Baculovirus-mediated Expression of the Epidermal Growth Factor-like Modules of Human Factor IX Fused to the Factor XIIIa Transamidation Site in Fibronectin

EVIDENCE FOR A DIRECT INTERACTION BETWEEN THE NH2-TERMINAL EPIDERMAL GROWTH FACTOR-LIKE MODULE OF FACTOR IXa AND FACTOR X*

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Factor IX is a vitamin K-dependent coagulant zymogen of a serine protease. In the presence of Ca2+ the active form of factor IX (factor IXa) forms a complex with factor VIIIa on suitable phospholipid surfaces such as aggregated platelets. This macromolecular complex rapidly activates factor X. We have previously provided data that suggest an interaction between the NH2-terminal epidermal growth factor (EGF)-like module of factor IXa and the substrate factor X. In an alternative approach to study this protein-protein interaction, we have expressed three recombinant baculovirus constructs encoding the EGF-like modules of human factor IX and a truncated form of fibronectin in a system based on the infection of insect cells (Spodoptera frugiperda 21). This strategy allows a simple one-step purification of the recombinant proteins on a gelatin-Sepharose column, followed by removal of the gelatin-binding part derived from fibronectin by proteolytic cleavage. The fusion proteins were isolated at yields of 20–50 μg/ml culture medium. The recombinant EGF-like modules contained 0.2–0.4 mol of erythro-δ-hydroxyaspartic acid/mol of protein, i.e., similar to the amount found in factor IX from human plasma, and appeared to be glycosylated at Ser-53. The NH2-terminal EGF-like module, which contained a transamidation acceptor site derived from fibronectin, was cross-linked by factor XIIIa in solution to intact and Gla-domainless factor X. There was no evidence of cross-linking to activated factor X or to factor X fragments containing only the γ-carboxyglutamic acid module and the two EGF-like modules. The cross-linking results suggest a specific interaction between the NH2-terminal EGF-like module of factor IXa and the heavy chain of unactivated factor X. This interaction, albeit weak as judged by competition experiments, may be important for the targeting of factor X to the factor IXa-factor VIIIa complex on biological membranes and for the subsequent dissociation of factor Xa from the complex after activation.

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1 The term module is used throughout this paper as suggested by P Athy (1) and by Baron et al. (2), rather than domain, repeat, or motif.
2 The abbreviations used are: EGF, epidermal growth factor; Gla, γ-carboxyglutamic acid; BfXa, residues 1–146 of bovine factor IX S-S-linked to residues 182–416; BfX, bovine factor X; BfXa, the β-form of activated bovine factor X; BfX-GlaEGFNC, residues 1–144 of bovine factor IX S-S-linked to residues 286–296; BfX-GlaEGFNC, the intact light chain of bovine factor X S-S-linked to residues 154–183 of the heavy chain; BfX-GlaEGFNC, residues 1–86 of the light chain of bovine factor X; Gb, pGE-1, residues 2356–2411 of mature fibronectin linked to the fibronectin mutant GAP 1–5; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; dansyl, 5-dimethylaminonaphthalene-1-sulfonil.
action between the recombinant NH₂-terminal EGF-like module of factor IXa and the heavy chain of factor X but not of factor Xa.

**EXPERIMENTAL PROCEDURES**

**Materials—**l-1-2-4-2-phenylphosphoryl phosphatase and leupeptin were from Sigma. The chromogenic substrate ω-naphthylamide-2-p-nitrophenylphosphatase was from Sigma. The chromogenic substrate ω-naphthylamide-2-p-nitrophenylphosphatase was from Sigma.

**Construction of Baculovirus Transfer Plasmids—**The baculovirus transfer plasmids were cotransfected with a previously described procedure (18) using Sequenase version 2 (U. S. Biochemical Corp.) to amplify the desired human factor IX cDNA sequences in a polymerase chain reaction (PCR) performed according to published procedures. The amplified DNAs were visualized on agarose gels, digested with EcoRI and XhoI, and ligated into the baculovirus transfer vector pAcYM1 (a gift from Dr. David Bishop, Institute of Virology, Oxford, UK) (Fig. 1) (47). Clones containing the insert in the correct orientation were identified by restriction enzyme analysis (XhoI) and characterized as described above. The two other transfer plasmids, pAcIXN and pAcIXaNC, were created in the same way.

**Isolation of Viruses—**Insect cells, Spodoptera frugiperda (Sf21), were generously provided by Dr. Paul Friesen (University of Wisconsin, Madison). The cells were cultured in TC100 medium (Life Technologies, Inc.) containing 10% fetal calf serum (HyClone, Logan, UT), supplemented with 2.5 μg/ml amphotericin B (Life Technologies, Inc.), 5 μg/ml streptomycin (Flow), 50 IU/ml penicillin (Flow), and 1% glucose. Restri ctant media were prepared from Boehmmering, Mannheim. Human factor IX cDNA was a kind gift from Dr. Kathleen Berkner (ZymoGenetics Inc., Seattle, WA). All other reagents were from sources listed in Refs. 22 and 26.

**Proteins—**Bovine factor X (BFX) (27), bovine factor Xa (BFXa) (28), Glu-domainless bovine factor X (29, 30), factor X-activating coagulant protein from Russell’s viper venom (31), fibronectin (32), bovine prothrombin (27), bovine protein Z (33), human α-thrombin (34), bovine factor X fragments (20, 35), and 1α/PG-1 (23) were isolated by published procedures. BFXa was from Enzyme Research Laboratories. Recombinant human factor XIII was from ZymoGenetics Inc. Human factor X was isolated from a factor IXA complex (Preconactase, Kabi), as previously described (18), using a monoclonal antibody against human factor X antigen (M5). Factor Xa was inactivated with 5-fold molar excess of dansyl-Glu-Gly-Arg chloromethyl ketone for 15 min and excess inhibitor removed by gel filtration. The remaining amidolytic activity against S-2765 was less than 0.01%. Marine mucosal mononuclear antibodies against human factor IX A were prepared by a previously described procedure (36), and polyclonal rabbit antibodies against the light chain of BFX were obtained by a sequential immunoadsorbative procedure (37). For the determination of protein concentrations the following absorption coefficients were used: BFXa, 14.3 and 46.500 (38); BFX, 12.4 and 55.000 (39); Glu-domainless bovine factor X, 11.9 and 50.000 (40); BFXa, 12.4 and 45.000 (41); human factor X, 11.6 and 59.000 (42); bovine prothrombin, 14.4 and 72.000 (43); bovine protein Z, 9.8 and 50.000 (44); BFX-Gla-fGln, 19.1 and 19.400 (45); BFX-GlaEGF1, 9.8 and 10.300 (20); fibronectin, 12.8 and 45.000 (45, 46), human α-thrombin, 19.5 and 37.400 (43); 1α/PG-1, 44.30; and BFX-GlaEGF1, 10.5 and 18.170 (36).

**Construction of Factor IX-containing Gelatin-Binding Expression Plasmids—**The following primers were synthesized in an Applied Biosystems 381 A DNA synthesizer to amplify the desired human factor IX cDNA sequences in a polymerase chain reaction (PCR) performed according to published procedures: GGGCATCCCCGGGATGGTGCTGGTTCACAGGACTTC (sense for pGE-IXaN); GGCCACGGGGATGGTGCTGGTTCACAGGACTTC (antisense for pGE-IXaN). Underlined sequences represent the 5' ends of the factor IX DNA to create a BamHI site for in-frame cloning into the gelatin-binding expression vector GE-1/pGEM4. This vector encodes the 32-amino acid preprodomain Acceptor Site (st-To prevent cleavage at lysine residues during tryptic cleavage, the purified recombinant proteins were cocrystallized with α-thrombin (23) and the sample chromatographed on a gelatin-Sepharose column (0.9 × 8 cm) before use (24). After 72 h the medium was collected and phenylmethylsulfonyl fluoride added to a final concentration of 2 mM. The conditioned medium was centrifuged at 28000 × g at 4°C until use. Sf21 cells were infected with dilutions of the transfection mixture as described by Summers and Smith (49) and the plates visually screened for recombinant occlusion-negative plaques after 3-4 days. Putative recombinant plaques were then plaque-purified three times to generate recombinant viruses free of contaminating wild-type virus (48, 49). The isolated recombinant viruses, designated as the rVIXaN, rIXN, and rIXaNC virus, respectively, were titred by the addition of 0.01% Neutral Red as described (50).

**Production and Purification of Recombinant Proteins—**The recombinant IXaNC (5 × 10⁸ cells/ml) was digested with recombinant factor IXa (5.0 pg/ml) and the sample chromatographed on a gelatin-Sepharose column (0.9 × 8 cm) as described above. The recombinant proteins were then gel filtered on a Sephadex G-50 column equilibrated in 0.1 M NaH₂PO₄ and eluted with 3 M guanidinium HCl. The protein-containing fractions were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. The proteins, designated according to the recombinant factor IX-containing virus used (1α/PG-IXaN/GAP-1, 1α/PG-IXA-1, and 1α/PG-IXaNC) were characterized with respect to their amino-terminal amino acid sequence, amino acid composition, and immunological properties.

**Treatment of Sf21 Cells with Pyridine-2,4-dicarboxylate—**The inhibitor pyridine-2,4-dicarboxylate was solubilized in deionized water and either added to the incubation medium or preincubated with the recombinant IXaNC for 1 h before infection or after 1 day. Prior to infection the medium was then harvested after 72 h and 5 ml were used for experiments where the inhibitor was added after 1 day, the cells were incubated with 4°C until use. Sf21 cells were infected with dilutions of the transfection mixture as described by Summers and Smith (49) and the plates visually screened for recombinant occlusion-negative plaques after 3-4 days. Putative recombinant plaques were then plaque-purified three times to generate recombinant viruses free of contaminating wild-type virus (48, 49). The isolated recombinant viruses, designated as the rVIXaN, rIXN, and rIXaNC virus, respectively, were titred by the addition of 0.01% Neutral Red as described (50).

**Isolation of EGF Fragment Containing the Factor XIIa Transamidination Acceptor Sites—**To prepare cleavage at lysine residues during tryptic cleavage, the purified recombinant proteins were cocrystallized with α-thrombin (23) and the sample chromatographed on a gelatin-Sepharose column (0.9 × 8 cm) as described above. The recombinant proteins were then gel filtered on a Sephadex G-50 column equilibrated in 0.1 M NaH₂PO₄ and eluted with 3 M guanidinium HCl. The amino acid sequence and composition of each protein fraction were determined.

**Cross-linking Reactions—**Cross-linking experiments were performed in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 10 mM CaCl₂. The incubation mixture (100 μl) contained the recombinant fusion proteins, the EGF-containing fragments, or 1α/PG-1 at a concentration of 2 μM, human α-thrombin (5 nM), recombinant factor XII (40 μg/ml), and either monodansylcadaverine (0.7 mM) or protein ligands as described. Incubation for 2 h at 37°C, the reactions were terminated by the addition of EDTA to 20 mM and 35 μl of a buffer, containing 3% SDS, 30% sucrose, and 0.01%
Recombinant EGF-like Modules of Human Factor IX

Expression and Characterization of Recombinant Proteins—Construction of the recombinant baculovirus rVIXaN is outlined in Fig. 1. The other two recombinant viruses, rVIXN and rVIXaNC, were created in the same way. Sf21 cells were infected with the purified viruses for 72 h in the presence of leupeptin to minimize the internal cleavage at Arg-259 that otherwise was present in 5–20% of the molecules (Fig. 3). The amount of fusion protein secreted into the culture medium at 72 h from a monolayer culture was estimated after purification on a gelatin-Sepharose column by amino acid analysis to be approximately 50 μg/ml with rVIXN, 40 μg/ml with rVIXaN, and 20 μg/ml with rVIXaNC. The recombinant fusion proteins were designated IXaN/GAP 1–5, IXaN/GAP 1–5, and IXaN/GAP 1–5. In all three proteins two NH2-terminal sequences were identified: Lys-Glu-Thr-Gly-Lys-Thr-Glu-Thr-Gly-Lys-Ser, corresponding to residues 1–6 in rat fibronectin and Glu-Ala-Gln-Gln-Ile-Val, corresponding to residues 1–6 of human factor IX. The relative amount of the first sequence, which probably reflects the beginning of the propeptide, varied between 10 and 70%. However, the amount of material without putative propeptide may have been underestimated due to cyclization of the NH2-terminal Gln to pyroglutamic acid (54).

The trypsin-sensitive site at Arg-259 (51) was used to remove the gelatin-binding part of each recombinant protein (residues 260–572 in rat fibronectin) (Figs. 2 and 3). To protect Lys-43 (residues numbered as in factor IX), the proteins were reversibly citraconylated before tryptic cleavage. Thrombin was unable to cleave the fusion proteins at Arg-259, although this cleavage has been described for native fibronectin (55). The material eluted with guanidine HCl from the gelatin-Sepharose column had a sequence corresponding to residues 260–265 in rat fibronectin (Thr-Ala-Ile-Tyr-Gln-Pro) and an amino acid composition corresponding to residues 260–572 (not shown). The flow-through fractions had an NH2-terminal sequence corresponding to residues 1–6 of rat fibronectin (see above). The amount of propeptide-containing material was reduced to less than 10%. The amino acid compositions were in good agreement with fragments consisting of the first 12 amino acids of mature fibronectin, including the factor XIIIa acceptor site at Gln-3, followed by residues 33–85 of human factor IX in αEGFα, residues 41–85 in EGFβ, residues 33–127 in αEGFαNC, and a 26-amino acid residue peptide of fibronectin at the COOH-terminal end of each fragment (Fig. 3; Table I). The proteins were found to contain 0.2–0.4 mol erythro-β-hydroxyaspartic acid/mol of protein. The hydroxylation was inhibited by pyridine-2,4-dicarboxylate, a known inhibitor of 2-oxoglutarate-dependent dioxygenases (56) (Table II). The absorption coefficients (ε1% at 280 nm) of the apoproteins were calculated to be 30 (EGFα), 28 (αEGFα), and 25 (αEGFβ) based on molecular weights of 9,100, 10,100, and 14,700, respectively.

The isolated EGFα fragment, subjected to NaOH/Na2SO4 treatment, was found to contain 1.2–1.4 mol of cysteic acid/mol of protein. When chemically synthesized factor X-EGFα was subjected to the same treatment, a background of 0.2–0.4

RESULTS

Recombinant Baculovirus rVIXaN

FIG. 1. Scheme for construction of the recombinant baculovirus rVIXaN. The cDNA coding for the COOH-terminal α-helical part of the EGF module and the NH2-terminal EGF-like module of human factor IX was amplified by PCR using human factor IX cDNA in the pUC8 vector as a template. The resulting PCR fragment was digested with BstXI and ligated into the BstXI site of the GE-1/pGEM4 vector described under "Experimental Procedures." The coding sequence of truncated fibronectin lacking the first five type I modules (GAP 1–5) is hatched. The cloned pGE-IXaN construct was digested with BamHI and the insert ligated into the BamHI site of the baculovirus transfer vector pAcYMI. The created plasmid designated pAcIXaN, thus contained the PCR-amplified human factor IX DNA linked to the DNA coding for the fibronectin mutant Gap 1–5 under the control of the viral polyhedrin promoter. This plasmid was then cotransfected with wild-type A. californica nuclear polyhedrosis virus DNA in Sf21 to allow the cloning of the recombinant virus rVIXaN. The sequences amplified by PCR were sequenced to ensure that no mutations had been introduced.
mol of cysteic acid/mol of protein was obtained. The results indicate the presence of O-linked carbohydrate side chain(s) in the recombinant factor IX fragment. Moreover, the serine residue in position 53 in the recombinant NH$_2$-terminal EGF-like module, known to be glycosylated in human factor IX (8), was undetectable on sequencing, whereas the following two residues were found in the expected amount, suggesting that Ser-53 was modified. Before reduction all three fusion proteins bound a monoclonal antibody recognizing the NH$_2$-terminal EGF-like module of human factor IX in Western blotting experiments (Fig. 4). The antibody did not bind the reduced proteins. Attempts to visualize the isolated EGF module-containing fragments by Western blotting were unsuccessful as they did not bind to the membrane. Functioning factor XIIIa transamidation acceptor site(s) were identified in all three fusion proteins, as well as in the isolated EGF-like fragments by cross-linking with the fluorescent amine monodansylcadaverine (Fig. 5).

**Effects of Recombinant EGF-like Fragments on Factor X Activation**—In previous papers, we have described the inhibitory effects of proteolytic fragments of bovine factor IX on factor X activation by factor IXa$\beta$ (22, 53). The effects of the recombinant EGF fragments were investigated using the same systems in the absence of factor VIIIa and phospholipid. The $\alpha$EGF$_{NC}$ fragment was the most potent inhibitor of factor Xa formation with a $K_s$ of $\approx 2 \mu M$ (Fig. 6). The fragments $\alpha$EGF$_N$ and EGF$_N$ also inhibited factor Xa formation but more weakly ($K_s$ $\approx 8$ and 20 $\mu M$, respectively). No inhibition was found with intact fibronectin. The inhibition data were best fit to a substrate depletion model in which the fragments bind to the substrate, factor X, forming a complex that is not available for activation by factor IXa$\beta$. The EGF-like fragments had similar effects on the activation of Gla-domainless Bx (not shown).

**Cross-linking of Recombinant EGF-like Modules to Factor X**—Cross-linking experiments with the fluorescent amine monodansylcadaverine demonstrated that the recombinant proteins contained functioning factor XIIIa transamidation acceptor site(s) (Fig. 5). Small amounts of cross-linked complexes were also found. It is noteworthy that, although trypsin was efficient in cleaving the recombinant native constructs, cleavage progressed much more slowly after incorporation of the dansyl group (not shown). This may be due to additional acceptor sites close to Arg-259 (Fig. 3) that have a sequence corresponding to that of reactive glutamines in $\beta$-casein (57).

To shed light on the nature of the putative interaction between the NH$_2$-terminal EGF-like module of factor IXa$\beta$ and the substrate factor X (22, 53), we have attempted to cross-link the recombinant EGF-like modules to proteolytic fragments of factor X and visualize the products by Western blotting. The recombinant EGF-containing fragments alone did not bind to the Immobilon membrane, a feature also observed for EGF-like fragments from factor X. The immunoblot, using a monoclonal antibody against the NH$_2$-terminal EGF-like module of factor IX, would therefore only be positive if this module had been linked to another molecule that binds to the membrane. Employing affinity-purified polyclonal antibodies against the light chain of bovine factor X, complexed and uncomplexed factor X (indicated by arrows in Figs. 7 and 9) were identified. The recombinant EGF$_N$ fragment was cross-linked to intact and as well to Gla-domainless factor X, both in the presence and absence of bovine serum

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**Fig. 2. Time course for the digestion of citraconylated recombinant IXN/GAP 1–5 protein with trypsin.** The digestion of citraconylated recombinant IXN/GAP 1–5 protein (0.3 mg/ml) was performed at 37°C with 0.1% trypsin in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 5 mM EDTA. Aliquots of 18 $\mu g$ were withdrawn from the incubation mixture and subjected to SDS-PAGE on a 10–15% gradient gel. Fifteen pg of the undigested protein was given, and on the left side of the gel molecular weight markers are shown. The samples were not reduced. The fusion protein and the gelatin-binding fragment of fibronectin have apparent molecular weights on the gel of 65,000 and 45,000, respectively. The EGF-like module (molecular weight of the apoprotein $\approx 9,100$; Fig. 3) was diffuse due to glycosylation, stained poorly by Coomassie Brilliant Blue, and is not shown on the gel.

**Fig. 3. Schematic model of the recombinant IXN/GAP 1–5 protein.** The NH$_2$-terminal EGF-like module in human factor IX was expressed as a fusion protein with the fibronectin mutant GAP 1–5 (see under “Experimental Procedures”). The arrow denotes the proposed beginning of the prepropeptide. The amino acids in the GAP 1–5 protein are numbered as in mature fibronectin, whereas the EGF$_N$ insert is numbered as in intact factor IX. The asterisks denote the proposed factor XIIIa transamidation sites and the shaded Asp residue in the EGF-like module the site of $\beta$-hydroxylation. The duplication of the PSPW sequence of fibronectin results from introduction of the BstXI site.
The composition according to the expected sequence is shown in parentheses.

| Amino acid | EGF<sub>N</sub> | αEGF<sub>N</sub> | αEGF<sub>NC</sub> |
|------------|----------------|----------------|----------------|
| Asp        | 9.5 (9)        | 10.1 (10)      | 17.9 (16)      |
| Thr        | 0.9 (1)        | 3.6 (4)        | 5.8 (6)        |
| Ser        | 9.6 (11)       | 9.8 (11)       | 11.0 (14)      |
| Glu        | 12.9 (12)      | 15.9 (15)      | 22.5 (21)      |
| Pro        | ND* (7)        | ND* (7)        | ND* (8)        |
| Gly        | 7.9 (7)        | 8.6 (7)        | 11.0 (9)       |
| Ala        | 3.8 (4)        | 3.8 (4)        | 6.2 (7)        |
| Cys        | ND* (6)        | ND* (6)        | ND* (12)       |
| Val        | 4.8 (5)        | 4.7 (5)        | 7.5 (8)        |
| Met        | <0.1 (0)       | <0.1 (0)       | <0.1 (0)       |
| Ile        | 1.9 (2)        | 2.3 (2)        | 3.0 (3)        |
| Leu        | 3.2 (3)        | 3.2 (3)        | 3.5 (4)        |
| Tyr        | 2.1 (2)        | 1.9 (2)        | 2.5 (3)        |
| Phe        | 3.2 (4)        | 4.0 (4)        | 4.9 (5)        |
| His        | <0.1 (0)       | <0.1 (0)       | <0.1 (0)       |
| Lys        | 3.8 (4)        | 3.9 (4)        | 7.6 (8)        |
| Arg        | 0.8 (1)        | 1.6 (2)        | 4.0 (4)        |
| Trp        | ND* (4)        | ND* (4)        | ND* (4)        |
| Hya        | 0.35           | 0.25           | 0.40           |

*ND,* not determined.

Determined after 72 h of hydrolysis.

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**Table II**

**Inhibition of β-hydroxylation with pyridine-2,4-dicarboxylate**

Sf21 cells were infected with a recombinant baculovirus encoding the NH<sub>2</sub>-terminal EGF-like module of factor IX embedded in the fibronectin mutant GAP 1-5 as described under "Experimental Procedures." The cells were then either incubated with pyridine-2,4-dicarboxylate immediately postinfection day 0 and the medium harvested after 72 h or incubated 24 h in the absence of the inhibitor followed by a 7-h preincubation period in the presence of the inhibitor before a change to fresh inhibitor-containing medium and a 40-h harvest period.

| Treatment        | Hya % of control mol/mol protein |
|------------------|---------------------------------|
| Control          | 0.32                            |
| 10 mM pyridine-2,4-dicarboxylate dl-3 | 44 | 0.14  |
| 20 mM pyridine-2,4-dicarboxylate dl-3 | 25 | 0.08  |
| 20 mM pyridine-2,4-dicarboxylate d0-3 | <6 | <0.02 |

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**FIG. 4. Immunoblotting of recombinant proteins.** The recombinant fusion proteins were purified on a gelatin-Sepharose 4B column, and aliquots of 5 μg of the IXN/GAP 1-5 (lane 2), IXαN/GAP 1-5 (lane 3), and IXαNC/GAP 1-5 (lane 4) fusion proteins were applied to a 10–15% SDS-PAGE gradient gel without prior reduction. In lane 1, 2 μg of purified human factor IX was applied. After transfer onto an Immobilon membrane, the proteins were incubated with a monoclonal antibody against the NH<sub>2</sub>-terminal EGF-like module of human factor IX, followed by an alkaline phosphatase-conjugated rabbit anti-mouse IgG fraction. The membranes were developed as described under "Experimental Procedures." The high molecular weight bands in lanes 2–4 correspond to multimers of the recombinant proteins and the band with an apparent molecular weight of approximately 60,000 in lane 1 to activated factor IXβ.

**DISCUSSION**

The baculovirus expression system has been used successfully to express several multidomain human proteins. We have now used this system to express the EGF-like modules of human factor IX and the adjacent α-helical part of the Glα module as fusion proteins with a truncated fragment of mature fibronectin that lacks the five NH<sub>2</sub>-terminal type 1 homology units (GAP 1–5) (23). This procedure endows the recombinant proteins with three important properties derived from fibronectin: secretory processing by insect cells, a gelatin-binding domain, which allows simple one-step purification of the recombinant proteins (24) and then can be removed by limited trypsic cleavage (51), and transamidation acceptor site(s), which allow factor XIIa-mediated cross-linking (25).

The occurrence of a sequence in the recombinant proteins beginning at residue –8 in fibronectin probably reflects incomplete proteolytic removal of the propeptide and that the insect cells process fibronectin in the way proposed based on the cDNA sequence, *i.e.* removal of a signal peptide followed by removal of the propeptide at the canonical KSRQ sequence (Fig. 3). The sequences LCLTG/L and LGT5/V have been proposed as likely sites for removal of the signal peptide (58). Our data, however, indicate that the site is SVRC/T. Signal peptidease cleavage carboxyl terminus of a Cys residue in the prepropeptide has also been described in other plasma proteins (59). We have three reasons to believe that the recombinant EGF-like modules were correctly folded: 1) the proteins were exocytosed, suggesting a native conformation; 2) a monoclonal antibody against the NH<sub>2</sub>-terminal EGF-like module in human factor IX bound to the unrecombined recombinant proteins but not to the reduced form (this antibody recognizes native plasma factor IX but not the reduced and carboxymethylated molecule); 3) the recombinant EGF-like module contained erthro-β-hydroxyaspartic acid in an
amount similar to that found in the native human factor IX molecule. As the Asp/Asn ϐ-hydroxylase requires a correctly folded EGF-like module as substrate, this implies a native conformation (60). Pyridine-2,4-dicarboxylic acid, an inhibitor of the Asp/Asn ϐ-hydroxylase in human cells (56), inhibited the ϐ-hydroxylation apparently without affecting the expression level. Recently, Monkvick and colleagues (61) purified an Asp/Asn ϐ-hydroxylase from insect cells that had a cofactor requirement and substrate specificity similar to that of the human enzyme. In addition, the recombinant NH2-terminal EGF-like module seemed to contain an O-linked carbohydrate moiety at Ser-53, a position where Iwanaga and co-workers (8) have found a disaccharide side chain with a unique structure.

Competition studies suggest that the NH2-terminal EGF-like module of factor IXaβ interacts directly with the substrate, factor X, either through an EGF-EGF interaction or through an interaction between the EGF-like module and the serine protease module of factor X (22, 53). We have now used the transamination acceptor site(s) in the recombinant proteins in conjunction with factor XIIIa to further investi-

FIG. 5. Identification of factor XIIIa transamination site(s) in the recombinant proteins. A, the isolated recombinant fusion proteins IXN/GAP 1–5 (lane 1), IXaN/GAP 1–5 (lane 2), and IXaNC/GAP 1–5 (lane 3), each at a concentration of 2 μM in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 10 mM Ca2+, were incubated with monodansylcadaverine (0.7 mM), human factor XIII (40 μg/ml), and human ϩ-thrombin (5 nM) in a total volume of 100 μl. The reactions were performed at 37°C and terminated after 2 h by the addition of 20 mM EDTA. The samples were reduced with 2-mercaptoethanol before electrophoresis on a 10–15% SDS-PAGE gradient gel and the proteins visualized by using an ultraviolet light source. B, the factor XIIIa transamination acceptor site(s) were also identified in the same way in the isolated EGFN-fragment (lane 2) after tryptic cleavage. In lane 1 the intact recombinant fusion protein IXN/GAP 1–5 is shown. The heterogeneity of EGFN is probably due to the carbohydrate side chain(s) and the weaker bands with higher molecular weights presumably correspond to multimers of the fragment. The samples were reduced before electrophoresis. The fluorescent band at the bottom of the gel represents unincorporated monodansylcadaverine.

FIG. 6. Effects of recombinant EGF-like fragments on factor X activation. The effect of the recombinant αEGFα (○), αEGFα (●), and EGFα (△) fragments and intact fibronectin (■) on the activation of factor X (1.2 μM) by factor IXαβ (0.1 μM) was measured in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 4.2 mM Ca2+, 1 mg/ml polyethylene glycol 6000. Increasing concentrations of the fragments were added and the initial rate of factor Xa formation measured through the hydrolysis of the chromogenic substrate S-2765. The data have been expressed as the percent of control activity, which corresponds to the ratio of v in the presence of 1 to v in the absence of I times 100. The solid lines were drawn using the substrate depletion model described in the accompanying paper (53). The dashed line represents no effect of inhibitor.

FIG. 7. Cross-linking experiments with the recombinant EGFα-fragment. The factor XIIIa-mediated cross-linking of recombinant EGFα-fragment (2 μM) to various concentrations of bovine factor X (A) and Gla-domainless bovine factor X (B) was performed in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, 10 mM Ca2+, 5 nM human ϩ-thrombin, and 40 μg/ml human factor XIII. Each incubation mixture was applied to a 10–15% SDS-PAGE gradient gel, transferred to an Immobilon membrane, and visualized by Western blotting using a monoclonal antibody against the NH2-terminal EGF-like module of human factor IX. The arrows indicate the position of the uncomplexed proteins. The protein concentrations were as follows: A, 0 μM (lane 1), 1 μM (lane 2), 5 μM (lane 3), 10 μM (lane 4), and 40 μM (lane 5) bovine factor X; B, 0 μM (lane 1), 2 μM (lane 2), 8 μM (lane 3), and 16 μM (lane 4) Gla-domainless bovine factor X.
Fig. 8. Effects of GlaEGF fragments from factors IX and X on cross-linking of recombinant GlaEGFα to factor X. Reombinant GlaEGFα fragment was cross-linked to bovine factor X as described in the legend to Fig. 7, both in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of GlaEGF fragments. The factor X concentration was 8 μM and that of BIX-GlaEGFα (lane 2) and BIX-GlaEGFβ (lane 4) 25 μM, respectively.

Fig. 9. Comparison of the cross-linking of recombinant GlaEGFα fragment to bovine factors X and Xa. The factor Xilla-mediated cross-linking of the recombinant GlaEGFα fragment (5 μM) to factors X and Xa was performed as described in the legend to Fig. 7. The arrows indicate the position of the uncomplexed proteins. The factor X concentrations were 0 μM (lane 1), 5 μM (lane 2), and 30 μM (lane 3), and the factor X concentrations were 5 μM (lane 4) and 30 μM (lane 5).

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action, albeit weak as judged by competition experiments, appears to be specific for thezymogen form of factor X. This may be important for the targeting of factor X to the active site of factor IXαβ in complex with factor VIIIa on biological membranes, i.e. the EGF-like modules of factor IXαβ can be regarded as part of the cofactor. The apparent lack of affinity of the EGF-like module for the serine protease module once factor X is activated suggests that this interaction is physiologically relevant. It is noteworthy that there is a precedence for a direct interaction between EGF-like modules and a serine protease module. In thrombomodulin two EGF-like modules bind thrombin with high affinity and even change the substrate specificity of the enzyme (10, 11).
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