Localizatión of the Human Tissue Factor Recognition Determinant of Human Factor VIIa*

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Tissue factor is an integral membrane glycoprotein that serves as an essential cofactor for the blood coagulation factor VIIa. Recent studies have attempted to localize the tissue factor recognition determinant of human factor VIIa. While several regions of factor VIIa have been implicated as important for tissue factor binding, the high affinity tissue factor recognition determinant of human factor VIIa is unknown. In order to define the determinant, we constructed a set of six chimeric proteins composed of portions of factor VII and factor IX. We then utilized the chimeras in competition experiments with 125I-labeled factor VIIa for recombinant tissue factor bound to an Immobilon-P membrane. The data indicate that the high affinity tissue factor recognition determinant of human factor VIIa is within the epidermal growth factor domains.

Factor VII is a vitamin K-dependent glycoprotein that plays a critical role in the early stages of blood coagulation. Within the vasculature, factor VII circulates as a zymogen serine protease. Conversion of factor VII to its activated form, factor VIIa, is achieved by proteolytic cleavage at a single site within the zymogen VII, resulting in a two-chain serine protease composed of an (NH2-terminal) light chain of 20 kDa and a heavy chain of 30 kDa connected by a single disulfide bond. The light chain is composed of a series of domains. The NH2-terminal domain is the γ-carboxyglutamic acid (Gla) domain that is believed to confer phospholipid binding capabilities to the protein (1). Following the Gla domain are an aromatic stack domain and two epidermal growth factor (EGF1 and -2)-like domains which prior to this report had no demonstrable function. The serine protease activity is conferred by the trypsin-like heavy chain.

Tissue factor (TF) is an integral membrane glycoprotein that is expressed in a variety of tissues where it serves as a cofactor for factor VII and VIIa. Factor VIIa possesses little or no catalytic activity in the absence of tissue factor and Ca2+ ions. When factor VIIa is complexed with tissue factor apoprotein, Ca2+ ions, and phospholipid, an ~57,000-fold increase in catalytic efficiency is observed when compared with the identical complex minus tissue factor (2). The interaction of factor VII with tissue factor has been explored by kinetic analysis (2) and by direct binding experiments (3–8). These studies established that factors VII and VIIa bind to tissue factor with comparable affinities. The VII(a)/TF dissociation constants vary with experimental conditions and range from 0.08 nM to 13 nM. Previous work has also established a factor VII/tissue factor stoichiometry of 1:1 and suggests that tissue factor exists as a dimer on the cellular surface (3). Recent studies suggest that the Gla domain and a 12-amino acid segment of the heavy chain are important in factor VII(a) binding to tissue factor (6–8).

In an effort to definitively delineate the tissue factor recognition determinant on factor VII(a), we prepared a series of chimeric proteins composed of regions of factors VII and IX. We then evaluated these factor VII/IX chimeras for their ability to interact with human tissue factor apoprotein. The data in this report suggest that the high affinity tissue factor recognition determinant of human factor VII(a) resides within the factor VII EGF domains and that the factor VII Gla domain and heavy chain do not directly participate in the high affinity binding to tissue factor.

EXPERIMENTAL PROCEDURES

Materials

Immobilon-P transfer membrane was purchased from Millipore. The Minifold I dot blot apparatus was purchased from Schleicher & Schuell. Nonfat dry milk is a product of the Carnation Co. Tissue factor apoprotein was a generous gift of Genentech, Inc. T-4 DNA ligase, restriction endonucleases, polynucleotide kinase, and Sequenase were purchased from U. S. Biochemical Corp. Calf intestine alkaline phosphatase was purchased from Boehringer Mannheim. Monoclonal antibodies A7, A5, and A1 were previously described (9, 10). The monoclonal antibody FxCO08 was a gift from S. Paul Bajaj and has also been previously described (10, 11). The two monoclonal antibodies to factor VII, one Ca2+-dependent (CDVII) and the other Ca2+-independent (CIVII), were gifts from Dr. U. Hedner (Novo Nordisk, Copenhagen) and Dr. Walter Kisiel (University of New Mexico School of Medicine, Albuquerque). Oligonucleotides were purchased from Bio-Rad, IODO-BEADS from Pierce Chemical Co., and Genetin (G418) from Gibco/IRL. Human factor IX was purchased from Enzyme Research Laboratories. 125I-Protein A, Na125I, and (α-25S)daTP were purchased from Amersham Corp. Vitamin K (Aquamethyl) was purchased from Merck Sharp and Dohme. Gla analysis was generously provided by Dr. Paul Friedman of Merck Sharp and Dohme (West Point, PA).

Methods

Cloning of the Factor VII cDNA—The factor VII cDNA was cloned by utilizing the polymerase chain reaction (PCR) (12). The complete nucleotide coding sequence of factor VII was assembled from five

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‡ The abbreviations used are: Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor; TF, tissue factor; rTF, recombinant tissue factor; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MoAb, monoclonal antibody.
factor VII fragments generated by PCR. The fragments were amplified from both a human liver cDNA preparation and from human genomic DNA. The oligonucleotides used to prime the amplification were designed by evaluating the previously published factor VII amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15). The program back-translates an amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15). The program back-translates an amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15). The program back-translates an amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15). The program back-translates an amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15). The program back-translates an amino acid and nucleotide sequence (13, 14) with the DNA analysis program of Little and Mount (15).

Consequently, this allowed the five factor VII PCR fragments to ultimately be connected into the full-length factor VII coding sequence. This full-length cDNA was then sequenced completely by the dideoxy chain termination method (16). In addition to the unique restriction sites, oligo 7-1, which represents the 5' end of the cDNA, also contains a Kozak sequence (GCCACC) (17) prior to the start site ATG.

Creation of the Chimeric cDNAs—In order to create the six chimeric cDNAs, compatible restriction sites were introduced into the cDNAs of factor VII and IX. The restriction sites were introduced at points within the cDNAs that correspond to portions of the two proteins that are identical. The modified cDNAs were then digested with the appropriate sets of restriction endonucleases. The various cDNA fragments that comprise the chimeric constructions were then purified, assembled into the complete chimeric cDNAs, and sequenced. The restriction sites were incorporated into the cDNAs of factors VII and IX either by site-directed mutagenesis (18, 19) or in some instances during the PCR amplification as in the case of factor VII cDNA. The factor IX cDNA was available in our laboratory and had already been partially modified to include two restriction sites at appropriate places within the cDNA (20). The design of the oligomers for mutagenesis was again guided by the program of Little and Mount (15) (Table I, B). A general representation of the chimeric protein constructions is shown in Fig. 1.

Expression and Purification of Recombinant Proteins—The factor VII, factor IX, and chimeric cDNAs were cloned into the mammalian expression vector PCMv5 (21). Each one of the expression constructs was then cotransfected (22) with pSV2-neo (23) into the human kidney cell line 293 (ATCC CRL 1573). Cells were maintained in Delbecco’s modified Eagle’s medium (DMEM) (high glucose) supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal calf serum. Stable transformants were selected with the antibiotic G418. Resistant colonies were subcloned and the culture media assayed for recombinant protein expression by immunoradiometric assay (20). The monoclonal antibodies used in these analyses (described above) were used in various combinations depending upon which recombinant protein was being detected. The colonies with the highest levels of recombinant protein expression were selected for expansion. The transformants were expanded and transferred to 850-cm² roller bottles for larger scale protein production. The expression medium was composed of DMEM/F-12 (high glucose) containing 1-1.5% fetal calf serum and 2 µg/ml vitamin K. The medium (175 ml/bottle) was harvested every 2 days and filtered through a 0.45-µm Durapore filter (Millipore) and then frozen at ~70°C. After several liters were collected from each transformant, the medium was thawed and the CaCl₂ concentration adjusted to 20 mM. The medium was applied directly to a column containing one of the Ca²⁺-dependent (conformation-specific) monoclonal antibodies (A7 or CDVII) coupled to either AffiPrep 10 or Affi-Gel 10 (~2-5 mg/ml) The column was washed initially with 20 mM Tris-Cl, pH 7.2, 0.05% Tween 20, 100 mM NaCl, 20 mM CaCl₂. This was followed by a second wash with 20 mM Tris-Cl, pH 7.2, 1.0 M NaCl, 20 mM CaCl₂. The proteins were eluted from the column with 20 mM Tris-Cl, pH 7.2, 100 mM NaCl, 20 mM EDTA and were then concentrated with a microconcentrator Centricon 30 (Amicon). The protein concentration was determined by utilizing the Bio-Rad protein assay kit.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis (PAGE) and Western

### Table I

Oligonucleotides used in the polymerase chain reaction (PCR) and mutagenesis A, oligomers used in the PCR amplification of fragments of the human factor VII cDNA. The underlined nucleotide sequence correponds to the respective restriction sites. Boldface nucleotides correspond to the Kozak sequence. The five fragments were subsequently connected into the full-length factor VII coding sequence. Fragment 1 lacks the 66 base pairs that correspond to the alternatively spliced exon 2, resulting in a 38-amino acid prepro leader. B, oligomers used in the mutagenesis of the factor VII and IX cDNAs.

| A | Fragment | 5' oligo | 3' oligo | Sequence | Restriction site | DNA template |
|---|----------|----------|----------|----------|-----------------|-------------|
| 1 | 7-1      | TCAGTCCGCCCAGCATGGTCTCCACGCGAGGCTCC | BamHI | Human liver cDNA |
| 2 | 7-2      | CCGGAATTTCGACACTGTCAGGACATCTACTGTAAGAATC | BstII |
| 3 | 7-3      | AGTGTAGTTGACGAGGATGGCCTCAAAGT | BstII |
| 4 | 7-4      | AGGTATGCGGCGGCGATGATAATTCAACTGTGG | Not1 |
| 5 | 7-5      | CCAATGCGGCGCCTACAATCTACATGAAAAAGA | Not1 |
| 6 | 7-6      | CTGCATGACGACTCCCTTTGGG | Neo1 |
| 7 | 7-7      | TGGCCTAGGAGCTGTGGTGGTGGG | Neo1 |
| 8 | 7-8      | CCTTCGCCGACGCGGATGACGTTCTCTCCA | Eco1 |
| 9 | 7-9      | GTGTCTGGGAGACGACCTACGAGGACAGCACAG | Eco1 |
| 10| 7-10     | TCGGATTCCATGAAATGGGGGTTGCCAGGAG | BamHI |

| B | Oligomer | Sequence | Template VII | cDNA IX | Result of mutagenesis |
|---|----------|----------|-------------|---------|----------------------|
| Mute 1 | GACATGTCTGAGGTTG | X | X | Created Xhol sites; used in the creation of the IX LC/VII HC |
| Mute 2 | GCAAACCAGAAATCCTGAGTTGGGGG | X | X | Created BglII sites; used in the creation of the VIII LC/IX HC |
| Mute 3 | AGTCCGACGACGCCCAGGGGCAATGGGGG | X | X | Reversed mutation resulting from PCR oligomer 7-4 |
Monoclonal Antibodies Used in Immunodot Assays and Western Blot Analysis—The specificities of the factor IX monoclonal antibodies (MoAb) are as follows: MoAb A7, specificity is between amino acids 1 and 42; MoAb A1, amino acids 147–153; MoAb A5, amino acids 180–310; MoAb FXc008, amino acids 180–310 (10). The specificity of the factor VII MoAb CDVII resides within amino acids 1–50 of factor VII. The specificity for MoAb CIVII resides within amino acids 50–152 of factor VII.

Iodination of Factor VII—Factor VIIa was iodinated with Na125I using IODOBeads according to the manufacturer's instructions. The radiolabeled factor VIIa was separated from free Na125I on a Sephadex G-25 (fine) column. The radiolabeled recombinant factor VIIa had a specific activity of ~1.0 µCi/µg and retained normal clotting activity by a prothrombin time assay.

Binding Studies—Recombinant human tissue factor apoprotein (rTF) was provided to us at a concentration of 0.1 mg/ml in 10 mM sodium phosphate, 150 mM NaCl, 0.9% octyl glucoside, pH 7.3. Just prior to use, the rTF was diluted to a concentration of 0.5 µg/300 µl in Western blot transfer buffer composed of 20 mM Tris, pH 7.5, 77 mM glycine, and 20% methanol. The rTF was then dot blotted on a prewet Immobilon-P transfer membrane. The Immobilon-P membrane was prewet according to the manufacturer's instructions. The dot blotting was conducted using a Minifold I dot blot apparatus. The membrane was prewet according to the manufacturer's instructions. The dot blotting was conducted using a Minifold I dot blot apparatus. The membrane was prewet according to the manufacturer's instructions. The dot blotting was conducted using a Minifold I dot blot apparatus. The membrane was prewet according to the manufacturer's instructions. The dot blotting was conducted using a Minifold I dot blot apparatus. The membrane was prewet according to the manufacturer's instructions.

Binding studies were performed as described (24).

Production and Characterization of Recombinant Proteins—In this study, eight recombinant proteins were generated. Six of the eight are chimeric proteins composed of portions of factors VII and IX. The other two remaining are wild-type factors VII and IX (Fig. 1). The proteins were produced in the human embryo kidney cell line 293 and then immunopurified with Ca2+-dependent monoclonal antibodies. The purified proteins were initially evaluated by SDS-PAGE followed by silver staining. Recombinant factor VII was activated to VIIa during the expression and purification. Because of the complexity of the different activators needed, the activation and the biological activity of these proteins will be described separately. The mobility of the eight recombinants relative to the plasma factors VII and IX were appropriate (data not shown). The recombinants were further characterized by Western blot analysis using monoclonal antibodies with recognition epitopes in the Gla/aromatic stack region of factor VII and IX, the activation peptide of factor IX, and the heavy chain of factor IX (Fig. 2). These characterizations indicate that the set of eight recombinants possesses the expected antibody recognition epitopes and is consistent with the proteins depicted in Fig. 1. The clotting activities of wild type recombinant factors VII and IX were normal.

Tissue Factor Binding—Other studies have shown that rTF will specifically interact with factor VII (2, 7). rTF enhances catalytic efficiency of factor VIIa by 5000-fold, which is ~5% of the enhancement observed in the presence of reconstituted tissue factor (2). In order to evaluate the tissue factor/factor VII interaction we chose to utilize the observation that tissue
factor and factor VII interact effectively and specifically in the absence of phospholipid. This capability allowed us to use purified components to study the interaction, thus eliminating the complicating features of nonspecific phospholipid binding in reconstituted tissue factor assays or internalization of ligand characteristic of cell binding assays. We examined the capability of the six factor VII/IX recombinant chimeras and the two wild-type protein factors VIIa and factor IX (Fig. 1) to bind to rTF bound to an Immobilon-P membrane. The initial experiments were to evaluate the specificity of binding of recombinant factor VIIa to rTF bound to an Immobilon-P membrane. This was demonstrated by incubating $^{125}$I factor VIIa with Immobilon-P membrane circles with and without blotted rTF (see “Methods”). The amount of $^{125}$I factor VIIa bound to the membrane in the absence of rTF was <10% of the amount bound in the presence of the blotted rTF. When EDTA was included in the washing solution, the amount of $^{125}$I factor VIIa that remained bound to the rTF circles was identical to that which bound to the circles in the absence of rTF. The binding of factor VIIa to the blotted rTF circles approaches equilibrium at ~3 h. The interaction appears saturable with half-maximal binding occurring at ~30 nM. This value is comparable with that published by Ruf et al. (7) with solubilized tissue factor (~10 nM) or a recombinant mutant tissue factor (minus the transmembrane region) (~100 nM). In an effort to generate relative binding affinities between the recombinant proteins and the bound rTF, competition experiments were performed as described (see “Methods”). The data from these experiments clearly demonstrate that the recombinant proteins (wild type) VIIa, VII LC/IX HC, VII-EGF2/IX, and IX-A/VII will compete with $^{125}$I factor VIIa for rTF, whereas factors IX, IX LC/VI1 HC, IX-EGF2/ VII, and VII-A/IX show no competition out to 1 μM (Fig. 3). The relative dissociation constants calculated from this data are compiled in Table II.

Gla Analysis—Native factor VII possesses 10 Gla residues while factor IX has 12. The Gla content of the recombinant proteins was evaluated by the method of Kuwada and Katayama (25). This analysis revealed that most of the recombinants possess 70% (+) of their expected Gla content. The two exceptions, factors VII LC/IX HC and IX-A/VII, possess 47 and 38%, respectively, of the theoretical Gla content (Table II). The Gla content values are determined from the total number of Gla residues in a fixed amount of protein. The minimum Gla content of a recombinant protein necessary for purification with the calcium-dependent antibodies A7 and CDVII is unknown.

DISCUSSION

Factor VII and Factor IX play a central role in the procoagulant aspect of human hemostasis. Their activity in blood coagulation requires that they interact specifically with a number of essential hemostatic proteins, from activating proteases and inhibitors to cofactors and receptors. In this report, we begin a study whose long-term goal is to determine what portions of factor VII and IX determine their respective specificities. In order to unravel these specific relationships, we chose to create a family of chimeric proteins composed of homologous regional exchanges between factors VII and IX. This approach is based on the observation that functionally related proteins with amino acid sequence identity in the range of 40–50%, such as trypsin and chymotrypsin, share similar tertiary structures. Factors VII and IX are functionally related homologous proteins with amino acid sequence identity of ~44–45%. It is therefore likely that factors VII and IX share a similar tertiary structure. Homologous exchanges between factors VII and IX are an attempt to preserve as nearly as possible the tertiary structure of a native coagulation protease, while simultaneously segregating specificity. Our goal was to generate chimeric proteins that would retain, lose, or acquire the functional characteristics of factors VII or IX, or both.

In this report, we begin a structure/function characterization of human factors VII and IX by utilizing a family of factor VII/IX chimeric proteins to localize the tissue factor recognition determinant of human factor VII. There have been several studies that have attempted to define the structural determinants on human factor VII that mediate its recognition of tissue factor (6–8). Two of these reports have implicated the factor VII Gla domain (6, 7) as being important for the interaction. In one case (6), Gla-domainless factor VII

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**FIG. 3. Displacement of $^{125}$I-factor VIIa from rTF bound to Immobilon-P.** Panel A, factor IX (○), Factor VIIa (■); panel B, IX LC/VI1 HC (△), VII LC/IX HC (▲); panel C, IX-EGF2/Ⅶ (■), VII-EGF2/IX (□); panel D, IX-A/VII (○), VII-A/IX (●), panel E, Factor VIIa (○), VII LC/IX HC (▲), VII-EGF2/IX (□), IX-A/VII (○).
TABLE II

| Ligands/controls | Gla* | $K_d$ values (nM) |
|------------------|------|------------------|
| Plasma factor X  | 10.2 ± 0.05 | ND |
| Plasma factor IX | 10.9 ± 0.1  | ND |
| Reombinant factor IX | ND | — |
| Reombinant factor VIIa | 7.15 ± 0.05 | 21 ± 8 |
| IX-EGF2/IX | 8.75 ± 0.05 | 79 ± 7.6 |
| VII-EGF2/IX | 7.65 ± 0.15 | 21 ± 4.5 |
| IX-A/VII | 4.65 ± 0.05 | 222 ± 22 |
| VII-A/IX | 8.3 | — |

*Values represent the total number of Gla residues per molecule.

was unable to interact at all with constitutively expressed tissue factor on a human bladder carcinoma cell line (J82). In contrast, in the other study, Gla-domainless factor VII was shown to bind to solubilized tissue factor and a recombinant mutant tissue factor (minus the trans-membrane region) with $K_d$ values of 112 and 120 nM, respectively (7). In a third study (8), a peptide corresponding to amino acids 195–206 of the factor VII heavy chain was shown to inhibit factor VII/tissue factor interaction with a $K_d$ of 1–2 mM. The data described in this report indicate the following. 1) The tissue factor/VIIa interaction is Ca$^{2+}$-dependent, in that the binding of factor VIIa to rTF is completely reversible in the presence of EDTA. This Ca$^{2+}$ dependence is consistent with the observations of others (6, 7). 2) The interaction of factor VIIa with tissue factor is predominantly mediated by the EGF domains. The competition experiments (Fig. 3) with factors IX LC/ VII HC and IX-EGF2/VII indicate that the heavy chain of factor VIIa does not contribute any high affinity specificity for tissue factor binding. The high affinity interaction is clearly mediated by the light chain as evidenced by the binding constants of wild-type factor VIIa (21 nM) and factor VII-EGF2/IX (21 nM). It also appears that the Gla domain of factor VII in and of itself does not mediate the interaction. Otherwise, one would have expected VII-A/IX to have displayed competition. We cannot exclude the Gla region from any participation due to the binding constants of VII LC/IX HC (80 nM) and IX-A/VI (220 nM). The extensive undercarboxylation of these chimeras excludes any definitive interpretation of these relative binding constants. The lower affinity for tissue factor with the undercarboxylated chimeras may suggest that the low affinity Ca$^{2+}$ binding ordinarily present in the Gla region has been perturbed. This would be consistent with the demonstration by Ruf et al. (7) that the low affinity calcium-binding sites of the VII Gla domain must be saturated for tight binding to tissue factor. In order to address this point of either direct or indirect participation by the factor VII(a) Gla domain in tissue factor specificity, we are presently attempting to purify fully carboxylated versions of these chimeras. One possible scenario is that a Ca$^{2+}$-dependent conformation of the Gla domain is providing some degree of structural integrity to the EGF domains, meaning that the role of the Gla domain may be a structural role and not one of direct specificity. It is clear that the majority of the binding affinity of factor VII(a) for rTF is within the EGF domains. Whether EGF1, EGF2, or both domains are required to mediate this interaction will be the subject of further investigation.

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