Activated Protein C (APC) inactivates factor VIIIa by cleavage at Arg\textsuperscript{336} and Arg\textsuperscript{562} within the A1 and A2 subunits, respectively, with reaction at the former site occurring at a rate \(\sim 25\)-fold faster than the latter. Recombinant factor VIII variants possessing mutations within the P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2 sites were used to determine the contributions of these residues to the disparate cleavage rates at the two P1 sites. Specific activity values for 336(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} and 336(P1\textsuperscript{-}P1\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{562} mutants, where indicated residues surrounding the Arg\textsuperscript{336} site were replaced with those surrounding Arg\textsuperscript{562}, were similar to wild type (WT) factor VIII; whereas 562(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} and 562(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} mutants showed specific activity values \(< 1\%\) the WT value. Inactivation rates for the 336 site mutants were reduced \(\sim 6-11\)-fold compared with WT factor VIIIa, and approached values attributed to cleavage at Arg\textsuperscript{562}. Cleavage rates at Arg\textsuperscript{336} were reduced \(\sim 100\)-fold for 336(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{562}, and \(\sim 9-16\)-fold for 336(P4-P2\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} and 336(P1\textsuperscript{-}P1\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{562} mutants. Inhibition kinetics revealed similar affinities of APC for WT factor VIIIa and 336(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} variant. Alternatively, the 562(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} variant showed a modest increase in cleavage rate (\(\sim 4\)-fold) at Arg\textsuperscript{562} compared with WT, whereas these rates were increased by \(\sim 27\)- and 6-fold for 562(P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336} and 562(P4-P2\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2)\textsuperscript{336}, respectively, using the factor VIII procofactor form as substrate. Thus the P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2\textsuperscript{-}P2 residues surrounding Arg\textsuperscript{336} and Arg\textsuperscript{562} make significant contributions to proteolysis rates at each site, apparently independent of binding affinity. Efficient cleavage at Arg\textsuperscript{336} by APC is attributed to favorable P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2\textsuperscript{-}P2 residues at this site, whereas cleavage at Arg\textsuperscript{562} can be accelerated following replacement with more optimal P4-P3\textsuperscript{-}P3\textsuperscript{-}P2\textsuperscript{-}P2\textsuperscript{-}P2 residues.

Factor VIII plays a critical role in the coagulation cascade evident from hemophilia A occurring in individuals with deficient or defective factor VIII. Factor VIII is synthesized as a single chain precursor (1, 2) consisting of three A domains homologous to the A domains in factor V, a unique B domain, and two homologous C domains (1, 3). A region rich in acidic amino acid residues designated a1 (residues 337–372), a2 (residues 711–740), and a3 (residues 1649–1689) borders each A domain. Thus, the factor VIII domain organization is represented as NH\textsubscript{2}-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH. Intra-cellular proteolytic processing generates the factor VIII procofactor composed of a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains) that are associated via a metal ion-dependent linkage. Thrombin activates the procofactor by limited proteolysis generating the active cofactor, factor VIIIa, a heterotrimer composed of A1, A2, and A3-C1-C2 subunits (4, 5). Factor VIIIa associates with the serine protease factor IXa in a phospholipid membrane-dependent interaction, forming the intrinsic factor Xase complex that efficiently activates factor X during the propagation phase of coagulation (see Ref. 6 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, a result of a weak affinity electrostatic interaction (5, 7). The second mechanism results from proteolytic inactivation of the cofactor catalyzed by APC.\textsuperscript{3} APC is a potent anticoagulant that proteolytically inactivates factor Va and factor VIIIa, and deficiencies in this proteinase are linked to thrombosis (see Ref. 8 for a review). APC cleaves both procofactor factor VIII and factor VIIIa, although the latter represents the more relevant, physiologic substrate, at Arg\textsuperscript{336} (A1 subunit) and at Arg\textsuperscript{562} (A2 subunit) (9, 10). Cleavage at the former site alters the interactions between A1 and A2 subunits yielding reduced \(k_{\text{cat}}\) values for factor Xase (11), as well as an increased \(K_{m}\) for factor X (12). Cleavage at the latter site occurs within an important factor IXa-interactive site, the 558-loop (13). Thus cleavage at either site contributes to cofactor inactivation. We recently demonstrated that these cleavages occur in an independent non-sequential fashion, with residue Arg\textsuperscript{336} being cleaved at a rate \(\sim 25\)-fold faster than Arg\textsuperscript{562} (14).

Several lines of evidence suggest that exosite interactions play a major role in substrate affinity and in enforcing the high specificity for coagulation proteases, whereas substrate docking at the active site contributes to efficient catalysis (see Ref. 15 for a review). Interactions at the active site include formation of a transition state analog consisting of several hydrogen bonds between the backbone of the peptide substrate and the protease

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\textsuperscript{3} The abbreviations used are: APC, activated protein C; WT, wild type.
and also interactions of the side chains of the peptide and the active site pockets. Hydrogen bonds formed minimally involve P3-P14 (16) residues flanking the scissile bond, that form an antiparallel β sheet with residues 214–216 of the chymotrypsin-like serine proteases (see Ref. 17 for a review). Although the interaction of P1′-P3′ residues of the substrate with the S1′-S3′ pockets of the enzyme are not very well defined, there is significant evidence indicating that these interactions are also important during substrate docking at the active site.

Whereas existing evidence implicates involvement of exosite-directed interactions in the catalytic mechanism leading to inactivation of factor VIIIa by APC (14, 18), another factor that may contribute to the observed disparate cleavage rates at Arg336 and Arg562 are residues surrounding these two P1 Arg residues. Little information exists relating the contribution of these residues to proteolysis of factor VIIIa by APC. In the current study we examine the contribution of the P4-P3′ residues surrounding residues Arg336 and Arg562 to catalysis by APC. Several recombinant variants were prepared where residues surrounding the faster reacting Arg336 site were replaced by those surrounding the slower reacting Arg562 site, and vice versa. Our results demonstrate marked cleavage rate reductions in the former set of variants, whereas rate increases were observed in the latter class, indicating that the P4-P3′ sequences surrounding the two P1 Arg residues make substantial contribution to the catalytic mechanism and appear directly responsible for the disparity in cleavage rates observed for the two sites.

MATERIALS AND METHODS

Reagents—The monoclonal antibody 58.12, which recognizes the NH2 terminus of the A1 domain of factor VIII, was a gift from Bayer Corp. (Berkeley, CA). The monoclonal antibody R8B12, which recognizes the COOH terminus of the A2 domain was obtained from Green Mountain Antibodies (Burlington, VT). The C5 antibody was a generous gift from Zaverio Ruggeri. The ESH8 antibody, which recognizes the light chain of factor VIII was obtained from American Diagnostica. Phospholipid vesicles containing 20% phosphatidylserine, 40% phosphatidylethanolamine, and 40% phosphatidylcholine, and 40% phosphatidylethanolamine were prepared using octylglucoside as described previously (19). The phospholipid vesicles containing 20% phosphatidylserine, 40% phosphatidylethanolamine, and 40% phosphatidylcholine were purchased from the indicated vendors. The B-domainless factor VIII proteins were typically 90% pure to catalysis by APC. Several recombinant variants were prepared where residues surrounding the faster reacting Arg336 site were replaced by those surrounding the slower reacting Arg562 site, and vice versa. Our results demonstrate marked cleavage rate reductions in the former set of variants, whereas rate increases were observed in the latter class, indicating that the P4-P3′ sequences surrounding the two P1 Arg residues make substantial contribution to the catalytic mechanism and appear directly responsible for the disparity in cleavage rates observed for the two sites.

Construction, Expression, and Purification of Recombinant Factor VIII Mutants—Recombinant factor VIII variants were prepared as B-domainless factor VIII forms, stably transfected into baby hamster kidney cells, and proteins expressed were purified as described previously (14). Factor VIII mutants, 336(P4-P3′)562, 336(P4-P2)562, and 336(P1′-P3′)562, were prepared by substituting the designated P4-P3′ residues surrounding the P1 Arg336 with the corresponding residues surrounding Arg562, whereas 562(P4-P3′)336 and 562(P4-P2)336 mutants were prepared by substituting the P4-P3′ residues surrounding Arg562 with the corresponding residues surrounding Arg336. Purified factor VIII proteins were typically >90% pure as judged by SDS-PAGE and staining with GelCode Blue (Pierce). Specific activity values for these proteins were calculated from activity and concentration values determined by a one-stage clotting assay and enzyme-linked immunosorbent assay, respectively, as previously described (14).

Reaction of Factor VIIIa with APC—Factor VIIIa (130 nM) was activated by addition of 10–20 nM thrombin in 20 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM CaCl2, 0.01% Tween 20, and 100 μg/ml bovine serum albumin (Buffer A) and reactions were run at 37 °C. Thrombin was inhibited after 2 min by the addition of 10–20 units/ml hirudin, and the resultant factor VIIIa was reacted with APC (2 or 40 nM) in the presence of 100 μM phospholipid vesicles. Aliquots were removed at the indicated times to assess residual factor VIIIa activity by factor Xa generation assay and proteolysis of subunits by Western blotting. The concentration of factor VIIIa employed represented the approximate Km value for inactivation by APC (100 nM) (14). Limitations in levels of several of the expressed proteins did not permit use of higher substrate concentrations.

Factor Xa Generation Assay—The rate of conversion of factor X to Xa was monitored in a purified system (20). Factor Xa generation was initiated by addition of factor IXa (40 nM) and factor X (400 nM) into the factor VIIIa reaction mixture. Aliquots 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 Xa substrate, Pefa-5523 (0.46 mM final concentration). Reactions were read at 405 nm for 5 min using a Vmax microtiter plate reader (Molecular Devices, Sunnyvale, CA). Factor VIIIa activity was determined based upon rates of factor Xa generated (nm) per minute, and this information was used to determine the concentration of residual, active factor VIII.

For each data set, control experiments assessing factor VIIIa stability were performed in the absence of APC 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% loss of the initial activity over a 20-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 this APC-independent mechanism. In addition, the correlation of APC concentration to rate of proteolysis (as judged by cleavage of the factor VIIIa A1 subunit by Western blotting, see below) was determined over the range of APC concentrations (from 2 to 40 nM). Deviations from linearity (≈2.9-fold) in the A1 subunit
cleavage rates observed for 40 nM APC compared with 2 nM APC were used in correcting calculations for inactivation and subunit cleavage rates.

Western Blotting—Aliquots from the APC cleavage reactions were removed at the indicated times and the reactions were stopped with SDS-PAGE buffer. Samples were subjected to SDS-PAGE using 8% acrylamide gels and Western blotting was performed as described previously (21). Cleavage at Arg336 was monitored using the SL.02 monoclonal antibody followed by a biotinylated goat anti-mouse secondary antibody, streptavidin, and biotinylated alkaline phosphatase (Bio-Rad) to enhance the detection of A1-containing bands. Cleavage at Arg562 was monitored using the R8B12 monoclonal antibody, followed by goat anti-mouse alkaline phosphatase-linked secondary antibody (Sigma). Signals were detected using the enhanced chemiluminescence system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices). Densitometric scans were quantified from linear density regions of the blots using ImageQuant software (Molecular Devices).

Data Analysis—All experiments were performed at least 3 separate times, and average values with standard deviations are shown. The concentration of factor VIIIa generated following reaction of factor VIII with thrombin was calculated from blotting data based upon density values for residual single chain and heavy chain compared with values for A1 and A2 subunits as previously described (14). Typically ~80–85% of factor VIII was converted to factor VIIIa using the conditions described above. Initial time points (where up to ~50% substrate was utilized) were fitted to the second order polynomial equation (Equation 1) using nonlinear least squares regression analysis,

\[
[VIIIa] = A + Bt + Ct^2 \quad \text{(Eq. 1)}
\]

where \([VIIIa]\) is factor VIIIa concentration in nM, \(t\) is time in minutes, and \(A, B,\) and \(C\) are coefficients of the quadratic equation. Specifically, \(A\) corresponds to the initial concentration of factor VIIIa or A1 or A2 subunit in nanomolar and \(B\) corresponds to the slope value at time 0. The absolute value of \(B\) represents the rate of factor VIIIa inactivation or the A1 or A2 subunit cleavage that was normalized by APC concentration and expressed in nanomolar FVIIIa/min/nM APC or nanomolar A1 or A2/min/nM APC, respectively.

The inhibition constant \((K_i)\) for 336(P4-P3')562 on APC-catalyzed cleavage of the WT factor VIIIa A1 subunit was determined by fitting the data using nonlinear least squares regression analysis according to a competitive inhibition model (Equation 2),

\[
\nu = V_{\text{max}} \times \left[ \frac{[WT]}{[K_m] \times (1 + [I]/K) + [WT]} \right] \quad \text{(Eq. 2)}
\]

where \(\nu\) is the initial velocity in nanomolar/min, \([WT]\) is the concentration of WT factor VIIIa A1 subunit in nanomolar, \([I]\) is the concentration of 336(P4-P3')562 factor VIIIa A1 subunit in nanomolar, and \(K_m\) is the Michaelis-Menten constant of WT factor VIIIa for APC, which we previously estimated as 102 nM (14).

### RESULTS

**Characterization of Recombinant Factor VIII 336(P4-P3')562 and 562(P4-P3')336 Mutants**—We recently demonstrated that APC-catalyzed cleavages of factor VIIIa at residues Arg336 and Arg562 occur independently with the rate of proteolysis at the former site ~25-fold faster than the latter (14). Whereas substantial evidence implies the involvement of exosite-directed interactions in the catalytic mechanism of APC, another factor that may contribute to the disparate reaction rates is the influence of residues surrounding the two P1 Arg residues. To examine the roles of these sequences in cofactor cleavage and inactivation, several recombinant B-domainless factor VIII mutants were prepared by replacing the P4-P3' sequence surrounding the faster-reacting Arg336 site with that surrounding the slower-reacting Arg562 site and vice versa (see Table 1). Additional variants representing partial sequence replacements were also prepared. The purified proteins revealed three bands of ~170, ~90, and ~80 kDa as visualized by SDS-PAGE and GelCode Blue staining (results not shown), which corresponded to the predicted molecular masses of the single chain factor VIII, and heavy chain and light chain of the factor VIII heterodimer, respectively. Specific activity values measured for the factor VIII mutants yielded similar values for the 336(P4-P3')562, 336(P4-P2)562, and 336(P1'-P3')562 variants as compared with WT (Table 1), indicating that residues surrounding Arg336 were not critical to cofactor function and that these positions tolerated sequence substitution. However, factor VIII 562(P4-P3')336 and 562(P4-P2)336 mutants revealed specific activity values <1% that of WT (Table 1). This dramatic decrease in specific activity for mutations surrounding Arg562 likely reflected the importance of these residues for factor VIII function inasmuch as residues 558–565 have been identified as comprising a site for interaction with factor IXa (13). However, all variants demonstrated similar interaction with thrombin that was indistinguishable from that for WT factor VIII, as judged by rates of cleavage of factor VIII and the generation of factor VIIIa subunits (results not shown). This observation further suggested that mutations surrounding Arg562 did not globally affect factor VIII conformation.

**Inactivation of 336(P4-P3')562 Factor VIIIa Mutants by APC**—Purified factor VIII variants (130 nM) were converted to the active factor VIIIa cofactor following thrombin activation. The resultant factor VIIIa was then reacted with the indicated levels of APC in the presence of phospholipid vesicles (100 μM)

### TABLE 1

Specific activity values of 336(P4-P3')562 and 562(P4-P3')336 factor VIII mutants

| Factor VIII | 336(P4-P3') sequence | 562(P4-P3') sequence | Specific activity (%) |
|------------|-----------------------|-----------------------|----------------------|
| WT         | PQLRMKN               | VDQRGNQ               | 100 ± 15             |
| 336(P4-P3')562 | VDQRGNQ           | VDQRGNQ               | 120 ± 13             |
| 336(P4-P2)562 | VDQ               | VDQ                   | 101 ± 25             |
| 336(P1'-P3')562 | GNQ              | GNQ                   | 126 ± 27             |
| 562(P4-P3')336 | PQLRMKN          | PQLRMKN               | 0.2 ± 0.1            |
| 562(P4-P2)336 | PQL                 | PQL                   | 0.3 ± 0.1            |
and cofactor activity was monitored over time using a factor Xa generation assay (Fig. 1). Significantly greater concentrations of APC were employed for inactivation experiments involving the mutant factor VIII forms (40 nM APC) compared with that used for WT factor VIIIa (2 nM APC) based upon reduced reactivity for the variants. The observed rate of spontaneous loss of factor VIIIa activity obtained in the absence of APC was similar for all three 336(P4-P3)562 variants and WT factor VIIIa forms (∼10% activity loss at 20 min, data 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 under “Materials and Methods.”

The inactivation rates for the set of 336(P4-P3)562 mutants were reduced ∼6–11-fold compared with WT factor VIIIa (Table 2). Rates were determined from the initial time points (up to 5 min) because significant deviation from the fitted curves occurred at more extended time points. The reason for this deviation likely resulted from depletion of substrate factor VIIIa. The extents for these rate reductions resulting from mutation around Arg562 approached the inactivation rate observed when Arg562 was replaced with a non-cleavable Gln residue (14), suggesting a greater contribution from cleavage at the Arg562 site to overall loss of cofactor activity. These results indicate that residues both NH2-terminal and COOH-terminal to the Arg562 site contribute to the efficient inactivation of factor VIIIa by APC.

**TABLE 2**

| Factor VIIIa | Inactivation | A1 cleavage | A2 cleavage |
|-------------|-------------|-------------|-------------|
| WT          | 5.4 ± 0.6   | 0.8 ± 0.1   | 0.9 ± 0.1   |
| 336(P4-P3)562 | 0.8 ± 0.1   | 0.5 ± 0.04  | 0.9 ± 0.1   |
| 336(P4-P2)562 | 0.5 ± 0.04  | 0.6 ± 0.1   | 1.1 ± 0.3   |
| 336(P1'-P3)562 | 0.9 ± 0.1   | 1.1 ± 0.3   | 0.3 ± 0.04  |

**FIGURE 1.** Inactivation of recombinant 336(P4-P3)562 factor VIIIa mutants by APC. WT (○), 336(P4-P3)562 (●), 336(P4-P2)562 (□), and 336(P1'-P3)562 (■) factor VIII forms (130 nM) were activated by thrombin (10 nM). Factor VIIIa inactivation was then monitored over time in the presence of APC (2 nM in WT reaction and 40 nM in mutant reactions) using a factor Xa generation assay. APC-catalyzed inactivation values were corrected by subtracting the corresponding values for factor VIIIa decay observed in the absence of APC and continuous lines were drawn through initial time points from the curve fitting as described under “Materials and Methods.”

**FIGURE 2.** Cleavage of A1 and A2 subunits of 336(P4-P3)562 factor VIIIa mutants by APC. WT (○), 336(P4-P3)562 (●), 336(P4-P2)562 (□), and 336(P1'-P3)562 (■) factor VIII forms (130 nM) were activated by thrombin (10 nM) and then reacted with APC (2 nM in WT reaction and 40 nM in mutant reactions). Aliquots were taken at various time points (0–60 min) and subjected to SDS-PAGE. A1 (and A1336) and A2 (and A2C) subunits were visualized by Western blotting using 58.12 (anti-A1) and R8B12 (anti-A2) monoclonal antibodies. Product concentrations were calculated based on the density values and plotted as a function of time (B and C). Continuous lines were drawn from the curve fitting as described under “Materials and Methods.”

**APC-Catalyzed Inactivation of Factor VIIIa**

The above results indicated reduced rates of APC-catalyzed inactivation of the factor VIIIa forms possessing mutations surrounding Arg562. Western blot analysis was performed to determine the rates of APC-catalyzed proteolysis at Arg562 and Arg562 for the 336(P4-P3)562, 336(P4-P2)562, and 336(P1'-P3)562 factor VIIIa variants and correlate these data to rates for factor VIIIa inactivation (Fig. 2). Cleavage at Arg562 within the factor VIIIa A1 subunit and generation of the A1336 product (residues 1–336) were detected by monoclonal antibody 58.12, which recognizes the NH2 terminus of this subunit. Cleavage at Arg562 within the A2 subunit and generation of the A2C product (residues 563–740) were detected by monoclonal antibody R8B12, which recognizes a discontinuous epitope.
within the factor VIII A2 domain (22). Concentrations of these substrates and products were quantitated based on linear density values determined from densitometry scans of the blots. Results from this analysis indicated that cleavage rates determined for the A1 subunit for the 336 mutants were significantly slower than that of WT factor VIIIa. Both the 336(P4-P2)562 and the 336(P1'-P3')562 mutants showed similar cleavage rates at the A1 site that were reduced ~16- and ~9-fold, respectively, as compared with the WT protein (Fig. 2, A and B, Table 2). However, the rate of cleavage for the A1 subunit of the fully substituted P4-P3’ mutant was reduced by ~100-fold relative to WT. These observations indicate residues both NH2- and COOH-terminal to this P1 site affect cleavage by APC, with more extensive mutation altering both sides of the scissile bond showing a maximal defect in cleavage rate.

On the other hand, cleavage rates for the A2 subunit in the 336(P4-P2)562 and 336(P1'-P3')562 variants were similar to that of WT (Fig. 2, A and C, Table 2) suggesting a normal interaction of APC and cleavage at the A1 site that was unperturbed by mutation at the A1 scissile bond. We did note that cleavage of the A2 subunit for the 336(P4-P3')562 appeared a few fold slower than that for WT, and the reason(s) for this disparity are not known.

Correlating the proteolysis and activity data suggested that cleavage of the A2 subunit becomes a more dominant mechanism for cofactor inactivation when cleavage at the A1 site is reduced by mutations surrounding Arg336. Separate mutations NH2- and COOH-terminal to Arg336 resulted in marked reductions in cleavage at this site, whereas minimally affecting reaction at the A2 site, and overall yielded significant reductions in rates for cofactor inactivation. Overall, these results suggest that P4-P3’ residues surrounding Arg336 make a prominent contribution to the mechanism of APC cleavage at Arg336 and cofactor inactivation by this pathway.

Inhibition of WT Factor VIIIa A1 Subunit Cleavage by the 336(P4-P3’)562 Mutant—To determine whether the ~100-fold slower cleavage at Arg336 for the 336(P4-P3’)562 factor VIIIa relative to WT resulted from a defect in the affinity of APC for this substrate, we used the mutant protein as an inhibitor of cleavage of the WT factor VIIIa. The rationale for this approach was that if proteinase binding to the mutant were unaffected, then it would efficiently compete with the WT substrate. Furthermore, because the mutant remains essentially uncleaved at the A1 site during a truncated time course, it would serve as an inhibitor of detected cleavage of the WT substrate. For these reactions, WT (130 nM) and the 336(P4-P3’)562 mutant (0–200 nM) factor VIII were simultaneously activated by thrombin (20 nM) and then reacted with a low concentration (2 nM) of APC in the presence of phospholipids (100 μM). Using band density values of the A1 substrate (WT and mutant) and A1336 product (WT) from Western blotting, cleavage rates were determined and plotted versus concentration of 336(P4-P3’)562 factor VIIIa mutant (Fig. 3). Control experiments showed no detectable cleavage of the mutant protein up to 40 min using these reaction conditions, whereas the WT protein was cleaved by >50% at the 4-min time point (Fig. 3, inset, panels b and a, respectively) thereby validating this approach. An inhibition constant (K_i) for 336(P4-P3’)562 of 36 ± 7 nM was determined by fitting these data to a competitive inhibition model and using a K_m of 102 nM as previously determined (14). This K_i value was ~3-fold less than the K_m for WT substrate indicating the binding of APC to the mutant was not diminished by the altered P4-P3’ sequence but modestly enhanced, possibly the result of the reduced reaction rate with this variant. This result is consistent with regions removed from the P4-P3’ making a primary contribution to the affinity of APC for factor VIIIa, and furthermore, suggests a primary role of the P4-P3’ sequence in affecting K_cat.

APC Cleavage of A1 and A2 Subunits of 562(P4-P3’336 Factor VIIIa Mutants—Although mutation within the P4-P3’ region of Arg562 yielded factor VIII forms lacking cofactor activity, likely due to alteration within a critical factor IXa-interactive site, these reagents could be evaluated as substrates for proteolysis by APC. Thus we performed a series of experiments to assess the effects of replacing residues around the P1 Arg562 with those that surround the more rapidly cleaved Arg336 site. Western blot analysis was performed to determine the rates of APC-catalyzed proteolysis within the A1 and A2 subunits for two variants, 562(P4-P3’)336 and 562(P4-P2)336 factor VIIIa forms (Fig. 4). Reactions were run using similar conditions as described above and cleavage rates were determined following Western blotting. Cleavage rates of the A1 subunit for both the A2 mutants were similar to that of WT factor VIIIa (Fig. 4, A and B, Table 3). This result was consistent with loss of activity due to mutation not resulting from changes in the gross factor VIII conformation, but rather restricted to an altered interactive site in and around Arg562. We observed the rate of A2
These results suggested that the P4-P3 residues (130 nM) were activated by thrombin (10 nM), then reacted by APC (2 nM) as described in the legend to Fig. 2. Aliquots were taken at various time points (0–60 min) and subjected to SDS-PAGE. A1 (and A1336) and A2 (and A2c) 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). Continuous lines were drawn from the curve fitting as described under “Materials and Methods.”

**TABLE 3**

Rates of A1 and A2 subunit cleavages for WT and 562(P4-P3’)336 factor VIIIa/IIIa mutants

Rates of factor VIIIa/IIIa A1 and A2 subunit/domain cleavages were estimated by nonlinear least squares regression analysis as described under “Materials and Methods.” Data points represent mean ± S.D. values of at least three separate experiments.

| Factor VIIIa/IIIa | Δt A1/min/nmol APC | Δt A2/min/nmol APC |
|-------------------|--------------------|--------------------|
| WT FVIIa          | 8.5 ± 1.8          | 0.5 ± 0.2          |
| 562(P4-P3’)336 FVIIa | 7.3 ± 1.5        | 1.9 ± 0.5          |
| 562(P4-P2’)336 FVIIa | 7.4 ± 1.6        | 1.7 ± 0.2          |
| WT FVIII          | 6.1 ± 0.6          | 0.1 ± 0.06         |
| 562(P4-P3’)336 FVIII | 2.7 ± 0.7        | 0.6 ± 0.3          |
| 562(P4-P2’)336 FVIII | 1.4 ± 0.6        |                    |

The A1 subunit cleavage for the 562(P4-P3’)336 mutant was increased ~4-fold, whereas the A2 cleavage rate for the 562(P4-P2’)336 mutant was similar to that of WT (Fig. 4, A and C, Table 3). These results suggested that the P4-P3’ residues surrounding the Arg562 site make a generally minor contribution to APC-catalyzed cleavage at this site in factor VIIIa.

**APC Cleavage of A1 and A2 Subunits of 562(P4-P3’)336 Factor VIII Mutants**—Cleavage of the A2 subunit in factor VIIIa is of apparent secondary importance to cofactor inactivation as compared with cleavage at the A1 site based on the significantly slower rate at this site (14). Furthermore, assessing the rate for cleavage within the A2 subunit is complicated by the tendency for this subunit to dissociate from factor VIIIa, and earlier results have indicated that free A2 subunit is a poor substrate for cleavage by APC (10). To better assess contributions of the P4-P3’ residues surrounding Arg562 to cleavage at this site, we examined the APC-catalyzed proteolysis of Arg562 and Arg562 in the heavy chain of the factor VIII procofactor for the WT and variant proteins. Use of this substrate stabilizes the inter-domain interactions involving A2 because the A1 and A2 domains are contiguous. Purified factor VIII (130 nM) was reacted with 2 nM APC in the presence of phospholipid vesicles (100 μg/mL) and subsequently subjected to SDS-PAGE and Western blot analysis. Cleavage of factor VIII a at Arg562 generates the A1336 fragment (residues 1–336) that is reactive with the 58.12 antibody and the rate of appearance of this fragment was similar in the WT and the two mutants (Fig. 5, A and B, Table 3). Inasmuch as APC rapidly attacks Arg562 in factor VIII (10), cleavage at Arg562 in the procofactor was monitored by the rate of appearance of the terminal product, the A2c fragment (residues 563–740), which is reactive with R8B12 antibody. Rates of generation of this fragment were ~27- and ~6-fold greater for 562(P4-P3’)336 and 562(P4-P2’)336 mutant factor VIII forms, respectively, compared with WT factor VIII (Fig. 5, A and C, Table 3). These results indicated that the P4-P3’ residues surrounding Arg562 indeed influence the mechanism for catalysis at this site in the factor VIII procofactor by APC and that replacement of residues at both NH2- and COOH-terminal positions relative to the P1 Arg with residues that appear more optimal for this interaction facilitate the cleavage reaction.

Western blotting of the factor VIII digest time course with the anti-A2 domain monoclonal antibody revealed two intermediates. One fragment of slightly greater mass (~48 kDa) than the A2 subunit and representing factor VIII residues 337–740 was predicted based upon cleavage at Arg562. Cleavage at this site generated the A1336 fragment indicated in the blots with the anti-A1 specific monoclonal antibody. However, a second, slightly smaller fragment (~43 kDa) was also noted. This band was of similar size to the A2 subunit (residues 337–740) derived from thrombin cleavage of factor VIII. Control experiments (not shown) indicated that this fragment did not react with C5 antibody, which recognizes an epitope within residues 351–365 (23), whereas the 48-kDa band did, confirming its origin. Furthermore, the 43-kDa band was not present following reaction of APC with a factor VIII variant possessing an R372Q mutation (data not shown), which would preclude cleavage at residue 372. Taken together, these results suggest APC catalyzes limited attack at Arg562 in the factor VIII procofactor.

The A1 subunit cleavage in the factor VIII procofactor does not comply with the second order kinetics (Fig. 5B). The reason(s) for this is(are) not clear but may suggest a more complex mechanism involved in A1336 product generation as a result of
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DISCUSSION

In this study we assessed the role of residues surrounding the two P1 Arg residues attacked by APC during inactivation of factor VIIIa. The rationale for this study was based upon our earlier observations indicating that proteolysis of these sites in the cofactor occurred independently and that Arg^{336} was cleaved at a rate ∼25-fold greater than that observed for cleavage at Arg^{562} (14). Results obtained evaluating rates of cleavage where selected P4-P3′ residues for one site were replaced with residues from the complementary site indicated that rapid cleavage at Arg^{336} resulted from favorable P4-P3′ residues surrounding this site because replacement of residues 333–339 with residues 559–565 yielded significantly diminished rates of cleavage. Conversely, the relatively slower rate of cleavage at Arg^{562} could be modestly accelerated following replacement of residues 559–565 with residues 333–339.

The wild type-like specific activity of factor VIII forms possessing mutations at P4-P3′ residues surrounding Arg^{336} indicated that these residues are not critical to factor VIIa cofactor function. This observation is supported by the absence of point mutations in the Hemophilia A data base over this region that yield a hemophilic phenotype (with the exception of mutation to stop codons), as well as no reports in the literature of this site yielding a hemophilic phenotype of varying severity (24). Recapitulating four of these point mutations in recombinant factor VIII expressed in heterologous mammalian cells for in vitro functional analyses demonstrated a significant reduction in the $k_{cat}$ values for factor IXa-catalyzed generation of factor Xa with essentially no observed cleavage at the Arg^{336} site (14). Thus proteolysis at Arg^{562} in the A2 subunit now contributes more heavily to the overall mechanism of cofactor inactivation.

Assessing APC-catalyzed proteolysis directed toward the A2 subunit in factor VIIIa is problematic due to the tendency for this subunit to dissociate and the earlier observation that the free A2 subunit is not efficiently cleaved by APC (10). Although the factor VIII heterodimer is a poorer substrate for APC than the factor VIIIa cofactor, as judged by reduced rates of cleavage at both the A1 and A2 sites, it does have the advantage of presenting the A2 domain as contiguous with A1, and in this regard approximates the structure of the factor Va heavy chain. Evaluation of both factor VIII and factor VIIIa substrates revealed that replacing residues 559–565 with residues 333–339 resulted in enhanced cleavage rates at the A2 site. The significantly greater effect on cleavage at Arg^{562} observed for the procofactor form may be attributed to structural differences sur-
rounding the scissile bond. Taken together with the above results, these data indicate residues flanking the P1 Arg336 as being more optimal for engaging the APC active site than those flanking the P1 Arg562.

APC-catalyzed inactivation of the homologous cofactor, factor Va, also results from cleavage at two sites in the protein with initial cleavage at Arg506 in the A2 domain preceding appreciable cleavage at Arg306 within the A1 domain (26). However, this cleavage order appears to be dictated in large part by conformational effects. Because the A1 and A2 domains are contiguous in cleavage order, the cleavage product Va, also results from cleavage at two sites in the protein with a P2 Arg adjacent to P1 Arg506 and the somewhat higher affinity of the former “inhibitor” for APC may reflect its full resistance to cleavage compared with the limited resistance of the latter.

In summary, these results demonstrate a primary role for flanking sequences of the P1 sites in modulating rates of inactivation of factor VIIIa by a direct contribution to APC active site engagement. Whereas the sequence surrounding Arg562 is important for cofactor function, thus precluding alteration at this site, mutagenesis at the faster-reacting Arg306 site appears refractory to specific activity concerns and could yield a mechanism to fine tune reductions in the rate of APC-catalyzed cofactor inactivation by selective mutation at this site.

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