Role of P1 residues Arg³³⁶ and Arg⁵⁶² in the activated-Protein-C-catalysed inactivation of Factor VIIIa

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APC (activated Protein C) inactivates human Factor VIIIa following cleavage at residues Arg³³⁶ and Arg⁵⁶² within the A1 and A2 subunits respectively. The role of the P1 arginine in APC-catalysed inactivation of Factor VIIIa was examined by employing recombinant Factor VIIIa molecules where residues 336 and 562 were replaced with alanine and/or glutamine. Stably expressed Factor VIII proteins were activated by thrombin and resultant Factor VIIIa was reacted at high concentration with APC to minimize cofactor inactivation due to A2 subunit dissociation. APC cleaved wild-type Factor VIIIa at the A1 site with a rate ~25-fold greater than that for the A2 site. A1 mutants R336A and R336Q were inactivated ~9-fold slower than wild-type Factor VIIIa, whereas the A2 mutant R562A was inactivated ~2-fold slower. No cleavage at the mutated sites was observed. Taken together, these results suggested that cleavage at the A1 site was the dominant

mechanism for Factor VIIIa inactivation catalysed by the proteinase. On the basis of cleavage at Arg³³⁶, a K_m value for wild-type Factor VIIIa of 102 nM was determined, and this value was significantly greater than K_i values (~9–18 nM) obtained for an R336Q/R562Q Factor VIIIa. Furthermore, evaluation of a series of cluster mutants in the C-terminal region of the A1 subunit revealed a role for acidic residues in segment 341–345 in the APC-catalysed proteolysis of Arg³³⁶. Thus, while P1 residues contribute to catalytic efficiency, residues removed from these sites make a primary contribution to the overall binding of APC to Factor VIIIa.

Key words: activated Protein C (APC), coagulation protein, exosite, Factor VIII, P1 arginine.

INTRODUCTION

The essential role of Factor VIII in blood coagulation is evident from the bleeding diathesis haemophilia A, which results from a deficiency or defect in Factor VIII. Factor VIII is synthesized as a single-chain precursor [1,2] consisting of three A domains homologous with the A domains in Factor V, a B domain, and two homologous C domains [1,3]. Each A domain is bordered by regions that are rich in acidic amino acid residues designated a1 (residues 337–372), a2 (residues 711–740) and a3 (residues 1649–1689). Thus the domain organization is as follows: A1-a1-A2-a2-B-a3-A3-C1-C2. The Factor VIII procofactor consists of a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains) associated in a non-covalent metal (Cu²⁺)-ion-dependent linkage. Thrombin activates the pro-cofactor by limited proteolysis generating a heterotrimer composed of A1, A2 and A3-C1-C2 subunits [4,5]. The A1/A3-C1-C2 dimer and A2 subunit are associated via weak electrostatic interactions [5,6]. The Factor VIIIa trimer (A1/A2/A3-C1-C2) binds the serine protease Factor IXa, forming the intrinsic Factor Xase complex which efficiently activates Factor X in a phospholipid- and Ca²⁺-dependent manner (see [7] for a review).

Down-regulation of the intrinsic Factor Xase complex is largely due to inactivation of Factor VIIIa, which is thought to occur by two independent mechanisms. The first reflects dissociation of the A2 subunit from the A1/A3-C1-C2 dimer. The second mechanism results from proteolytic inactivation of the cofactor catalysed by APC (activated protein C). APC is a potent anticoagulant that proteolytically inactivates Factor Va and Factor VIIIa, and deficiencies in this proteinase are linked to thrombosis (see [8] for a review). APC inactivates Factor VIIIa by cleaving the A1 subunit at Arg³³⁶ and the A2 subunit at Arg⁵⁶² [9,10]. Cleavage at

the former site alters the interactions between A1 and A2 subunits yielding reduced k_{cat} values for Factor Xase [11], as well as resulting in a severalfold increase in the K_m for Factor X [12]. Cleavage at the latter site occurs within an important Factor IXa-interactive site, the 558-loop [13]. Although limited information exists on the roles of these cleavage reactions in cofactor function, the relative contributions they make to residual cofactor activity are poorly understood.

In the present study, we examined the mechanism for APC-catalysed cleavage at residues Arg³³⁶ and Arg⁵⁶², and the effect of each cleavage on the cofactor activity. Recombinant human Factor VIII mutants, R336A, R336Q, R562A and R336Q/R562Q were constructed and the proteins were stably expressed in BHK (baby-hamster kidney) cells. Detailed kinetic evaluation and correlation of substrate cleavage with changes in activity employed the physiologically relevant activated cofactor Factor VIIIa as a substrate for APC. Our results demonstrate an independent non-sequential mechanism for cleavage at the A1 and A2 sites, with cleavage at the A1 site making the dominant contribution to inactivation of the cofactor. Furthermore, while the P1 residues are primary determinants for catalytic efficiency, sites removed from the P1 residues account for the overall binding of APC to Factor VIIIa. One such site may be represented by the acidic residues in segment 341–345, which appears to make a specific contribution to APC-catalysed proteolysis at Arg³³⁶.

MATERIALS AND METHODS

Reagents

The monoclonal antibody 58.12, which recognizes the N-terminus of the A1 domain of Factor VIII, was a gift from Bayer. The

Abbreviations used: APC, activated Protein C; BHK, baby-hamster kidney; WT, wild-type.

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monoclonal antibody R8B12, which recognizes the C-terminus of the A2 domain, was obtained from Green Mountain Antibodies. The ESH8 antibody, which recognizes the C2 domain of the light chain of Factor VIII, was obtained from American Diagnostica. Phospholipid vesicles containing 20 % phosphatidylserine, 40 % phosphatidylcholine and 40 % phosphatidylethanolamine were prepared using octyl glucoside as described previously [14]. The reagents α -thrombin, Factor IXa β , Factor X and human APC were purchased from Enzyme Research Laboratories; hirudin and phospholipids were purchased from Sigma; the chromogenic APC substrate S2366 (L-pyroglyutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride) was purchased from Chromogenix Instrumentation Laboratory S.p.A; chromogenic Factor Xa substrate Pefa-5523 (Pefachrome®FXa) was purchased from Centerchem. The B-domainless Factor VIII expression vector (RENeo FVIII) and Bluescript Factor VIII vector (pBS Factor VIII) were gifts kindly provided by Dr Pete Lollar and Mr John Healey (both at Winship Cancer Institute, Emory University, Atlanta, GA, U.S.A.).

Construction, expression and purification of recombinant Factor VIII mutants

B-domainless Factor VIII cDNA was restricted from the mammalian expression vector, FVIIIHSQ-MSAB-NotI-RENeo (containing Factor VIII residues 1–745). Cassettes for coding regions 176–526 and 526–745 were prepared by further restriction of the B-domainless Factor VIII cDNA using endonucleases SpeI/SacII and SacII/NotI respectively, and were subcloned into the pBluescript II KS(–) vector. Mutations were introduced into the shuttle constructs using the Stratagene QuikChange™ site-directed mutagenesis kit as described previously [15]. Factor VIII mutants, R336A/K338A, R336Q/K338Q, R562A and R336Q/K338Q/R562Q were constructed by substituting alanine or glutamine for Arg³³⁶ and Lys³³⁸ and/or Arg⁵⁶². Lys³³⁸ was mutated in concert with Arg³³⁶ to prevent potential cleavage at this site, on the basis of an earlier observation suggesting that cleavage occurred at Lys³³⁸ by APC when Arg³³⁶ was mutated [16]. Factor VIII expression vector constructs were transfected into BHK cells using FuGENE 6 (Roche). Factor VIII cluster mutants within the 337–372 region, E341A/E342A/E344A/D345A, D347A/D348A/D349A and D361A/D362A/D363A utilized in the present study were constructed and transfected as described in [17]. Cloning and selection of stable transfectants and protein expression were performed as described previously [15]. The conditioned medium was collected daily, and the expressed proteins were purified following chromatography on an SP (sulphopropyl)-Sephacrose column (Amersham Biosciences) as described previously [18]. Active fractions were detected using the one-stage clotting assay, pooled and dialysed against 20 mM Hepes, pH 7.2, 0.1 M NaCl, 5 mM CaCl₂ and 0.01 % (v/v) Tween 20. Resultant Factor VIII forms were typically > 90 % pure as judged by SDS/PAGE (8 % gels). Factor VIII samples were quick-frozen and stored at –80 °C.

ELISA

Factor VIII concentration was determined using a sandwich ELISA using ESH8 as a capture antibody and biotinylated R8B12 as the detection antibody. The amount of bound Factor VIII was determined optically using streptavidin-linked horseradish peroxidase (Calbiochem) with the substrate *o*-phenylenediamine dihydrochloride (Sigma) as described previously [15]. Purified commercial recombinant Factor VIII (Kogenate; Bayer Corporation) was used as the standard. Factor VIII specific activity was determined using the one-stage clotting assay and ELISA.

Factor Xa generation assay

The rate of conversion of Factor X into Factor Xa was monitored in a purified system [19]. Factor VIII (130 nM) in the presence and absence of R336Q/R562Q mutant (0–100 nM) was activated by addition of 20 nM thrombin in 20 mM Hepes, pH 7.2, 50 mM NaCl, 5 mM CaCl₂, 0.01 % (v/v) Tween 20 and 100 µg/ml BSA (buffer A) and reactions took place out at 37 °C. Thrombin was inhibited after 2 min by addition of 10 units/ml hirudin, and the resultant Factor VIIIa was reacted with APC (2–5 nM) in the presence of 50–100 µg/ml phospholipid vesicles. Aliquots of 1 µl were removed at the indicated times to assess residual Factor VIIIa activity. Factor Xa generation was initiated by the addition of Factor IXa (150 nM) and Factor X (1 µM) into the reaction mixture. Aliquots of 4 µl were removed at appropriate times and added to tubes containing EDTA (50 mM final concentration) to assess initial rates of product formation. Rates of Factor Xa generation were determined by the addition of the chromogenic Factor Xa substrate, Pefa-5523 (0.46 mM final concentration). Samples were read at 405 nm for 5 min using a $V_{\max}$ microtitre plate reader (Molecular Devices). Factor VIIIa activity was determined as the amount (in nmol) of Factor Xa generated per min and converted into the amount (in nmol) of Factor VIIIa.

For each data set, control experiments assessing Factor VIIIa stability were performed in the absence of APC in order to determine the rates of Factor VIIIa activity loss resulting from A2 subunit dissociation. At the concentrations of Factor VIIIa employed, this value approximated a 10–15 % loss of the initial activity over a 30 min time course. Thus, for each time point in the time-course experiments including APC, the observed residual activity was corrected for the contribution of activity loss from the APC-independent mechanism.

One-stage clotting assay

Factor VIII was activated by thrombin and then reacted with APC as described above. Factor VIII-deficient pooled human plasma was incubated with activated partial thromboplastin reagent (General Diagnostics) for 7 min at 37 °C at which time a dilution of Factor VIIIa was added to the cuvette. After 1 min, CaCl₂ (6 mM final concentration) was added to the mixture, and time to clot formation was measured using a clotting instrument (Diagnostica Stago) and compared with a pooled normal plasma standard.

Western blot analysis of A1 and A2 subunit cleavages

Factor VIII (130 nM) in the presence and absence of R336Q/R562Q mutant (0–100 nM) was activated by the addition of 20 nM thrombin in buffer A and reactions took place at 37 °C. Thrombin was inhibited after 2 min by addition of 10 units/ml hirudin, and the resultant Factor VIIIa was reacted with APC (2 nM) in the presence of 50–100 µg/ml phospholipid vesicles. Aliquots were removed at the indicated times, and the reactions were stopped with SDS/PAGE buffer. Samples were subjected to SDS/PAGE using 8 or 10 % polyacrylamide gels and Western blotting as described previously [20]. Blots were probed using the 58.12 and R8B12 monoclonal antibodies, followed by a goat anti-mouse alkaline-phosphatase-linked secondary antibody (Sigma). The signal was detected using the ECF® (enhanced chemifluorescence) system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices). Densitometric scans were quantified using Image Quant software (Molecular Devices).

Chromogenic assay for APC

The amidolytic activity of APC (1 nM) was monitored at increasing concentrations of S2366 substrate in buffer A. Rates

of substrate hydrolysis were measured by reading the samples at 405 nm for 5 min using a $V_{\max}$ microtitre plate reader. The K_m for cleavage of this substrate by APC was calculated by fitting the data to the Michaelis–Menten equation according to a non-competitive inhibition model using non-linear least-squares regression analysis.

Data analysis

All experiments were performed at least three separate times, and average values are given. The concentration of A1 or A2 subunit generated by thrombin activation of Factor VIII was estimated by density values for all Factor VIII bands and was used to calculate the concentration of Factor VIIIa in Factor Xa generation or one-stage clotting assays. The concentration of uncleaved subunit was calculated by multiplying the initial substrate concentration by the band density of the uncleaved subunit divided by the sum of density values for the cleaved and uncleaved subunits. Initial time points were fitted to the single-exponential decay (eqn 1) using non-linear least-squares regression analysis:

$$A = A_0 \times e^{-kt} \quad (1)$$

where A_0 is the initial concentration of Factor VIIIa or A1 or A2 subunit in nM, k is the rate constant in minutes^{-1} and t is time in minutes. The rates of Factor VIIIa inactivation and A1 or A2 cleavage by APC correspond to the absolute slope value at time 0 and were calculated from $A_0 \times k$. Kinetic parameters were determined by Western blot analysis, monitoring time courses for cleavage of WT (wild-type) A1 subunit at Arg³³⁶. Values for K_m were calculated by fitting the data using non-linear least-squares regression analysis to the Michaelis–Menten equation (eqn 2):

$$v = V_{\max} \times [\text{WT}] / (K_m + [\text{WT}]) \quad (2)$$

where v is the initial velocity in nmol/min, and $[\text{WT}]$ is the concentration of WT Factor VIIIa in nM.

The inhibition constant (K_i) for R336Q/R562Q on A1 subunit cleavage of Factor VIIIa by APC was determined by fitting the data using non-linear least-squares regression analysis according to a competitive inhibition model (eqn 3):

$$v = V_{\max} \times [\text{WT}] / \{K_m \times (1 + [\text{I}] / K_i) + [\text{WT}]\} \quad (3)$$

where $[\text{I}]$ is the concentration of R336Q/R562Q Factor VIIIa.

RESULTS

Characterization of recombinant Factor VIII P1 mutants

To elucidate the role of the P1 residues in APC-catalysed inactivation of Factor VIIIa, recombinant B-domainless Factor VIII mutants were prepared by replacing the P1 arginine residues at positions 336 and/or 562. The arginine residues at these sites were replaced with glutamine, creating a mutant analogous to the naturally occurring Factor V Leiden (R506Q) mutant [21]. Two additional mutants were prepared by replacing each APC cleavage site with alanine. The purified proteins, visualized by SDS/PAGE, revealed three bands of 170, 90 and 80 kDa (results not shown), which corresponded to the expected molecular mass of the single-chain Factor VIII, and heavy chain and light chain of the Factor VIII heterodimer respectively. Specific-activity values measured for recombinant molecules revealed modestly higher values (~ 30 – 60%) for R336Q, R336Q/R562Q and R562A forms compared with WT, whereas the R336A mutant was ~ 2 -fold greater than WT. These disparities may reflect low levels of activated cofactor in the protein preparations, since Western blot

Figure 1 Inactivation of recombinant WT and P1 Factor VIIIa mutants by APC

WT (○), R336A (●), R336Q (□), R562A (■) and R336Q/R562Q (△) Factor VIII forms (130 nM) were activated by thrombin (20 nM). Factor VIIIa inactivation was then monitored over time in the presence of APC (2 nM) using a Factor Xa generation assay. APC-catalysed inactivation values were corrected by subtracting the corresponding values for Factor VIIIa decay observed in the absence of APC, and lines were drawn from the curve fitting as described in the Materials and methods section.

analysis revealed the presence of small, but variable, amounts of A2 subunit in the unactivated samples (results not shown). However, the efficiency of thrombin activation for all P1 mutants was similar to that of WT Factor VIII (results not shown). These parameters suggest that the P1 mutants employed in the present study are not defective in macromolecular interactions related to cofactor function.

Inactivation of Factor VIIIa P1 mutants by APC

Purified Factor VIII (130 nM) was converted into Factor VIIIa by 20 nM thrombin. The resultant Factor VIIIa was reacted with 2 nM APC in the presence of phospholipids (50–100 $\mu\text{g/ml}$), and activity was monitored for 30 min using the Factor Xa generation assay (Figure 1). Factor VIIIa was used at high concentration (> 100 nM) to minimize its inactivation due to dissociation of A2 subunit from the A1/A3-C1-C2 dimer. The observed loss of activity obtained in the absence of APC was similar for the P1 mutant and WT Factor VIIIa forms ($\sim 10\%$ activity loss at 30 min; results not shown), and all data obtained in the presence of APC were corrected for the contribution of inactivation due to this APC-independent mechanism as described in the Materials and methods section. Inactivation studies using Factor VIIIa substrates possessing a single susceptible bond (see below) allowed for evaluation of the contribution of cleavage at either site to the loss of cofactor activity. The inactivation rate for the R562A mutant was ~ 2 -fold less than that of WT Factor VIIIa, whereas rates for R336A and R336Q were reduced ~ 8 – 9 -fold compared with the WT Factor VIIIa (Table 1). These results suggest that proteolytic attack at the A1 site represents the predominant mode of cofactor inactivation. The inactivation rate observed for the R336Q/R562Q double mutant was ~ 10 -fold less than that of WT Factor VIIIa, and this rate was indistinguishable from the activity loss due to non-proteolytic decay of Factor VIIIa under the reaction conditions employed. APC-catalysed inactivation rates and the extent of inactivation determined using a one-stage clotting assay yielded values that were ~ 2 -fold greater than the corresponding values determined using the Factor Xa generation assay (results not shown). This disparity probably reflects the lower concentration of substrate Factor X present in the plasma-based assay (~ 40 nM) compared with its concentration used in Factor Xa generation assay (1 μM) and the observation that cleavage at the A1 site increases the K_m for substrate Factor X [12]. The

Table 1 Rates of P1 mutant Factor VIIIa inactivation and A1 and A2 subunit cleavages

Rates of Factor VIIIa inactivation, and A1 cleavage and A2 cleavage were estimated by non-linear least-squares regression analysis of the data shown in Figures 1(A), 2(B) and 2(C) respectively, as described in the Materials and methods section.

Factor VIIIa	Inactivation (nmol of Factor VIIIa/min per nmol of APC)	A1 cleavage (nmol of A1/min per nmol of APC)	A2 cleavage (nmol of A2/min per nmol of APC)
WT	3.04 ± 0.44	8.33 ± 0.90	0.34 ± 0.20
R336A	0.40 ± 0.12	< 0.002	0.24 ± 0.18
R336Q	0.34 ± 0.11	< 0.002	0.25 ± 0.19
R562A	1.70 ± 0.23	7.91 ± 0.92	< 0.002
R336Q/R562Q	0.30 ± 0.06	< 0.002	< 0.002

observation that residual Factor VIIIa activity does not go to zero is consistent with earlier results of ~60 % activity for Factor VIIIa reconstituted with the truncated A1³³⁶ (residues 1–336) compared with the intact A1 subunit [12], indicating that the cleaved protein retains a reduced level of cofactor activity.

Cleavage of A1 and A2 subunits of P1 mutant and WT Factor VIIIa by APC

Western blot analysis was performed to examine the rates of APC-catalysed proteolysis at either cleavage site and correlate these events to Factor VIIIa inactivation (Figure 2). The intact A1 subunit substrate and A1³³⁶ product, resulting from cleavage at Arg³³⁶ were detected by monoclonal antibody 58.12, which recognizes the N-terminus of A1 subunit. The intact A2 substrate and A2_c product (residues 563–740), resulting from cleavage at Arg⁵⁶² were detected by monoclonal antibody R8B12, which recognizes the C-terminus of A2 subunit. The amounts of these substrates and products were quantified on the basis of the density values determined from the blots.

The cleavage rate for A1 subunit of the R562A mutant was equivalent to that for WT Factor VIIIa, whereas R336A, R336Q and R336Q/R562Q mutants showed no detectable cleavage at the 336 site (Figures 2A and 2B, and Table 1). Conversely, the cleavage rates for the A2 subunit of R336A and R336Q were similar to that for WT Factor VIIIa, whereas R562A and R336Q/R562Q Factor VIIIa mutants showed no detectable cleavage at the 562 site (Figures 2A and 2C, and Table 1). The observations that R336A and R336Q mutants were cleaved at the 562 site, and that R562A mutant was cleaved at the 336 site, indicate that A1 and A2 cleavages occur independently of one another in Factor VIIIa. In addition, the cleavage-resistance observed at both P1 sites suggested stringency at these positions. Furthermore, while cleavage at the susceptible A1 site was essentially complete during the time course, significant (>60 %) intact A2 subunit remained at the end of the time course for those susceptible Factor VIIIa forms. We have observed this result in the past and attributed it to dissociated A2 subunit, which is a poor substrate for APC [10]. This observation probably explains the deviation from the fitted curves at later time points illustrated in Figure 2(C).

Inhibition of WT Factor VIIIa inactivation by the R336Q/R562Q mutant

The R336Q/R562Q Factor VIIIa, which is cleavage-resistant at both A1 and A2 sites (Figure 2A), was used to probe the role of sites other than the P1 residue in the recognition of substrate Factor VIIIa. WT (130 nM) and R336Q/R562Q mutant (0–100 nM) Factor VIII were simultaneously activated by thrombin

Figure 2 Cleavage of A1 and A2 subunits of WT and P1 Factor VIIIa mutants by APC

WT (○), R336A (●), R336Q (□), R562A (■) and R336Q/R562Q (△) Factor VIII mutants were reacted as described in the legend to Figure 1. Aliquots were taken at various time points (0–180 min) and were subjected to SDS/PAGE. A1 (and A1³³⁶) and A2 (and A2_c) subunits were visualized by Western blotting using 58.12 (anti-A1) and R8B12 (anti-A2) monoclonal antibodies (A), and their concentrations were calculated based on the density values and plotted as a function of time (B and C). Lines were drawn from the curve fitting as described in the Materials and methods section. The inset shows the first 20 min of the assays in detail.

(20 nM) and then reacted with APC (2 nM) in the presence of phospholipids (100 µg/ml). Inactivation of WT Factor VIIIa was monitored over time in the presence of increasing concentrations of the R336Q/R562Q (Figure 3A). This mutant inhibited APC-catalysed inactivation of WT Factor VIIIa in a dose-dependent manner (Figure 3B). Using a K_m of 102 nM for APC inactivation of WT Factor VIIIa, as estimated from A1 subunit cleavage (Figure 4A), a K_i of 17.5 ± 2.4 nM was estimated from the observed inhibition of the cleavage-resistant protein. Comparison of these values suggested that APC possessed

Figure 3 Effects of the R336Q/R562Q mutant on APC-catalysed inactivation of WT Factor VIIIa

WT Factor VIII (130 nM) in 0–100 nM R336Q/R562Q mutant was activated by thrombin (20 nM) and then reacted with APC (5 nM). (A) Results obtained in the presence of 0 (○), 10 (●), 40 (□) or 80 (■) nM R336Q/R562Q mutant. Factor VIIIa activity was measured at indicated times using a Factor Xa generation assay and lines were drawn from the curve fitting as described in the Materials and methods section. (B) Inactivation rates are plotted as a function of the concentration of the R336Q/R562Q mutant. Lines were drawn from the curve fitting as described in the Materials and methods section.

~6-fold greater affinity for the R336Q/R562Q mutant compared with WT Factor VIIIa. While the reason for this enhanced affinity is not clear, these results confirm that the binding energy for the enzyme–substrate interaction is mediated by sites independent of the P1 residue.

Inhibition of WT Factor VIIIa A1 subunit cleavage by the R336Q/R562Q mutant

The inhibitory effect of R336Q/R562Q on WT inactivation rates was examined further by monitoring A1 subunit cleavage of WT Factor VIIIa by APC using Western blot analysis. Cleavage of WT Factor VIIIa A1 subunit (~40–300 nM) by APC (2 nM) was monitored in the absence and presence of fixed concentrations (15 and 60 nM) of R336Q/R562Q mutant (Figure 4A). The inset shows the representative Western blot data obtained with 105 nM WT Factor VIII. Substrate concentrations (A1 subunit) were corrected for efficiency of thrombin activation of Factor VIII in each reaction, which varied from ~75–90%. Using band density values of the A1 substrate and A1³³⁶ product, the initial velocity of A1 subunit cleavage was estimated and plotted against A1 subunit concentration. The A1 cleavage data obtained in the absence of R336Q/R562Q were fitted to the Michaelis–Menten equation using non-linear least-squares regression analysis to determine K_m (102.3 ± 16.7 nM) and k_{cat} (0.66 ± 0.04 s⁻¹) parameters. Lineweaver–Burk plots indicated that inhibition of A1 cleavage by R336Q/R562Q was competitive (Figure 4B). We estimated the K_i for R336Q/R562Q (9.0 ± 0.8 nM) by fitting the data to a competitive inhibition model (K_m value was fixed as 102 nM) as described in the Materials and methods section. The K_i estimated

Figure 4 Effects of the R336Q/R562Q mutant on A1 subunit cleavage of WT Factor VIIIa by APC

(A) WT Factor VIII (40–300 nM) in the presence of 0 (○), 15 (●) and 60 (□) nM R336Q/R562Q mutant was activated by thrombin (20 nM) and then reacted with APC (2 nM). Aliquots were taken at various time points (0–20 min) and were subjected to SDS/PAGE. The A1 subunit and A1³³⁶ fragment were visualized by Western blot analysis using 58.12 (anti-A1) monoclonal antibody, and their concentrations were calculated on the basis of the scanned density values. The A1 band represents the A1 subunit of uncleaved WT and R336Q/R562Q mutant, whereas the A1³³⁶ band represents the product fragment of the WT A1 subunit. The amount of the A1³³⁶ (nmol) generated over time was determined based on the amount of WT A1 substrate, calculated by subtracting the amount of the mutant A1 from the total amount of A1 subunit. The initial velocity of A1 cleavage was estimated and plotted as a function of the concentration of the R336Q/R562Q mutant, and lines were drawn from the curve fitting as described in the Materials and methods section. The inset represents one set of Western blot data using 105 nM WT. (B) is the Lineweaver–Burk plot of the data shown in (A). Symbols represent the same data sets as indicated in (A).

from the cleavage data was in good agreement with the value estimated from the inactivation data, and both methods indicated a significantly reduced K_i value compared with the K_m for the WT substrate.

Effect of R336Q/R562Q on the APC active site

To determine whether the inhibition of APC-catalysed cleavage of WT Factor VIIIa observed for R336Q/R562Q resulted from occlusion of the active site, we examined the effects of R336Q/R562Q on the APC-catalysed hydrolysis of the chromogenic substrate S2366. The hydrolysis rate of S2366 substrate (0–1.6 mM) by APC (1 nM) was monitored in the presence and absence of R336Q/R562Q mutant Factor VIIIa (100 nM) (Figure 5). The R336Q/R562Q Factor VIIIa was employed at high concentration to effectively saturate the enzyme on the basis of the K_i values. The apparent K_m value estimated for hydrolysis of the chromogen (0.82 mM) was similar to that reported by the manufacturer for human APC, and this value was independent of the presence of the R336Q/R562Q mutant. This result indicated that binding of R336Q/R562Q Factor VIIIa does not occlude the S1–S3 subsites within enzyme, and was consistent with dominant

Figure 5 Effects of the R336Q/R562Q mutant on the amidolytic activity of APC

Initial velocity values for catalysis of 0–1.6 mM chromogenic substrate S2366 by APC (1 nM) in the presence (●) and absence (○) of R336Q/R562Q mutant Factor VIIIa (100 nM) were measured by reading the samples at 405 nm for 5 min using a V_{max} microtitre plate reader. Data were fitted to Michaelis–Menten equation using non-linear regression analysis as described in the Materials and methods section.

binding interactions between APC and Factor VIIIa involving sites other than those proximal to the scissile bond.

Identification of an APC-interactive site within residues 337–372 of Factor VIIIa

The C-terminal region of A1 subunit following the P1 Arg³³⁶ (residues 337–372) contains a high concentration of acidic residues. This region has been shown to contribute to Factor Xa-catalysed cleavage at Lys³⁶, leading to cofactor inactivation [22] and to Factor Xa-catalysed activation of Factor VIII following cleavage at Arg³⁷² [17]. The latter study indicated specific involvement of an acidic cluster of residues at 361–363 in the catalytic mechanism. To determine whether acidic residues within this region influenced cleavage by APC, we utilized a panel of B-domainless Factor VIII cluster mutants, where alanine was substituted for glutamate and aspartate residues at 341–345, 347–349 and 361–363 [17].

Factor VIII cluster mutants (130 nM) were activated by thrombin (20 nM) and then reacted with APC (5 nM) in the presence of phospholipids (100 µg/ml) using the Factor Xa generation assay. APC-catalysed inactivation values were corrected by subtracting the corresponding values for Factor VIIIa decay as described in the Materials and methods section. The inactivation rate for the 341–345A mutant was ~6-fold slower than WT, whereas the difference in inactivation rates between WT and 347–349A or 361–363A mutants were ~2-fold or less (Figure 6A and Table 2).

Cleavage rates for the A1 subunit (Figure 6B) and A2 subunit (results not shown) of the cluster mutants were determined using Western blot analysis, and values are listed in Table 2. The cleavage rate of A1 subunit for the 341–345A mutant was ~3-fold lower than that of WT Factor VIIIa, whereas the A1 subunit cleavage rates of 347–349A and 361–363A mutants were similar to that of WT. Cleavage rates of the A2 subunit for all mutants were similar to that of WT Factor VIIIa. Overall, these results indicate that mutation within the 341–345 region affects APC interactions with Factor VIIIa and suggest a role for the acidic residues in this region in the catalytic mechanism for cleavage at the Arg³³⁶ site.

DISCUSSION

In the present study, we prepared APC-cleavage resistant Factor VIII mutants by substituting alanine and/or glutamine for P1

Figure 6 Effects of cluster mutations within the A1 subunit C-terminal region on the inactivation and A1 subunit cleavage of Factor VIIIa by APC

(A) WT (○), 341–345A (●), 347–349A (□) and 361–363A (■) Factor VIII forms (130 nM) were activated by thrombin (20 nM). Factor VIIIa inactivation was then monitored over time in the presence of APC (5 nM) using a Factor Xa generation assay. APC-catalysed inactivation values were corrected by subtracting the corresponding values for Factor VIIIa decay observed in the absence of APC. (B) WT (○), 341–345A (●), 347–349A (□) and 361–363A (■) Factor VIII forms (130 nM) were activated by thrombin (20 nM) and then reacted with APC (2 nM). Aliquots were taken at various time points (0–60 min) and were subjected to SDS/PAGE. The A1 subunit and A1³³⁶ fragment were visualized by Western blot analysis using the 58.12 monoclonal antibody, and a representative blot is shown in the inset. The concentration of the A1 subunit was calculated on the basis of the scanned density values. Lines were drawn from the curve fitting as described in the Materials and methods section. The initial velocity values for the A1 cleavage was estimated and plotted as a function of time.

Table 2 Rates of cluster mutant Factor VIIIa inactivation and A1 and A2 subunit cleavages

Rates of Factor VIIIa inactivation, and A1 and A2 subunit cleavages were estimated by non-linear least-squares regression analysis as described in the Materials and methods section.

Factor VIIIa	Inactivation (nmol of Factor VIIIa/min per nmol of APC)	A1 cleavage (nmol of A1/min per nmol of APC)	A2 cleavage (nmol of A2/min per nmol of APC)
WT	4.3 ± 0.7	8.1 ± 0.8	0.55 ± 0.28
341–345A	0.7 ± 0.2	2.6 ± 0.2	0.30 ± 0.10
347–349A	1.9 ± 0.2	7.3 ± 0.8	0.50 ± 0.24
361–363A	2.8 ± 0.2	8.4 ± 0.9	0.26 ± 0.13

Arg³³⁶ (A1 subunit) and Arg⁵⁶² (A2 subunit). Kinetic analyses of single-site mutants using the physiologically relevant activated cofactor as substrate revealed that cleavage of either site is independent of the other and contributes to Factor VIIIa inactivation, with a predominant role for the A1 site in this mechanism. Interestingly, the double mutant R336Q/R562Q, in which both sites are resistant to cleavage, was an effective competitor of APC-catalysed inactivation of WT Factor VIIIa, indicating that the dominant binding interactions are removed from the P1 sites. This observation was supported further following kinetic analyses of acidic cluster mutations within the 337–372 region of the A1 subunit, which revealed that residues 341–345 contribute to specific interactions of APC with Factor VIIIa facilitating cleavage at Arg³³⁶.

Inactivation studies using the single point mutations revealed that cleavage events at Arg³³⁶ and Arg⁵⁶² occurred independently and were not linked in a sequential mechanism, although the rate at the former site was ~25-fold that of the latter. This result is in contrast with mechanisms observed for the APC-catalysed inactivation of Factor Va, where a glutamine replacement at Arg⁵⁰⁶ yields a markedly reduced rate of cleavage at Arg³⁰⁶ [23] and results in a thrombophilia due to APC-resistance [24]. This discrepancy in reaction mechanisms is not fully explained by the location of the scissile bonds on separate subunits in Factor VIIIa, whereas the sites are located on a contiguous chain in Factor Va, since earlier results examining point mutations in the unactivated Factor VIII suggested that cleavages were not ordered [16]. While an independent cleavage mechanism was observed, the maximum rate of inactivation was obtained when both cleavages occurred supporting previous findings [10,25] for a synergistic effect in Factor VIIIa inactivation.

Comparison of cofactor inactivation and cleavage rates resulting from proteolysis solely at the Arg³³⁶ site showed that the latter rate was ~3-fold higher than the inactivation rate. This observation of a lag between cleavage and altered function suggests a time-dependent change in conformation and/or loss of a functional fragment as a result of proteolysis. Several lines of evidence suggest that loss of the 337–372 fragment at the C-terminal end of the A1 subunit alters Factor VIII structure and yields a reduction in cofactor activity. CD spectral analysis demonstrated altered conformation of the APC-cleaved dimer (A1³³⁶/A3-C1-C2) compared with the intact one (A1/A3-C1-C2), suggesting that residues 337–372 are important in maintaining the active conformation of the cofactor [25]. In addition, this cleavage diminishes the effect of the A1/A3-C1-C2 dimer on the fluorescence anisotropy of fluorescein-Phe-Phe-Arg Factor IXa and increases the inter-fluorophore distance between A1 and A3-C1-C2 subunits as judged by fluorescence resonance energy transfer [11]. Furthermore, reconstitution studies have shown removal of this fragment alters the interactions between the A1 and A2 subunits that are necessary for maximal stimulation of Factor IXa-catalysed activation of Factor X [12]. While recent studies have shown no direct contribution of residues 337–372 to retention of A2 subunit in Factor VIIIa [11, 12], this region does appear necessary to properly orient A2 subunit in Factor VIIIa [11]. Whereas cleavage at this site yielded a ~50% loss in cofactor activity as measured in a Factor Xa generation assay ([12], and the present study), a markedly greater activity loss (~95%) has been observed in a one-stage clotting assay [11]. This disparity in activity values reflects in part a ~5-fold increase in the K_m value for Factor X observed with cleavage at this site [12] and the markedly lower substrate levels in the plasma-based assay. This K_m increase is likely to be due to the loss of a Factor X-binding site localized to this region [26,27]. Thus we speculate the lag between cleavage at Arg³³⁶ and activity loss reflects the dissociation

rate for the 337–372 fragment from the truncated A1 subunit.

On the other hand, cleavage at the Arg⁵⁶² site closely paralleled the rate of cofactor inactivation, suggesting that this cleavage is of itself inactivating. While the exact mechanism for this action remains to be determined, earlier studies indicated that this scissile bond was located within a Factor IXa-interactive site mapping to residues 558–565 [13]. Inasmuch as point mutations within this sequence yield reduced k_{cat} values for Factor Xase [15], it is reasonable to speculate that cleavage at this site would also be detrimental to this parameter. However, the apparent ~2-fold reduction in the rate of inactivation of the single point mutant Factor VIII R562A was largely overshadowed when this mutant was combined with that at Arg³³⁶.

Our results parallel an earlier study by Amano et al. [16] that examined APC-catalysed inactivation of P1 residue point mutations in the unactivated pro-cofactor. Although kinetics of inactivation were not determined, these investigators noted partial resistance to inactivation for the single point mutations and full resistance with mutation at both P1 sites [16]. That study noted that both cleavage events occurred with similar rates based on inactivation data [16]. However, we observed a significantly greater cleavage rate at the A1 site. This disparity may be due to use of activated Factor VIIIa substrate, a more physiologically relevant form, in the present study rather than the pro-cofactor Factor VIII form. APC-catalysed inactivation of Factor VIIIa has been observed to be significantly more efficient than inactivation of the pro-cofactor form [10], and this observation may reflect differences in the structures of Factor VIII and Factor VIIIa; most notably the contiguous and non-contiguous A1 and A2 domains respectively. It is interesting to speculate that thrombin-catalysed cleavage of the Factor VIII heavy chain, which exposes Factor IXa-interactive sites in the A2 subunit that are otherwise cryptic when A2 is contiguous with A1 [28], may also generate and/or enhance interactive sites in these domains for APC.

A dominant role for interactive sites for APC removed from the P1 sites in Factor VIIIa was supported by results obtained from competition studies using the R336Q/R562Q double mutant. Indeed, we observed a K_i value for inhibition of cleavage of WT Factor VIIIa at Arg³³⁶ that was ~6–10-fold lower than the K_m value for this substrate. The reason for this substantially greater affinity of APC for the inhibitor compared with the substrate is not clear, but indicated that binding is largely independent of the P1 residues. Furthermore, the observation that a high concentration of this non-cleavable Factor VIIIa form did not affect the rate of APC-catalysed hydrolysis of a small-molecule substrate indicated that the S1-S3 subsites of the enzyme do not appreciably contribute to binding the macromolecular substrate. This result parallels earlier observations from Orcutt et al. [29] for the activation of prothrombin by prothrombinase and is consistent with a role for exosite interactions in the catalytic mechanism of APC.

Kinetic analyses of cluster mutants within the C-terminal of A1 subunit revealed that residues 341–345 were important for APC catalysis at Arg³³⁶. Interestingly, this cluster of acidic residues showed little effect on Factor Xa-catalysed proteolysis of Factor VIII heavy chain at Arg³⁷², whereas a more dominant role was observed for the cluster at residues 361–363 [17]. These observations suggest unique specificities for APC and Factor Xa in Factor VIIIa recognition dependent upon interaction with acidic residues in the substrate. In addition, the observation by Amano et al. [16] of cleavage by APC at Lys³³⁸ when Arg³³⁶ was mutated is consistent with the participation of conserved acidic residues at the P6' and P9' positions, specifically residues Glu³⁴² and Asp³⁴⁵ following cleavage at Arg³³⁶ and residues Glu³⁴⁴ and Asp³⁴⁷ following cleavage at Lys³³⁸.

Basic residues on three surface loops of APC, the 39-loop, the 60-loop and the 70–80-loop form an exosite analogous to the anion-binding exosite I of thrombin. Previous evidence has indicated that point mutations in selected basic residues in these structures cause a reduction in inactivation rates of both Factor Va [30,31] and Factor VIIIa [32]. Examination of cleavage patterns showed that these mutations primarily affected cleavage of Factor Va at Arg⁵⁰⁶ with little, if any, effect on specificity at Arg³⁰⁶. On the other hand, these same basic residues appeared to be critical for cleavage specificity at both sites in Factor VIIIa. Results in the present study support the speculation by Manithody et al. [32] for possible interaction of loop 39 residues of APC with Glu³⁴¹ (P5') and/or Glu³⁴² (P6') of Factor VIIIa. However, further investigation is required to determine the complementarity for these interactions.

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