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The binding of natural variants of human factor IX to endothelial cells

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Abstract

The Gla-domain of human factor IX contains a specific element required for the binding of factor IX to an endothelial cell surface protein. We have investigated the dependence of this interaction on the structural integrity of the adjacent hydrophobic stack and epidermal growth factor-like domains. The ability of purified natural variants of human factor IX to compete with wild-type factor IX binding to the endothelial cell surface was used to obtain apparent K_i values of the variants. Our data suggest that the functional integrity of the Gla domain, enabling factor IX to specifically interact with an endothelial cell surface protein, depends on the structural and functional integrity of both the hydrophobic stack domain and the first epidermal growth factor-like domain.

Key words: Factor IX; Endothelial cell binding; γ-Carboxyglutamate; EGF-like domain

1. Introduction

Human factor IX is a 56 kDa glycoprotein that plays a key role in the blood coagulation cascade [1]. Activated factor IX (FIXa) catalyses the conversion of factor X to its active form, factor Xa, in a reaction requiring calcium ions, phospholipid (a membrane surface) and the cofactor factor VIIIa. Defective factor IX or a deficiency thereof results in the blood clotting disorder haemophilia B. The mature form of human factor IX is made up of five domains [2]. The amino-terminal domain contains 12 glutamate residues which are γ-carboxylated in a vitamin K-dependent reaction giving γ-carboxyglutamate (Gla), a post-translational modification which is essential for activity [3]. The C-terminal end of the Gla domain consists of eight residues which are frequently referred to as the hydrophobic stack domain and are coded by a separate exon. Both of the domains that follow the hydrophobic stack contain the highly conserved arrangement of 6 cysteine residues characteristic of human epidermal growth factor. The first of these epidermal growth factor-like (EGF-like) domains contains a high-affinity calcium-binding site [4]. Furthermore, this domain contains three post-translational modifications. It has been demonstrated that Ser-53 and and Ser-61 are O-glycosylated [5,6], while 30% of all human factor IX molecules are β-hydroxylated at Asp-64 [7]. The second EGF-like domain is followed by the activation peptide which contains two N-linked carbohydrates at residues Asn-157 and Asn-167. The activation peptide is cleaved between residues 145–146 and residues 180–181 either by the action of factor VIIa or by factor XIa resulting in the active, two-chain form of factor IX, factor IXa. Finally, the carboxy-terminal domain of human factor IX contains the catalytic site and has recently been shown to contain a high-affinity calcium-binding site [8].

After activation, the four amino-terminal domains form the light chain of factor IX which is disulphide-bonded to the heavy chain containing the catalytic domain. While the function of the catalytic domain is clear, the functions of the light chain domains are less well understood. Mutagenesis and domain-exchange experiments have implicated both EGF-like domains in the interaction between factor IX with both factors VIII and X [9,10]. The Gla domain is known to be required for the interaction of factor IX with phospholipid [11]. The Gla residues, which form low-affinity calcium-binding sites [12,13], are believed to form an area of high local negative charge which could allow non-specific calcium bridging with the negatively charged head groups of the endothelial membrane surface [14].

The Gla domain has also been shown to contain a recognition element for the specific binding of factor IX to an endothelial cell surface protein [9,15,16]. The presence of a human factor IX binding protein on the endothelial cell surface was initially described by Stern et al. [17]. These studies demonstrated that both the zymogen and activated forms of human factor IX bound to cultured endothelial cells with a K_d of 2.3 nM. The isolated

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Gla domain from bovine factor IX inhibited the binding of full-length factor IX to endothelial cells with a K_i of 60 nM while a synthetic peptide corresponding to the first EGF-like domain of factor IX inhibited the binding with a K_i of only 35 μM [15]. The role of the Gla domain in endothelial cell binding was confirmed by Astermark and Stenflo [9], who demonstrated that while a proteolytically-derived peptide corresponding to the Gla domain linked to both the EGF-like domains inhibited the binding of factor IX to endothelial cells, Gla-domainless factor IX did not inhibit the binding. More recently, it was shown that a chimeric factor IX molecule containing the Gla domain of factor VII failed to inhibit factor IX binding to endothelial cells, while a chimeric factor VII containing the Gla domain from factor IX inhibited binding [18]. Finally, Cheung et al. [16] have pin-pointed the specific binding region to residues 3–11 of human factor IX using a combination of chimeric coagulation factors and site-directed mutagenesis.

A specific role of the first EGF-like domain in the interaction of factor IX with the endothelial cell surface protein has been ruled out [19]. However, an emerging feature from studies on coagulation factors is the interdependence of the domains [20]. To investigate whether the Gla domain is dependent on any properties of the adjacent EGF-like domains for endothelial cell binding, we have studied the ability of 5 natural variants of factor IX to inhibit the binding of wild-type factor IX to endothelial cells.

2. Materials and methods

2.1. Protein purification

All five factor IX mutants were obtained as 25 ml plasma samples from the patients listed in Table 1. Each of the plasma samples was assayed for factor IX antigen by ELISA [21] using the antibody 3A6 [22]. The factor IX variants were purified in a single step by affinity purification on affigel-10 (Bio-Rad) to which either 3A6 or the divalent cation-dependent antibody A7 [23] had been covalently coupled (according to the manufacturer's instructions). Each plasma sample was buffer exchanged into 1 × TBS (150 mM NaCl, 20 mM Tris-HCl pH 7.2), run onto a 0.5 ml affinity column which was subsequently washed with 1 × TBS. For each A7 column, the 1 × TBS was supplemented with 20 mM $MgCl_2$. Factor IX bound to the A7 column was eluted with 1 × TBS containing 20 mM EDTA while factor IX bound to the 3A6 column was eluted with 3 M NaSCN. The factor IX samples were then dialysed against 1 × TBS and concentrated in a centricon-30 microconcentrator (Amicon). The clotting activities of the purified factor IX mutants were assayed in a one stage clotting assay [24]. Wild-type factor IX was labelled with ^{125}I using the enzymobead reagent (Bio-Rad) according to the manufacturer's instructions.

2.2. Western blot analysis

After 12% polyacrylamide gel electrophoresis [25] proteins were transferred onto nitrocellulose by electroblotting in 25 mM Tris, 150 mM glycine, 20% methanol at 100 V for 1 h. The filters were blocked in 1 × TBS (150 mM NaCl, 20 mM Tris-HCl pH 7.2) containing 0.1% Triton X-100 and 5% low fat milk powder and incubated for 1 h with either the monoclonal antibody 3A6 or A7. The filters were washed with 1 × TBS and incubated for one hour with a 1 in 1000 dilution of anti-mouse IgG conjugated to horse radish peroxidase (Sigma) in 1 × TBS/0.1% v Triton X-100/5% milk powder. All solutions were supplemented with 5 mM $CaCl_2$ if the antibody A7 was used. The filters were developed using the ECL detection reagent (Amersham).

2.3. Endothelial cell culture

Human umbilical vein endothelial cells were isolated by standard methods [26,27]. The cells were cultured on gelatin-coated tissue culture flasks in M199 (Gibco-BRL) supplemented with 20% heat-inactivated foetal calf serum. The cells were subcultured by trypsin treatment. All experiments were performed on passage 3 cells.

2.4. Endothelial cell binding assay

Endothelial cells were grown in 6 mm wells until they were 1 day pre-confluent. The medium was then replaced with serum-free medium containing 4% Ultroser G (Gibco-BRL) and the cells incubated for another 24 h. The cells were washed once with binding buffer (137 mM NaCl, 4 mM KCl, 5.5 mM glucose, 5 mg/ml bovine serum albumin, 2.5 mM $CaCl_2$ and 10 mM HEPES, pH 7.45) and incubated with 50 μl of binding buffer containing 3.5 nM [^{125}I]factor IX and increasing amounts of the competing factor IX variants. After 60 min incubation at room temperature, the cells were washed extensively with binding buffer and then solubilised by incubation in 50 μl solubilisation buffer (0.2 M NaOH, 1% SDS, 10 mM EDTA). The cell extracts were counted in an LKB 1261 Multigamma counter.

3. Results and discussion

The natural variants of factor IX were all selected from the haemophilia B database [28] according to a number of criteria. Since only 25 ml of plasma was available, we selected patients with near normal factor IX antigen levels (100% or 5 μg/ml), so that sufficient factor IX could be purified for our studies. Also, the ease of obtaining the plasma samples and the patients' HIV status had to be taken into account. Table 1 shows the 5 mutants selected. All, except Lys-43→Glu, were puri-

Table 1
Characteristics of the factor IX variants

Factor IX variants	Factor IX antigen[a]	Specific clotting activity of plasma[b]	Specific clotting activity of purified factor IX[d]	Patient identification number[e]
Wild-type	100	100	100	–
Phe-9→Ile	100	14	90	51
Lys-43→Glu	90	15.5	150	83
Asp-47→Glu	84	1.2 or 16.7[c]	10	90
Asp-64→Asn	110	2.7	10	114
Val-107→Ala	114	17.5	20	133

[a]Determined by 3A6 ELISA (see section 2), and expressed as a percentage of the antigen found in a pool of normal human plasma (5 μg/ml). ll values compare well with the published figures [28].
[b]The percent clotting activities [28] were recalculated as specific clotting activities, defined as percent clotting activity divided by percent antigen (results column 2). Results are expressed as a percentage.
[c]Two different figures have been calculated [28] the first from patient number 90 (used here) and the second from patient number 89 with an identical mutation. An independent assay of the plasma of patient 90 (see section 2) gave a figure of 8.3%.
[d]The specific clotting activities of the purified factor IX variants expressed as a percentage of normal factor IX (100%).
[e]According to [28].

fied using an affinity column of coupled monoclonal antibody A7 which recognises a divalent metal ion-dependent epitope (see section 2). Lys-43→Glu does not retain the A7 epitope and was, therefore, purified using an affinity column of coupled monoclonal antibody 3A6 (see methods). The specific clotting activities of the purified factor IX variants were assayed and compared to the specific clotting activities calculated from the haemophilia B database [28] as shown in Table 1. Interestingly, both purified Phe-9→Ile and Lys-43→Glu had clotting activities within the normal range despite the much lower specific clotting activities of the plasma samples. It is possible that the divalent metal ion-dependent antibody, A7, used in the purification of Phe-9→Ile, selected for the more active subset of factor IX molecules with higher levels of γ-carboxylation. However, this apparent selection would not diminish any local structural changes that the mutation might cause, and therefore this variant remained of interest.

Western blot analysis (Fig. 1) of the five purified factor IX variants demonstrated that each of the mutants, except Lys-43→Glu, was predominantly present in its zymogen form. Lys-43→Glu, however, had been almost completely activated during the purification procedure, giving heavy chain and light chain products (Fig. 1). This activation could account for the observed high specific clotting activity (150%) since the lag phase of the one stage clotting assay [24] would be dramatically reduced. Wild-type factor IX purified on a 3A6 column was also partly activated suggesting that the method of purification may cause part activation. Since both factor IX and its activated form, factor IXa, have the same affinity for the endothelial cell surface binding protein [17], this Lys-43→Glu variant remained of interest.

The ability of the five factor IX variants to inhibit the binding of iodinated wild-type factor IX to endothelial cells was assayed as before [29]. All binding assays were carried out on confluent monolayers of passage 3 endothelial cells in 6 mm wells (see section 2). The apparent affinity of the endothelial cells for factor IX was determined by performing a simple saturation binding experiment in which half maximal binding occurred at 15 nM factor IX (not shown). A wild-type factor IX control competition experiment was carried out in parallel with each factor IX variant tested. The data were analysed by plotting the fractional inhibition of [^{125}I]factor IX binding to endothelial cells against the concentration of competitor added. Apparent K_i values were obtained by fitting the data iteratively to

$$\delta = \frac{[\text{Competitor}]_{\text{Total}}}{K_i + [\text{Inhibitor}]_{\text{total}}}$$

where δ = fractional inhibition. Fig. 2 illustrates such curves derived for the mutants Phe-9→Ile and Val-107→Ala. The apparent K_i values obtained are listed in Table 2. All of the variants except Val-107→Ala, which

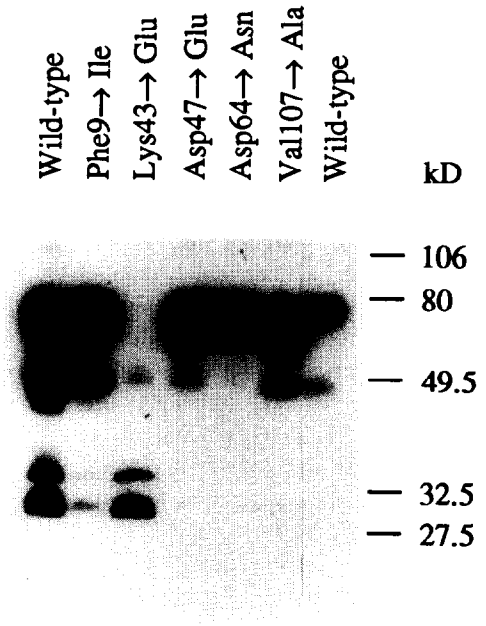

Fig. 1. Western blot of the purified factor IX variants. Two wild-type factor IX controls are included. The wild-type factor IX in the far left lane was purified on a 3A6 affinity column, while that in the far right lane was purified on an A7 affinity column (see section 2).The positions of control molecular weight protein standards are indicated. Due to the presence of N- and O-linked carbohydrate, intact factor IX has an apparent molecular weight of ~ 75 kDa, the light chain ~ 30 kDa, the heavy chain ~ 34 kDa.

is a mutation within the second EGF-like domain, are impaired in their ability to compete with wild-type factor IX for endothelial cell surface binding. Val-107→Ala competed as well as wild-type factor IX for endothelial cell surface binding (Fig. 2b). This result is important in two respects. Firstly, it acts as a positive control for the binding of the plasma-purified mutants to the endothelial cell surface, and secondly it suggests that amino acid 107 of the second EGF-like domain is unlikely to be involved directly in the binding of factor IX to the endothelial cell surface.

The factor IX variant Lys-43→Glu had the highest apparent K_i (Table 2) suggesting that the structural integrity of the hydrophobic stack domain of factor IX is important in maintaining the recognition element for endothelial cell binding. While it is possible that this residue is involved directly in an interaction with endothelial cells, this is improbable in light of the data from Astermark et al. [30], who show that a peptide comprising residues 1–37 of bovine factor IX competes efficiently (K_i~180 nM) with intact bovine factor IX for endothelial cell surface binding. The variants Phe-9→Ile, Asp-47→Glu and Asp-64→Asn also have increased apparent K_i values. Although these increased values cannot be interpreted as identifying the residues directly involved in the binding to endothelial cells, they do suggest that changes in local structure or chemical character at these

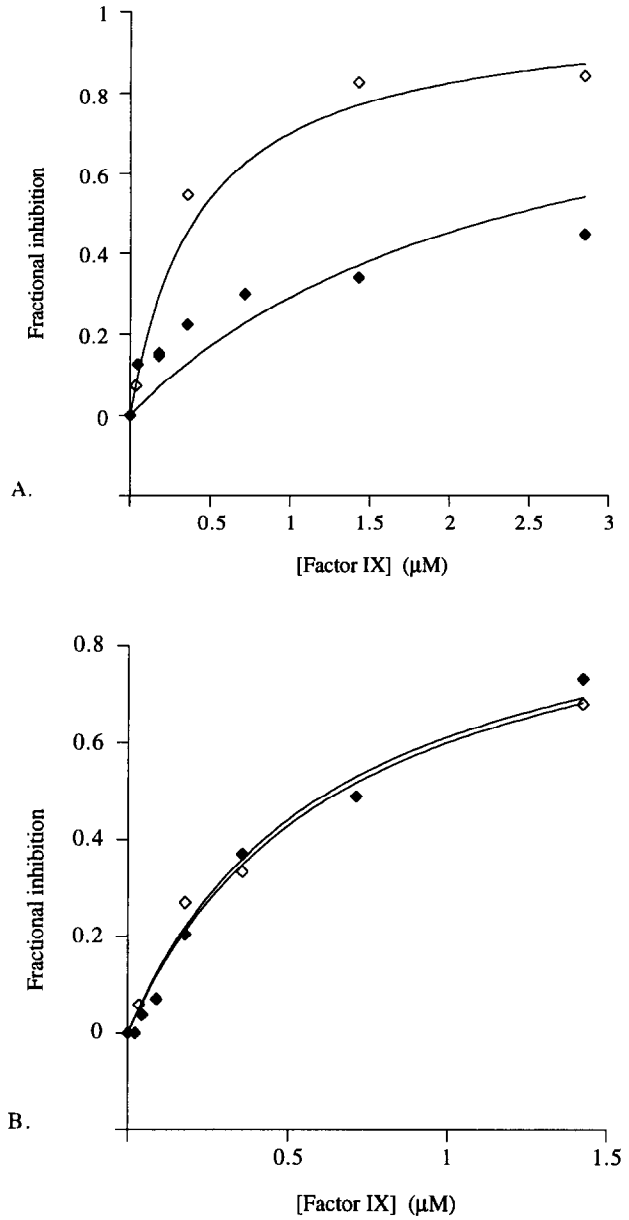

Fig. 2. Inhibition of human [^{125}I]Factor IX binding to endothelial cells by the human factor IX variants (A) Phe-9→Ile and (B) Val-107→Ala. ◇ = wild-type human factor IX, ◆ = variant factor IX. The curves were fitted as described in the text. Each experiment was performed once in triplicate and the mean of the values obtained plotted.

positions indirectly effect this binding. Residues Asp-47 and Asp-64 of the first EGF-like domain have both been identified as direct ligands in a calcium ion binding site [31]. The changes Asp-47→Glu and Asp-64→Asn are known to reduce and abolish calcium binding, respectively, [31]. Although the first EGF-like domain is not known to contain any elements that confer specificity of binding to the endothelial cell surface binding protein [16], our data suggest that the structural integrity or stability of this domain, conferred by calcium-binding, is important for optimal binding of factor IX to the endothelium.

It has recently been demonstrated by two different approaches that the Gla domain interacts with the first EGF-like domain. Firstly, studies of calcium-binding to the Gla domains of bovine factors IX and X have shown that the isolated domains bind calcium less well than the intact proteins. However, when the Gla domains are linked to their respective EGF-like domains, the affinity of the calcium-binding sites are similar to those of the intact proteins [30,32]. Thus, the first EGF-like domain cooperates with the Gla domain in forming the optimal structure for calcium binding. Secondly, thermodynamic analysis of the temperature-induced melting of factor IX has demonstrated that there is a strong interaction between the Gla and first EGF-like domains, but that the interaction between the two EGF-like domains is much weaker [20].

Thus the reduced ability of the natural factor IX variants studied here to compete with wild-type factor IX for the binding to the endothelial cell surface suggests that these mutations indirectly affect the recognition element within factor IX by disrupting the stability of the Gla-first EGF-like domain interaction. Since the specific binding of human factor IX to endothelial cells is reported to be mediated by residues 3–11 of the Gla domain [16], the mutation Phe-9→Ile may affect the binding site directly, while the mutations Lys-43→Glu, Asp-47→Glu and Asp-64→Asn may affect the interaction between factor IX and the endothelial cell surface binding protein by destabilising the interaction between the Gla domain and the first EGF-like domain, as discussed above. The study by Vysotchin et al. [20] demonstrated that the second EGF-like domain only interacts weakly with the first EGF-like domain and therefore, it is unlikely that small structural alterations in the second EGF-like domain will have an effect on the endothelial cell binding determinant of factor IX. This is indeed what we have observed with variant Val-107→Ala. Furthermore, it should be noted that this variant retains only 20% of normal clotting activity implying that the mutation is not simply silent but has a detrimental effect on a different aspect of factor IX function.

In summary, our results are consistent with previously published data describing the identification of the bind-

Table 2
Apparent K_i values for the inhibition of [^{125}I]factor IX binding to the endothelial cell surface

Competitor	Apparent K_i (μM)
Wild-type factor IX	0.4–0.6
Phe-9→Ile	2.4
Lys-43→Glu	7.4
Asp-47→Glu	3.5
Asp-64→Asn	2.9
Val-107→Ala	0.66

ing determinants on factor IX for the endothelial cell surface ([15,16,18,19]. Our results suggest that a precise interaction between the first EGF-like domain and the Gla domain is required for optimal binding. Furthermore, we have demonstrated a mutation in the second EGF-like domain, while clearly being detrimental to factor IX activity, has no effect on the interaction of factor IX with the endothelial cell surface. This is consistent with Vysotchin et al. [20], suggesting a minimal functional interaction between the second EGF-like domain and those domains N-terminal to it. Thus, we conclude that while a single domain may contain the element that confers specificity of binding, the optimal conformation required by the binding element may depend on an interaction with a number of adjacent domains.

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