Defining the Factor Xa-binding Site on Factor Va by Site-directed Glycosylation*

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Activated Factor V (FVa) functions as a membrane-bound cofactor to the enzyme Factor Xa (FXa) in the conversion of prothrombin to thrombin, increasing the catalytic efficiency of FXa by several orders of magnitude. To map regions on FVa that are important for binding of FXa, site-directed mutagenesis resulting in novel potential glycosylation sites on FV was used as strategy. The consensus sequence for N-linked glycosylation was introduced at sites, which according to a computer model of the A domains of FVa, were located at the surface of FV. In total, thirteen different regions on the FVa surface were probed, including sites that are homologous to FIXa-binding sites on FVIIIa. The interaction between the FVa variants and FXa and prothrombin were studied in a functional prothrombin activation assay, as well as in a direct binding assay between FVa and FXa. In both assays, the four mutants carrying a carbohydrate side chain at positions 467, 511, 652, or 1683 displayed attenuated FXa binding, whereas the prothrombin affinity was unaffected. The affinity toward FXa could be restored when the mutants were expressed in the presence of tunicamycin to inhibit glycosylation, indicating the lost FXa affinity to be caused by the added carbohydrates. The results suggested regions surrounding residues 467, 511, 652, and 1683 in FVa to be important for FXa binding. This indicates that the enzymecofactor assembly of the prothrombinase and the tenase complexes are homologous and provide a useful platform for further investigation of specific structural elements involved in the FVa/FXa complex assembly.

The formation of thrombin from prothrombin is a key event in the coagulation process. In this reaction, the activated form of coagulation Factor V (FVa) functions as a cofactor to the enzyme Factor Xa (FXa). The two proteins are assembled on the surface of negatively charged phospholipid membranes to the highly efficient prothrombinase complex (1–4).

Factor V (FV) is a single-chain procofactor (330 kDa), having the domain organization A1-A2-B-A3-C1-C2 in common with the homologous Factor VIII (FVIII) (5–7). FV circulates in human plasma at a concentration of 7 μg/ml but is also present in platelets from where it is released and activated during hemostasis (8). FX is converted to its activated form FXa upon proteolytic cleavage mediated by either thrombin or FXa. During this reaction, the B domain of FV is released from the active FXa molecule (9–12). FVa is composed of the 105-kDa heavy chain (A1-A2 domains) and the 71/74-kDa light chain (A3-C1-C2 domains), the two chains being held together by non-covalent, calcium-ion dependent forces (1, 11, 12). The activity of the prothrombinase complex is regulated by activated protein C (APC), which inhibits FXa by proteolytic cleavage at Arg-306, Arg-506, and Arg-679 (13).

Factor X (FX) is a zymogen to a serine protease. It is composed of a Gla domain, two epidermal growth factor-like domains, an activation peptide, and a serine-protease domain. During blood coagulation, FX is activated by either the extrinsic pathway Factor VIIa-tissue factor complex or the intrinsic pathway tenase complex that comprises the phospholipid-bound Factor IXa (FIXa) and activated FVIII (FVIIIa) (14). FVIIIa is a cofactor to FIXa in the activation of FX, a reaction that in many respects is very similar to the prothrombin activation (2). After activation of FX, the light chain (18 kDa) containing the Gla and two epidermal growth factor-like domains remains associated via a disulfide bridge to the heavy chain (27 kDa) composed of the serine-protease domain (15, 16).

Even though the kinetics of the prothrombinase complex have been well characterized, surprisingly little is known about structure-function relationships of the complex and the sites of protein-protein interaction between FVa and FXa. Two regions in the A2 domain of FVa, 493–506 (17) and 311–325 (18), have, based on peptide studies, been suggested to be important for binding of FXa. In addition, removal of the C-terminal region of the A2 domain, 684–709, has been shown to result in reduced FXa affinity (19). In contrast to the FVa-FXa complex, extensive interaction studies have been carried out on the structurally and functionally homologous FVIIIa-FIXa complex. Various interaction sites for FIXa have been identified on FVIIIa. Three of these binding sites, comprising residues 511–530, 558–565, and 692–710, are located in the A2 domain (20–22). Furthermore, a FIXa binding sequence, including residues 1811–1818 in the A3 domain of the FVIIIa light chain, has also been identified previously (23).

To map the regions on FVa that are important for binding of FXa, targeted glycosylation created by site-directed mutagenesis was used as the experimental strategy. It has been shown that mutations aiming at introduction of N-linked glycosyla-
tion at selected residues can be a useful way to probe molecular regions involved in protein-protein interaction (24–26). The size of a carbohydrate chain is convenient for searching binding site regions, because it probes larger areas than can be achieved via amino acid scanning, while it is more directed than peptide or antibody inhibition studies. The targeted glycosylation was achieved by introduction of the sequence signaling for N-linked glycosylation, as has been shown to result in a higher degree of glycosylation than when serine occupies the third position (27, 28).

A recently created three-dimensional model of FVAs was very helpful for the selection of adequate sites for the novel carbohydrate side chains (29, 30). We focused on introducing the glycosylation sites at residues that are surface-exposed in the three-dimensional model of FVAs. Unfortunately, in the model, the end of the A2 domain (after residue 656) is missing. In addition to the computer model of the A domains of FVAs, we designed our mutations taking into account documented binding sequences of FVIIa and corresponding homologous sequences in FVAs (20–23). The results of this study show that regions surrounding residues 467, 511, 652, and 1683 of FVa were involved in protein-protein interaction (24–26). Bsu36I and Bsp12I (2 kb) were purchased from Roche Molecular Biochemicals (Penzberg, Germany). A double-stranded DNA sequencing kit was obtained from PerkinElmer Life Sciences. Cell culture media (Optimem Glutamax) were from Invitrogen. Tunicamycin was from Calbiochem (La Jolla, CA). A monoclonal antibody (Mk1) directed to the B domain of Factor V has been described before (31). A monoclonal antibody (AHV-5101) was from Hematologic Technologies Inc. Bovine serum albumin (BSA), ovalbumin, soybean trypsin inhibitor, and leupeptin were purchased from Sigma. Aporphin was from Bayer (Leverkusen, Germany). Bovine FX (bFX) was purified as described (32) and activated using Russell’s viper venom (33). Bovine Factor Xa (bFXa) was labeled with [35S] using the chloramine T method. The specific activity was 20,000 cpm/ng, equal to 0.4–0.5 MBq/μg of protein. The labeled bFXa was characterized on SDS-PAGE and functionally analyzed using a prothrombinase assay and appeared unaffected by the labeling procedure.

### EXPERIMENTAL PROCEDURES

**Materials**—Bin1 was from Roche Molecular Biochemicals (Germany), and Bsu36I and Bsp12I were from New England BioLabs (Beverly, MA). Pfu polymerase and T4 DNA ligase were purchased from Roche Molecular Biochemicals (Penzberg, Germany). A double-stranded DNA sequencing kit was obtained from PerkinElmer Life Sciences. Cell culture media (Optimem Glutamax) were from Invitrogen. LipofectAMINE 2000 was from Invitrogen. Tunicamycin was from Calbiochem (La Jolla, CA). A monoclonal antibody (Mk1) directed to the B domain of Factor V has been described before (31). A monoclonal antibody (AHV-5101) was from Hematologic Technologies Inc. Bovine serum albumin (BSA), ovalbumin, soybean trypsin inhibitor, and leupeptin were purchased from Sigma. Aporphin was from Bayer (Leverkusen, Germany). Bovine FX (bFX) was purified as described (32) and activated using Russell’s viper venom (33). Bovine Factor Xa (bFXa) was labeled with [35S] using the chloramine T method. The specific activity was 20,000 cpm/ng, equal to 0.4–0.5 MBq/μg of protein. The labeled bFXa was characterized on SDS-PAGE and functionally analyzed using a prothrombinase assay and appeared unaffected by the labeling procedure.

**Expression of Recombinant FV (rFV)**—Expression plasmids containing the various FV cDNA constructs were transfected into COS 1 cells using both the DEAE-dextran method as described (34) and the LipofectAMINE 2000 (Invitrogen) method as described by the manufacturer. Proteins were collected 72 h after transfection in serum-free media (OptiMEM Glutamax). For the expression in the presence of tunicamycin, cells were pretreated with 10 μg/ml tunicamycin prior to protein collection in serum-free media in the presence of 10 μg/ml tunicamycin.

**Concentration and Quantification of rFV**—Media containing the recombinant proteins were concentrated—fold using Vivaspin 100,000 molecular weight cut off, on the day of harvest, and the FV concentration was measured using an ELISA, after minor modifications (35). In brief, microtiter plates were coated overnight with 10 μg/ml monoclonal Mk1 and then blocked for 30 min. Samples were diluted in ELISA buffer supplemented with 10 mM benzamidine and 2 mM CaCl2, and incubated in 4°C overnight. Standard curves were created using pooled normal citrated plasma, assuming the FV concentration to be 7 ng/ml (8). Biotinylated monoclonal HV-1 (0.1 μg/ml) was used as secondary antibody, and after 2 h streptavidin-peroxidase was added. After 30 min, plates were developed for 3 min, and absorbance was measured at 490 nm. To rule out that the cell culture medium interfered with the ELISA, plasma-purified FV was diluted in mock medium or in buffer. No difference was detectable, indicating that the medium does not interfere.

**Pulse Labeling of rFV**—A pulse labeling experiment was performed.
24 h after transfection by radioactive labeling with [35S]methionine and [35S]cysteine as previously described with some modifications (36). In brief, cell extracts were prepared by lysis in a cell lysis buffer supplemented with inhibitors. Cell lysate was preclarified with protein A-Sepharose (Amersham Biosciences) for 2 h at 4 °C and immune precipitated overnight at 4 °C using a polyclonal anti-FV antibody, 8806 (20 μg). The immune complexes were precipitated and washed with immune precipitate washing buffer. After elution by boiling, samples were subjected to 2 units/ml thrombin for 30 min in an activation buffer. Proteins were deglycosylated by endoglycosidase H digestion (1 unit/ml) overnight at 37 °C in an endoglycosidase H buffer, supplemented with inhibitors. Boiling the sample terminated the digestion. Proteins were separated by electrophoresis on a 7.5% SDS-PAGE (37). The gels were then exposed in a sequencer and finally scanned using a PhosphorImager (Amersham Biosciences).

Purification of FVa—The purification was performed as described by Heeb et al. (38) after some modifications. The supernatant of conditioned medium of rFV (100 ml) was concentrated to 3 ml using the Vivaspin 100,000 molecular weight cut off. Concentrated medium was treated with 10 μM benzamidine and 2 μg FPPACK and incubated on ice for 30 min. The supernatant was loaded onto a 5-ml Hi-trap column (Amersham Biosciences) coupled with 2 mg of anti-(FV light chain) monoclonal antibody AHV 5101 at a flow rate of 0.18 ml/min. The column was then washed with 20 ml of buffer at a flow rate of 0.18 ml/min. Finally, the column was eluted with buffer B at a flow rate of 0.5 ml/min and collected in 1-ml fractions. Fractions were analyzed by gel filtration on a Superdex 200 column (Amersham Biosciences) and stored at −80 °C.

Phospholipid Vesicle Preparation—Phospholipids dissolved in chloroform/methanol (9:1, v/v) were dried in a glass tube under a mild flow of nitrogen. The phospholipids were suspended in 25 mM Hepes, 150 mM NaCl, pH 7.5, vigorously vortexed for 5 min at room temperature at amplitude 3, using an XL 2020 sonicator (Misonix). For the binding experiments, the phospholipid composition of the vesicles was PE/PS/PC at molar ratio of 10/20/70. A trace amount of [14C]PC was not dependent on FVa was determined in parallel from reactions containing a 100-fold excess of unlabeled bFXa, and then bound FXa was subtracted from the total binding. In addition, binding that was not dependent on FVa was determined in parallel from reactions lacking added FVa. To estimate the Kd of the FXa binding to FVa, the amount of bound FXa was plotted as a function of added FXa concentration. The data were fitted to the above equation for a single site binding isotherm via non-linear least squares regression analysis.

Determination of Apparent Kd of FXa for FVa Using the Prothrombinase Assay—Recombinant FV proteins were incubated with 0.5 unit/ml of α-thrombin (Hematologic Technologies Inc.) for 10 min at 37 °C prior to analysis in the prothrombinase assay. Typically, if not specifically noted, the conditions for the prothrombinase assay were 50 μl FXa, 5 μM FVa, 0.5 μM prothrombin, and 50 μM phospholipid vesicles (10/90/PS/PE) at 37 °C for 1 min before the reaction was stopped by dilution with ice-cold EDTA buffer. The amount of generated thrombin was quantified using a chromogenic substrate S-2238. In control experiments, plasma-treated with 10 mM benzamidine and 2 mM PPACK and incubated on ice for 2 h, and then thrombin was added and further functional characterization (Table II). These FV variants were expressed transiently in COS 1 cells, and their concentrations were determined with ELISA. All of the variants yielded expression levels similar to that of WT FV, around 200 ng/ml. The procoagulant activity was tested in a prothrombinase-based system at two different conditions, one with limiting concentrations of FVa (5 pm) and saturating concentrations of FXa (5 nm), and the other with saturating concentrations of FVa (100 pm) and limiting concentration of FXa (5 pm). Four of the mutants, having their glycosylations located at positions 467, 511, 652, and 1683 in the A2 domain comprising regions 511–558, 565–692, 530–558, and 692–710 and one in the A3 domain composed of residues 1811–1818 (20–23). Corresponding regions in FVa include residues 455–547, 502–509, and 638–655 in the A2 domain and 1676–1683 in the A3 domain. Therefore, carbohydrate side chains were introduced at FVa residues 467, 511, 652, 655, 1677, and 1683 to probe the different regions for FXa interaction sites. In addition, six other carbohydrate side chains were introduced in areas surrounding these segments. These sites were at residue 172, 319, 345, 373, 435, and 450. In all cases, the consensus sequence for glycosylation was introduced within solvent exposed regions in areas in which substitutions and glycan grafting should be structurally tolerated.

FV Variants with Attenuated Procoagulant Activity—The different FV variants were expressed transiently in COS 1 cells, and their concentrations were determined with ELISA. All of the variants yielded expression levels similar to that of WT FV, around 200 ng/ml. The procoagulant activity was tested in a prothrombinase-based system at two different conditions, one with limiting concentrations of FVa (5 pm) and saturating concentrations of FXa (5 nm), and the other with saturating concentrations of FVa (100 pm) and limiting concentration of FXa (5 pm). Four of the mutants, having their glycosylations located at positions 467, 511, 652, or 1683, demonstrated decreased FXa cofactor activity (less than 50% of WT) and were chosen for further functional characterization (Table II). These FV vari-
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The procoagulant activity of the mutants were tested in a prothrombinase-based system. The activity of the mutants were determined at two different conditions: with limiting concentrations of FVa (5 pM) and saturated concentration of FXa (5 nM) and with saturated concentrations of FVa (140 pM) and limiting concentration of FXa (5 pM).

| FV mutant   | Procoagulant activity |
|-------------|-----------------------|
|             | 5 nM FXa, 5 pM FVa | 5 pM FXa, 100 pM FVa |
| 172 NGT     | 103                   | 85                   |
| 319 NKT     | 104                   | 74                   |
| 345 NGT     | 102                   | 96                   |
| 373 NET     | 100                   | 100                  |
| 435 NTT     | 99                    | 100                  |
| 450 NET     | 96                    | 108                  |
| 461 NFT     | 99                    | 82                   |
| 467 NNT     | 7                     | 25                   |
| 511 NAT     | 44                    | 32                   |
| 652 NDT     | 43                    | 47                   |
| 655 NCT     | 105                   | 79                   |
| 1677 NTT    | 105                   | 89                   |
| 1683 NAT    | 14                    | 26                   |

Screening to find mutants with attenuated procoagulant activity

The procoagulant activity of the mutant were tested in a prothrombinase-based system. The activity of the mutants were determined at two different conditions: with limiting concentrations of FVa (5 pM) and saturated concentration of FXa (5 nM) and with saturated concentrations of FVa (140 pM) and limiting concentration of FXa (5 pM).

SDS-PAGE Analysis of the Pulse-labeled FV Variants—To assess whether the FV variants had the additional carbohydrate side chain or not, the mobility on SDS-PAGE of pulse-labeled thrombin-activated FV variants was compared. In this experiment, pulse-labeled FV immunoprecipitated from the cell lysates was chosen rather than FV secreted in the medium, because the proteins in the cell lysate carry relatively homogeneous carbohydrate side chains, which facilitate the analysis. As judged by the electrophoretic analysis, an extra glycan was present in all mutants having impaired procoagulant activity (Fig. 1A). Thus, the apparent molecular weights of the heavy chains of FV variants 467 NNT, 511 NAT, and 652 NDT were increased as compared with that of WT FV. The light chain of the 1683 variant demonstrated altered mobility compatible with the presence of an extra carbohydrate side chain. After deglycosylation of the FV variants with endoglycosidase H, no differences were observed between the mutants having an extra carbohydrate in the heavy chain, i.e. 467 NNT, 511 NAT, and 652 NDT, and WT FV. After endoglycosidase H treatment, the heavy chains migrated as multiple closely spaced bands, suggesting partial deglycosylation (Fig. 1B). After the deglycosylation, the light chains migrated as a single band indicating that the partial glycosylation disappeared. The light chain of the 1683 NAT variant migrated as the light chain of WT FV, demonstrating release of the extra carbohydrate side chain. The additional nine FV mutants that had full biological activity were also pulse-labeled and analyzed on SDS-PAGE after activation with thrombin to investigate whether the mutagenesis indeed resulted in the attachment of an extra carbohydrate side chain. The heavy chains of two of these mutants, 435 NET and 511 NCT, migrated like the heavy chain of WT FV, indicating that they did not carry an extra carbohydrate, whereas the other seven mutants appeared to be glycosylated at the sites of mutation (Fig. 2, A and B). The sequence for deglycosylation at position 655 (NCT) is not optimal for the glycosylation reaction, which might explain why position 655 was not glycosylated. Only a fraction of the 319 NKT variant demonstrated increased molecular weight on the SDS-PAGE analysis, suggesting partial glycosylation at position 319.

Apparent Kd for FXa Binding to FVa as Determined by Prothrombinase Assay—Impaired binding of FXa or decreased interaction with prothrombin may be the cause of the poor FXa cofactor activity of the FV variants that was observed in the prothrombinase assay. In addition, a decreased catalytic activity of the bound FXa, as reflected by a low kcat value, could potentially result in decreased biological activity of the FV variants. To characterize the nature of the attenuated cofactor activity of the FV mutants, their ability to support prothrombin activation at increasing FXa concentrations was studied (Fig. 3). Three of the FV mutants, 467 NNT, 511 NAT, and 1683 NAT, demonstrated poor FXa cofactor activity, which was particularly pronounced in the case of variants 467 NNT and 1683 NAT. The 511 NAT variant demonstrated a different pattern with the highest concentrations of FXa tested yielding around 70% of the maximum activity of WT FVAs. In contrast, the 652

![Fig. 1. SDS-PAGE analysis of the mutants with attenuated procoagulant activity. COS 1 cells expressing the WT, 467 NNT, 511 NAT, 652 NDT, and 1683 NAT FV variants were radiolabeled for 1 h (pulse). The radiolabeled FV in the cell lysates was immunoprecipitated and incubated with thrombin (2 units/ml) for 30 min at 37 °C (A). The positions of the heavy (HC) and light (LC) chains are indicated. In B, the thrombin-treated immune precipitates were subjected to endoglycosidase H (1 unit/ml) digestion overnight at 37 °C. The sharp upper band represents the deglycosylated B domain. The HC is partially deglycosylated as illustrated by the multiple closely spaced bands, whereas the LC appears to be fully deglycosylated. Proteins were separated on 7.5% SDS-polyacrylamide gel electrophoresis and detected using a PhosphorImager.](image-url)
NDT variant reached a plateau of maximum thrombin generation, which was ~50% of that of WT FVa. The results of this experiment allowed calculation of apparent Kd for WT FVa and the 652 NDT and 511 NAT variants (Table III). Wild-type FVa demonstrated an apparent Kd of 0.2 nM, which agrees with results on record from similar experiments (19, 40, 41). The 652 NDT variant yielded a similar Kd as WT FVa, but the maximum rate of thrombin generation was only half of that of WT FVa. The Kd for the 511 NAT variant was estimated to be 6 nM. The experiment illustrated in Fig. 3A was performed at 50 pM FVa. To ensure that the low maximum thrombin generation rate observed for several of the variants was not the result of low FVa levels, the FXa concentration was kept constant at 5 pM and the FVa increased up to a maximum of 2 nM (Fig. 4). In this case, the thrombin-generation rate of the 652 NDT variant was still around 50% of that of WT FVa. The 511 NAT variant also reached around 60% of the WT FVa thrombin generation, whereas the 467 and 1683 variants were less efficient in supporting prothrombin activation.

In the described experiments, the recombinant FV were not purified from the condition medium. To ensure that components of the medium did not affect the results, control experiments in which plasma-purified FVa was added to mock medium were performed. These experiments demonstrated that the medium did not affect the ability of FVAs to express FXa cofactor activity. In addition, the recombinant mutants demonstrating low activity and WT FVAs were analyzed after purification (Fig. 3B). Because the concentrations of the recombinant mutants were considerably lower after purification (0.1 nM), the FVa variants were allowed to generate thrombin for longer duration of time (8 min). The results obtained were similar to those derived from experiment with non-purified FV, suggesting that it is adequate to study the FV variants without prior purification.

Restored Procoagulant Activity upon Synthesis in the Presence of Tunicamycin—To ensure that the attenuated FXa affinity of the FVa variants was the result of the added carbohydrate side chain rather than a direct effect of the mutations, the recombinant FVa variants were expressed in the COS 1 cells in the presence of tunicamycin, which inhibits N-linked glycosylation. The expression levels obtained in the presence of tunicamycin were considerably lower than in its absence but enough FVa was recovered for analysis in the prothrombinase assay (Fig 5). The rate of thrombin generation was measured at increasing concentrations of FXa, which allowed estimation of the binding ability of FXa to the recombinant FVAs. Of the investigated FV variants, only the 511 NAT variant demonstrated reduced ability to bind FXa. All the other variants yielded similar profiles as WT FV, suggesting tunicamycin to restore the procoagulant activities of the mutants. Thus, the deteriorated FXa affinity of the FV variants observed with FV expressed in the absence of...
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The apparent \( K_m \) for binding of FXa to FV mutants estimated in the prothrombinase assay and in the magnetic bead assay

| Mutant     | \( K_m \) estimated in the prothrombinase assay | \( k_m \) estimated in the prothrombinase assay | \( K_m \) estimated in the magnetic bead-based assay | \( K_m \) estimated in the prothrombinase assay |
|------------|-----------------------------------------------|-----------------------------------------------|--------------------------------------------------|-----------------------------------------------|
| WT FVa     | 200                                          | 170                                          | 1100                                            | 200                                          |
| 467 NNT    | ND                                           | ND                                           | ND                                               | ND                                           |
| 511 NAT    | 6000                                         | 120                                          | ND                                               | 200                                          |
| 652 NDT    | 200                                          | 80                                           | 3800                                            | 200                                          |
| 1683 NAT   | ND                                           | ND                                           | ND                                               | 200                                          |

\( a \) \( k_m \) values were calculated from the absolute rate of maximal thrombin observed in the experiment presented in Fig. 3.

\( b \) \( K_m \) values were calculated from data of Fig. 6 fitted to the Michaelis-Menten equation.

\( c \) ND, not determined.

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**FIG. 4.** FVa titration in the prothrombinase assay. The recombinant FV mutants were incubated with thrombin for 10 min at 37 °C. The activated mutants (20–200 pM) were then incubated for 4 min with FXa (5 pM) and 10/90, PS/PC phospholipid vesicles (50 °C) at 37 °C. Thrombin generation was started by the addition of thrombin (0.5 μM). After 5 min, the reactions were stopped by dilution with ice-cold EDTA buffer. Thrombin generation obtained in the absence of FVa was subtracted. WT FVa (■), 467 NNT (□), 511 NAT (▲), 652 NDT (▲), and 1683 NAT (●).

**FIG. 5.** Expression of FV variants in the presence of tunicamycin-restoring procoagulant activity. The recombinant FV mutants were expressed in COS 1 cells in the presence of tunicamycin. After thrombin activation, the mutants were incubated for 4 min with FXa (1–50 000 pM) and 10/90, PS/PC phospholipid vesicles (50 °C) at 37 °C. Thrombin generation was started by the addition of thrombin (0.5 μM). After 1 min, the reactions were stopped by dilution with ice-cold EDTA buffer. The generated thrombin was determined with the chromogenic substrate S-2238. The activity was expressed as a percentage of maximum activity generated by WT FVa. WT FVa (■), 467 NNT (□), 511 NAT (▲), 652 NDT (▲), and 1683 NAT (●). Each data point represents the mean of three independent experiments performed in duplicate. Error bars represent ± S.E.

tunicamycin was caused by the added carbohydrate side chains rather than by the amino acid replacements per se.

**Prothrombin Titrations to Determine \( K_m \).**—To estimate the interaction between prothrombin and the prothrombinase complexes formed by FXa and the various FV variants, the rate of thrombin generation was determined at increasing concentrations of prothrombin (Fig. 6). To ensure that the prothrombin interacted specifically with the FV-FXa complex rather than with the phospholipid surface, high concentrations of Va (200 pM) and suboptimal vesicles were used. The phospholipids had a composition of 5/95 PS/PC and was used at a concentration of 25 μM. Control experiments showed that, in the absence of Va, the \( K_m \) for prothrombin activation was severalfold higher. This indicates that prothrombin interacted specifically with the FVa-FXa complex. The results allowed determination of the \( K_m \) for each reaction. All the FV mutants, when assembled in the prothrombinase complex, yielded similar values of \( K_m \) for prothrombin (Table III).

**FXa Binding to FVa Variants in the Presence of Phospholipid Vesicles.**—The functional studies were performed in the presence of prothrombin and therefore reflected the ability of FXa to interact with FVa/prothrombin rather than with FVa alone. A direct binding assay was therefore established to investigate the specific interaction between FXa and the phospholipid membrane-bound FVa variants (Fig. 7). In the assay, phospholipid-coated magnetic beads were incubated with the thrombin-treated recombinant FV variants and then with radiolabeled bFXa. To estimate the level of nonspecific binding and to allow calculation of binding affinities, increasing amounts of unlabeled bFXa was added. The direct binding assay demonstrated no binding of FXa to the 467 NNT, 511 NAT, and 1683 NAT variants. The binding of FXa to the 652 NDT mutant was only slightly lower than that of WT FVa and the \( K_d \) was estimated to be around 4-fold lower than that of WT FVa (Table III).

**DISCUSSION**

To date, three regions in FVa involved in the binding of FXa have been identified, including residues 311–325, 493–506, and 584–709, all of which are located in the A2 domain (17–19). The first two regions were identified using synthetic peptides, whereas the last was the result of specific proteolysis that liberated the 684–709 peptide. To enhance our understanding of the FVa-FXa interaction, we screened putative binding regions in FVa by introducing N-glycosylations using site-directed mutagenesis. With this approach, we have now identified three previously unknown interaction sites for FXa. The A2 domain carried two of the sites, at residue 467 and 652, whereas the third was localized in the A3 domain at residue 1683.

To investigate whether the method we have used, i.e. to introduce novel N-linked glycans to probe binding regions, was able to confirm regions suggested by peptide studies, the 319 NKT and 511 NAT variants were created. The 511 NAT variant
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**FIG. 6. Prothrombin titration in the prothrombinase assay.** The FV mutants were incubated with thrombin as described in the legend to Fig. 3 and then diluted to 200 pM and incubated for 4 min with prothrombin (5–2500 nM) and 5/95, PS/PC phospholipid vesicles (25 μM) at 37 °C. Thrombin generation was started by addition of FXa (5 nM final concentration). After 1 min, the reactions were stopped with EDTA, and the generated thrombin was determined as described in legend to Fig. 3. The activity was expressed as the percentage of maximum activity generated in the presence of WT FVa. WT FVa (●), 467 NNT (□), 511 NAT (▲), 652 NDT (△), and 1683 NAT (○). Each data point represents the mean of three independent experiments performed in duplicate. Error bars represent ± S.E.

**FIG. 7. Direct binding of radiolabeled FXa to membrane-bound FVa.** Phospholipid-coated beads were incubated for 10 min in the presence of the FVa variants (0.5 nM). Increasing concentrations of 125I-Fxa (0.2–6 nM) were added. After 30-min incubation, the beads were isolated and washed before being counted. The amount of bound 125I-Fxa was determined and plotted versus the amount of added 125I-Fxa. Binding determined from parallel incubation mixtures containing a 100-fold excess of unlabeled FXa was subtracted from all reactions. WT FVa (●), 467 NNT (□), 511 NAT (▲), 652 NDT (△), and 1683 NAT (○). Each data point represents the mean of three independent experiments performed in duplicate. Error bars represent ± S.E.

**FIG. 8. Proposed binding site for FXa at the surface of FVa.** The three-dimensional model for the three A domains of FV is presented as a solid surface (the A1 domain is blue, A2 is white, and A3 is yellow). Only two of the three cleavage sites for APC can be visualized on this structure, namely at positions Arg-306 and Arg-506. The solvent-exposed surface of the residues expected to be glycosylated are colored in red. The residues identified to be important for the interaction with FXa in the present study are enclosed in boxes (magenta). The overall dimension of the surface delineated by residues 467, 511, 652, and 1683 is compatible with the size of the binding region identified on the FXa side. Our data are crucial for the future building of models of the prothrombinase complex, because we defined important regions for the contact as well as areas that should not be directly involved.

...away from the main binding surface for FXa, whereas the entire 311–325 segment or part of it is indeed involved in the interaction. It is also noteworthy that a homologous binding site in FVIIIa has not been identified.

There is no consensus at present as to whether FXa interacts with the light chain of FVa. The observation, that a monoclonal antibody directed against the A3 domain of FVa inhibits the FVa-FXa interaction, supports this direct interaction (43). However, the membrane-bound light chain of FVa has been proposed to not interact with FXa (44). The now observed inhibitory effect on FXa binding of the introduction of a carbohydrate side chain at 1683 supports the idea that the light chain carries an interaction site for FXa. It is interesting to note that a homologous FXa interaction site on the light chain of FVIIIa is suggested to confer a most important interaction between FIXa and FVIIIa. The light chain of FVIIIa binds FIXa with a $K_d$ of about 15 nM, whereas a $K_d$ of around 300 nM characterized the binding of FXa to the A2 domain (45–47). The proposed FIXa-binding region in FVIIIa includes residues 1811–1818 (the equivalent residues in FV are 1676–1683), a region that is rich in basic Lys residues (46). FXa does not carry such a cluster of Lys residues, but it is still possible that binding sites are present at this location in both cofactors. In this context it is noteworthy that the mutation introducing an N-linked glycosylation at 1677 did not affect the FXa binding. Residue 1677 is about 20 Å away from amino acid 1683 and could thus be outside the key interaction area.

The 652 NDT variant demonstrated an interesting functional pattern in the prothrombinase complex with a decreased $k_{cat}$, whereas the $K_d$ for FXa and the $K_m$ for prothrombin seemed to be unaffected. This suggests that the extra carbohy-
drate side chain affects the catalytic activity of the bound FXa. The exact underlying mechanism is unknown. Proteolytic removal of residues 683–709 in FVa results in impaired FVa-FXa interaction, and it is possible that the region surrounding 652 is part of an interaction site that also includes residues 683–709. It is not possible to estimate how close residue 652 is to the 683–709 region, because the present three-dimensional models end at residue 656. Therefore, the region around residue 652 is difficult to analyze structurally. However, it is important to note that the equivalent region of the A2 domain of FVIIIa, encompassing residues 698–712, has been reported to play a crucial role for the binding of FIXa (22).

A third A2 domain region of FVIIIa involved in FIXa binding was found after identification of a naturally occurring substitution at residue 527. This FIXa-binding site was confirmed by peptid inhibition studies, demonstrating a peptide comprising 511–530 to inhibit FIXa binding (20). In the present investigation, introduction of a carbohydrate at residue 467, which is located at the equivalent segment of FV, showed a remarkably attenuated FXa binding. In fact, the 467 NNT mutant appears to have lost almost all FXa-binding ability. In the three-dimensional model of FIXa, residue 467 is strikingly close to the reported FXa binding region at 493–520 and as such could be part of an extended FIXa interaction site.

Our ultimate goal is to understand the structure-function relationships of the fully assembled prothrombinase complex and to create a three-dimensional model for the complex. This requires information about the binding site on FIXa for FVa. Recently, a FIXa-binding site in FVa was identified and found to be a homologous counterpart to the FIXa-binding site of FIXa (48). In FVIIa the two regions at 517–527 and 556–569 are suggested to bind to a surface loop at 330–339 in the catalytic domain of FIXa (45, 49). If the situation would be similar for the FVaFIXa complex, the FVa region surrounding residue 467 (residues 461–471) would together with FVa residues 500–513, bind to a surface loop of FIXa, including amino acids 344–352.

Although the identified FXa binding regions in FVas are far apart in the primary amino acid sequence, i.e. residues 467, 652, and 1683 and regions 311–325, 493–520, and 683–709, they are relatively close in space according to the three-dimensional model predictions (Fig. 8). The three-dimensional model has some inherent limitations, e.g. some segments are missing and the resolution of some loops is low. Despite this, the model suggests that the identified multiple binding sites form an extended interaction site for FXa. This is compatible with the extended surface predicted to be involved in the interaction from the FVas side (48). The residues that were probed in this investigation but with negative results, i.e. with no measurable effect on FXa binding, also help define the binding surface.

In conclusion, we have defined three previously unidentified interaction sites for FXa on FVa, which are located close to residues 467 and 652 in the A2 domain and to residue 1683 in the A3 domain. This information provides a useful platform for further investigation of the specific structural elements that are involved in the FVa-FXa complex assembly. Our data suggest the enzyme cofactor assembly of the prothrombinase and the tenase complex to be homologous, demonstrating the rational use of comparisons between the two complexes when mapping interaction sites.

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