The Endothelial Cell Binding Determinant of Human Factor IX Resides in the γ-Carboxyglutamic Acid Domain†

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ABSTRACT: The blood coagulation factor IX(a) binds specifically to a site on endothelial cells with a K_d of 2.0–3.0 nM. A number of previous studies have attempted to define the region(s) of factor IX(a) that mediate this interaction. These studies suggested that there are two regions of factor IX(a), the γ-carboxyglutamic acid (Gla) domain and the epidermal growth factor like (EGF-like) domains, that mediate high-affinity binding to endothelial cells. Recently, however, the participation of the EGF1 domain has been excluded from the interaction. This indicated that if there was an EGF component of factor IX contributing to the binding affinity, then it must be in the second EGF-like domain. In order to further evaluate this relationship, we performed competitive binding experiments between ^{125}I plasma factor IX and a set of six chimeric proteins composed of portions of factor VII and factor IX. Our data suggest that the high-affinity interaction between factor IX and the endothelial cell binding site is mediated by the factor IX Gla domain and that the factor IX EGF domains are not involved in binding specificity.

Human factors IX and IXa possess a specific binding site on human and bovine endothelial cells (Stern et al., 1983; Heimark & Schwartz, 1983). The binding site is thought to be a 140 kDa protein (Rimon et al., 1987). The site is half-maximally occupied at 2.3 nM factor IX(a) (Stern et al., 1983) and preferentially binds factor IXa in the presence of the clotting factors VIII and X (Stern et al., 1985). Additionally, the bound factor IXa retains coagulant activity (Stern et al., 1984). Several studies have been conducted in an effort to determine the region of factor IX(a) mediating this interaction. Peptides representing the factor IX γ-carboxyglutamic acid (Gla) domain and the first loop of the first epidermal growth factor like (EGF-like) domain have been shown to specifically compete with plasma factor IX for the binding site with K_i's of ~60 nM and 10 μM, respectively (Ryan et al., 1989). Another study utilizing a Gla peptide (Derian et al., 1989) reported an IC_{50} of 30 nM. In the same study, Gla-domainless factor IX and descarboxy factor IX (uncarboxylated factor IX) showed no competitive binding for the site. Recently, Astermark et al. (1991), using proteolytic fragments of factor IX corresponding to the Gla domain or both EGF-like domains, obtained binding constants of 100 nM and 10 μM, respectively. Additionally, by comparing the binding constants of a factor IX peptide representing the entire factor IX light chain with that of native factor IX, they inferred that the heavy chain of factor IX did not possess a high-affinity recognition determinant for the endothelial cell binding site. The combined data suggested that there were two regions of factor IX that provide endothelial cell binding specificity, the Gla domain and one or both of the EGF domains. However, shortly thereafter, Cheung et al., (1991), using factor IX/X chimeric proteins in competitive binding experiments, showed that the aromatic stack domain and the first EGF-like domain of factor IX do not participate in the specificity of interaction with the endothelial cell binding site. Thus, if the EGF domains are involved in endothelial cell binding as has been reported (Astermark et al., 1991), then it follows that the determinant is in the second EGF domain. In an attempt to resolve the question of factor IX EGF2 domain participation in endothelial cell recognition, we used six factor VII/IX chimeric proteins (Toomey et al., 1991) in competitive binding experiments with ^{125}I plasma factor IX for the endothelial cell binding site. The data gleaned in this study establish the Gla domain as the high-affinity endothelial cell recognition determinant of factor IX(a) and suggest that the factor IX EGF domains do not play a role in the specificity of interaction.

Materials and Methods

Materials

Tissue culture plasticware was purchased from the Costar Corp. (Cambridge, MA). Human factor IX was purchased from Enzyme Research Laboratories (Southbend, IN). Na^{125}I was purchased from the Amersham Corp. (Arlington Heights, IL). Iodobeads were purchased from Pierce (Rockford, IL). Bovine aortic endothelial cells were a gift from Dr. Charles Esmon and Dr. Naomi Esmon of the Oklahoma Medical Research Foundation (Oklahoma City, OK).

Methods

Creation of the Recombinant Proteins. The molecular biology, expression, purification, and partial characterization of the chimeric proteins were previously described (Toomey et al., 1991).

Iodination of Factor IX. Plasma factor IX was labeled with Na^{125}I using Iodobeads according to the manufacturer's in-

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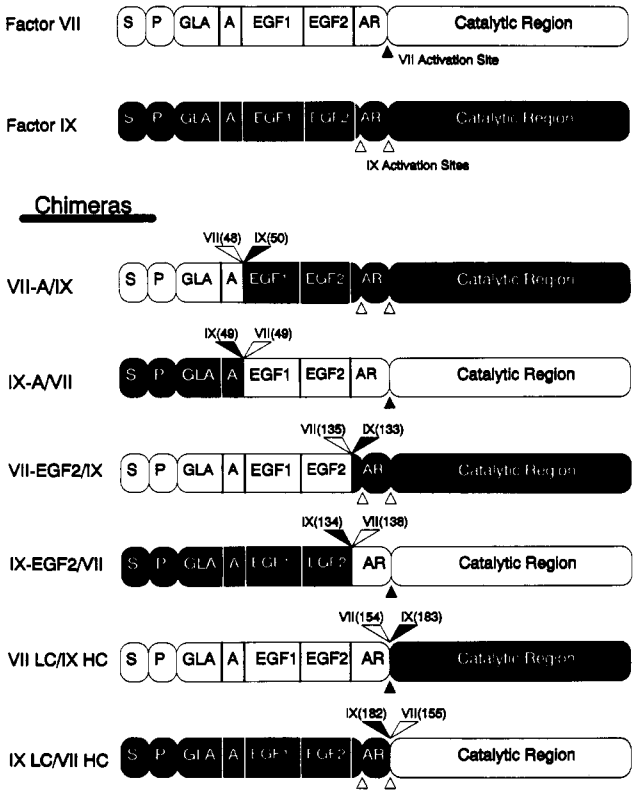

FIGURE 1: Homologous exchanges between factors VII and IX: There are eight recombinant proteins, two wild type and six chimeric. The protein domains are as follows: (S) signal peptide; (P) propeptide; (Gla) γ-carboxyglutamic acid domain; (A) aromatic stack; (EGF1 and EGF2) epidermal growth factor domains; (AR) activation region and a catalytic region. The signal peptide and the propeptide are proteolytically removed during expression. Factor VII and portions of factor VII are in white, whereas factor IX and portions of factor IX are in black. Within the chimeras, the specific adjacent amino acid residues from factors VII and IX that comprise the exchange sites are indicated by numbers. The activation sites are represented as follows: VII activation site (▲); IX activation sites (△).

structions. The radioactively labeled proteins were separated from free ^{125}I on a Sephadex G-25 column. The radiolabeled plasma factor IX had a specific activity of ~2.0 μCi/μg and retained normal clotting activity by activated partial thromboplastin time (aPTT) assay.

Binding Studies. The binding experiments were based upon a previously established method (Stern et al., 1983). Briefly, bovine aortic endothelial cells were grown to confluence in 48-well tissue culture plates in DMEM supplemented with penicillin, streptomycin, and 20% fetal calf serum. The confluent plates were washed 4 times with 0.3 mL of 10 mM Hepes, pH 7.5, 137 mM NaCl, 4 mM KCl, and 11 mM glucose (buffer A) at room temperature. Cells were then incubated in 0.5 mL of incubation buffer [buffer A containing 2 mg/mL bovine serum albumin (BSA) and 2 mM $CaCl_2$ for 15 min at 4 °C]. The incubation buffer was replaced with 0.2 mL of the same buffer, but now containing ^{125}I plasma factor IX (0.5 or 2.0 nM) and increasing concentrations of competitor (plasma factor IX or a recombinant protein from 0.1 to 150 nM). The plates were incubated at 4 °C for 2.5 h. The plates were then washed 5 times with 0.5 mL of 5 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2 mM $CaCl_2$, and 2 mg/mL BSA (buffer B) at 4 °C. The cells were then dissolved in 0.5 mL of 200 mM NaOH, 10 mM EDTA, and 1% sodium dodecyl sulfate for 10–20 min at room temperature. The radioactivity per well was assessed with an LKB minigamma counter (Model 1275). The binding data were evaluated and

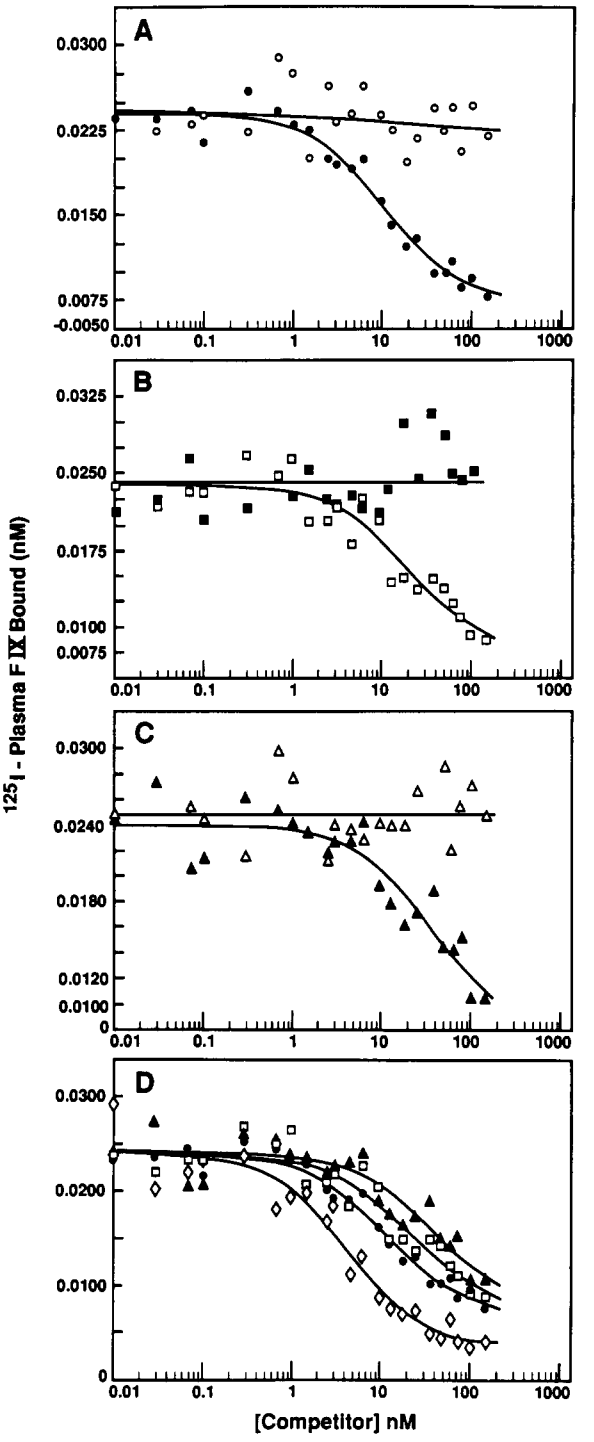

FIGURE 2: Displacement of ^{125}I-labeled plasma factor IX from endothelial cells by (panel A) VII LC/IX HC (○) and IX LC/VII HC (●), (panel B) VII-EGF2/IX (■) and IX-EGF2/VII (□), (panel C) VII-A/IX (△) and IX-A/VII (▲), and (panel D) plasma factor IX (◇), IX LC/VII HC (●), IX-EGF2/VII (□), and IX-A/VII (▲).

K_d values calculated by employing the nonlinear regression program MK model.

Results

Competition for the Endothelial Cell Receptor. In this study, competition experiments for the endothelial cell receptor between the eight recombinant proteins depicted in Figure 1 and ^{125}I plasma factor IX were performed. The competition took place on monolayers of bovine aortic endothelial cells. A fixed concentration of ^{125}I-labeled plasma factor IX was mixed with increasing concentrations of recombinant com-

Table I: K_d Values for Plasma Factor IX, Recombinant Factor IX, and the Six Chimeras[a]

competitors	K_d values (nM)
plasma factor IX	3.4 ± 1.4
recombinant factor IX	2.5 ± 0.14
IX LC/VII HC VII LC/IX HC	7.3 ± 3.4
IX-EGF2/VII VII-EGF2/IX	9.4 ± 2.4
IX-A/VII VII-A/IX	12.5 ± 2.2

[a] Each competition experiment was performed in triplicate for a total of 60 points/competitor. The measure of variation is a standard deviation.

petitors. After an incubation and a series of washes, the concentration of ^{125}I-labeled plasma factor IX remaining bound to the endothelial cells was determined. The initial competition experiments were performed at two concentrations of ^{125}I plasma factor IX, 0.5 and 2.0 nM. The data obtained at either concentration were comparable. A concentration of 2.0 nM was chosen for the remainder of the experiments. The K_d values determined from these experiments are listed in Table I. Recombinant factor VII was included in these experiments but showed no competition at a concentration of 150 nM (not shown). The number of binding sites per cell ranged from 24 000 to 50 000. This range is similar to previously published values (Stern et al., 1985). A best-fit presentation of representative competition experiments is shown in Figure 2. The data indicate that the chimeras IX-A/VII, IX-EGF2/VII, and IX LC/VII HC compete for the endothelial cell binding site comparably to plasma factor IX(a) whereas the chimeras VII-A/IX, VII-EGF2/IX, and VII LC/IX HC do not compete for the site.

Discussion

There are two regions of factor IX(a) that are thought to participate in high-affinity binding to endothelial cells, the Gla domain and the EGF domains. The participation of the Gla domain is clear, and the literature on its involvement is consistent. The participation of the EGF domains is less clear. Interpretation of the data on the EGF contribution suggests two conclusions. Either the EGF component of specificity resides within the second EGF domain or the EGF domains are not involved in interaction specificity. The evidence in this report suggests that there is a single high-affinity factor IX endothelial cell recognition determinant and that the determinant is within the Gla domain.

The binding affinities of the chimeras present a consistent picture of the interaction between factor IX(a) and its endothelial cell binding site. The importance of the factor IX(a) Gla domain is reaffirmed with the chimeras VII LC/IX HC, VII-EGF2/IX, and VII-A/IX. These chimeras representing the entire factor IX protein with the exception of factor IX's Gla domain are uniformly unable to compete for the binding site out to 150 nM. Although this observation with these three chimeras does not address the possibility of a 10 μM binding constant within the EGF domains, it is consistent with the existing literature that places the vast majority of the binding specificity to within the Gla domain. The extent of participation of the EGF domains emerges with the binding affinities of the reciprocal chimeras. This is best illustrated with the two chimeras IX-A/VII and IX-EGF2/VII which have nearly identical binding affinities. The K_d's of these two chimeras clearly indicate that the replacement of factor IX's EGF domains with factor VII's EGF domains has not affected the specificity nor the affinity of the chimeras for the binding site. This suggests that neither of the factor IX EGF domains are directly contributing to binding specificity. Thus, while isolated EGF domains may bind to the cellular site (Astermark et al., 1991), it does not appear that these domains contribute to the affinity of factor IX(a) as a whole for its binding site. This observation is consistent with the report of Cheung et al. (1991) and makes a compelling case for a single recognition determinant within the Gla domain. We attribute the 3–5-fold difference in K_d between native factor IX and the positively binding chimeras to marginal perturbations of this Gla binding determinant.

The factor VII and factor IX Gla domains share amino acid sequence identity of 63%. This amounts to a difference of 14 amino acids which must comprise the recognition site. Working on this assumption, we are currently examining mutations in this region in an effort to define the amino acids that constitute the binding determinant.

Registry No. Factor IX, 9001-28-9.

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