Communication

The Binding of Human Factor IX to Endothelial Cells Is Mediated by Residues 3–11*

Wing-Fai Cheung‡, Nobuko Hamaguchi‡, Kenneth J. Smith§¶, and Darrel W. Stafford‡∥

From the ‡Department of Biology and Center for Thrombosis and Hemostasis, University of North Carolina, Chapel Hill, North Carolina 27599 and the §Departments of Medicine and Pathology, University of New Mexico School of Medicine and United Blood Services, Albuquerque, New Mexico 87131

We have used chimeras and point mutations of recombinant coagulation factor IX to examine factor IX's specific interaction with bovine endothelial cells. Previously (Toomey, J. R., Smith, K. J., Roberts, H. R., and Stafford, D. W. (1992) *Biochemistry* 31, 1806–1808), we restricted the region of factor IX responsible for binding to endothelial cells to its Gla domain. Molecular modeling of the Gla domain of factor IX using the coordinates of the Gla domain of bovine prothrombin-(1–145) (Soriano-Garcia, M., Padmanabhan, K., deVos, A. M., and Tulinsky, A. (1992) *Biochemistry* 31, 2554–2566) reveals two major surface determinants whose sequences differ among factors IX, X, and VII. A chimeric protein comprised of the Gla domain of factor VII with the remainder of the molecule of factor IX did not bind to the endothelial cell binding site. We changed residues 33, 34, 35, 39, and 40 to those of factor IX without restoring endothelial cell binding. Replacement of amino acid residues 3–10 with those of factor IX restored normal binding. With the knowledge that specific binding was localized to the first 11 amino acids, point mutations were made at residues predicted to be on the surface in this region of the factor IX molecule. Changing lysine 5 to alanine (K5A) or valine 10 to lysine (V10K) resulted in loss of binding with total retention of *in vitro* clotting activity. The lysine 5 to arginine (K5R) mutation also was fully active *in vitro* but displayed 3-fold tighter binding. In addition to defining the sequence of factor IX necessary for binding to endothelial cells, these results suggest that the binding site is not phospholipid but instead is specific, and in all likelihood, protein.

Blood coagulation protein factor IX, whose absence causes hemophilia B, binds to a specific receptor on the surface of endothelial cells (1, 2). Binding is calcium-dependent, satu-

rable, and reversible (2). The receptor binds factor IX and factor IXa equally well in the absence of its cofactor and substrate; however, in the presence of factors VIII and X, the K_d value of factor IXa is reduced about 20-fold (3). The endothelial cell receptor has been reported to be a protein of about 140 kDa, but little else is known of its properties (4).

It has been shown that factor IX bound to endothelial cells can be activated by exogenous factor XIa and that, once activated, addition of the cofactor, factor VIIIa, and the substrate, factor X, leads to activation of factor X (5). The physiological significance of these interactions is unclear; however, the presence of a specific binding site on the lining of the vasculature suggests that it may play an important role in hemostasis.

We have previously reported the construction of several factor IX-factor VII chimeras and their specific binding to endothelial cells (6, 7). Our previous work narrowed the binding region to the Gla domain of factor IX (7). In this paper we describe a computer-generated model for the Gla domain of factor IX and demonstrate that the endothelial cell binding determinant resides on a prominent surface in the first 11 amino acids of factor IX.

EXPERIMENTAL PROCEDURES

Materials

Escherichia coli DNA polymerase I (Klenow fragment), calf intestine alkaline phosphatase, polynucleotide kinase, and T4 DNA ligase were obtained from either GIBCO-BRL, New England Biolabs, or Boehringer Mannheim. Na^{125}I and ^{35}S-dATP were purchased from Amersham Corp. Affi-Prep 10 was obtained from Bio-Rad, Iodobeads was from Pierce Chemical Co., and Geneticin (G418) was from GIBCO-BRL. Human factor IX was purchased from Enzyme Research Laboratories (South Bend, IN) or purified from plasma as described (8).

Factor IX-deficient plasma was obtained from Sigma. Platelin Plus Activator was purchased from General Diagnostics (Morris Plains, NJ). Human factor XIa was purified according to Braunstein *et al.* (9). Vitamin K (Aquamephyton) was obtained from Merck Sharp and Dohme. 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

Production of Mutant Proteins—Mutant proteins were made and characterized as previously described (6). All mutations were completely sequenced (10) to ensure that no inadvertent mutations had been created.

Gla Analysis—Gla analysis was performed at Merck Sharp and Dohme according to the procedure of Przysiecki and Friedman (11).

Binding of Normal and Chimeric Factor IX to Endothelial Cells—Bovine aortic endothelial cells were seeded at 3×10^4 cells/cm^2 and grown to confluence in 48-well microtiter plates in Dulbecco's modified Eagle's medium containing penicillin, streptomycin, and 20% fetal calf serum. Confluent wells (1 cm^2) contained an average of 2.2 × 10^5 cells. Binding assays and analysis of data were as previously described (6).

Calculation of Binding Constants—MK Model (Biosoft, Cambridge, United Kingdom), which uses Feldman's algorithm (12), was used for calculating the binding constants of factor IX and its mutants to bovine aortic endothelial cells.

Molecular Modeling—The model of the factor IX Gla domain was constructed from residues 1 to 50 of the coordinates of the bovine prothrombin fragment 1 crystal structure (13). The amino acid sequence was changed to that of factor IX and the structure energy minimized; the positions of the common atoms in the backbone, the

FIG. 1. **Summary of the constructs used and the results of binding studies, coagulation assays, and Gla analysis.** *Black* on *white* depicts factor VII sequences while white on *black* depicts factor IX sequences. *Line 1* is the beginning construct, which consists of the Gla domain and aromatic stack of factor VII attached to factor IX. The construction is at the *Bst*EII site described in our previous paper (21). *Line 10* is factor IX's Gla domain. In *lines 14* and *15*, residues were changed to those found at the corresponding position in human factor X. The K_d values are nM; *ND* , not detectable at the concentrations of competitor used; *APTT*, activated partial thromboplastin time with reference to a pooled normal plasma; *Gla*, mol of Gla/mol of factor IX; -, analysis was not done.

side chains, and seven calcium ions were fixed during this initial minimization. The structure was then solvated with an 8-Å layer of TIP3P water, and 20 ps of dynamic simulation was performed. Finally, the entire system was minimized to a convergence criterion of 0.1 kcal/mol·Å root mean square gradient. All of the calculations were performed with AMBER version 3A (14, 15). All covalent bonds involving hydrogen were constrained, and 3 atomic mass units (amu) was used for all hydrogens. For the energy minimization calculations, a 9-Å cutoff was used for non-bonded interactions with a distance-dependent dielectric constant. The partial charges and parameters for calcium ions and Gla residues were taken from Charifson *et al.* (16) and Maynard *et al.* (17). All computations were done on either the CRAY Y-MP supercomputer at the North Carolina Supercomputing Center (Research Triangle Park, NC) or the Convex 240 at the University of North Carolina-Chapel Hill.

RESULTS AND DISCUSSION

In our previous studies we have shown that factor IX's major determinant for binding to its endothelial cell receptor resides on its Gla domain (6, 7). To further characterize the residues of factor IX responsible for its binding to the specific site, we have made a number of mutations in the Gla domain of a chimeric factor IX, which contained the Gla domain and aromatic stack of factor VII attached to the EGF-like domains, activation peptide, and heavy chain of factor IX. The binding constants of recombinant factor IX and the chimeric molecules were determined by their ability to compete with purified ^{125}I plasma factor IX for binding to endothelial cells.

The constructions used in these experiments as well as summaries of K_d values, Gla analysis, and clotting times are shown in Fig. 1 and will be referred to by line number. *Line 1* shows factor IX with the Gla domain of factor VII. *Line 2* shows that adding factor IX's residues 33, 34, 35, 39, and 40 fails to restore binding, although it does confer the ability to bind to the monoclonal antibody A-7.[1] Similarly, the constructs shown in *lines 3–7* failed to compete. In contrast, those

constructs depicted in *lines 8* and *9* competed very effectively for binding to factor IX's endothelial cell binding site. Because we could show that A-7 had no effect on binding to endothelial cells (not shown) we concluded that the binding site was in residues 1–11. We also knew, from the construct shown in *line 8*, that the amino-terminal tyrosine of factor IX was not critical. We therefore made several point mutations of amino acids predicted to be on the surface of this region. Lysine 5 was changed to Arg (K5R) or Ala (K5A) (*lines 11* and *12*, respectively) and Val-10 to Lys (V10K) (*line 13*). In addition the surface residues at 9, 10, and 11 (*line 14*) or 4 and 5 (*line 15*) were changed to the comparable residues in human factor X. An additional change, Phe-9 to Ala (F9A) also failed to compete for binding (not shown). A summary of the relevant results of the data from which the K_d values in Fig. 1 were extracted is shown in Fig. 2. K5R gives a 3-fold tighter binding constant, while all of the others fail to compete. Binding studies were done on all of the constructs; however, of those that failed to bind, only K5A was plotted, as the others which failed to bind had essentially identical curves. Significantly, K5A, K5R, and V10K all have 100% *in vitro* clotting activity.

We attempted to understand the results of these mutations by reference to a computer-generated structure of human factor IX. This model was prepared from the coordinates of bovine calcium prothrombin-(1–146) (13). The root mean square deviation of backbone atoms of the energy-minimized factor IX Gla domain, compared to the x-ray structure of prothrombin's Gla domain, was 0.56. All of the calcium ions maintained their ligations to the Gla residues as observed in the crystal structure of the prothrombin Gla domain. The amino terminus of factor IX is tyrosine, while the comparable position of the other vitamin K-dependent proteins is alanine. Although this tyrosine hydroxyl is exposed to solvent, the electrostatic interactions of the amino group of Tyr-1 to Gla-17, -21, and -27 were maintained in the minimized model. Thus the Ω loop is also present in the factor IX Gla domain. Overall, the model of the factor IX Gla domain predicts that the structure of the Gla domain is similar among the vitamin K-dependent blood proteins.

The largest backbone movement was observed around residues 4 and 5; at residue 5, factor IX has Lys in place of the Phe residue in bovine prothrombin. These residues are disordered in the x-ray structure of bovine prothrombin fragment 1 and are thus likely to be part of a flexible region.

Because factor IX has 2 more Gla residues in the α-helical region of residues 35–48 than prothrombin, there may be an additional calcium binding site in factor IX (13) which mediates A-7 binding. Gla-15 is near the carboxyl terminus of helix 35–48 and could, with the aid of a calcium ion, form a
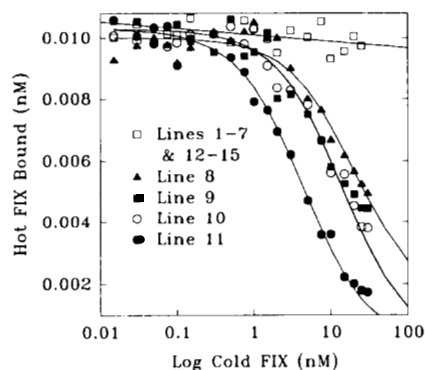
[1] W.-F. Cheung, D. W. Stafford, and K. J. Smith, manuscript in preparation.

FIG. 2. **Results from competition binding studies.** The results were analyzed with the program MK model as described under "Methods."
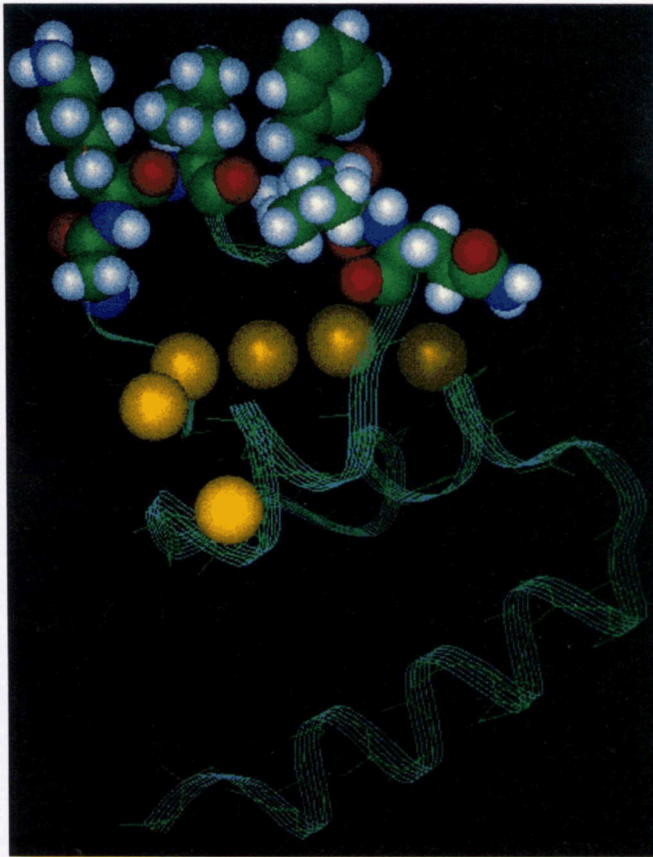
FIG. 3. **Model of the factor IX Gla domain with the relevant amino acids shown as space-filling and with the inclusion of the calcium ions.** From *left* to *right* the amino acids shown are Gly-4, Lys-5, Leu-6, Phe-9, Val-10, and Gln-11.

bridge to the first epidermal growth factor-like domain of factor IX. This awaits further structural information.

When one puts the mutagenesis data into the context of the modeled structure, a plausible picture of the interaction between factor IX and its endothelial cell binding site emerges. Residues 5, 9, and 10, which are prominent surface residues on the Ω loop, are critical. In Fig. 3, lysine 5 and phenylalanine 9 are pointing upward, whereas valine 10 is projecting out from the page. Glutamine 11 is also shown, although it is apparently not part of the binding site, as bovine factor IX has arginine at this position and bovine and human factor IX bind equally well to bovine endothelial cells (2, 18). Residues 4–6 and 9–10 form a water-accessible surface

area of approximately 800 Å^2, which is consistent with the surface areas of known protein-protein interactions (19). It is significant that point mutations at 5 and 10, which eliminate endothelial cell binding, do not affect *in vitro* clotting activity. Thus, because *in vitro* clotting activity requires phospholipid binding, the phenomenon of endothelial cell binding appears to be independent of phospholipid binding. Moreover, it is significant that a change of lysine to arginine results in about 3-fold tighter binding. This is consistent with the increased fractional charge of Arg compared to Lys (20). Because of the specificity we observed for this interaction and because interactions with phospholipids are unlikely to be so specific, these results support the conclusions of Rimon *et al.* (4) that the binding site is a protein.

The function of the endothelial cell binding site remains speculative and further progress will depend upon its purification and (or) cloning.

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