Identification of the endothelial cell binding site for factor IX

collagen/coagulation

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ABSTRACT We previously demonstrated that the primary region of factor IX and IXa responsible for saturable specific binding to bovine aortic endothelial cells resides in residues 3–11 at the amino terminus of factor IX. We also demonstrated that mutations of lysine to alanine at residue 5, factor IX K5A, or valine to lysine at residue 10, factor IX V10K, resulted in a molecule unable to bind to endothelial cells. Moreover, a mutation with lysine to arginine at residue 5, factor IX K5R, resulted in a factor IX molecule with increased affinity for the endothelial cell binding site. In this paper we report that collagen IV is a strong candidate for the factor IX binding site on endothelial cells. Factor IX and factor IX K5R compete with 125I-labeled factor IX for binding to tetrameric collagen IV immobilized on microtiter plates, while factor X, factor VII, and factor IX K5A or V10K fail to compete. The Kd for wild-type factor IX binding to collagen IV in the presence of heparin was 6.8 ± 2 nM, and the Kd for factor IX K5R was 1.1 ± 0.2 nM, which agrees well with our previously published Kd values of 7.4 and 2.4 nM for binding of the same proteins to endothelial cells. Our working assumption is that we have identified the endothelial cell binding site and that it is collagen IV. Its physiological relevance remains to be determined.

Factor IX is the zymogen of a serine protease which is active in normal hemostasis. Mutations in factor IX result in the bleeding disease hemophilia B. Factor IX is activated by factor Xa or factor VIIIa-tissue factor to yield factor IXa; this reaction results from cleavage between residues 145/146 and 180/181 of the factor IX. Activated factor IX, together with its nonenzymatic cofactor factor VIIIa, cleaves factor X to factor Xa. Factor IX, like factors X and VII, is a vitamin K-dependent clotting factor which contains multiple 4-carboxyglutamic acid (Gla) residues at its amino terminus. The factor IX Gla domain is followed by two epidermal growth factor-like domains, an activation peptide, and a serine protease domain. For a recent review see Roberts (1).

The binding of factor IX to a specific saturable site on endothelial cells was first described by Heimark et al. (2) and Stern et al. (3). Heimark et al. found that bovine endothelial cells had approximately 280,000 sites per cell with a Kd for factor IX of about 5 nM. The factor IX binding sites on the endothelial cell were subsequently characterized in great detail by Stern et al. (4). They reported about 40,000 sites per cell and a Kd for factor IX or IXa of about 2 nM. Moreover, it was found that factor IXa in the presence of factor VIII and factor X binds with a 10-fold increased affinity. The factor X-cleaving (“tenase”) complex on endothelial cells could catalyze the formation of factor Xa with a greater Vmax than a tenase complex assembled upon phospholipids. Finally, Brinkman et al. (5) showed that the endothelium may serve as an effective surface for formation of a tenase complex.

We (6) determined that the primary region of factor IX and IXa responsible for binding to bovine aortic endothelial cells resides in residues 3–11 at the amino terminus of factor IX. We also demonstrated that mutations of lysine to alanine at residue 5, factor IX K5A, or valine to lysine at residue 10, factor IX V10K, result in a molecule unable to bind to endothelial cells. Moreover, a mutation of lysine to arginine at residue 5, factor IX K5R, results in a factor IX molecule that has a 3-fold increased affinity for the endothelial cell binding site. Our observation that factor IX residues 3–11 are critical for the factor IX endothelial cell interaction have recently been confirmed by Castellino’s laboratory (7). They demonstrated that converting the first 11 amino acids of protein C to those of factor IX resulted in a protein C chimera that bound to endothelial cells.

In an attempt to identify the molecule on bovine aortic endothelial cells to which factor IX was binding, Rimon et al. (8) cross-linked factor IX to a 140-kDa protein on the surface of endothelial cells. In spite of this initial success, a number of laboratories have failed to identify the molecule to which factor IX binds, probably because the cross-linked factor IX was bound to a proteolytic fragment of collagen IV.

In this paper we report that collagen IV is a strong candidate for the factor IX binding site on endothelial cells. Collagen IV is an extracellular matrix (ECM) protein which is one of the major components of the basement membrane of endothelial cells (9, 10). It is found arranged in a two-dimensional array in the lamina densa of basement membranes and is usually isolated from placenta (11) or the mouse tumor cell line EHS (12). The isolation includes a limited pepsin digestion which leads to a significant fraction of tetrameric collagen. Tetrameric collagen IV is composed of four triple-helix collagen molecules joined by their amino-terminal 7S globular domains (13). The triple-helical molecule, which consists of two α1 chains and one α2 chain, constitutes the collagen-like domain of collagen IV. The collagenous domain of collagen IV differs from fibrillar collagens in that its Gly-Xaa-Yaa sequence is interrupted by noncollagen-type sequences. Therefore the triple helix is not in perfect register, a condition which appears to provide flexibility for supercoiling of triple helices. Moreover, collagen IV has noncollagenous domains at both the amino and carboxyl termini of its constituent polypeptide chains.

Collagen IV may also be extracted by limited cleavage with collagenase to yield dimeric collagen. Dimeric collagen is composed of two triple-helical collagen molecules which are attached at their carboxyl termini by their NC1 domains and

Abbreviations: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm.

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which lack the 7S domain (14). A cyanogen bromide fragment from the triple-helical region of collagen IV (15, 16) possesses high-affinity binding sites for α1β1 and α2β1 integrins. The major collagen-binding integrin of platelets (17) is gp Iα/IIα, which is a synonym for α2β1.

We report here that the triple-helical region of collagen IV possesses all of the attributes previously described for the factor IX binding site on endothelial cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, fetal bovine serum, and porcine intestinal mucosa heparin were from GIBCO/BRL. Removable microtiter wells were from Corning. Iodobeads were from Pierce. Sodium bromide fragment (CB3) of human placenta collagen IV (16) was expressed and purified as previously described (21). Binding of Factor IX to Collagen Type IV. Five micrograms of human placental collagen IV in 100 μl was added to each well of a 96-well microtiter plate and incubated at 4°C overnight. After three washes with TBS, the coatings in some wells were digested with 2.5 μg of recombinant collagenase-I in 100 μl of TBS containing 2 mM CaCl2, 1 mM MgCl2, and 0.2% BSA at 37°C for 2 hr. After three additional washes with TBS, binding experiments were performed at 4°C for 2.5 hr as described above.

Factor X Activation on Endothelial Cells and ECMs. Confluent bovine endothelial cells were seeded at 1.5 × 105 cells per well and grown in 24-well plates for 2 days. The ECM of endothelial cells was prepared as described above by removing the cells with 0.5% deoxycholate. Factor IXα (0.5 nM) was incubated with intact endothelial cells and ECM as in a binding experiment, and the tenase activity was measured on both the matrix and washed cells. The reaction was started by the addition of 400 μl of a solution containing 5 units/ml factor VIIIa and 0.2 μM factor X in 5 mM Tris-HCl/140 mM NaCl/2 mM CaCl2/0.2% BSA, pH 7.5. Factor VIIIa was preactivated with thrombin at 37°C for 5 min and stored on ice until use. Aliquots (40 μl) were collected at timed intervals over 25 min. Each aliquot was added to a well of a 96-well microtiter plate containing 60 μl of 50 mM Tris-HCl/175 mM NaCl/6 mM EDTA/0.05% ovalbumin, pH 7.5. Factor Xa activity was assayed by adding 100 μl of 0.2 mM chromogenic substrate, Spectrozyme Xa, and the absorbance at 405 nm was monitored with a plate reader (Labsystems, Chicago) for 3 min at room temperature.

RESULTS AND DISCUSSION

Our initial stimulus to examine the role of the ECM in factor IX binding arose from the observation that heparin sulfate and heparin affected the binding of factor IX to endothelial cells. To examine the possible role of the ECM in factor IX binding, endothelial cells were removed by exposure to 0.5% sodium deoxycholate as described in Materials and Methods. Fig. 1 demonstrates that, at the time of measurement, 2 days, more factor IX binding sites are on the matrix than on intact endothelial cells. When the cells reach confluence, however, the situation is reversed. Therefore, although this experiment clearly demonstrates that the ECM binds factor IX, it is likely that collagen IV or an epitope of collagen IV is also present on the surface of endothelial cells. To examine if the ECM maintained the same specificity as that observed previously for endothelial cells we demonstrated that plasma factor IX or factor IX K5R competed for binding, while factor IX with the point mutations previously shown to lack the ability to bind endothelial cells (6) failed to compete (Fig. 2).

This observation suggests that the binding site that we have previously characterized on endothelial cells is actually found in the ECM secreted by endothelial cells. We therefore tested a number of known ECM proteins for their ability to bind factor IX. Human fibronectin, mouse laminin, mouse entactin (nidogen), mouse heparan sulfate proteoglycan, and both human and bovine collagen I, III, and V were tested; all failed to bind factor IX (Table 1).
In the absence of heparin, factor IX bound to collagen IV, although with lower affinity ($K_d = 43$ nM) than to the ECM; in the presence of heparin at 1 $\mu$g/ml, however, collagen IV bound with an affinity similar to that previously determined for endothelial cells (4, 6). Therefore, heparin was used in all subsequent experiments. Fig. 3 shows that the $K_d$ for wild-type factor IX binding to collagen IV in the presence of heparin was 6.8 ± 2 nM and the $K_d$ for factor IX K5R was 1.1 ± 0.2 nM. These values agree well with our previously published $K_d$ values of 7.4 and 2.4 nM for binding of the same factor IX variants to endothelial cells (6). The $K_d$ values were obtained by analyzing three independent experiments simultaneously by the program $MK$ model (Biosoft, Ferguson, MO). There are uncertainties in the determination of the $K_d$ values because the concentration of competitor was frequently insufficient to allow the program to predict the nonspecific background. Therefore, the nonspecific value calculated for the factor IX K5R mutant, which did reach a plateau, was used as a constant value for those experiments.

To determine if the specificity of binding observed with the ECM was retained with the purified collagen IV, competition experiments with wild-type and variant factor IXs were done. Fig. 4 shows that factor IX and factor IX K5R compete with specificity of factor IX binding to the ECM. Confluent bovine endothelial cells were treated as described in the legend to Fig. 1 to remove cells. The remaining ECM was washed three times with 2 mM Tris-HCl/1 mM phenylmethylsulfonyl fluoride, pH 8, to remove cells. The remaining ECM was washed three times with 2 mM Tris-HCl/1 mM phenylmethylsulfonyl fluoride, pH 8. Binding experiments were done on both the intact cells and the remaining ECM.

### Table 1. Binding of ECM proteins to factor IX

<table>
<thead>
<tr>
<th>ECM protein</th>
<th>Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human fibronectin</td>
<td>No</td>
</tr>
<tr>
<td>Mouse laminin</td>
<td>No</td>
</tr>
<tr>
<td>Mouse nidogen</td>
<td>No</td>
</tr>
<tr>
<td>EHS cell heparan sulfate proteoglycan</td>
<td>No</td>
</tr>
<tr>
<td>Bovine collagen I</td>
<td>No</td>
</tr>
<tr>
<td>Human collagen V</td>
<td>No</td>
</tr>
<tr>
<td>Lathyritic mouse EHS collagen IV</td>
<td>No</td>
</tr>
<tr>
<td>Human placental collagen IV titeram (Kuhn)</td>
<td>Yes</td>
</tr>
<tr>
<td>Human placental collagen IV dimer (Kuhn)</td>
<td>Yes</td>
</tr>
<tr>
<td>Human placental collagen IV 7S domain (Kuhn)</td>
<td>No</td>
</tr>
<tr>
<td>Human placental collagen IV CNBr fragment (binds to a1B1 and a2B1 integrins) (Kuhn)</td>
<td>No</td>
</tr>
<tr>
<td>Human placental collagen IV (Sigma)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The major known constituents of the ECM were coated on microtiter plates and tested to determine which was responsible for factor IX binding. $^{125}$I-factor IX for binding to tetrameric collagen IV immobilized on microtiter plates, whereas factor X, factor VII, and factor IX K5A or V10K fail to compete. The relative specificity is exactly that observed for endothelial cells (6).

To further examine the interaction between collagen IV and factor IX, monolayers of endothelial cells were incubated with 0.5 nM $^{125}$I-factor IX and the competition was done with dimeric or tetrameric collagen IV instead of nonradioactive factor IX. The results for dimeric and tetrameric collagen were similar. Therefore, all four sets of data were analyzed simultaneously by the $MK$ model, and a $K_d$ value of 36 nM was obtained (see Fig. 5). This $K_d$ value approximates the $K_d$ determined for factor IX's binding to collagen IV in the absence of heparin. Therefore, these results appear to confirm the role of collagen IV in binding to endothelial cells.

Collagen IV can be dissected into several fragments. Neither the amino-terminal 7S globular domain nor the carboxy-terminal noncollagenous domain one (NC1) competed with $^{125}$I-factor IX binding to tetrameric collagen IV immobilized on microtiter plates, whereas factor X, factor VII, and factor IX K5A or V10K fail to compete. The relative specificity is exactly that observed for endothelial cells (6). To further examine the interaction between collagen IV and factor IX, monolayers of endothelial cells were incubated with 0.5 nM $^{125}$I-factor IX and the competition was done with dimeric or tetrameric collagen IV instead of nonradioactive factor IX. The results for dimeric and tetrameric collagen were similar. Therefore, all four sets of data were analyzed simultaneously by the $MK$ model, and a $K_d$ value of 36 nM was obtained (see Fig. 5). This $K_d$ value approximates the $K_d$ determined for factor IX's binding to collagen IV in the absence of heparin. Therefore, these results appear to confirm the role of collagen IV in binding to endothelial cells.
endothelial cells for binding to factor IX (Table 1); moreover, both dimeric and tetrameric collagen bind 125I-factor IX. Because dimeric collagen IV lacks the 7S domain while tetrameric collagen IV lacks the NC1 domain, this provides additional positive data that the binding site resides in the collagenous domain of collagen IV. The collagenous domain of collagen IV can be cleaved with cyanogen bromide to give several fragments. One of these, designated CB3, has been shown to bind the integrins α1β1 and α2β1 (16). The integrin α2β1 is also known as glycoprotein IIa/IIia, which is a major platelet integrin. Patients with a defect in α2/31 are impaired in collagen-stimulated platelet aggregation (22). Furthermore, monoclonal antibodies against α2β1 inhibit specifically the collagen-induced aggregation of platelets (23, 24). Although

Fig. 4. Specificity of factor IX binding to tetrameric collagen IV. Two independent experiments are separated by a space. Lyophilized tetrameric collagen IV was dissolved to 3 mg/ml in 10 mM Tris-HCl/150 mM NaCl/2 mM CaCl2/1 mM MgCl2, pH 8, then diluted to 50 μg/ml. One hundred microliters of this solution was incubated at 4°C overnight in 96-well microtiter plates, blocked with ovalbumin, and used for binding studies.

The evidence that factor IX is not binding to a phospholipid membrane contaminant is shown in Fig. 7. Either heat denaturation or treatment with 10 mM dithiothreitol results in loss of binding of factor IX to collagen IV; this result is consistent with our failure to obtain positive results with ligand blotting experiments. Furthermore, we have found that mutant factor IX K54A or V10K binds phospholipid membranes normally (Kd = 1.5 and 0.5 μM, respectively) while failing to compete with

Fig. 5. Competition by collagen IV for factor IX binding to endothelial cells. In this experiment collagen IV was used as a competitor for the binding of 125I-factor IX to intact endothelial cell monolayers. Heparin was not included and the methods are as described in the legend for Fig. 4.

Fig. 6. Treatment of collagen IV with recombinant collagenase eliminates binding of 125I-factor IX. Collagen IV was applied to microtiter plates and a portion of the wells were treated with recombinant collagenase from Sigma. Treatment was with 2.5 μg of collagenase at 37°C for 2 hr.

The binding site for factor IX does not appear to reside in fragment CB3 of collagen IV (Table 1), it is interesting that factor IX and platelets both have binding sites on this prevalent ECM protein.

It is always difficult to prove that the binding one observes is not the result of some minor contaminating protein. However, several lines of evidence point toward collagen IV as the molecule to which factor IX binds. First, a polyclonal antibody to collagen IV inhibited the binding of 125I-factor IX (data not shown). Second, recombinant collagenase treatment of the purified collagen IV totally eliminated binding (Fig. 6). We also treated endothelial cells directly with collagenase and found that factor IX binding was reduced to nearly background levels. More severe treatment of cells with collagenase would result in their release from the plates. Third, collagen IV from several different sources—for example, dimeric or tetrameric collagen IV prepared from human placenta or bovine testes—binds factor IX specifically. Finally, none of the other major constituents of the ECM bind factor IX (Table 1). This suggests that it is not an expected contaminant to which factor IX is binding.
125I-factor IX for binding to endothelial cells (unpublished results).

When the ability of the ECM of bovine endothelial cells to function as a surface for tenase activity is compared with that of endothelial cells per se it is found that there is essentially no tenase activity on the ECM (Fig. 3) by the level of tenase activity on intact endothelial cells is similar to that observed by others (4, 5) (about 8 nmol of factor Xa generated per min). While these experiments are qualitative, it is clear that the activity of the matrix is much diminished compared with intact endothelial cells; this may simply reflect the need of a cooperative effect for phospholipid. Preliminary experiments from several industrial laboratories suggest that, while phospholipids do stimulate activity in the presence of collagen IV, the stimulation does not exceed that of phospholipid alone.

At present it is difficult to assess the functional significance of these observations. Briet (25) reported that more infused factor IX could be recovered from CRM+ than from CRM− patients (CRM, cross-reacting material). He did mixing experiments to demonstrate that the cellular components did not bind the infused factor IX and postulated that the endothelial cell lining of the blood vessel was the surface to which the infused factor IX was adsorbed. Therefore it is possible that collagen IV functions merely as a storage site and is the "extravascular space" where infused factor IX disappears. This scenario would, at a minimum, put the factor IX where it is needed upon rupture of a vessel.

In retrospect it is perhaps not surprising that collagen IV appears to be the factor IX binding site. Collagen IV is the major ECM protein, and one would expect coagulation to proceed after endothelial cell disruption rather than on the endothelial cell surface. Several collagens have been shown to activate platelets (26, 27), and there are a number of experiments implicating the ECM in coagulation (26). Moreover, leech and tick anticoagulants that act by inhibiting the interaction between collagen and platelets have been identified (28, 29). The interactions are complex and are not restricted to collagen IV; however, the leech anticoagulant protein does inhibit the binding of platelets to collagen IV (30). Collagen type IV has also been shown to bind fibrin and cause extensive structural alterations (31). Another coagulation-related ligand which binds with high affinity to collagen IV is protease nexin-I, which binds specifically to collagen IV with a $K_d$ of approximately 15 nM (32). This interaction decreases protease nexin-I's rate constant for the inhibition of urokinase or plasmin.

Because collagen IV appears to have so many properties identical with those of the previously characterized factor IX binding site—that is, the $K_d$ is nearly identical, the specificity of mutant factor IX is identical, and factor IX and IXa bind indistinguishably—it seems highly probable that collagen IV is the factor IX endothelial cell binding site that we have previously characterized. However, we cannot eliminate the possibility that there is yet another binding site for factor IX that resides upon the endothelial cell surface, that has different binding specificity, and that is responsible for the observed tenase activity of endothelial cells.

One aspect of our work is in contradiction to the results of Rimon et al. (8), who reported that there was no binding to the plates after cells had been removed by the detergents CHAPS or Triton X-100. In our hands removing cells with either detergent results in a surface with specific factor IX binding activity. In addition, they reported that the binding specificity was maintained by proteins in the cellular extracts. We also observed that partially purified endothelial cell membranes adsorbed to microtiter plates resulted in a factor IX binding surface. We made large-scale membrane preparations from a bovine placenta but found that, while there was binding activity, there was no discrimination between factors IX, X, and VII. These differences remain unexplained.

The observation that collagen IV has a specific binding site for factor IX demands a host of additional physiological and biochemical experiments on the interaction between factor IX and the ECM as well as factor IX and collagen IV. Furthermore, it may be profitable to look at the effect of collagen IV on other blood coagulation factors.


![Fig. 8. Tenase activity of factor IX bound to collagen IV of ECM or intact endothelial cells. Factor IXa (final 0.5 nM) was bound to endothelial cells or matrix for 3 min at room temperature. A, Tenase activity on endothelial cells; B, tenase activity on the ECM.](image-url)