX, to which a third antiparallel strand (equivalent to residues 47–50) is loosely attached. The structure of these β sheets is such that the side chains of residues 47, 49 and 64 in factor IX would be expected to lie on the same face of the sheet, in close proximity to one another. However, residue 78 is located on the opposite face of the structure and cannot interact with the other three residues, unless major conformational changes occur.

The presence of β-hydroxyaspartate in homologous domains in a number of plasma proteins and the correlation between the occurrence of these sequences and Ca^{2+} binding sites with dissociation constants of 10–100 μM, suggests that β-hydroxyaspartate may be involved as a ligand for Ca^{2+}. Evidence that β-hydroxyaspartate, alone, is not critical for high affinity Ca^{2+} binding (Morita and Kisiel, 1985) may be rationalized by suggesting that either aspartate or β-hydroxyaspartate can bind Ca^{2+} equally effectively. The presence of the two conserved aspartate residues on the same face of the β sheet would provide additional ligands for a Ca^{2+}. In the EGF structures the third strand of the β sheet may only exist in this conformation for a small proportion of the time (Cooke *et al.*, 1987) and it is possible therefore that the role of a bound Ca^{2+} ion at this position could be to stabilize the interaction between the strands of the β sheet and maintain the active conformation of the protein in this region. The evidence presented here suggests that alteration of the residues forming this structure interferes with the interaction between factor IXa and factor VIIIa and this may be a consequence of the destabilization of the active tertiary conformation resulting from the loss of Ca^{2+} binding. This Ca^{2+} dependent conformation would therefore differ significantly from those involving the gla domain (Nemerson and Furie, 1980; Liebman *et al.*, 1897) in that with a K_d of 10–100 μM the Ca^{2+} binding site would essentially be saturated at the normal plasma concentration of Ca^{2+} (~1.5 mM) and would therefore act as a structural determinant only. By contrast, the lower affinity Ca^{2+} sites in the gla domain appear to trigger the association of vitamin K-dependent proteins with negatively charged phospholipids in a fashion which may require conformational changes.

Equivalent sites in other proteins

The third type C group of sequences shown in Figure 3 are of EGF-related domains from a variety of proteins, none of which is vitamin K-dependent. All contain aspartate or asparagine at the consensus position for hydroxylation and at least two and usually three of the other consensus residues seen in the EGF, type B sequences. Except for the sea urchin uEGF-1 sequence, these represent only some of the EGF-related domains in these proteins. In the *Notch* product in *Drosophila* a further 12 domains have these consensus residues, with another 19 domains having the characteristics of the type A EGF sequences. The other EGF-related domains in thrombospondin, thrombomodulin, the LDL receptor, the EGF precursor and uromodulin are all more closely related to the type A sequences. We predict that these type C sequences are hydroxylated on the appropriate aspartate or asparagine residue (see Figure 3) and bind Ca^{2+}. Recently amino acid analysis has shown that uromodulin, thrombospondin and thrombomodulin (Przysiecki *et al.*, 1987) contain detectable levels of β-hydroxyasparagine, although the exact location remains to be determined.

The potential role of Ca^{2+} in these proteins is not known, but it is of interest that several of the mutations characterized in the *Notch* gene (Kelley *et al.*, 1987; Hartley *et al.*, 1987) affect residues which would be predicted, from our analysis, to be important for Ca^{2+} binding. This may prevent correct protein–protein interactions and so affect the interaction of the product of *Notch* with the products of other neurogenic loci, resulting in the mutant phenotypes observed. The presence of similar sequences in the product of the *Delta* gene in *Drosophila* (Vassin *et al.*, 1987) would suggest that these structures may be widespread in proteins with functions in development, as well as having important roles in protein–protein interactions in the coagulation and complement systems of higher organisms.

Materials and methods

Cell culture

MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FCS). 24 h after transfection, the medium was replaced by DMEM/10% FCS containing geneticin (Gibco) at 800 μg/ml and the cells grown until colonies were visible. Single colonies were then picked, cloned and assayed by ELISA (see below) for secretion of factor IX antigen into conditioned medium. Cloned cell lines producing the largest amounts of antigen were selected for larger-scale 1 l spinner culture on Cytodex 3 microcarrier beads (Pharmacia) and conditioned medium was harvested every 5–10 days, centrifuged and stored at −20°C in 0.4% trisodium citrate, 1 mM phenylmethylsulphonyl fluoride (PMSF) before processing.

Purification of human factor IX, ELISA and clotting assays

Human factor IX was purified from normal human plasma, or from conditioned medium by adding trisodium citrate to 0.4% w/v and PMSF to 1 mM (Radcliffe and Nemerson, 1976). 1/10th vol of 30% w/v barium chloride was added slowly and stirred for 30 min at 4°C. The precipitate was recovered by centrifugation and resuspended in 5 mM $BaCl_2$, 1 mM PMSF and stirred for 20 min at 4°C. The precipitate was recovered by centrifugation and washed again in 5 mM $BaCl_2$, 1 mM PMSF. Adsorbed proteins were eluted from the barium citrate with 1/10 vol of 0.1 M EDTA pH 8.0, 1 mM PMSF at 4°C for 20 min and the remaining insoluble material was removed by centrifugation. The supernatant was made 10% with saturated ammonium sulphate and the resulting barium sulphate precipitate removed by centrifugation. The supernatant was then dialysed against 100 mM EDTA, 20 mM Tris–Cl pH 7.2, then against 150 mM NaCl, 20 mM Tris–Cl pH 7.2, 1 mM EDTA, and then twice against 150 mM NaCl 20 mM Tris–Cl pH 7.2 (TBS). $MgCl_2$ was added to 10 mM final concentration and the solution was passed over an immunoaffinity column, prepared using monoclonal antibody A7 (Smith and Ono, 1984) coupled to Affigel-10 (BioRad), at approximately four column volumes per hour. The column was then washed in 150 mM NaCl, 20 mM $MgCl_2$, 20 mM Tris–Cl, pH 7.2 (TBS/Mg) for five column volumes, and then in 1 M NaCl, 20 mM $MgCl_2$, 20 mM Tris–Cl pH 7.2 for 20 column volumes, and finally in TBS/Mg until the A_{280nm} of the eluate reached a constant baseline. The factor IX was then eluted from the column using 150 mM NaCl, 20 mM EDTA, 20 mM Tris–Cl pH 7.2 at 0.5 column volume per hour, in 1 ml fractions made 1 mg/ml bovine serum albumin (BSA) to stabilize the factor IX against denaturation. Factor IX was assayed by ELISA (Anson *et al.*, 1985) using the monoclonal antibody 3A6 and those fractions containing significant amounts of factor IX were pooled, dialysed twice against TBS and stored at −20°C. The final antigen concentration of the pooled, dialysed samples was determined by ELISA. Factor IX clotting assays were performed in duplicate using a one-stage assay (Austen and Rhymes, 1975) using pooled normal human plasma as a standard.

Expression constructs and site-directed mutagenesis

The factor IX expression plasmid pKG5IX1 was constructed by ligating the 1.55-kb *Bam*HI to *Hin*dIII full length factor IX cDNA clone cVI (Anson *et al.*, 1984) into the *Bam*HI–*Hin*dIII polylinker of the expression plasmid pKG5 (Gould *et al.*, 1987), such that the factor IX expression is under control of the SV40 early promoter/enhancer and poly(A) site. cVI contains nucleotide residues 25–1572 of the mRNA with residues 25–40 inverted, due to a cloning artefact. The first methionine available for translation is amino acid −41 (see Anson *et al.*, 1984). Site-directed mutagenesis was carried out in M13K19 (Carter *et al.*, 1985), after transferring the full length factor IX cDNA insert as an *Xho*I to *Hin*dIII fragment from pKG5IX1. After selecting and identifying mutant M13 phage by hybridization to the mutagenic primer, RF DNA was isolated, restriction mapped and the *Hin*dIII to *Bam*HI insert was recloned into the expression plasmid pKG5. The *Xho*I–*Hin*dIII inserts from these mutants were sequenced completely except for the region corresponding to the 3'-non-coding region of the mRNA, by recloning into M13K19 followed by dideoxy sequence analysis using a set of eight factor IX-specific oligonucleotide primers placed at ~150 nt intervals along the cDNA coding sequence (Rees, 1986).

Western blotting

Monoclonal antibody A7 (Smith and Ono, 1984) was labelled with ^{125}I using sodium 125-iodide and chloramine T. The labelled protein was separated from the reaction by gel filtration on Sephadex G-25 and the peak fractions of the flow-through were pooled. Specific activities of 10^7 d.p.m./μg protein were obtained. 10% SDS–polyacrylamide (Laemmli) gels were run and proteins were transferred to nitrocellulose (Amersham) by electroblotting in 20 mM Tris, 150 mM glycine, 20% methanol at 200 mA/50 V for 6 h. After transfer the filters were blocked with 5% w/v dried milk powder in 150 mM NaCl, 20 mM Tris–Cl pH 7.4, 1 mM $MgCl_2$, 5 mM $CaCl_2$ (TBS/Ca/Mg). The filters were then incubated with ^{125}I-labelled monoclonal antibody A7 for 4 h and then the filters were washed extensively in TBS/Ca/Mg before autoradiography.

β-Hydroxyaspartate analysis

This was carried out according to Fernlund and Stenflo (1983) on purified recombinant factor IX from clone C6 (as above except that no BSA was added to the purified factor IX), and on heparin–Sepharose purified factor IX from human blood.

Factor IX activation analysis

'Contact product' was prepared from human factor IX-deficient plasma (Austen and Rhymes, 1975). Factor IX activation was analysed using 20 μl of contact product and ~500 ng of purified test factor IX in 150 mM NaCl, 15 mM $CaCl_2$, 20 mM Tris–Cl, pH 7.4 at 37°C in 200 μl reaction. Duplicate 15 μl aliquots were removed at 15 min to 2 h time intervals stopping the reaction by adding 5 μl 0.1 M EDTA, pH 7.4. Samples were then analysed on 10% SDS–PAGE and immunoblotted with ^{125}I-labelled monoclonal antibody A7. Control reactions were performed with 15 mM EDTA replacing 15 mM $CaCl_2$. Selected mutants (19, 56, 220 and 223) were also analysed with 0.5 mM $CaCl_2$ replacing 15 mM $CaCl_2$.

Factor X activation assay

Between 150 and 750 ng of activated factor IX (see above) was used to activate [^3H]bovine factor X in a reaction mixture containing 2.5 units/ml human factor VIII (gift from Dr Rizza), 0.2 mg/ml inosithin (Uniscience Ltd), 50 μg/ml [^3H]bovine factor X (sp. act. 1.23×10^8 d.p.m./μg), in 1 ml of 16.6 mM Tris–Cl, pH 7.5, 5 mM $CaCl_2$ at 37°C. 100 μl were removed at various times, added to 800 μl 50 mM EDTA, 100 mM NaCl, 50 mM Tris–Cl, pH 7.5, 0.1% BSA and precipitated with 5% trichloroacetic acid (TCA). The precipitate was centrifuged and an aliquot of the supernatant analysed by liquid scintillation counting. Counts per minute were corrected for the background by subtracting the 0 time point and converted to ng factor X divided by ng factor IXa, as determined by ELISA (see above).

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