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The first epidermal growth factor domain of human coagulation factor VII is essential for binding with tissue factor

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The intrinsic pathway of coagulation is initiated when zymogen factor VII binds to its cell surface receptor tissue factor to form a catalytic binary complex. Both the activation of factor VII to factor VIIa and the expression of serine protease activity of factor VIIa are dependent on factor VII binding to tissue factor lipoprotein. To better understand the molecular basis of these rate-limiting events, the interaction of zymogen factor VII and tissue factor was investigated using as probes both a murine monoclonal antibody and a monospecific rabbit antiserum to human factor VII. To measure factor VIIa functional activity, a two-stage chromogenic assay was used; an assay which measures the factor Xa generated by the activation of factor VII to factor VIIa. Purified immunoglobulin from murine monoclonal antibody 231-7, which was shown to be reactive with amino acid residues 51–88 of the first epidermal growth factor-like (EGF) domain of human factor VII, inhibited the activation of factor VII to factor VIIa in a dose-dependent manner. The mechanism of this inhibition was demonstrated using a novel solid-phase ELISA which quantitatively measured the binding of purified factor VII zymogen to tissue factor adsorbed onto microtiter wells. Thus, the binding of factor VII zymogen to immobilized tissue factor was inhibited by antibody 231-7, again in a dose-dependent manner. Similar results were obtained using a monospecific rabbit antiserum to human factor VII which also reacted with the β-galactosidase fusion proteins containing amino acid residues 51–88 (exon 4) of human factor VII. We conclude therefore that the exon 4-encoded amino acids of the first EGF domain of human factor VII constitute an essential domain participating in the binding of factor VII to tissue factor.

Blood coagulation; Extrinsic pathway; Human factor VII, tissue factor

1. INTRODUCTION

The initiation of blood coagulation is generally thought of as occurring either by the intrinsic or extrinsic pathways, but the precise series of molecular events initiating either pathway have not been completely defined [1]. Activation of factor VII zymogen occurs on the surface of cells expressing the lipoprotein receptor tissue factor, which is dependent on the presence of both Ca^{2+} and activated factor Xa [2,3]. Structural integrity of the γ-carboxylated glutamic acid (Gla) residues, the calcium-binding Gla domain situated at the amino terminus of factor VII, has been shown to be a prerequisite for factor VII–tissue factor interaction [4]. However, other vitamin K-dependent coagulation proteins including prothrombin, factor IX, factor X, protein C, protein S and protein Z also contain Gla domains which are highly similar in amino acid sequence to factor VII [5–7] yet do not bind tissue factor. In an attempt to further define this interaction, the binding of factor VII to tissue factor was therefore examined using both monoclonal and monospecific polyclonal antibodies to factor VII. Our results provide evidence that the first EGF domain of factor VII is essential for the binding of tissue factor to factor VII and thus is critical for the initiation of extrinsic coagulation. These results were presented in part at the annual meeting of the American Society of Hematology [8].

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2. MATERIALS AND METHODS

2.1. *Materials*

Human factor VII, factor VIIa, and rabbit polyclonal anti-human factor VII antiserum were purchased from Diagnostica Stago (Wellmark Diagnostics, Guelph, ONT). Human factor VII was also purified in our laboratory from factor VII concentrate (generously provided by Immuno AG, Vienna, Austria) on a Sepharose 4B immunoabsorbent column containing covalently attached immunoglobulin G (IgG) from murine monoclonal antibody 231-7 to factor VII [9]. For some experiments, human factor VII prepared by Enzyme Research Laboratories Inc. (Southbend, IN) was used. Thromborel S (tissue factor from human placenta) was obtained from Behring Diagnostics (Hoechst Canada Inc., Montreal, QUE). S-2222 (*N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide), a chromogenic substrate for factor Xa, was obtained from Kabivitrum (Helena Laboratories, Mississauga, ONT). Recombinant tissue factor apoprotein was a generous gift of Genentech Inc., (San Francisco, CA) and was reconstituted into phosphatidylcholine-phosphatidyl serine vesicles

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(40:60 by wt.) as described by Rao and Rapaport [10]. Factor X was purified from normal human plasma as previously described [11]. Russell's Viper Venom (RVV) was obtained from Enzyme Research Laboratories, Inc. Isopropyl-β-D-thiogalactopyranoside (IPTG), p-aminobenzyl-1-thio-β-D-galactopyranoside (ABTG)-agarose, Tween-20, disodium-p-nitrophenyl phosphate and bovine serum albumin (BSA), fraction V, were from Sigma Chemical Company (St. Louis, MO). Goat anti-rabbit IgG and goat anti-mouse IgG conjugated with alkaline phosphatase was from Jackson Immuno Research Laboratories (West Grove, PA). ELISA assays were performed in 96-well flat bottomed well polystyrene microtitre plates (Immulon II, Dynatech Laboratories, Inc., Chantilly, VA). All other chemicals were analytical grade reagents.

2.2. *Mapping of the MAb [231-7] binding epitope on factor VII*

Construction of a 200–300 base pair (bp) factor VII cDNA library in lambda gt11 was performed exactly as previously described [12]. Full-length factor VII cDNA in plasmid pDx [5,13] was a gift from Dr. Earl Davie (Seattle, Washington). Approximately 1×10^5 bacteriophage from the factor VII λgt11 cDNA library was plated on *E. coli* 1090γ(−) (Promega Biotec) and fusion proteins were induced with IPTG [12]. Twenty strongly positive MAb [231-7] immunoreactive plaques were identified on 10 separate discs. Eighteen of the twenty initial phage population identified were successfully plaque-purified. DNA was isolated from each of the phage populations, the putative factor VII cDNA inserts were released from the vector DNA by digestion with *Eco*RI, the DNA was end-labelled with [α-^{32}P]dATP and sized by electrophoresis on a 5% polyacrylamide gel [14]. The seven shortest putative factor VII cDNA inserts were subcloned into the *Eco*RI site of bacteriophage M13mp18 and the DNA sequence of the inserts determined as described previously [12]. Each insert contained the DNA coding for a region of factor VII.

2.3. *Chromogenic assay for factor VII*

Factor VII activation or factor VIIa enzyme activity were assayed using minor modifications of a two-step coupled amidolytic assay, employing the chromogenic substrate S-2222 [15]. The buffer used to prepare all reagents was 0.05 M Tris, pH 8.3, containing 0.15 M NaCl and 1 mg/ml BSA. Thromborel S (tissue factor) was reconstituted in H_2O as suggested by the manufacturer and diluted 2.5 times in buffer just before use. In the first step of the assay, the reaction mixture contained tissue factor (50 μl), factor VII (88.9 ng/ml) or factor VIIa (22 ng/ml), calcium chloride (5.5 mM) and purified factor X (4 μg/ml) in a final volume of 0.225 ml. Conditions were standardized so that the amount of factor Xa generated depended upon the concentration of factor VII (or factor VIIa) in the reaction mixture. After incubation for 3 min at 37°C, the reaction was stopped by the addition of 25 μl of 0.3 M Na_2EDTA and cooled on ice. In the second step, the generated factor Xa activity was measured by adding a subsample (62.5 μl) of the incubation mixture to 437.5 μl of 0.2 mM S-2222 in 0.05 M TBS, pH 8.3. The initial rate of cleavage of S-2222 was determined by following the ΔA_{405} nm/min in a Delta 1000 Spectrophotometer (Smith- Kline Instruments Inc., Sunnyvale, CA).

2.4. *Antibody inhibition of factor VII activation*

The chromogenic assay was modified slightly to study the effect of anti-human factor VII antibodies. In the first step, factor VII (or factor VIIa), calcium, and varying amounts of anti-factor VII antibody were incubated for 1 h at room temperature. Purified factor X was then added followed by tissue factor and the reaction allowed to proceed for 3 min at 37°C. Subsequent operations were as described above.

2.5. *Binding assay for factor VII*

A solid-phase enzyme-linked immunoadsorbent assay (ELISA) to measure binding of factor VII to tissue factor was developed. Tissue factor (TF) was reconstituted in antigen coating buffer (0.05 M Na_2CO-$NaHCO_3$, pH 9.6) and 100 μl was coated onto wells of Immulon II plastic microtitre plates overnight at 4°C. The plates were subsequently washed three times with TBS-T buffer (10 mM Tris, pH 8.0, 0.9% NaCl, 0.05% (v/v) Tween-20, 5 mM Ca^{2+}) and non-specific binding sites were blocked by incubation with 0.35 ml of TBS-T buffer, containing 1 mg/ml BSA for 2 h at 20°C. Human factor VII was incubated alone or in the presence of various concentrations of factor VII antibody in TBS-T buffer containing 1 mg/ml BSA for 2 h at 4°C. Triplicate 100 μl aliquots were then added to microtitre plate wells coated with tissue factor and incubated for 2 h at 20°C. The amount of factor VII bound to the tissue factor-coated wells was determined by the sequential addition and incubation for 1 h at 20°C each of 100 μl of rabbit polyclonal antisera to human factor VII (1:2000 dilution) and alkaline phosphate-conjugated goat anti-rabbit IgG (1:5000 dilution). Microtiter plates were washed 3–5× after the addition of each of the above reagents. Finally, 100 μl of a 1 mg/ml solution of p-nitrophenyl phosphate in substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.02% NaN_3, pH 9.8) was added to wells and incubated for 1 h at 20°C. Absorbance at 405 nm was determined in an automated ELISA plate reader. The amount of free factor VII in the antibody-containing samples was expressed as a percent of control.

2.6. *Isolation of β-galactosidase fusion proteins containing human factor VII*

Lysogens of λgt11-factor VII recombinant bacteriophage were grown in culture and fusion protein production induced by IPTG as previously described [12,16]. Cells were harvested by centrifugation and β-galactosidase fusion proteins extracted and isolated as described [16,17]. Final purification of the β-galactosidase fusion proteins was achieved by affinity chromatography on an ABTG-agarose column as described [17,18]. Purity of the eluted fusion protein was greater than 95% as analysed by SDS-polyacrylamide gel electrophoresis. Two β-galactosidase fusion proteins containing human factor VII amino acids 25–88 (fusion protein 8-9) and factor VII amino acids 46–107 (fusion protein 9–2) were thus purified. Both proteins had in common a region of sequence encompassing the first EGF domain of human factor VII. Control β-galactosidase encoded by wild-type λgt11 was prepared and purified in a similar manner. The immunogenicity of the two β-galactosidase fusion proteins, containing portions of human factor VII, was verified by solid-phase ELISA using both the murine monoclonal antibody 231–7 and the rabbit polyclonal antiserum to human factor VII as described above.

3. RESULTS

3.1. *Specificity of anti-factor VII antibodies*

As shown in Table I, the two shortest factor VII inserts contained in phage clones 9 (amino acid residues 25–88) and 11 (amino acid residues 51–114) limit the

Table I

DNA sequence analysis of factor VII recombinant bacteriophage

Phage clone	Factor VII amino acids encoded
2	43–105
3	43–113
8	46–107
9*	25–88
11*	51–114
18	36–95
19	21–89
Consensus sequence	51–88

*These two clones define the maximum limits of the epitope recognized by monoclonal antibody 231-7.

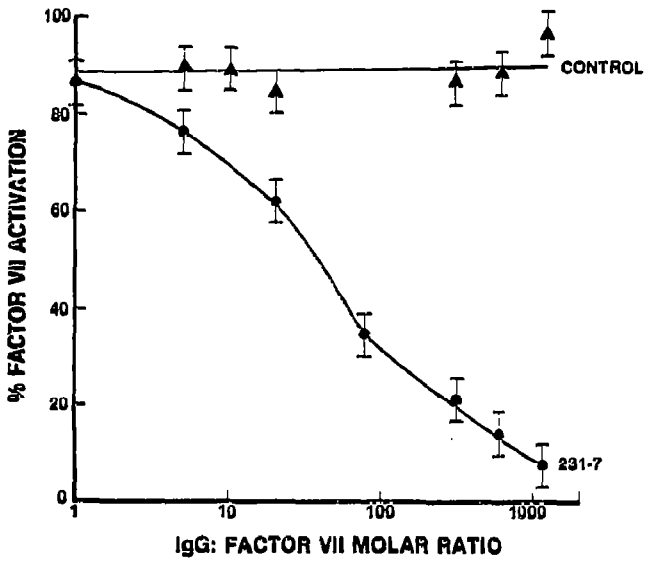

Fig. 1. Effect of MAb 231-7 IgG (●) and control mouse immunoglobulin (▲) on the activation of factor VII as measured by chromogenic assay. Control reaction mixture without any antibody is taken to give 100% activation of factor VII and values for samples containing antibody are expressed as a percentage of control. Error bars are S.E.M. of triplicate determinations.

MAb 231-7 binding epitope to amino acid residues 51–88 of human factor VII. This epitope is part of the first EGF domain of factor VII. The MAb 231-7 belonged to the IgG_1 subclass and its specificity was confirmed by ELISA experiments using purified β-galactosidase fusion proteins 8-9 and 9-2 containing the first EGF domain amino acid residues 46–88. Similarly, the monospecific polyclonal rabbit antiserum to factor VII also strongly reacted with the two fusion proteins, indicating the presence of antibodies against the first EGF domain of factor VII (data not shown).

3.2. *Antibody-mediated inhibition of factor VII activation*

Initial experiments were performed to determine whether antibodies to factor VII might be useful in mapping the tissue factor binding domain(s). The effect of varying doses of MAb 231-7 on factor VII activation was studied in a conventional two-stage chromogenic assay system. As seen from Fig. 1, MAb 231-7 markedly inhibited factor VII activation. The dose-response curve was sigmoidal suggesting that the inhibition observed was co-operative in nature. Half-maximal inhibition occurred at a 44:1 molar ratio of antibody to factor VII. Control mouse immunoglobulin of the same IgG subclass had no effect on activation of factor VII. Similarly, MAb 231-7 had no effect on either activation of factor X to factor Xa by RVV or the cleavage of the chromogenic substrate S-2222 by purified factor Xa (data not shown). These results indicated that the first EGF domain of factor VII is involved in the activation of factor VII zymogen to factor VIIa.

Similar results were obtained using the rabbit polyclonal antiserum monospecific for human factor VII. Half maximal inhibition of factor VII activation by rabbit polyclonal antibody occurred at 1:10,000 dilution of antisera. Normal rabbit serum had no effect on the activation of factor VII. Neither RVV activation of factor X to Xa nor purified factor Xa activity was affected by the presence of rabbit antibodies (data not shown).

3.3. *Antibody-mediated inhibition of the binding of tissue factor to factor VII*

In order to test the hypothesis that the above antibodies to factor VII were inhibiting the interaction of factor VII with its essential co-factor tissue factor, we devised a simple, solid-phase ELISA to measure factor VII binding to immobilized tissue factor. The assay was shown to be linear over the range of 3–20 ng factor VII. However, when preincubated with MAb 231-7, the binding of factor VII to tissue factor decreased progressively as the molar ratio of MAb 231-7 IgG to factor VII increased from 1:1 to 100:1 (Fig. 2). Half-maximal inhibition of binding of factor VII to immobilized tissue factor occurred at about 10:1 molar ratio of IgG to factor VII. Control mouse immunoglobulin of the same IgG subclass had no effect in the concentration range tested. Similar results were obtained with rabbit polyclonal serum monospecific for factor VII, i.e. after preincubation of the antiserum with factor VII the binding of factor VII to tissue factor decreased progressively as the antiserum dilution was varied from 1:5,000 to 1:400. Half-maximal inhibition of binding of factor VII

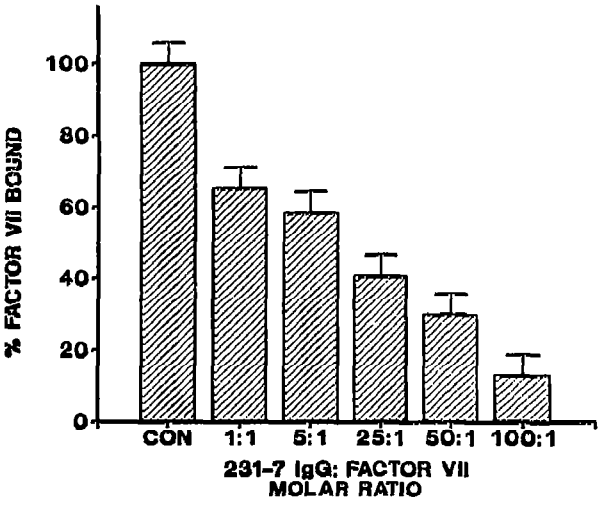

Fig. 2. Effect of MAb 231-7 IgG on the binding of factor VII to immobilized tissue factor. Control reaction mixture without any antibody is taken to give 100% binding of factor VII to TF and values for samples containing antibody are expressed as a percentage of control. Erros bars are S.E.M. of triplicate determinations. Control mouse immunoglobulin did not have any effect on factor VII binding to TF.

to immobilized tissue factor by rabbit antiserum occurred at 1:1,000 dilution of the serum (data not shown). Normal rabbit serum had no significant effect in the assay system. When purified relipidated recombinant tissue factor was substituted for crude tissue factor in the assay system, identical results were obtained (data not shown).

3.4. *Purified tissue factor blocks interaction of MAb 231-7 and factor VII*

In an ELISA-type assay, relipidated recombinant tissue factor was found to block the binding of MAb 231-7 to purified factor VII immogilized on a microtiter plate (data not shown). The inhibition of MAb 231-7 binding by TF was dose-dependent and reached a steady level of about 40% inhibition at 25 ng TF. In a converse of this experiment, MAb 231-7 failed completely to bind factor VII, which was preincubated and bound to purified TF immobilized on a microtiter plate. These experiments further support the concept that both TF and MAb 231-7 compete for the same binding site on factor VII.

4. DISCUSSION

A number of investigators have recently addressed the issue of the tissue-factor-binding domain(s) of factor VII. It has been known for a long time that γ-carboxylation of the glutamic acid residues of the vitamin K-dependent coagulation factors is a necessary feature for the manifestation of their biological activity [19]. Moreover, there is now direct evidence indicating that the Gla domain of factor VII is essential for interaction with tissue factor [4]. In addition, the two EGF domains of factor VII have been implicated recently as comprising an important tissue factor recognition determinant on factor VII [20]. These studies were based on observations with chimeric proteins composed of domains from human factor VII linked to human factor IX. Only chimeric proteins containing both EGF domains of factor VII bound tissue factor [20]. Other investigators, using synthetic peptides corresponding to various regions of human factor VII, reported 2 regions to be of importance for tissue factor binding; those comprising amino acid residues 44–50, and 285–305 [21,22]. In the latter report [22], evidence was provided that the latter domain was critical for factor X activation. Another group of investigators reported amino acid residues 195–206 to be critical for factor VII binding to tissue factor [23]. Thus, the Gla domain, the EGF domain, and the heavy chain of human factor VII have been implicated in the binding of tissue factor with factor VII. It is likely that the Gla-domain interacts with the phospholipid moiety of tissue factor while the latter two interact with the apoprotein [4,24].

Conversely, investigations exploring the recognition domains on the tissue factor apoprotein have delineated two domains within the extracellular portion of the tissue factor molecule that interact with factor VII [24–26]. Thus, from the available evidence, there appear to be at least two interactive domains between human tissue factor apoprotein and the human factor VII molecule, which facilitate the activation of factor VII by factor Xa. For factor VII it is likely that one of these binding domains is contained within the light and the other within the heavy chain. With regard to the domain within the light chain, the domains comprising the two EGF structures have been implicated [8,20]. The evidence provided in this communication pinpoints this tissue factor binding region to the first EGF domain. Direct evidence that the first EGF domain participates in tissue factor binding has recently been provided by the natural mutation factor VII-Charlotte [27,28]. This mutant factor VII has an $Arg^{79} \rightarrow Gln$ mutation within the first EGF domain [27,28]. Finally, we have shown recently that both plasma-derived and recombinant factor VII-Charlotte do not bind to tissue factor (High, Chaing, Sridhara, Clarke and Blajchman, unpublished results).

In summary, all of available evidence provided in this report points to the first EGF domain of factor VII to be a critical tissue factor binding domain on human factor VII. Studies are required to further delineate this and other critical binding domains for this important interaction.

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