Identification of Surface Epitopes of Human Coagulation Factor Va That Are Important for Interaction with Activated Protein C and Heparin*

Kenneth Segers†, Björn Dahlböck‡, Jan Rosing¶, and Gerry A. F. Nicolaes§†

From the §Department of Biochemistry, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, 6200MD, The Netherlands and the ¶Department of Laboratory Medicine, Clinical Chemistry, Lund University, the Wallenberg Laboratory, University Hospital, Malmö, SE-205 02 Malmö, Sweden

Inactivation of factor Va (FVa) by activated protein C (APC) is a key reaction in the down-regulation of thrombin formation. FVa inactivation by APC is correlated with a loss of FXa cofactor activity as a result of three proteolytic cleavages in the FVa heavy chain at Arg306, Arg506, and Arg679. Recently, we have shown that heparin significantly inhibits the APC-mediated cleavage at Arg506 and stimulates cleavage at Arg306. Three-dimensional molecular models of APC docked at the Arg306 and Arg506 cleavage sites in FVa have identified several FVa amino acids that may be important for FVa inactivation by APC in the absence and presence of heparin. Mutagenesis of Lys320, Arg321, and Arg400 to Ala resulted in an increased inactivation rate by APC at Arg306, which indicates the importance of these residues in the FVa-APC interaction. No heparin-mediated stimulation of Arg306 cleavage was observed for these mutants, and stimulation by protein S was similar to that of wild type FVa. With this, we have now demonstrated that a cluster of basic residues in FVa comprising Lys320, Arg321, and Arg400 is required for the heparin-mediated stimulation of cleavage at Arg306 by APC. Furthermore, mutations that were introduced near the Arg506 cleavage site had a significant but modest effect on the rate of APC-catalyzed FVa inactivation, suggesting an extended interaction surface between the FVa Arg506 site and APC.

The protein C pathway provides a major anticoagulant mechanism for the regulation of hemostasis by nullification of the procoagulant activities of FVIIa and FVa, the cofactors in the tenase and prothrombinase complexes, respectively. Protein C is a vitamin K-dependent protein composed of a heavy chain and a light chain that are connected through a single disulfide bridge (1). The light chain is composed of an N-terminal γ-carboxyglutamic acid-rich domain and two epidermal growth factor-like domains. The heavy chain contains a short 3-carboxyglutamic acid-rich domain and two epidermal growth factor-like domains. The heavy chain contains a short disulfide bridge (1). The light chain is composed of an N-terminal γ-carboxyglutamic acid-rich domain and two epidermal growth factor-like domains. The heavy chain contains a short 3-amino acid (12 amino acid) signal peptide and the serine protease (peptidase) domain.

1 To whom correspondence should be addressed. Tel.: 31-43-388-1539; Fax: 31-43-388-4159; E-mail: G.Nicolaes@bioch.unimaas.nl.

2 The abbreviations used are: APC, activated protein C; DOPS, 1,2 dioleoyl-sn-glycero-3-phosphoserine; DOPC, 1,2 dioleoyl-sn-glycero-3-phosphocholine; FV, coagulation factor V; FVa, activated FV; HBS, Hepes-buffered saline; QRR, recombinant FV carrying the R306Q/R679Q mutations; RQQ, recombinant FV carrying the R306Q/R679Q mutations; UFH, unfractionated heparin; ELISA, enzyme-linked immunosorbent assay.

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† To whom correspondence should be addressed. Tel.: 31-43-388-1539; Fax: 31-43-388-4159; E-mail: G.Nicolaes@bioch.unimaas.nl.
Identification of Surface Epitopes of Human Coagulation FVα

Besides its anticoagulant and anti-inflammatory functions, heparin also regulates APC activity by stimulating the inhibition of APC by the serpin protein C inhibitor (2, 19, 20). In addition, it has been shown that heparin alters the pathway of APC-catalyzed FVa inactivation by inhibition of the Arg<sup>306</sup> cleavage and stimulation of the Arg<sup>506</sup> cleavage in FVa (9). This latter inhibition has been suggested to contribute to the less favorable effects of APC in heparin-cotreated sepsis patients, as were studied in the PROWESS trial (18, 21). Three-dimensional models of APC docked at the Arg<sup>306</sup> and Arg<sup>506</sup> cleavage sites in FVα suggest that heparin prevents optimal docking of APC at Arg<sup>506</sup> and stimulates cleavage at Arg<sup>306</sup> by bridging electrostatic regions in FVa and APC (9).

In this study, we have used site-directed mutagenesis to show that FVa residues Lys<sup>320</sup>, Arg<sup>321</sup>, and Arg<sup>400</sup> are important for the heparin-mediated stimulation of FVa cleavage at Arg<sup>506</sup> by APC. In addition, we observed minor but significant effects of mutations that were introduced near the Arg<sup>506</sup> cleavage site on the APC-catalyzed FVa inactivation. The most prominent effect was observed for the FVa mutants with mutations in an acidic cluster spanning from residues 659 to 663, which is likely part of an extended FVa:APC binding interface.

EXPERIMENTAL PROCEDURES

Reagents—Serum-free cell culture media (Opti-MEM Glutamax) were from Invitrogen. Heparin, sodium chloride, calcium chloride, and bovine serum albumin were from Sigma. Unfractionated heparin (UFH) was obtained from LEO Pharma (Ballerp, Denmark); 1 IU/ml UFH contains ~5.7 μg/ml UFH/ml (22). The chromogenic substrates S-2238 and S-2366 were supplied by Chromogenix (Milan, Italy). Phospholipid vesicles were prepared as described (23). Synthetic lipids used were 1,2 dioleoyl-sn-glycero-3-phosphoserine (DOPS) and 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC), and these were obtained from Avanti Polar Lipids (Alabaster, AL).

Proteins—Human α-thrombin and protein S were from ERL (Swansea, UK). Hirudin was purchased from Kordia Laboratory Supplies (Leiden, The Netherlands). Bovine FX was purified according to Fujikawa et al. (24). Human FXa was prepared from FX after activation by RVV-X (24, 25). Human prothrombin was purified according to the method of Di Scipio et al. (26). Monoclonal antibody 3B1 directed against the heavy chain of human FVa was a kind gift from Prof. B. N. Bouma. The anti-human protein C polyclonal antibodies Dako A0370 and Dako P0374 were obtained from Dako (Germany).

Site-directed Mutagenesis—FV cDNA constructs containing either combined mutations at the Arg<sup>306</sup> and Arg<sup>679</sup> APC cleavage sites (FV R506Q/R679Q, abbreviated as RQQ) or at the Arg<sup>306</sup> and Arg<sup>679</sup> (FV R306Q/R679Q, abbreviated as QRQ) were prepared as described, using the pMT2-V vector as template (27). Amino acid substitutions (Tables 1 and 2) were introduced near the Arg<sup>306</sup> or Arg<sup>506</sup> cleavage sites by PCR-based site-directed mutagenesis of the expression vectors encoding the RQQ and QRQ mutants by use of the Quick-Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). For reasons of clarity, recombinant FV molecules given in Table 1 and 2 are identified only by their mutations other than the R306Q, R506Q, or R679Q that were present in the template that was used in their construction.

Transient Expression of Recombinant FV Variants—Plasmid DNA containing the various FV cDNA constructs was transfected into COS-1 cells using the DEAE-dextran method (28). As a control, a pMT2-V vector, of which the FV cDNA had been excised, was used to transfect cells. Proteins were collected in serum-free medium (Optimem Glutamax) 72 h after transfection. Cell debris was removed by centrifugation, and the media containing the recombinant proteins were concentrated ~10-fold using Vivaspin concentrators having a molecular weight cut off of 100,000 (Vivascience, Hannover, Germany). Expression levels of all FV variants were determined using an FVa activity assay, and FV antigen was determined with the FV-specific ELISA described below.

FV ELISA—FV expression levels were determined by an FV-specific enzyme-linked immunosorbent assay, essentially as described (29). Briefly, microtiter plates (Greiner Bio-one, Frickenhausen, Germany) were coated overnight with the monoclonal antibody 3B1. The plates were blocked for 1 h at room temperature with 50 nM Heps, pH 7.5, 150 mM NaCl, 1% bovine serum albumin (Heps-buffered saline, HBS/bovine serum albumin). A standard curve was prepared using different dilutions of pooled normal citrated plasma, assuming a plasma FV concentration of 21.3 nM (30). Standard and samples (diluted in HBS/bovine serum albumin) were incubated for 2 h at room temperature, and the plates were then washed three times with 0.05% Tween 20 in HBS. The biotinylated monoclonal antibody HV1 (0.1 μg/ml) was used as secondary antibody. After 2 h, avidin-horseradish peroxidase (Strep ABC complex/HRP from DAKO, Denmark) was added for 30 min, and plates were developed for 10 min with peroxidase substrate solution (TMB substrate kit, Pierce). The reaction was stopped, and the absorbance was measured at 490 nm.

To exclude the possibility that the cell culture medium interfered with the ELISA, plasma-purified FV and plasma were diluted in mock medium or in buffer. No difference was detected between these diluted samples, indicating that the cell culture medium did not interfere with the FV quantitation.

FVa Assay—Expression levels of the various FV variants were also determined with a functional assay. Recombinant FV proteins were incubated with 2 nM α-thrombin for 20 min at 37 °C. FVa cofactor activity was determined by measuring the rate of FVa-catalyzed prothrombin activation as described (11). Typically, reaction conditions for the prothrombinase assay were as follows: 0–20 pM FVa, 5 nM FXa, 0.5 μM prothrombin, 40 μM phospholipid vesicles (10% DOPS, 90% DOPC; mol/mol), and 0.5 mg/ml ovalbumin in 25 mM Heps, pH 7.5, 150 mM NaCl, and 3 mM CaCl<sub>2</sub>. Thrombin generation was allowed to continue for 2 min at 37 °C before the reaction was stopped with ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 (31). The molar factor Va concentration in the assay mixture was calculated as described (32).

In control experiments, equal antigenic amounts of plasma-purified FVa and recombinant wild type FVα in conditioned medium or in buffer gave the same rates of prothrombin activation, indicating that conditioned medium did not interfere
with FVa functional activity. Moreover, no FV(a) was detectable in the conditioned medium, as judged by both ELISA and functional activity assay.

Expression and Purification of Recombinant Human Protein C Variant K37S/K38Q/K39Q/K62N/K63D—Recombinant human protein C variant K37S/K38Q/K39Q/K62N/K63D was expressed after PCR-based site-directed mutagenesis of the eukaryotic expression vector pGT-hyg (Lilly). The recombinant protein was expressed in 293 cells (CRL-1573; ATCC), purified, and activated as described (2, 6). Protein C was quantified by measurement of absorbance at 280 nm and by a chromogenic assay as described (2).

APC-catalyzed Inactivation of FVa in the Absence and Presence of UFH and Protein S—Time courses of FVa inactivation by APC were determined in the absence and presence of UFH and protein S by following the loss of FXa cofactor activity of FVa in the prothrombinase complex as a function of time. To this end, 20 pM of the activated FVa variants was incubated with 40 μM phospholipid vesicles (10% DOPS, 90% DOPE; mol/mol) at 37 °C in the absence or presence of 25 units/ml UFH or 150 nM protein S in 25 mM Hepes, pH 7.5, 150 mM NaCl, and 3 mM CaCl₂. Next, 3 aliquots of 115 μl were taken from the reaction mixture to determine the FVa activity in the absence of APC in triplicate. Then APC was added to the reaction mixture, and the mixture was subsequently separated into several 115-μl aliquots, one for each time point measured. For the QRQ and RQQ variants, final APC concentrations of 0.085 and 0.75 nM were used, respectively. At different time points after the addition of APC, the residual FVa activity was measured in the prothrombinase assay described above, with minor modifications. Prewarmed FXα (0.5 mM for the QRQ variants; 5 mM for the RQQ variants, unless otherwise indicated) and prothrombin (1 μM) were added to the FVa aliquots, and thrombin generation was allowed to continue during 2 min after which the reaction was stopped by >40-fold dilution in ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 (31). We verified that the formation of thrombin was linearly dependent on the FVa concentration present in the prothrombinase mixture and that heparin (25 units/ml) did not interfere with the FVa assay. The stability of the various FVa variants was assessed by following the loss of FVa activity as a function of time in a reaction mixture to which no APC had been added.

Analysis of Kinetic Data—We have previously shown that the loss of FVa cofactor activity during the APC-catalyzed inactivation of membrane-bound wild type FVa can proceed via two pathways (11) as shown in Reactions 1 and 2,

\[
\begin{align*}
\text{factor Va} & \rightarrow \text{factor Va}_{\text{int}} & \rightarrow & \text{factor Va}, \\
\text{REACTION 1} & \\
\text{k}^{306} & \text{factor Va} & \rightarrow & \text{factor Va}, \\
\text{REACTION 2}
\end{align*}
\]

Initial cleavage at Arg⁵⁰⁶ (with a corresponding rate constant of \(k^{306}\)) results in a reaction intermediate (FVa_{int}) with partial cofactor activity, which must be further cleaved at Arg⁵⁰⁶ (k₃⁰₆) to completely abolish FVa cofactor activity (pathway 1, Reaction 1). Alternatively, initial cleavage at Arg⁵⁰⁶ (k₃⁰₆) results in complete loss of FVa cofactor activity (pathway 2, Reaction 2).

For calculation of the pseudo first-order rate constant for cleavage at Arg⁵⁰⁶ in the FVa mutants, time curves obtained for the FVa RQQ variants were fit to Equation 1,

\[
V_{a,t} = V_{a,0} \cdot e^{-k_{306} \cdot t} + A \cdot V_{a,0} \cdot \frac{k_{506} \cdot e^{-k_1 \cdot t}}{(k_{506} + k_1 - k_2)} \cdot (1 - e^{-k_{306} + k_1 - k_2} \cdot t) \quad \text{(Eq. 1)}
\]

in which \(V_{a,t}\) is the FVa cofactor activity determined at time \(t\); \(V_{a,0}\) is the cofactor activity before APC is added; \(A\) is the fraction of remaining procoagulant cofactor activity of the FVa variant cleaved at position 506; \(k_{506}\) is the observed pseudo first-order rate constant for cleavage at position 506; and \(k_1\) and \(k_2\) are the first-order rate constants of spontaneous inactivation of intact FVa and of the FVa intermediate, respectively. The value for \(k_1\) was determined by following the loss of FVa activity as a function of time in a reaction mixture that did not contain APC. The stability of the FVa intermediate \((k_2)\) was assessed in a separate experiment in which time courses of APC-catalyzed inactivation were monitored as follows. Wild type recombinant FVa was inactivated with APC, and at the beginning of the observed second slow phase, the reaction volume was divided into two portions, each transferred into a new tube. To one reaction mixture, anti-APC polyclonal antibodies were added that completely block APC-catalyzed FVa inactivation and to the other an equal volume of buffer was added. Loss of FVa was monitored in both samples, and the resulting time courses of FVa inactivation were compared and found to be superimposable. This observation further rules out the possibility that APC-mediated cleavages at alternative cleavage sites contribute to the observed loss of FVa activity. The time courses were fit to a biphasic exponential from which the value for \(k_2\) was derived.

Time curves of inactivation of the FVa RQQ variants were fit to a single exponential that describes the loss of FVa cofactor activity resulting from a single cleavage at Arg⁵⁰⁶, as shown in Equation 2,

\[
V_{a,t} = V_{a,0} \cdot e^{-k_{306} \cdot t} \quad \text{(Eq. 2)}
\]

in which \(V_{a,t}\) and \(V_{a,0}\) are the FVa cofactor activities determined at time \(t\) and before APC addition, respectively; \(k_{306}\) is the observed pseudo first-order rate constant of cleavage at position 306, and \(k_1\) is the first-order rate constant of spontaneous inactivation of intact FVa.

RESULTS

Expression of FV RQQ and FV QRQ Variants—Three-dimensional models of APC-heparin complexes and of APC alone docked at either the Arg⁵⁰⁶ and the Arg⁵⁰⁶ cleavage sites in FVa provided a molecular basis for the experimentally observed heparin-mediated inhibition of Arg⁵⁰⁶ cleavage and stimulation of the Arg⁵⁶ cleavage in FVa by APC (9). On the basis of
Identification of Surface Epitopes of Human Coagulation FVa

TABLE 1
Activity to antigen ratio and relative rate constants ($k_{306}$) for the APC-catalyzed inactivation of FV QRQ variants by wild type APC and mutant APC (K375/K380/K394/K62N/K63D)

| FV mutant FV mutation | Activity/antigen | Wild type APC $k_{306}$ | APC mutant $k_{306}$ |
|------------------------|------------------|-------------------------|-----------------------|
| QRQ R306Q/R679Q        | 0.82             | 1                       | 1                     |
| 1 D513N                 | 1.09             | 1.83                    | 7.71                  |
| 2 D577N/D578N           | 0.01             | 0.86                    | 1.25                  |
| 3 D513N/D577N/D578N     | 0.02             | 1.21                    | 1.08                  |
| 4 E323Q                 | 1.25             | 0.99                    | 0.78                  |
| 5 E372Q/D373N/E374Q     | 1.19             | 0.63                    | 0.88                  |
| 6 E323Q/E372Q/D373N/E374Q | 0.74           | 0.68                    | 0.90                  |
| 7 D659N/D660N           | 0.56             | 0.50                    | 1.47                  |
| 8 D661N/E662Q/D663N     | 0.87             | 0.52                    | 1.52                  |
| 9 D659N/D660N/D661N/E662Q/D663N | 1.20            | 0.49                    | 1.57                  |

Note that because FV mutants D577N/D578N and D513N/D577N/D578N had a low activity to antigen ratio, their activity loss could not be followed under the same experimental conditions as used for the other FV QRQ variants. To this end, the final concentrations of 100 pM FV and 5 nM FXa were used in experiments in which these mutants were investigated. We verified that under the experimental conditions used, FV inactivations were determined under pseudo first-order reaction conditions in which the initial rate of FV inactivation was linearly proportional to the concentration of FV and APC.

TABLE 2
Activity to antigen ratio and relative rate constants ($k_{306}$) for the APC-catalyzed inactivation of FV QRQ variants

| Mutant | Mutation | Activity/antigen | $k_{306}$ |
|--------|----------|------------------|-----------|
| RQQ    | R306Q/R679Q | 0.87             | 1         |
| 10     | K320A/R321A | 1.09             | 7.20      |
| 11     | R400A      | 0.77             | 6.87      |
| 12     | K320A/R321A/R400A | 0.84           | 0.83      |
| 13     | K320E/R321E | 1.27             | 3.83      |
| 14     | K320E/R321E/R400E | 0.35           | 0.72      |
| 15     | K320E/R321E/R400E/R506E | 0.35       | 0.89      |
| 16     | K320A/R321A/R400A/R501A | 0.32       | 0.96      |
| 17     | K320E/R321E/R400E/R501E | 0.29       | 0.88      |

these molecular models, several charged FVa residues located near the Arg$^{306}$ and Arg$^{506}$ cleavage sites were identified that may play a role in the interactions between FVa, APC, and heparin. To investigate whether these FVa residues are indeed important for the recognition of the Arg$^{306}$ and Arg$^{506}$ cleavage sites by APC in the absence and/or presence of heparin, we have constructed a panel of FV mutants in which the charge of these surface residues was neutralized or reversed (Tables 1 and 2). To facilitate the kinetic analysis of APC-mediated cleavage at these sites, we have used FV variants that have only the Arg$^{306}$ or the Arg$^{506}$ cleavage site available for proteolysis by APC (see Fig. 1).

After transfection of COS-1 cells, conditioned media containing the FV mutants were collected, centrifuged, concentrated, and stored at −80 °C. The activity to antigen ratio of the various FV variants was determined using a quantitative FV ELISA and a functional assay (Tables 1 and 2). Expression levels for the different recombinant FV forms, as determined by FV ELISA, were all approximately equal (~100 pM), indicating that no major structural abnormalities were introduced by mutation of the wild type amino acid sequence. Remarkably, the activity to antigen ratio of FV mutants D577N/D578N and D513N/D577N/D578N was ~50-fold lower than that of FV QRQ. Both FVa variants express a reduced affinity for FXa as a result of the replacement of Asp$^{577}$ and Asp$^{578}$ for an asparagine residue.3

APC-catalyzed Inactivation of QRQ FVa Variants—The importance of the amino acids listed in Table 1 for APC-catalyzed cleavage at Arg$^{306}$ was evaluated by following time courses of inactivation of FV variants that can only be cleaved by APC at this single peptide bond. In these experiments, loss of FVa cofactor activity, as a result of cleavage at Arg$^{506}$ by APC, was followed in a prothrombinase assay. Single cleavage at Arg$^{506}$ in FV results in the formation of FV$_{int}$, which exhibits ~40% partial FVa cofactor activity (11) at high FXa and negligible cofactor activity at low FXa concentrations. We have performed the FVa assay at a lower FXa concentration (0.5 nM) than routinely used (5 nM FXa), as a result of which the FV$_{int}$ activity is <20% of initial FVa activity.

Note that because FV mutants D577N/D578N and D513N/D577N/D578N had a low activity to antigen ratio, their activity loss could not be followed under the same experimental conditions as used for the other FV QRQ variants. To this end, the final concentrations of 100 pM FV and 5 nM FXa were used in experiments in which these mutants were investigated. We verified that under the experimental conditions used, FV inactivations were determined under pseudo first-order reaction conditions in which the initial rate of FV inactivation was linearly proportional to the concentration of FV and APC.

Apparent second-order rate constants for APC-mediated cleavage at Arg$^{506}$ were derived from fitting the obtained time courses to Equation 1 using nonlinear least squares regression analysis. FV mutant QRQ, which can only be cleaved at Arg$^{506}$ and does not contain any other amino acid substitutions, was inactivated at a rate of 6.25 × 10$^7$ M$^{-1}$ s$^{-1}$ (see also Table 1).

For the FV QRQ variants that contain additional mutations around the Arg$^{506}$ cleavage site, the rate constants for cleavage at Arg$^{506}$ ($k_{306}$) were determined likewise, and the resulting rates were expressed relative to the inactivation rate of QRQ (Fig. 2 and Table 1). Substitution of residues Glu$^{372}$, Asp$^{373}$, and Glu$^{374}$ (mutants 5 and 6) resulted in a reduction of about 40% of the inactivation rate compared with QRQ. For the FVa variants

3 M. Steen and B. Dahlbäck, manuscript in preparation.
resulted in an approximate 2-fold increase in $k_{506}$. As described above, reaction conditions for studying the APC-catalyzed inactivation of mutants D577N/D578N and D513N/D577N/D578N were different from those of the other FV variants, which explains the different shape of the inactivation curve for FV variants D577N/D578N and D513N/D577N/D578N.

**Inactivation of FV Variants R306QR679Q by APC Mutant K37S/K38Q/K39Q/K62N/K63D**—To further probe the importance of negatively charged residues in FV that surround the Arg506 cleavage site, we monitored time courses of inactivation of FV QRQ variants by APC mutant K37S/K38Q/K39Q/K62N/K63D (Fig. 3 and Table 1), a mutant that has reduced activity toward the Arg506 cleavage site and that shows no detectable binding to heparin. Compared with the inactivation by wild type APC, FVa QRQ was inactivated at an ~15-fold slower rate by APC mutant K37S/K38Q/K39Q/K62N/K63D (Table 1). All FVa QRQ variants were inactivated by the APC mutant at a rate similar to QRQ, except for FVa D513N, for which an ~8-fold increase in inactivation rate was observed (Table 1 and Fig. 3).

**Effect of Heparin and Protein S on the APC-catalyzed Inactivation of FV Variants QRQ**—To evaluate the effect of heparin and protein S on the APC-mediated cleavage at Arg506, time courses of inactivation of the FV R306QR679Q variants were monitored in the presence of 25 units/ml heparin or 150 nM protein S. Apparent second-order rate constants for APC-mediated cleavage at Arg506 in the presence of heparin or protein S were derived from fitting time courses of inactivation to the same equation that was used to determine $k_{506}$ in the absence of heparin or protein S (see Equation 1). We verified that heparin and protein S did not alter the cofactor activity and stability of the FVa intermediate ($k_2$). For each FVa QRQ variant, the Arg506 cleavage rate in the presence of heparin or protein S was expressed relative to the Arg506 cleavage rate in the absence of heparin or protein S (see Equation 1). We verified that heparin and protein S did not alter the cofactor activity and stability of the FVa intermediate ($k_2$). For each FVa QRQ variant, the Arg506 cleavage rate in the presence of heparin or protein S was expressed relative to the Arg506 cleavage rate of the mutant in the absence of heparin or protein S (Fig. 4). The inactivation of QRQ, which does not contain amino acid substitutions in the Arg506 region, was 3–4-fold inhibited by heparin but was unaffected by protein S. Heparin and protein S had a similar effect on the inactivation of the FV variants, indicating that these mutations have no influence on the heparin-mediated inhibition of cleavage at Arg506 and also that they do not notably contribute to protein S cofactor activity in the APC-catalyzed cleavage at Arg506 in FVa.

**APC-catalyzed Inactivation of FVa RQQ Variants**—A three-dimensional model of APC docked at the Arg306 cleavage site in FVa is shown in ribbon (blue), and the side chains of mutated residues are shown as a colored ball-and-stick representation. The Arg306 and Arg506 APC cleavage sites are colored in red. Residues that were mutated near (<25 Å) the Arg506 and Arg506 cleavage site (see also Table 1) are shown in green and magenta, respectively. The coordinate files of the FVa model were provided by Dr. B. Villoutreix. The putative C-terminal region of the FVa heavy chain, for which no template structure is available, is indicated as a cylinder, positioned as was proposed by Orban et al. (41). The figure was created with the YASARA/WhatIf Twinset program.
Identification of Surface Epitopes of Human Coagulation FVa

FIGURE 3. A, time courses of inactivation of FVa QRQ variants (20 pm) by APC mutant K375/K380/K390/K62N/K63D (0.56 nm) were determined as described under “Experimental Procedures.” FVa cofactor activity was determined at 0.5 nm FXa, except for FVa mutants D577N/D578N and D513N/D577N/D578N, which were assayed at a final concentration of 100 pm FVa and 5 nm FXa (see “Results”). The figure represents the average of two experiments, and standard deviations were <10% for all of the FVa variants. ●, QRQ; ○, D513N; △, D577N/D578N; ▲, D513N/D577N/D578N. Data were fit to an equation describing the loss of FVa cofactor activity as a result of Arg306 cleavage by APC (see Equation 1 under “Experimental Procedures”). Rates for cleavage at Arg306 (k_{inact}) in the presence of heparin or protein S were expressed relative to the inactivation rate of the FVa mutant in the absence of heparin or protein S. Values are the average of two experiments, and standard deviations were less than 10% for all FVa variants.

FIGURE 4. Effect of heparin and protein S on the APC-catalyzed inactivation of FVa QRQ variants. Time courses of inactivation of FVa QRQ variants (20 pm) by APC (0.75 nM) were determined in the absence or presence of UFH (25 units/ml; open bars) or protein S (150 nm; shaded bars). Data were fit to an equation describing the loss of FVa cofactor activity as a result of Arg306 cleavage by APC (see Equation 2 under “Experimental Procedures”). Rates for cleavage at Arg306 (k_{inact}) in the presence of heparin or protein S were expressed relative to the inactivation rate of the FVa mutant in the absence of heparin or protein S. Values are the average of two experiments, and standard deviations were less than 10% for all FVa variants.

FIGURE 5. Effect of heparin and protein S on the APC-catalyzed inactivation of FVa QRQ variants. Time courses of inactivation of FVa QRQ variants (20 pm) by APC (0.085 nM) were determined in the absence or presence of UFH (25 units/ml; open bars) or protein S (150 nm; shaded bars). Data were fit to an equation describing the loss of FVa cofactor activity as a result of Arg306 cleavage by APC (see Equation 2 under “Experimental Procedures”). Rates for cleavage at Arg306 (k_{inact}) in the presence of heparin or protein S were expressed relative to the inactivation rate of the FVa mutant in the absence of heparin or protein S. Values are the average of two experiments, and standard deviations were less than 10% for all FVa variants.

rate constants for cleavage at Arg306 were determined (see Equation 2). FVa mutant RQQ, which can only be cleaved at Arg306 and which does not contain any further amino acid substitutions in this region, was inactivated with a rate of 1.1 \times 10^6 \text{M}^{-1} \text{s}^{-1} (Table 2). In the absence of heparin or protein S, the inactivation rate (k_{inact}) of FVa variants K320A/R321A, R400A, and K320E/R321E was 4–7-fold increased compared with RQQ (Table 2). All other FVa variants were inactivated at Arg306 with a rate similar to RQQ.

Effect of Heparin and Protein S on the APC-catalyzed Inactivation of FVa RQQ Variants—In the presence of heparin, the inactivation of RQQ was 3–4-fold stimulated (Fig. 5). However, none of the FVa RQQ variants showed a heparin-mediated stimulation of cleavage at Arg306, but even a mild inhibition (−2-fold) (see also Fig. 5). Because the stimulating effect of heparin on the inactivation of FVa mutants K320A/R321A and R400A was completely abolished, we conclude that residues Lys320, Arg321, and Arg400 constitute a heparin-binding region in FVs that is important for the accelerated cleavage at Arg306 by APC in the presence of heparin.

Protein S stimulated APC-mediated cleavage at Arg306 for all FVa RQQ variants (Fig. 5). The stimulating effect of protein S was similar for all FVa variants and slightly lower (1.5–2-fold) compared with the effect of protein S on RQQ. This indicates that the mutated residues play only a minor role in the cofactor activity of protein S in the cleavage at Arg306 by APC.

DISCUSSION

We have recently shown that heparin alters the APC-catalyzed inactivation of FVa by specifically inhibiting cleavage at Arg306 and stimulating cleavage at Arg306 (9). On the basis of the available kinetic data and three-dimensional molecular models of the APC-heparin complex and of APC alone docked...
at the Arg<sup>306</sup> and Arg<sup>506</sup> site in FVa, several amino acid residues were identified in FVa that may be important for the FVa-APC interaction and its modification by heparin. To investigate the role of these residues in APC-catalyzed FVa inactivation, we have replaced these amino acids by neutral or charge-reversed residues by site-directed mutagenesis (Table 1). Because all target amino acid residues are solvent-exposed and most of the introduced residues can be considered as conservative, we expected that these mutations had no adverse effects on protein folding and that the effects of the substitution would be limited to the interaction under study. The interaction surfaces between FVa and APC are likely not limited to single amino acid contacts, and therefore we chose to produce both single as well as multiple mutations to probe the FVa-APC interaction.

Upon calculation of activity/antigen ratios for the different FV variants, we noticed a remarkable ratio for the FV mutants D577N/D578N and D513N/D577N/D578N. Although ratios for all mutants were within a 2–3-fold range, the activity/antigen ratio in this case was ~50-fold lower than that of FV QRQ. We ascribe this discrepancy to a reduced affinity for FXa as a result of the replacement of Asp<sup>577</sup> and Asp<sup>578</sup> for an asparagine residue. Several data in the literature, both structural (33) and functional (34), agree with a role for Asp<sup>577</sup> and Asp<sup>578</sup> in the FVa-FXa interaction. In addition, FVa sequences between residues 311 and 325 (35) and 493 and 506 (36) have also been reported earlier to interact with FXa, which may explain the observed lower activity/antigen ratio of FVa variants bearing combined mutations of these residues (Table 1).

Analysis of the time courses of APC-catalyzed inactivation of the FV variants that can only be cleaved at Arg<sup>506</sup> showed only minor effects of the mutations that were introduced near the Arg<sup>506</sup> cleavage site. Significant differences were noted, however, for several of the mutants tested, which suggests that there is an extended interaction surface between FVa and APC. The most prominent effect was observed for the FVa mutants with mutations in an acidic cluster spanning from residues 659 to 663, for which an ~2.5-fold reduction in the second-order rate constant of inactivation (k<sub>506</sub>) was observed. A possible role of this cluster of negatively charged residues has been proposed by Pellequer et al. (37), who suggested that this cluster could interact with APC residues Arg<sup>75</sup> and Arg<sup>75</sup>, in addition to Arg<sup>499D</sup> and Arg<sup>151</sup> from the autolysis loop from APC.

None of the introduced mutations had an effect on the heparin-mediated inhibition of Arg<sup>506</sup> cleavage by APC. Together with the observation that heparin does not inhibit FVa inactivation by APC variants with a defective heparin-binding site (9), these data suggest that the inhibition of the Arg<sup>506</sup> cleavage is not mediated by a direct interaction of heparin with FVa. Heparin binds to three conserved surface loops in APC (loops 37, 60, and 70) that contain a cluster of basic residues. This positively charged exosite partially overlaps with an FVa-binding region (loops 37 and 70) crucial for cleavage at Arg<sup>506</sup> by APC (2, 3, 20). Our data indicate that binding of heparin to APC likely prevents appropriate docking of APC at Arg<sup>506</sup> because of steric hindrance by the heparin chain, which had also been observed in in silico docking studies (9). In contrast to the Arg<sup>506</sup> cleavage site, there are no negatively charged residues present in the close vicinity of the Arg<sup>506</sup> site. This suggests that cleavage at Arg<sup>306</sup> by APC is not merely driven by an exosite-mediated interaction, based on mostly electrostatic forces, given the fact that a cluster of net positively charged surface loops on the serine protease domain of APC have been shown to be required for interaction of APC with its substrates. However, inspection of the APC/heparin and APC models docked at the FV Arg<sup>506</sup> cleavage site suggested that although direct contacts between FVa and APC are sparse, heparin could bridge the heparin-binding region of APC and a positively charged region comprising residues Lys<sup>320</sup>, Arg<sup>321</sup>, Arg<sup>400</sup>, and Arg<sup>501</sup> near the Arg<sup>306</sup> cleavage site (9). To investigate whether the predictions based on the theoretical model were correct and to obtain detailed information on the role of a basic cluster of surface-exposed residues in the APC-mediated cleavage at Arg<sup>506</sup>, several FVa variants that can only be cleaved at Arg<sup>506</sup> were made in which these positively charged amino acids were replaced for neutral or negatively charged residues.

Remarkably, the rate of Arg<sup>306</sup> cleavage by APC of FVa mutants K320A/R321A, R400A, and K320E/R321E was 4- to 7-fold increased compared with FVa RQQ. Residues Lys<sup>320</sup>, Arg<sup>321</sup>, and Arg<sup>400</sup> are located on the C terminus of the Arg<sup>306</sup> loop and are involved in hydrogen bonds and salt bridges with surrounding residues Glu<sup>315</sup>, Met<sup>319</sup>, Glu<sup>323</sup>, Ile<sup>359</sup>, and Glu<sup>461</sup>. Because the distance between the N and C terminus of the Arg<sup>306</sup> loop is much larger than that of the Arg<sup>506</sup> loop, although both loops involve about the same number of residues, it was proposed that the structure of the Arg<sup>306</sup> loop may be more constrained. Local melting of the Arg<sup>306</sup> loop therefore could be required for proper presentation of the Arg<sup>306</sup> loop to the active site of APC, which in turn could contribute to the slower cleavage rate at Arg<sup>506</sup> compared with Arg<sup>506</sup> (37). Because replacement of residues Lys<sup>320</sup>, Arg<sup>321</sup>, and Arg<sup>400</sup> for Ala disrupts hydrogen bonding and substitution by Glu residues introduces local electrostatic repulsion, these mutations may result in a less constrained Arg<sup>306</sup> loop that hypothetically can explain the observed increase of the rate of cleavage at Arg<sup>306</sup> in these FVa mutants. More experimental data are needed to confirm this hypothesis. Our data are in agreement with a reported global involvement of the area around Ile<sup>311</sup>–Phe<sup>325</sup> in the FVa-APC interaction as was recently shown by the inhibitory activities of a peptide, comprising residues 311–325 of FVa, in the inactivation of FVa by APC (38). One might expect that the inactivation rate of the other FVa variants carrying mutations at Lys<sup>320</sup>, Arg<sup>321</sup>, and Arg<sup>400</sup> would also be increased, but this was not the case. An explanation for this observation cannot be provided at this point however, other than that local differences in the conformation of the Arg<sup>306</sup> loop structure as a result of different combinations of mutations in these FVa variants may explain this apparent discrepancy. In addition to the arguments given here for the relatively slow cleavage at Arg<sup>306</sup>, as compared with Arg<sup>506</sup>, it is noteworthy that in a number of new structures, the two dominant APC cleavage sites (i.e. Arg<sup>306</sup> and Arg<sup>506</sup>) are present at ~80 Å above the membrane (33, 39–42). This observation would argue against an earlier hypothesis, which proposed that the 20-fold difference in cleavage rate at Arg<sup>306</sup> and Arg<sup>506</sup> results from a different position of both cleavage sites with respect to the membrane (and consequently also with respect to the APC.
Identification of Surface Epitopes of Human Coagulation FVa

active site). More experimental data are needed, however, to verify the relative positions of Arg\(^{306}\) and Arg\(^{506}\) in human FvAs. The fact that mutagenesis can increase the rate of cleavage at Arg\(^{306}\) strongly suggests that differences in cleavage between Arg\(^{306}\) and Arg\(^{506}\) are likely to be caused by differences in the local interactions independent of the cleavage site/membrane distance. The stimulating effect of protein S, mainly on Arg\(^{306}\), would then be explained by improved contacts between residues probed in this study in the Arg\(^{306}\) region (see also below) and the serine protease domain of APC.

In the presence of heparin, the rate of inactivation of FvA RQQ was increased almost 4-fold. Replacement of amino acid residue Arg\(^{400}\) or Lys\(^{320}\) and Arg\(^{521}\) nullified the heparin-mediated stimulation of cleavage at Arg\(^{306}\), indicating that these amino acid residues are pivotal for the FvA-heparin interaction. When no bridging between APC and FvA with heparin can occur, because of mutation of the Arg\(^{400}\)-Lys\(^{320}\)-Arg\(^{321}\) mutants, heparin even mildly (~2-fold) inhibited the cleavage at Arg\(^{306}\). Similar to the heparin-mediated inhibition of cleavage at Arg\(^{506}\), the inhibition of cleavage at Arg\(^{306}\) by APC can likely be explained by an overall dominant steric hindrance of the APC-bound heparin chain, thus preventing a correct positioning of the APC serine protease domain at the Arg\(^{306}\) cleavage site.

All FvA variants that can only be cleaved at Arg\(^{306}\) were stimulated by protein S (3–5-fold), although to a lesser extent when compared with FvA RQQ, which was about 8-fold stimulated. Possibly, the positively charged region in FvA comprising residues Lys\(^{320}\), Arg\(^{321}\), Arg\(^{400}\), and Arg\(^{501}\) are part of or close to a proposed protein S-binding site (36), which is very close (15–20 Å) and includes FvA residues 493–506. However, because protein S did not protect the inactivation of the FvA mutants that can only be cleaved at Arg\(^{306}\), a protein S-binding site different from the 493–506 region is likely present in FvA. In line with this, protein S does not ablate the protective effect of FXa on the heavy chain and that facilitates the heparin-mediated stimulation of Arg\(^{306}\) cleavage by APC.

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