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The R306G and R506Q mutations in coagulation Factor V reveals additional cleavage sites for Activated Protein C in the R313-R321 region and at R505

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ABSTRACT

The procoagulant function of activated factor V (FVa) is inhibited by activated Protein C (APC) through proteolytic cleavages at R306, R506 and R679. Recombinant FVa mutated at all three APC-cleavage sites, FVa-GQA, was still inactivated by APC through at least two cleavages in the heavy chain of FVa; relatively rapid cleavage at R_{x1} close to residue 506 and slower cleavage at R_{x2} nearby residue 306. We investigated the exact location of these two cleavages, by substitution of arginines by glutamine within the R_{x1}-region (R501, R505 or R510) and the R_{x2}-region (R313, R316, R317 or R321). Immunoblot and kinetic analyses of the inactivation of activated R_{x1}-mutants by APC revealed that using mutant FVa-GQA-505Q no R_{x2}-R_{x1} fragment was formed and that the inactivation reaction was first order with a rate constant of 1.0×10^4 M^{-1} s^{-1}, similar to the rate constant of R_{x2} cleavage ($k_2 = 1.3\times10^4$ M^{-1} s^{-1}). No single arginine could be pinpointed identified as R_{x2}. Individual replacement of arginine by glutamine at positions 313, 316, 317 or 321 in FV-GQA-505Q did not result in the disappearance of R_{x2} as judged from kinetic and immunoblot analyses. However, replacement of all four arginines by glutamine completely prevented formation of the R_{x2}-R_{709} fragment.

We conclude that substitution of arginine 506 by glutamine as in FV-Leiden, leads to the detection of a novel cleavage site at arginine 505 (R_{x1}). Substitution of arginine 306 by glycine, like in FV-Cambridge, reveals several alternative cleavage sites near arginine 306, which together constitute a secondary cleavage site.

Introduction

Human coagulation factor V (FV) is a glycoprotein with a molecular mass of ~330 kD which is mainly synthesized in the liver [1]. In plasma, FV circulates at a concentration of ~20 nmol/L. FV is synthesized as a single-chain procofactor, with a domain structure (A1-A2-B-A3-C1-C2) similar to that of factor VIII [2]. Full length FV acts together with Protein S as a cofactor for activated Protein C (APC) in the inactivation of factor VIIIa [3]. During coagulation, activated factor V (FVa) is formed by thrombin via limited proteolysis. FVa consists of a heavy chain (M_r ~105 kD) and a light chain (M_r ~71-74 kD), held together by divalent metal ions [4]. Together with factor Xa (Xa), calcium ions and a negatively charged membrane surface, FVa forms the prothrombinase complex. In this complex, FVa acts as a non-enzymatic cofactor that accelerates Xa-catalyzed prothrombin activation 10^3-10^5 fold [5–7].

Proteolytic inactivation of FVa by APC is an essential reaction in the protein C anticoagulant pathway [8]. Cleavage by APC of peptide bonds at R306, R506 and R679 in the heavy chain of FVa results in inactivation of FVa. [9]. Several mutations have been reported in these APC-cleavage sites, from which the R506Q (FV Leiden) [10] is the most important one due to its association with APC-resistance and an increased risk of deep-vein thrombosis [10,11]. Two different mutations have been found in the APC cleavage site at R306 [12,13]. None of these seems to be associated with an increased risk of venous thrombosis [12,14].

Several studies have been performed to obtain more insight into the relative importance of the different APC-cleavages. In the presence of negatively charged phospholipids, inactivation of FVa by APC proceeds via a rapid and a slow phase, which are associated with cleavages at R506 and R306, respectively [9,15]. The cleavage at R679 is much slower than the cleavage at R506 and R306 and seems to play a marginal role in the inactivation of FVa in vitro [15]. Recently, we studied recombinant FV, triple-mutated at all three APC-cleavage sites (R306G, R506Q, R679A) and with a major part of the B-domain deleted (rFV-ΔB-GQA) [16]. This study showed that rFVa-GQA was still inactivated by cleavage by APC. The inactivation was sensitive to the protease inhibitor APMSF and an inhibitory monoclonal antibody against protein C, indicating that the inactivation was the result of the proteolytic action of APC [16]. Kinetic analysis of the inactivation curves showed a similar, but much slower inactivation pattern as was reported for the inactivation of wild-type FVa. A rapid phase (k_{x1}) and a slow phase (k_{x2}) could be recognized. The latter was stimulated by Protein S. After comparison of the 30 kD fragments generated during

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APC-catalyzed inactivation of rFVa-GQA, rFVa-wt and rFVa-GRA, it was concluded that two additional APC-cleavage sites did exist, one (R_{x1}) very close to position 506 and another (R_{x2}) at ~1.5-2 kD carboxyterminal to position 306 [16]. In the present study we have studied the position of these novel APC cleavage sites by replacing arginine-residues by glutamine within the regions of rFV-ΔB-GQA where these sites were expected to be located.

Experimental Procedures

Materials

Restriction enzymes were from New England Biolabs, Beverly, MA, USA. Rapid ligation kit was from Boehringer Mannheim, Mannheim, Germany. Plasmid isolation kits were from Qiagen, Chatsworth, CA, USA. DNA restriction fragments were purified from agarose gel using the "cleanmix kit", Talent, Trieste, Italy. SP-Sepharose was from Pharmacia, Uppsala, Sweden. Bovine serum albumin (BSA), minimally 98% pure and fatty acid free, ovalbumin, benzamidine and Hepes were from Sigma, St. Louis, MO, USA. Dioleoylphospatidylcholine (DOPC) and dioleoylphospatidylserine (DOPS) were from Avanti Polar Lipids, Alabaster, Al, USA. EDTA was from Serva, Heidelberg, Germany. NH_4Cl, Tris and $CaCl_2$ were purchased from Merck, Whitehouse Station, NJ, USA. S-2238 was from Chromogenix, (Instrumentation Laboratories, Breda, The Netherlands). Western Blocking Reagent and (4-amidinophenyl)-methanesulfonylfluoridehydrochloride monohydrate (APMSF) were from Roche Diagnostics, Mannheim, Germany. SuperSignal® West Pico chemiluminescent substrate was from Pierce, Rockford, IL, USA.

Proteins

Human APC, Protein S, prothrombin and thrombin were purchased from Enzyme Research Laboratories (South Bend, IN, USA). The APC concentration was determined from APC activity measurements with S2366 using previous reported kinetic parameters ($K_m = 0.20$ mM, $k_{cat} = 190\ s^{-1}$) [17]. Human factor Xa and monoclonal antibody AHV-5146 were from Haematologic Technologies Inc., Essex, VT, USA. Goat-anti-mouse IgG conjugated with horse-radish peroxidase (GAMPO) was from Bio-Rad Laboratories Inc., Hercules, CA, USA.

Mutagenesis

An expression vector (pMT2) was used containing the cDNA of FV [18] in which three APC-cleavage sites had been mutated (R306G, R506Q and R679A) and a major part of the B-domain (amino acids 827 to 1499) had been removed. (pMT2FV-ΔB-GQA) [16,19]. Further mutations were introduced using the Quikchange™ Site-Directed Mutagenesis Kit from Stratagene, La Jolla, CA, USA. Arginines at position 501, 505, 510, 313, 316, 317 and 321 in the GQA-construct were individually and in certain combinations replaced by glutamine using mutagenic primers (sequences available on request). After mutagenesis the presence of the mutations was confirmed by direct DNA sequencing using the CEQ2000™ Dye Terminator Cycle Sequencing Kit from Beckman Coulter Inc., Fullerton, CA, USA. Heavy chain mutations were inserted into pMT2FV-ΔB-GQA by exchange of appropriate restriction enzyme fragments.

After identification of R_{x1}, mutations R313Q, R316Q, R317Q, R316Q-R317Q, R321Q or R313Q-R316Q-R317Q-R321Q were build in this Q_{x1} mutant for investigation of R_{x2} by exchange of restriction enzyme fragments (Table 1).

Transient expression and purification of rFVΔB mutants

rFV mutants were transiently expressed in COS-1 cells using the calcium phosphate precipitation method [20]. Conditioned media were collected, centrifuged and stored at -20 °C. FV expression was measured by functional FV assay and ELISA [16]. After thawing and pooling of conditioned media, mutated rFVs were (semi)-purified by ion-exchange chromatography only (R_{x2} mutants) or by ion-exchange- and immunoaffinity-chromatography (R_{x1} mutants) [19]. FV containing fractions were supplemented with 2 mg/ml BSA, dialyzed against 25 mM Hepes, 50 mM NaCl (pH 7.3), and stored at –80 °C. After purification, specific activities of the rFV mutants were about 20% lower than in the culture supernatant, which might be due to minor conformational changes. rFV mutants (8 nM) were activated by incubation with thrombin (5 nM) at 37 °C for 10 min[15,21].

Factor V assays

FV activity was measured in a two step procedure as previously described with minor modifications [19]. In the first step, rFV (9 pM) was completely activated with 1 nM thrombin in HBS-Ca (25 mM Hepes, 175 mM NaCl, 3 mM $CaCl_2$, 5 mg/ml BSA, pH 7.5) during a 10 min incubation at 37 °C in the presence of 10 μM DOPS:DOPC (10:90 molar ratio) in a final volume of 230 μl. Subsequently 10 μl Xa (f.c. 5 nM) and 10 μl prothrombin (f.c. 1 μM) were added to start the prothrombinase reaction. After 2 min, 10 μl of the prothrombinase mixture was diluted in 490 μl TN-EDTA (50 mM TRIS, 175 mM NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9) and the amount of thrombin formed was quantified as descripted before [7,19,22].

FV antigen was measured by ELISA as previously described using two different monoclonal antibodies against the light chain of FVa [16,22]. Both assays were calibrated with dilutions of pooled normal plasma containing one unit (U) FV activity or FV antigen per ml plasma. One U/ml FV antigen corresponds to 20 nM FV. One U/ml FV

Table 1
Recombinant FV molecules used in this study. Recombinant proteins were named after the amino acids present at the indicated positions using the one letter code for amino acids. The symbol *ΔB* indicates that a major part of the B-domain (Δ 827-1499) is lacking. Levels of FV activity and FV antigen were measured in conditioned medium. The activity/antigen (Act/Ag) ratio for FV in pooled normal plasma is 1.00. n refers to the number of independent transfections used for the measurements.

Factor V Mutations	Abbreviation	Activity (U/ml) ± SD (n)	Antigen (U/ml) ± SD (n)	Act/Ag ratio
ΔB R306G,R506Q,R679A	FV-GQA	0.064	0.056	1.14
ΔB GQA,R501Q	FV-GQA-501Q	0.111 ± 0.03 (2)	0.12 ± 0.032 (2)	0.92
ΔB GQA,R505Q	FV-GQA-505Q = FV-GQQA	0.108 ± 0.007 (2)	0.123 ± 0.009 (2)	0.88
ΔB GQA,R510Q	FV-GQA-510Q	0.062 ± 0.009 (2)	0.073 ± 0.009 (2)	0.84
ΔB GQA,R313Q,R505Q	FV-GQQA-313Q	0.037 ± 0.025 (3)	0.096 ± 0.053 (3)	0.38
ΔB GQA,R316Q,R505Q	FV-GQQA-316Q	0.037 ± 0.026 (3)	0.101 ± 0.055 (3)	0.36
ΔB GQA,R317Q,R505Q	FV-GQQA-317Q	0.021 ± 0.006 (4)	0.070 ± 0.029 (4)	0.30
ΔB GQA,R316Q,R317Q,R505Q	FV-GQQA-316QQ	0.005 ± 0.003 (4)	0.058 ± 0.006 (4)	0.086
ΔB GQA,R321Q,R505Q	FV-GQQA-321Q	0.027 ± 0.012 (4)	0.073 ± 0.017 (4)	0.37
ΔB GQA, R313Q,R316Q, R317Q,R321Q, R505Q	FV-GQQA-313/316/317/321Q	0.005 ± 0.002 (16)	0.027 ± 0.01 (16)	0.20