Site-directed mutagenesis but not γ-carboxylation of Glu-35 in factor VIIa affects the association with tissue factor

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Abstract Factor VIIa is a vitamin K-dependent enzyme whose γ-carboxyglutamic acid (Gla)-containing domain is important for calcium ion-dependent binding to the cofactor tissue factor and membrane surfaces. This domain contains 10 Gla residues, the individual roles and importance of which are not known. Comparisons with the homologous protein C, factor IX and prothrombin may provide functional information on the first nine Gla residues, whereas no data can be extrapolated to Gla-35 in factor VIIa. Therefore, the effects of posttranslational γ-carboxylation and site-directed mutagenesis of Glu-35 were investigated. Mutations to Asp, Gln or Val all lead to a lower affinity for tissue factor by decreasing the rate of association, in the case of the Val mutant by a factor of 200, as measured by surface plasmon resonance. In contrast, Glu or Gla side chains at position 35 appear to fulfil the functional roles equally well.

Key words: Factor VIIa; Gla mutation; γ-carboxylation; Gla domain; Tissue factor binding; Surface plasmon resonance

1. Introduction

Coagulation factor VII (fVII) belongs to the family of vitamin K-dependent plasma proteins characterized by their posttranslationally γ-carboxylated Glu residues located in well-conserved positions [1]. The N-terminal domain, encoded on a separate exon, contains the first 37 amino acid residues including all 10 γ-carboxyglutamic acid (Gla) residues [2]. This so-called Gla domain binds seven Ca^{2+} ions [3,4], an event mediated by the Gla residues, and thereby attains a membrane-interactive ability through the exposure of hydrophobic side chains [4,5]. Studies of the roles of the individual Gla residues in factor IX [6], protein C [7-10] and prothrombin [11], all of which are homologous to fVII, have been conducted. They demonstrated functional differences between various Gla residues but similar, although with a few differences, patterns for the three proteins and this presumably also applies to fVII. Thus, the anticipated roles of the first 9 Gla residues in fVII regarding properties common to all vitamin K-dependent plasma proteins, such as basic structure maintenance, Ca^{2+} and phospholipid binding, can be inferred. However, protein C and prothrombin have no Gla residue corresponding to Gla-35 in fVII and the corresponding factor IX mutant was not included in that study. In addition, residue 35 was poorly ordered in the crystal structure of the complex between fVIIa and tissue factor (TF) [4]. Therefore, it was of interest to us to investigate the consequences of mutating this residue in fVII. In addition, the production of factors VII and IX in mammalian cell cultures is fraught by the problem of partial γ-carboxylation of the most C-terminal Gla residues [12,13] and we also wanted to determine the effects of this specific modification of Glu-35 in fVII.

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Abbreviations: fVII(a), (activated) factor VII; Gla, γ-carboxyglutamic acid; γ35D, γ35Q, γ35V, mutations of Glu35 in factor VIIa resulting in the substitutions of Asp, Gln and Val, respectively, for Gla in the mature protein; TF, tissue factor; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction

2. Materials and methods

2.1. Proteins, materials and standard methods

Recombinant fVIIa was purified according to Thim et al. [12]. The γ35 and E35 isoforms of fVIIa were separated by ion-exchange chromatography on a NucleoPac PA-100 column using a 0–0.85 M linear gradient of ammonium acetate in 10 mM Tris, 10 mM Bis-Tris propane, pH 9.2. The two forms were identified by mass spectrometry and N-terminal amino acid sequencing (with positive identification of Gla) of peptic and tryptic peptides (manuscript in preparation). The ELISA employing the monoclonal antibodies F1A2 and F6A4 was carried out as described [14]. SDS-PAGE was run according to Laemmli [15].

Materials for cell culturing were from Gibco. Taq polymerase was from Perkin-Elmer and Pwo polymerase was from Boehringer Mannheim.

2.2. fVII cDNA constructions

For cDNA constructions the following PCR primers were used:

1. (A) AAC GGA TCC ACC ATG GTC TCC CAG GCC CTC AGG
2. (B) ACG GAA TTC ACT AGT CTA GGG AAA TGG GGC TCG CAG GA
3. (C) CGT CCT GTC CGC GTC CTT GAA
4. (D) TTC AAG GAC GCG GAC AGG ACG
5. (E) CGT CCT CTG CGC GTC CTT GAA
6. (F) TTC AAG GAC GCG CAG AGG ACG
7. (G) CGT CCT AAC CGC GTC CTT GAA
8. (H) TTC AAG GAC GCG GTT AGG ACG

A wild-type fVII cDNA for expression was prepared by PCR with Taq polymerase using primer A as 5′ primer, primer B as 3′ primer and the human cDNA [16] as a template. This adds a *Bam*HI site to the 5′ end and an *Eco*RI site followed by an internal *Spe*I site to the 3′ end. At the same time 5′ and 3′ non-coding regions are deleted. The PCR fragment was cloned as a *Bam*HI-*Eco*RI fragment into the vector pBluescript II KS+ (Stratagene). The coding region was sequenced by the enzymatic chain termination method [17] and was finally transferred as a *Bam*HI-*Spe*I fragment to the mammalian cell expression plasmid Zem219b [18] which carries a mouse metallothionein promoter for transcription of the inserted cDNA and the dihydrofolate reductase cDNA under the control of an SV40 early promoter for use as a selectable marker. The resulting plasmid was designated pLN174.

The γ35D mutant was constructed by overlap extension using PCR [19]. Briefly, two PCR fragments were prepared with Pwo polymerase using the human cDNA [16] as a template and primers A+C (5′ fragment) and B+D (3′ fragment). The two fragments were purified

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by agarose gel electrophoresis and overlapped by PCR using Pwo polymerase and primers A+B. The resulting PCR fragment was cloned into the vector pBluescript II KS+ as described above for the wild-type cDNA. The region between the *BglII* and *XbaI* sites (base pairs 201–534 of the fVII coding sequence) was verified by sequencing and the internal *BglII-XbaI* fragment was removed from the plasmid pLN174 and replaced with the corresponding fragment from the mutant plasmid. The presence of the mutation in the final expression construct was verified by sequencing. The resulting expression plasmid was designated pLN198.

The γ35Q and γ35V mutants were constructed in a similar manner with primers A+E (γ35Q) and A+G (γ35V) for the 5′ fragments and primers B+F (γ35Q) and B+H (γ35V) for the 3′ fragments. Preparation of fragments, overlapping, cloning into pBluescript II KS+ and preparation of the final expression construct were performed as described above for the γ35D mutant. The resulting expression plasmids were designated pLN199 (γ35Q mutant) and pLN200 (γ35V mutant).

DNA for transfection was prepared using the Qiagen maxiprep kit.

2.3. Transfection

The baby hamster kidney cell line BHK570 (ATCC CRL 1632) was transfected with the mutant fVII expression plasmids (pLN198, pLN199 and pLN200) using a modified calcium phosphate method [20]. Briefly, 2.5×10^5 cells were seeded on day 0 in a 6 cm Petri dish. On day 1, the medium (5 ml of Dulbecco-modified Eagle's medium containing 10% fetal bovine serum) was changed and the DNA mixture (500 μl containing 20 μg DNA, 0.12 M $CaCl_2$, 0.82% NaCl, 0.6% HEPES, and 0.02% Na_2HPO_4) was added. Before incubation, 50 μl chloroquine (10 mM) was added to the dish. On day 2 the medium was changed and later the same day the cells were transferred to a T80 culture flask. Selection pressure (1 μM methotrexate) was applied on day 3 and the cultures were incubated until clones were visible with the naked eye. The cultures were then transferred to T80 flasks for further culture and freezing.

2.4. Expression and purification of mutant fVII

Pools of transfectants prepared as described above were seeded into two three-layer T175 culture flasks (Nunc) and allowed to grow to confluency in Dulbecco-modified Eagle's medium containing 10% fetal bovine serum. The medium was aspirated and replaced by 100 ml of the same medium containing 2% fetal bovine serum and 5 μg/ml vitamin K. The medium was harvested and fresh medium added after 2 or 3 days of incubation. Medium was harvested 4 times from each pool of transfectants.

Prior to purification, $CaCl_2$ was added to the harvested media to a final concentration of 10 mM and pH was adjusted to 8.0. The mutant proteins were purified by affinity chromatography employing the monoclonal antibody F1A2 [14]. The F1A2-Sepharose column was equilibrated with 20 mM Tris, pH 8.0, containing 0.1 M NaCl and 10 mM $CaCl_2$, and washed with the same buffer after sample application. The proteins were eluted with the same buffer containing 10 mM EDTA instead of $CaCl_2$. After purification, the concentrations of all mutants were determined in an ELISA.

2.5. Activity assays

The amidolytic activity was measured by mixing fVIIa (final concentration 100 nM) with the chromogenic substrate S-2288 (Chromogenix, final concentration 2 mM) in 50 mM HEPES, pH 7.5, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml BSA. The mixture was incubated for 50 min at ambient temperature and the absorbance at 405 nm was read every 10 min.

The clotting activity was determined by adding various concentrations of fVIIa sample to fVII-deficient plasma and then mixing 50 μl plasma with 50 μl relipidated TF (Innovin, Baxter, diluted 12.5-fold after dissolution prior to the assay) and 50 μl buffer (50 mM HEPES, pH 7.5, 0.1 M NaCl). This mixture was incubated at 37°C for 1 min, clot formation was initiated by the addition of 50 μl buffer supplemented with 20 mM $CaCl_2$ and the clotting time was recorded on an Amelung KC4A coagulometer.

Table 1
Kinetic and equilibrium constants for the interactions between soluble TF and fVIIa molecules substituted in position 35

Complex	k_a ($\times 10^5$) (M^{-1} s^{-1})	k_d ($\times 10^{-4}$) (s^{-1})	K_d (nM)
fVIIa:TF	3.4	11	3.2
γ35D-fVIIa:TF	0.86	12	14
γ35Q-fVIIa:TF	0.44	8.5	19
γ35V-fVIIa:TF	0.015	9.8	650

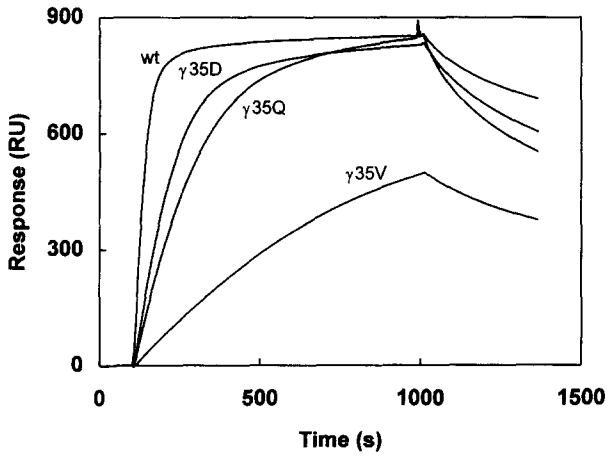

Fig. 1. Corrected sensorgrams for the interactions between the indicated variants of fVIIa and immobilized soluble TF. The analyte concentration was 100 nM.

2.6. Tissue factor binding experiments

The analyses were performed using a BIAcore instrument (Pharmacia Biosensor) as described [21] with a few minor modifications: Soluble TF was coupled to the sensor chip at a level of 740 resonance units and 100 nM of the different fVIIa forms in 20 mM HEPES, pH 7.5, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 0.02% Tween 80, was injected. The association and dissociation phases were 15 and 6 min, respectively. Binding data was evaluated using a one-site model and the BIAevaluation 2.1 software.

3. Results and discussion

According to SDS-PAGE, all fVII species had been converted to two-chain fVIIa during the purification procedure. They displayed similar amidolytic activities (80–95% of wild-type) and similar specific procoagulant activities were measured in a clot assay (data not shown). After this initial characterization we went on to specifically characterize the TF and Ca^{2+} binding properties of the different fVIIa species.

The binding of fVIIa to soluble TF was monitored using surface plasmon resonance, allowing both association and dissociation rates to be estimated. The recorded sensorgrams after background subtraction are shown in Fig. 1. The sensorgrams acquired with the isolated γ35 and E35 forms of fVIIa were identical to that of fVIIa (which is a mixture of the two forms) and are omitted. Taking into account the molecular masses of fVIIa and soluble TF, the maximal response signal indicated that approx. 60% of the immobilized TF molecules were able to bind fVIIa. Evaluation of the binding data revealed that the apparently slower association of the mutant forms of fVIIa with soluble TF indeed was solely due to decreased association rates, whereas the dissociation rates were similar for all fVIIa forms (Table 1). The association rate decreased in the order fVIIa>γ35D-fVIIa>γ35Q-fVIIa>γ35V-fVIIa, with the big drop occurring between the two last mutants. The structure of the fVIIa:TF complex reveals no direct contribution of residue 35 to the interaction with TF [4] and apparently the two relatively conservative

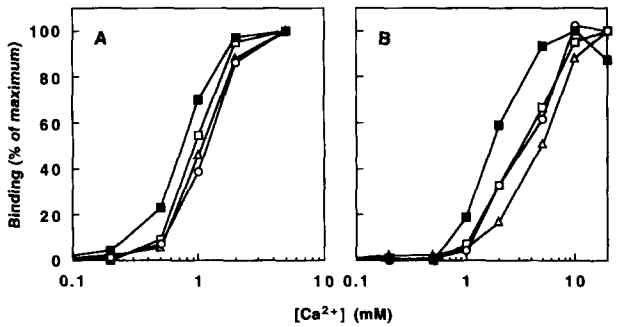

Fig. 2. Ca^{2+}-dependent binding of the various factor VIIa muteins to monoclonal antibodies directed against the Gla domain. The binding of wild-type factor VIIa (■), γ35D-fVIIa (□), γ35Q-fVIIa (○) and γ35V-fVIIa (△) to F6A4 (A) and F1A2 (B) was measured at the indicated Ca^{2+} concentrations. The data points represent the mean of duplicate experiments.

substitutions are reasonably tolerated. However, the γ35V mutation may distort the α-helical environment of residue 35, perhaps influencing the presentation of Phe-31 and Phe-40 [4], leading to a decreased rate of association. At present, we do not know why the impaired TF binding was not revealed in the clot assay. A comparison with a previous study of the kinetics of the binding of N-terminally truncated forms of fVIIa to soluble TF [21] generates two very interesting observations apart from the fact that both the mutations and truncations decreased the affinity for TF. Firstly, whereas truncations in the N-terminus of fVIIa cause an increased dissociation rate without affecting the association process the mutations at position 35 have the opposite effect. Secondly, it appears as if γ35V-fVIIa has a lower affinity for TF than does a fVIIa molecule lacking the first 44 amino acid residues, i.e. the entire Gla domain and hydrophobic stack.

The recognition of wild-type and mutant fVIIa by two Ca^{2+}-dependent monoclonal antibodies, F1A2 and F6A4, was investigated in order to characterize the Ca^{2+} binding properties of the mutant proteins. Again, no difference was observed between the γ35 and E35 forms of fVIIa (not shown). A lower apparent binding of F6A4 was observed for γ35V-fVIIa. Residue 35 is either part of or in close proximity of the epitope [14] and the lower response supports the assumption that the γ35V mutation introduces structural changes that also may cause the decreased TF binding. The Ca^{2+} dependencies of all fVIIa mutants for binding to F6A4 were nevertheless similar to that of the wild-type protein (Fig. 2). In contrast, binding to F1A2, with its epitope in residues 3–10 of fVIIa [22], required higher Ca^{2+} concentrations for the mutants and in particular for γ35V-fVIIa. Thus, mutagenesis of residue 35 appeared to affect the Ca^{2+}-mediated induction of structural order in the N-terminal part of the Gla domain. This part of the Gla domain is not involved in the contact with TF [4] and the suboptimal folding of this region of the γ35V-fVIIa molecule at 5 mM Ca^{2+} is presumably not what caused the reduced affinity for TF. In addition, the binding of γ35V-fVIIa to TF in the BIAcore was the same at 5 and 10 mM Ca^{2+} (not shown). It may, however, bear consequences on the ability to bind phospholipids.

Our results, in agreement with data obtained for the two C-terminal Gla residues in factor IX, show that γ-carboxylation of Glu-35 in fVIIa appears to be of no functional importance and do not encourage efforts to optimize this modification during the mammalian cell production of recombinant vitamin K-dependent clotting factors. In contrast, mutations at position 35 in fVIIa, which is occupied by a Gla residue in plasma fVIIa and by Gla or Glu in recombinant fVIIa, affect the affinity for TF. Indeed, a γ35V substitution results in lower TF affinity than does removal of the entire Gla domain. The effect is caused solely by a decreased association rate. Conceivably, an improperly structured Gla domain, perhaps also with suboptimal relative orientations of the Gla and first epidermal growth factor-like domains, may obstruct the association of fVIIa with TF.

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