The Role of the Epidermal Growth Factor-1 and Hydrophobic Stack Domains of Human Factor IX in Binding to Endothelial Cells*

(Received for publication, August 23, 1990)

Wing-Fai Cheung‡, David L. Straight‡, Kenneth J. Smith‡, Shu-Wha Lin¹, Harold R. Roberts**, and Darrel W. Stafford‡‡

From the †Department of Biology and Center for Thrombosis and Hemostasis and the **Department of Medicine and Division of Hematology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, ‡‡The Departments of Medicine and Pathology, University of New Mexico School of Medicine and United Blood Services, Albuquerque, New Mexico 87131, and the ¶Graduate Institute of Medical Technology, College of Medicine, National Taiwan University Hospital, Taipei, Taiwan

To determine the function and specificity in factor IX of the first epidermal growth factor (EGF)-like domain and the eight-amino acid hydrophobic stack encoded by exon C (residues 39–46), these domains were replaced by the corresponding polypeptide regions of factor X and chimeric proteins were produced in human embryo kidney cells. Both chimeras were activated by factor XIa at a rate similar to plasma factor IX and exhibited calcium-dependent fluorescence quenching similar to plasma factor IX. Both chimeras competed equally for binding to the endothelial cell receptor. Our findings make it unlikely that the first EGF-like domain or the hydrophobic stack of factor IX are responsible for the specific binding of factor IX to its endothelial cell receptor.

Blood coagulation protein factor IX, the absence of which causes hemophilia B, binds to a specific receptor on the surface of endothelial cells (1, 2). This binding is calcium-dependent, saturable, and reversible (2). The receptor binds factor IX and factor IXa indistinguishably, but in the presence of factors VIII and X the $K_d$ value is reduced about 20-fold (3). The endothelial cell receptor has been shown to be a protein of about 140 KDa, but little else is known about its properties (4). Although the physiological significance of this receptor is unclear, its presence on the lining of the vasculature suggests that it may play an important role in hemostasis. It has been shown that factor IX bound to endothelial cells can be activated by exogenous factor Xla and that, once activated, addition of factor VIIIa and factor X leads to activation of factor X (5).

Factors IX, X, VII, and protein C are closely related members of a family of plasma serine proteases. The functional domains of these four proteins (from amino to carboxyl terminus) are: the vitamin K-dependent domain, containing $\gamma$-carboxyglutamic acid (Gla) residues; two EGF-like (epidermal growth factor-like) domains; an activation peptide region; and the catalytic domain, which confers the protease function. The presence of growth factor-like domains in coagulation proteins was first noticed by Doolittle (6). The function of these domains is unknown, but EGF itself, as well as the EGF-like domains of other proteins, has been shown to bind to receptors on the cell surface (7). Furthermore, it has been suggested that the first EGF-like domain of factor IX plays a role in binding to its endothelial cell receptor (8).

We have previously reported the construction and partial characterization of factor IX with its first EGF-1-like domain replaced by the highly similar domain of factor X (16). In this paper we describe an additional chimeric protein, one with its eight-amino acid hydrophobic stack (residue 39–46 encoded by exon C) changed to amino acids found in factor X. We report additional characterizations of these proteins and their effectiveness as competitors of $^{125}$I-labeled plasma factor IX for binding to the endothelial cell receptor.

**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-Bethesda Research Laboratories, New England Biolabs (Beverly, MA), or Boehringer Mannheim. $^{125}I$-Protein A, $^{125}I$-Na, $[^{35}]S$-dATP, and $[^{32}]P$-dATP were purchased from Amersham Corp. Affi-Gel 10 was obtained from Bio-Rad, Iodobeads from Pierce Chemical Co., and Geneticin (G418) from Gibco-Bethesda Research Laboratories. Human factor IX was purchased from Enzyme Research Lab (South Bend, IN) or purified from plasma as described (9). Factor IX-deficient plasma and factor XI-deficient plasma and DEAE-Sephrose CL-6B were obtained from Sigma. Platein Plus Activator was purchased from General Diagnostics (Morris Plains, NJ). Human factor Xla was purified according to Braunstein et al. (10). This factor Xla preparation corrected the clotting defect of factor XI-deficient plasma. According to the criteria of both clotting assays and polyacrylamide gel analyses, the factor Xla activated human factor IX in the presence of calcium. Vitamin K (Aquamephyton) was obtained from Merck Sharp & 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**

In Vitro Mutagenesis and Construction of the Expression Plasmid—Site-directed mutagenesis was accomplished by the "gapped duplex" method (11). Each mutated molecule was sequenced by the dideoxy-chain termination method (12) to confirm that the sequence was as expected and that no inadvertent mutations had been introduced.

Cell Culture and Transfection by the CaPO₄ Method—Human embryo kidney 293 cells (ATCC CRL1573) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (growth media). Transfection was performed using the calcium phos-
Results

The factor IX chimera, with its eight-amino acid hydrophobic stack replaced by the homologous sequence from human factor X, was constructed and purified as previously described (16). The region of changed amino acids is shown in Fig. 1. The purified factor IX(XexonC) chimera was fully carboxylated with 10.4 Gals/mol compared with 10.5 Gals/mol for purified plasma factor IX analyzed concurrently. The clotting activity of factor IX(XexonC) was about 25–30% of the activity of plasma factor IX. Factor IX(W1) and factor IX(XexonC) were reported previously to be nearly fully carboxylated and have full activity (16).

We have reported previously that factor IX(XexonC), and factor IXa each activates normally (16). These experiments were done by polyacrylamide gel electrophoresis analysis of recombinant molecules at specific times after adding the activators. To evaluate activation more quantitatively, we examined the activation of factor IX(XexonC) as well as the previously reported chimeric molecules by the reaction of the activated factor IX with p-aminobenzamidine (21). As depicted in Fig. 2, the rate of activation of all of the recombinant molecules is similar to purified plasma factor IX. The $t_{1/2}$ values for activation in pseudo first-order reaction rate conditions were 1228, 1123, 1012, and 877 s for plasma, recombinant wild-type, factor IX(XexonC), and factor IX(XexonC), respectively. Thus, they appear to behave normally by this criterion. The extent of reaction, measured using the active site probe p-aminobenzamidine as described previously (21). The concentration of each factor IX molecule was 2.0 μM, and the amount of factor XIa was the same in each case (1:25, W/W).

Calculation of Binding Constants—Two programs, both of which employ Feldman’s algorithm (19), were used for calculating the binding constants of factor IX and its chimeras to bovine aortic endothelial cells. The results were the same but the MK model program allowed more flexibility in plotting and analyzing the data. The figures and constants presented here were derived from MK model.

Fluorescence Measurements—Fluorescence quenching by calcium was determined with factor IX preparations which had been eluted from a calcium-specific (A-7) antibody column with EDTA followed by dialysis against 20 mM Tris-HCl, 100 mM NaCl, pH 7.5. Measurements were made in a Shimadzu RF-5000 spectrophotometer with an excitation wavelength of 280 nm and an emission wavelength of 340 nm. Factor IX concentration was 20 μg/ml.

Activation—The activation of factor IX and its chimeras was
measured by fluorescence change, was about 25% less for the factor IX(Exonc) and about 15% less for factor IX(Exonf). Polyacrylamide gel electrophoresis analysis, however, indicates that activation is complete. We interpret the combined data to mean that activation is nearly normal.

Factor IX has been shown to exhibit fluorescence quenching upon the addition of Ca$^{2+}$ ions. Another indication of the similarity of the chimeric molecules to purified plasma factor IX is therefore found in comparing fluorescence quenching between purified plasma and recombinant molecules. Fig. 3 shows that each of the chimeric proteins exhibits fluorescence quenching. The calcium required for half-maximal quenching of plasma and recombinant factor IX is 0.6 mM; it is decreased to 0.1 mM for factor IX(Exonc), and to 0.35 mM for factor IX(Exonf). The extent of quenching also differs, with the two chimeric molecules exhibiting the greatest degree of quenching.

The binding constant of recombinant factor IX and the chimeric molecules were determined by their ability to compete with the binding of purified $^125$I plasma factor IX to endothelial cells. The results from one representative set of experiments are shown in Fig. 4. It is evident that there is no significant difference between the binding of normal recombinant factor IX, factor IX(Exonc), and factor IX(Exonf). Factor X m50 nM showed no competition. The $K_d$ values (nanomolar) obtained from experiments done at two concentrations of $^{125}$I-factor IX are shown in Table I. It is evident that the values for $K_d$ are not significantly different. MK model treats labeled and unlabeled ligands independently and allows one to calculate $K_d$ values for radioactive and unlabeled molecules. There was essentially no effect of iodination on binding by this criterion as the $K_d$ values for plasma factor IX and iodinated plasma factor IX were within 0.3 nM. The range of values for the maximum binding of factor IX to endothelial cells was 0.072 to 0.14 nM for twelve determinations. This corresponds to a range of receptors per cell of 35,000 to 67,000.

The best fit to the data is 50,000 $\pm$ 12,000 receptors/cell, which is in reasonable agreement with the estimates of Stern (3), who estimated that the number of receptors per cell lay between 25,000 and 37,000.

![Fig. 3. Calcium-dependent quenching of fluorescence. FIX (O), FIX(Exonc) (B), FIX(Exonf) (V), and FIX(Exonc) (V), purified by elution from the calcium-dependent antibody A-7, were dialyzed into a Tris-HCl buffer with 0.1 M NaCl. Increasing concentrations of calcium chloride were added and the fluorescence emitted at 340 nm was measured after 2 min.](image)

![Fig. 4. Displacement of $^{125}$I-labeled plasma factor IX from endothelial cells by FIX (O), FIX(Exonc) (B), FIX(Exonf) (V), and FIX(Exonc) (V). Both "hot" and "cold" concentrations are nanomolar. The concentration of $^{125}$I-labeled plasma factor IX was 0.5 nM.](image)

**Table I**

| Factor IX | $K_d$ values (nanomolar) |
|-----------|-------------------------|
| Plasma    | $2 \text{ nM}$ | $0.5 \text{ nM}$ | $0.01 \text{ nM}$ |
| FIX(Exonc)| 4.1 ± 0.7 | 3.2 ± 0.4 | 2.0 ± 0.5 |
| FIX(Exonf)| 3.8 ± 0.4 | 4.3 ± 0.6 | 2.8 ± 0.7 |
| FIX(Exonf)| 3.7 ± 0.6 | 3.7 ± 0.8 | 2.5 ± 0.7 |
| FIX(Exonc)| 3.9 ± 0.6 | 4.0 ± 0.6 | 3.2 ± 0.5 |

**DISCUSSION**

These experiments were stimulated in part by reports that a decapeptide corresponding to residues 41-50 of human factor IX would inhibit binding of factor IX to its endothelial cell receptor (8). Because this peptide included six residues corresponding to the hydrophobic stack encoded by exon c as well as four residues from the beginning of the first EGF-like domain of factor IX, we changed the sequences codon for each of these regions to factor X sequences.

Analogous to the structure of prothrombin (22), the hydrophobic stack should interact with the Gla domain. Therefore, the tryptophan molecule responsible for the calcium-induced fluorescence change probably would be residue 42 of human factor IX. We are reluctant to draw conclusions from the differences in the calcium concentrations required for half-maximal binding or from the differences in the extent of fluorescence quenching in the chimeric molecules. Very slight changes in the environment of the tryptophan molecule could account for these differences. It is, in our view, more significant that all of these molecules exhibit fluorescence quenching and that the interactions that normally accompany the calcium-induced conformational change in factor IX must be nearly normal in the recombinant chimeras. This argument is strengthened by our observations (16) that the chimeras that exhibits the most difference in fluorescence quenching has normal clotting activity. Why then is the clotting activity of factor IX(Exonc) only 25-30% of the activity of plasma factor IX? It has been suggested that interactions between the hydrophobic stacks of pairs of proteins such as factor IX and factor X are necessary for correct alignment of enzyme and
Factor IX Domains Binding to Endothelial Cell Receptor

substrate (23). If this were so, it could explain the decreased clotting activity of factor IX<sub>Xenon</sub>. Further experiments are required to address this point.

Ryan <i>et al.</i> (24) extended their earlier work on the role of the first EGF-like domain of factor IX in binding to endothelial cells and found that a synthetic peptide completely encompassing the first EGF-like domain inhibited the binding of factor IX to endothelial cells. Moreover, a peptide corresponding to the first loop of EGF-1 was inhibitory, with a K<sub>i</sub> of about 10 μM.

Our results appear to rule out any specific binding role for either the first EGF-like domain or the eight-amino acid hydrophobic stack of factor IX in its interaction with its endothelial cell receptor. EGF-1 alone contains a calcium-binding site (25, 26), and there is independent evidence that modification of residue 47 in the EGF-1 domain of factor IX alters the calcium-binding properties of factor IX (27). Additionally, there is evidence that EGF-1 influences the structure and calcium binding of the Gla domain (28) of the highly homologous protein C molecule. The results obtained by Ryan <i>et al.</i> (24) could reflect an interaction of their peptide with the Gla domain of the intact factor IX. For example, at the high peptide concentrations used, it is possible that the structure of factor IX is sufficiently flexible to allow the synthetic peptides to displace the normally interacting residues. This would then prevent formation of the normal conformation of factor IX. A proper conformational change appears to be necessary for binding, as factor IX lacking its γ-carboxy groups fails to interact with its receptor (29). The failure of the acarboxy factor IX to bind to its receptor could also be explained if part of the binding energy for the interaction was provided by Gla residues.

We have eliminated the possibility that EGF-1 functions in binding either to factor VIII (16) or to the endothelial cell receptor. No other function is known, except for calcium binding (26). If the domains of factors IX and X were so similar that they were functionally equivalent and if EGF-1 played a role in binding the receptor, we would have seen competition with factor X. However, factor X showed no competition at a concentration of 50 nM. Our opinion is that there is likely to be an additional, as yet unknown, interaction between the EGF-1 domain of factor IX and some other component of the coagulation pathway, but that is conjecture.

The recent papers by Derian <i>et al.</i> (29) and Ryan <i>et al.</i> (24) suggest that the major determinant for endothelial cell binding lies in the Gla domain of factor IX. There are only a few regions of amino acid differences between the Gla domains of factor IX and factor X and the binding specificity must lie in one of these regions. Furthermore, as the bovine receptor exhibits no discrimination between bovine and human factor IX (2, 29), additional residues may be removed from consideration as candidates for determining specificity. We imagine that the amino acids responsible for binding factor IX to its receptor are on the surface of the Gla domain in the middle of the cluster of six Gla residues defined by the crystal structure of fragment one of prothrombin (22). Thus, to understand further the interaction of factor IX with its receptor, we are currently examining the effects of specific amino acid changes in the Gla domain on its binding to its receptor.

REFERENCES

1. Heimark, R. L., and Schwartz, S. M. (1983) Biochem. Biophys. Res. Commun. 111, 723-731
2. Stern, D., Drillings, M., Nossel, H. A., Hurlet-Jensen, A. La Gamma, K. and Owen, J. (1983) Proc. Natl. Acad. Sci. U.S.A 80, 4118-4123
3. Stern, D. M., Narworth, P., Kisiel, W., Vehar, G. and Eason, C. T. (1985) J. Biol. Chem. 260, 6717-6722
4. Rimon, S., Melamed, R., Savion, N., Scott, T., Narworth, P. P., and Stern, D. M. (1987) J. Biol. Chem. 262, 6023-6031
5. Stern, D. M., Drillings, M., Kisiel, W., Narworth, P., Nossel, H. L., and La Gamma, K. S. (1984) Proc. Natl. Acad. Sci. U.S.A 81, 913-917
6. Doolittle, R. F., Feng, D. F., and Johnson, M. S. (1984) Nature 307, 558-560
7. Appella, E., Weber, I. T., and Blasi, F. (1988) FEBS Lett. 231, 1-4
8. Ryan, J., Woltitzky, B., Heimer, E., Felix, A., Huang, L., Tam, J., Kisiel, W., Narworth, P., and Stern, D. (1989) FASEB J. 3, A1050
9. Smith, K. J., and Ono, K. (1984) Thromb. Res. 33, 211-223
10. Braunstein, K. M., Noyes, C. M., Griffith, M. J., Lundblad, R. L., and Roberts, H. R. (1981) J. Clin. Invest. 68, 1420-1428
11. Kramer, W., and Fritz, H. J. (1987) Methods Enzymol. 154, 350-367
12. Sanger, F., Nicklen, S., and Coulson, R. (1977) Proc. Natl. Acad. Sci. U.S.A 74, 5463-5467
13. Graham, F. L., and vas der Eb, A. J. (1973) Virology 52, 456-461
14. Anderson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) J. Biol. Chem. 264, 8222-8229
15. Berk, A. J., and Sharp, P. A. (1978) Proc. Natl. Acad. Sci. U.S.A 75, 1274-1278
16. Lin, S.-W., Smith, K. J., Welsch, D., and Stafford, D. W. (1990) J. Biol. Chem. 265, 144-150
17. Przysiecki, C. T., Staggers, J. E., Ramjit, H. G., Musson, D. G., Stern, A. M., Bennett, C. D., and Friedman, P. A. (1987) Proc. Natl. Acad. Sci. U.S.A 84, 7856-7860
18. Smith, K. J., Singaraju, C., and Smith, L. F. (1987) Am. J. Clin. Path. 87, 370-376
19. Feldman, H. A. (1972) Anal. Biochem. 48, 317-338
20. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220-239
21. Munson, D. M., Sherrill, G. B., and Roberts, H. R. (1988) Anal. Biochem. 172, 427-435
22. Soriano-Garcia, M., Park, C. H., Tulinsky, A., Ravichandran, K. K., and Skrzypczak-Jankun, E. (1989) Biochemistry 28, 6805-6810
23. Klonis, K., Holland, S. K., Boys, C. W. G., Burgess, A. L., Esnouf, M. P., and Blake, C. C. F. (1987) Nature 330, 82-84
24. Ryan, J., Woltitzky, B., Heimer, E., Lambrusee, T., Felix, A., Tam, J. P., Huang, L. H., Narworth, P., Wilner, G., Kisiel, W., Nelsutsch, G. L., and Stern, D. M. (1986) J. Biol. Chem. 261, 20293-20297
25. Persson, E., Selander, M., Linsen, S., Drakenbert, T., Ohlin, A. K., and Stenflo, J. (1989) J. Biol. Chem. 264, 16987-16994
26. Handford, P. A., Baron, M., Mayhew, M. Willis, A., Beesley, T., Brownlee, G. G., and Campbell, I. D. (1990) EMBO J. 9, 475-480
27. McCord, D. M., Monroe, D. M., Smith, K. J., and Roberts, H. R. (1990) J. Biol. Chem. 265, 10250-10254
28. Ohlin, A.-K., Björk, L., and Stenflo, J. (1990) Biochemistry 29, 644-651
29. Derian, C. K., VanDusen, W., Przysiecki, C. T., Walsh, P. N., Berkner, K. L., Kaufman, R. J., and Friedman, P. A. (1989) J. Biol. Chem. 264, 6615-6618