Identification of Plasmin-interactive Sites in the Light Chain of Factor VIII Responsible for Proteolytic Cleavage at Lys36*

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We have recently reported that plasmin likely associates with the factor VIII light chain to proteolyze at Lys36 within the A1 domain. In this study, we determined that the rate of plasmin-catalyzed inactivation on the forms of factor VIIIa containing A1-(1–336) and 1722A3C1C2, reflecting Lys36 cleavage, was reduced by ~60%, compared with those containing 1649A3C1C2 and 1690A3C1C2. SDS–PAGE analysis revealed that Lys36 cleavage of factor VIIIa with 1722A3C1C2 was markedly slower than those with 1649A3C1C2 and 1690A3C1C2. Surface plasmon resonance-based assays, using active site-modified anhydro-plasmin (Ah-plasmin) showed that 1722A3C1C2 bound to Ah-plasmin with an ~3-fold lower affinity than 1649A3C1C2 or 1690A3C1C2 (Kₚ 176, 68.2, and 60.3 nM, respectively). Recombinant A3 bound to Ah-plasmin (Kₚ 44.2 nM), whereas C2 failed to bind, confirming the presence of a plasmin-binding site within N terminus of A3. Furthermore, the Glu-Gly-Arg active site-modified factor IXa also blocked 1722A3C1C2 binding to Ah-plasmin by ~95%, supporting the presence of another plasmin-binding site overlapping the factor IXa-binding site in A3. In keeping with a major contribution of the lysine-binding sites in plasmin for interaction with the factor VIII light chain, analysis of the A3 sequence revealed two regions involving clustered lysine residues in 1690–1705 and 1804–1818. Two peptides based on these regions blocked 1649A3C1C2 binding to Ah-plasmin by ~60% and plasmin-catalyzed Lys36 cleavage of factor VIIIa with A1-(1–336) by ~80%. Our findings indicate that an extended surface, centered on residues 1690–1705 and 1804–1818 within the A3 domain, contributes to a unique plasmin-interactive site that promotes plasmin docking during cofactor inactivation by cleavage at Lys36.

Factor VIII circulates as a complex with von Willebrand factor and functions as an essential cofactor in the tenase complex responsible for anionic phospholipid surface-dependent conversion of factor X to Xa by factor IXa (1). Molecular defects in factor VIII result in the congenital bleeding disorder, hemophilia A. Factor VIII is composed of 2,332 amino acid residues with a molecular mass of ~300 kDa and contains three types of structural domain, arranged in the order of A1-A2-B-A3-C1-C2 (2, 3). Mature factor VIII is processed to a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, together with heterogeneous fragments of a partially proteolyzed B domain, linked to a light chain consisting of the A3, C1, and C2 domains (2–4).

Factor VIII is converted into an active form, factor VIIIa, by limited proteolysis catalyzed by either thrombin or factor Xa (5). Cleavages at Arg372 and Arg740 in the heavy chain produce 50-kDa A1 and 40-kDa A2 subunits. Cleavage of the 80-kDa light chain (1649A3C1C2) at Arg1689 produces a 70-kDa A3C1C2 subunit (1690A3C1C2). Additional cleavage by factor Xa at Arg721 produces a 67-kDa A3C1C2 subunit (1722A3C1C2). Proteolysis at Arg722 and Arg1689 is essential for generating factor VIIIa cofactor activity (6). Cleavage at the former site exposes a functional factor IXa-interactive site within the A2 domain that is cryptic in the unactivated molecule (7). Cleavage at the latter site liberates the cofactor from its carrier protein, von Willebrand factor (8), and contributes to the overall specific activity of the cofactor (9, 10).

APC2 (5), factor Xa (5), and factor IXa (11) are serine proteases that inactivate factor VIII(a) by cleavage at Arg36 within the A1 subunit. This inactivation appears to be associated with an altered interaction between the A2 subunit and truncated A1 and is coupled with an increase in the Kₚ value for the substrate, factor X (12, 13), reflecting loss of a factor X-interactive site within residues 337–372 (14). In addition, a second specific cleavage site for factor Xa, Lys36, was identified within the A1 subunit (13). Attack at this site also results in factor VIII inactivation mediated by an altered conformation of the A1 subunit limiting productive interaction with the A2 subunit (13).

Plasmin is a potent fibrinolytic protease and is composed of a heavy chain consisting of five kringle domains and a light chain containing the catalytic domain. The protease associates with numerous proteins via the LBS on the exposed surface (15). Several reports have shown that plasmin proteolytically inactivates the cofactor VIIIa, and our findings support this observation.

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‡ The abbreviations used are: APC, activated protein C; LBS, lysine-binding site; EGR-factor IXa, Glu-Gly-Arg active site-modified factor IXa; mAb, monoclonal antibody; rA3, recombinant A3; rC2, recombinant C2; Ah-plasmin, anhydro-plasmin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; SPR-based assay, surface plasmon resonance-based assay; ELISA, enzyme-linked immunosorbent assay.

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vates several coagulation proteins, including factors Va (16, 17), VIII (18), IXa (19), and X (20). In detail, we have demonstrated that plasmin rapidly inactivates factor VIII by cleavage at Arg336 (18). Direct binding assays using Ah-plasmin, a catalytically inactive derivative of plasmin, revealed that plasmin interacts with the factor VIII heavy chain, predominantly the A2 domain, with high affinity (K_D ~ 6 and ~20 nM, respectively), in a mechanism largely independent of LBS (21). Our findings demonstrated, in particular, that Arg484 in the A2 domain significantly contributes to a unique plasmin-interactive site within the heavy chain that promotes plasmin docking during cleavage of the heavy chain (21).

In contrast, plasmin interacts with the factor VIII light chain with moderate affinity (K_D ~ 70 nM), predominantly through LBS-dependent mechanisms (21). Our previous data suggested that plasmin cleavage at Lys36 within the A1 domain appears to be selectively regulated by the light chain (18). In this study, we have expanded our studies using truncated light chain, recombinant factor VIII subunits, and synthetic peptides, and identified plasmin-interactive sites within the light chain responsible for cleavage at Lys36. Our results indicate that an extended surface centered on lysine residues involving the 1690–1705 and 1804–1818 regions in the A3 domain contributes to a unique plasmin-interactive site that promotes plasmin docking during cofactor inactivation by cleavage of the heavy chain at Lys36.

**Materials and Methods**

Reagents—Purified recombinant factor VIII preparations and the monoclonal antibody (mAb58.12) recognizing the N terminus of the A1 domain (22) were generous gifts from the Bayer Corp. (Osaka, Japan). The mAb NMC-VIII/5 recognizing the C2 domain was purified as described previously (23). Purified human plasmin (Lys-plasmin) devoid of factor Xa or APC was purchased from Sigma. Ah-plasmin, a catalytically inactive derivative of plasmin in which the active site serine is replaced by dehydroalanine, was prepared as described previously (21). The modified product demonstrated ~1% plasmin activity, and its molecular weight was similar to that of native plasmin. Factor IXa, Glu-Gly-Arg active site-modified factor IXa (EGR-factor IXa), factor Xa, and thrombin were obtained from Hematologic Technologies Inc. (Essex Junction, VT). Horseradish peroxidase-labeled streptavidin were purchased from Amersham Biosciences (Arlbor, MI). DNA fragments encoding the A3 domain were generated by PCR using pMT2/factor VIII as a template and a pair of corresponding primers. The amplified fragments were ligated with pET-20b(+) expression vectors. Plasmid DNA was purified, and the sequence was confirmed by direct sequencing in both directions using Applied Biosystems technology (Foster City, CA). The plasmid was used for transformation of Origami(DE3)pLysS E. coli cells (Novagen), the host strain for the protein expression. The protein was expressed and subsequently purified using a His-Select affinity cartridge (Sigma). Proper folding of the rA3 fragment was confirmed by determination of the affinities for conformationally sensitive anti-A3 mAb CLB-CAGA (30). The cDNA coding the C2 domain sequence of human factor VIII was constructed, transformed into Pichia pastoris cells, and expressed in a yeast secretion system as described previously (31). The rC2 protein was purified by ammonium sulfate fractionation and CM-Sepharose chromatography (Amersham Biosciences) as described previously (31).

Reconstitution of Factor VIIIa Form—The A1/A3C1C2 dimer was reconstituted by mixing 500 nM of A1 form (A1–1–372), A1–1–336, and A1–37–336) and an equimolar amount of A3C1C2 form (1649A3C1C2, 1690A3C1C2, and 1722A3C1C2) overnight at 4 °C in 20 mM HEPES, pH 7.2, 0.3 M NaCl, 25 mM CaCl_2, and 0.01% Tween 20 (13). Furthermore, the reconstituted A1/A3C1C2 dimer forms (100 nM) were reacted with the excess amounts (300 nM) of A2 subunit for >15 min at 22 °C to minimize the A2 subunit dissociation from factor VIIIa. Reaction for >15 min generated the maximal factor VIIIa activity.

Factor Xa Generation Assay—The rate of conversion of factor X to factor Xa was monitored in a purified system (13). Plasmin-catalyzed inactivation of factor VIIIa was performed in HBS buffer (20 mM HEPES, pH 7.2, 0.1 M NaCl, 5 mM CaCl_2, 0.01% Tween 20) containing 0.1% bovine serum albumin and phospholipid vesicles (10 μM). Samples were removed from the mixtures at the indicated times, and plasmin reaction was immediately quenched by the addition of 0.2 μl Pefabloc and dilution. All reactions were performed at 37 °C. Factor Xa generation was initiated by the addition of factor IXa (20 nM) and factor X (400 nM) in the presence of phospholipid (10 μM). The reaction was quenched by addition of EDTA (100 μM). Rates of factor Xa generation were determined at 405 nm using a microtiter plate reader after the addition of chromogenic substrate.
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S-2222 (0.46 mM final concentration). A control experiment showed that the presence of plasmin and Pefabloc in the diluted samples did not affect this assay (data not shown). Factor VIIIa activity was determined as the amount (in nanomoles) of factor Xa generated per min and converted into the amount (in nanomoles) of factor VIIIa.

Experiments assessing the stability of factor VIIIa were performed in the absence of plasmin to determine the rates of factor VIIIa activity loss resulting from the A2 dissociation. At the concentrations employed, ~5% loss of the initial activity was observed over a 20-min time course. Thus, for each time point in this experiment, including plasmin, the observed residual activity was corrected for the contribution of activity loss from the plasmin-independent mechanism.

Cleavage of Factor VIIIa Forms by Plasmin—Human plasmin (4 nM) was added to the reconstituted factor VIIIa forms (100 nM) at 37 °C in HBS buffer containing phospholipid vesicles (10 μM). Samples were obtained at the indicated times, and the reactions were immediately terminated and prepared for PAGE by adding SDS and 2-mercaptoethanol and boiling for 3 min.

Electrophoresis and Western Blotting—SDS-PAGE was performed using 8% gels by the procedure of Laemmli (32). Electrophoresis was carried out at 150 V for 1 h. For Western blotting, the proteins were transferred to a polyvinylidene difluoride membrane at 50 V for 2 h in buffer containing 10 mM CAPS, pH 11, and 10% (v/v) methanol. Proteins were probed using anti-A1 mAb58.12, followed by goat anti-mouse peroxidase-linked secondary antibody (MP Biomedicals, Aurora, OH). The signals were detected using the enhanced chemiluminescence system (PerkinElmer Life Sciences).

Kinetics Measurements Using Real Time Biomolecular Interaction Analysis—The kinetics of factor VIII light chain and plasmin interaction were determined by SPR-based assays using a BIAcore X instrument (BIAcore AB, Uppsala, Sweden) as reported previously (21). Ah-plasmin was covalently coupled (∼7 ng/mm²) to a CM5 sensor chip surface. Association of the ligand was monitored at a flow rate of 20 μl/min for 4 min. The dissociation of bound ligand was recorded over a 4-min period by replacing the ligand-containing buffer with buffer alone. The level of nonspecific binding corresponding to ligand binding to the uncoated chip was subtracted from the signal. The reactions were run at 37 °C. The rate constants for association (kₐ) and dissociation (kₐ) were determined by nonlinear regression analysis using the evaluation software provided by BIAcore AB. Equilibrium dissociation constants (Kd) were calculated as kₐ/κₐ.

ELISA Binding Assays Using Immobilized Ah-plasmin—These assays were performed as reported previously (21). Briefly, Ah-plasmin (200 nM) was coated onto microtiter wells overnight at 4 °C. The wells were blocked with 5% bovine serum albumin for 2 h at 37 °C, and various concentrations of the A3C1C2 subunits were added and incubated for 2 h at 37 °C. Biotinylated anti-C2 NMC-VIII/5 mAb IgG (1 μg) was added to each well, and bound IgG was detected by addition of horseradish peroxidase-labeled streptavidin. The absorbance was measured at 492 nm with a Labsystems Multiskan Multispec microplate reader (Labsystems, Helsinki, Finland). The amount of nonspecific binding of biotinylated IgG, observed in the absence of A3C1C2, was <5% of the total signal, and the amount of specific binding was obtained by subtracting the amount of nonspecific binding of biotinylated IgG.

Data Analysis—All experiments were performed at least three separate times. The parameters and their standard errors are shown. Nonlinear least squares or linear regression analysis was performed by KaleidaGraph (Synergy Reading, PA). The rates of the slope of first several points (within 5 min) in the time course of the plasmin-catalyzed inactivation of factor VIIIa form were fitted to a straight line from linear regression, and the obtained values were expressed as the inactivation rate. All correlation values (r) were >0.99.

Analyses of the interactions between the different forms of A3C1C2 and Ah-plasmin in ELISA were performed by a single-site binding model using Equation 1,

\[ A = \frac{A_{\text{max}} \cdot [S]}{K_d + [S]} \]  

where [S] is the concentration of A3C1C2 form in the solid-phase binding assay; K_d is the dissociation constant; and A_{\text{max}} represents maximum absorbance signal when the site is saturated by the A3C1C2 form.

Data from studies assessing the EGR-factor IXa or A3 synthetic peptide-dependent inhibition of plasmin interaction with A3C1C2 form were fitted by nonlinear least squares regression by using Equation 2,

\[ \% \text{ binding} = \frac{B_{\text{max}} \cdot [\text{A3C1C2 form}]}{K_d \cdot \left(1 + \frac{[L]}{K_i}\right) + [\text{A3C1C2 form}]} + C \]  

where L represents the concentration of EGR-factor IXa or A3 peptide; B_{\text{max}} represents maximum binding; K_d is the dissociation constant for the interaction between the A3C1C2 form and Ah-plasmin; K_i is the apparent inhibition constant for L; and C is a constant for binding of the A3C1C2 form and Ah-plasmin that was unaffected by L.

RESULTS

Plasmin-catalyzed Inactivation of Factor VIIIa Reconstituted with the A11–336 and Various A3C1C2 Forms—We have recently reported that plasmin appears to associate with the light chain of factor VIII to regulate the proteolytic cleavage at Lys^{36} within the A1 subunit by its protease (18). To investigate whether the light chain contributes to plasmin-catalyzed factor VIIIa inactivation because of Lys^{36} cleavage within the A1 subunit by its protease (18). To investigate whether the light chain contributes to plasmin-catalyzed factor VIIIa inactivation because of Lys^{36} cleavage, we first examined the effect on plasmin-catalyzed inactivation of factor VIIIa forms reconstituted with various A3C1C2 forms. Intact A1-(1–372) subunit is proteolyzed at Arg^{336} and Lys^{36} by plasmin, and the former cleavage significantly contributes to the factor VIIIa inactivation (18). Therefore, the A1-(1–336) subunit was utilized instead of the A1-(1–372) to observe the effect of Lys^{36} cleavage alone. Factor VIIIa forms were reconstituted in two-step procedures. The A1-(1–336)/A3C1C2 dimer forms were prepared by reacting the equimolar concentrations (500 nM) of the A1-(1–336) and isolated A3C1C2 subunits (1649A3C1C2,
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Addition of Various A3C1C2 Forms on Plasmin-catalyzed Inactivation of Factor VIIIa with A11–336—To confirm that the N terminus of the A3C1C2 domain is responsible for plasmin-catalyzed inactivation of factor VIIIa, we examined the inhibitory effects by the addition of various A3C1C2 forms on these reactions. The A1-(1–336)/1649A3C1C2 dimer was reconstituted with excess amounts of A2 subunit, and factor VIIIa inactivation was then monitored by the addition of plasmin (1 nM) in the presence of various A3C1C2 in a factor Xa generation assay as described under “Materials and Methods.” The presence of competitors, A3C1C2 forms, did not affect this assay (data not shown). These data are illustrated in Fig. 1 and Table 1.

In the absence of plasmin, the maximal generated factor Xa activity was similar (V_{max}, ~55 nM/min and ~5% activity loss at 20 min, respectively), independent of various A3C1C2 forms (data not shown). The activity of factor VIIIa forms with 1649A3C1C2 subunit was rapidly reduced in a time-dependent manner by the addition of plasmin, similar to that of factor VIIIa with 1690A3C1C2, with similar inactivation rates. The reduced activity reached ~55% of initial activity at 20 min. Of interest, slower inactivation of factor VIIIa with 1722A3C1C2 by plasmin was observed, compared with 1649A3C1C2 and 1690A3C1C2. The activity was reduced by ~30% at 20 min, and the inactivation rate was ~45% that observed with 1649A3C1C2 and 1690A3C1C2. These results suggested that the N terminus of the light chain might be associated with plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336) mediated by cleavage at Lys356.

To further examine that inactivation of factor VIIIa with A1-(1–336) subunit by plasmin attributed to Lys356 cleavage, we repeated the same experiments using factor VIIIa forms reconstituted with the A1-(37–336) subunit, deleting the A1-(1–36). Factor VIIIa forms with the A1-(37–336), and various A3C1C2 subunits were reconstituted by the same approach. Independent of A3C1C2 forms, the maximally generated factor Xa activity loss (decay) of factor VIIIa forms were similar (V_{max} ± ~25 nM/min and ~5% activity loss, respectively) in the absence of plasmin (data not shown). Three factor VIIIa forms with A1-(37–336) were, however, little inactivated (by ~5%) by plasmin even at over a 20-min reaction (Fig. 1, inset). Inactivation rates were similar, supporting the view that plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336) subunit was regulated by Lys356 cleavage.

Addition of Various A3C1C2 Forms on Plasmin-catalyzed Inactivation of Factor VIIIa with A11722A3C1C2—To confirm that the N terminus of the A3C1C2 domain is responsible for plasmin-catalyzed inactivation of factor VIIIa, we examined the inhibitory effects by the addition of various A3C1C2 forms on these reactions. The A1-(1–336)/1649A3C1C2 dimer was reconstituted with excess amounts of A2 subunit, and factor VIIIa inactivation was then monitored by the addition of plasmin (1 nM) in the presence of various A3C1C2 in a factor Xa generation assay as described under “Materials and Methods.” The presence of competitors, A3C1C2 forms, did not affect this assay (data not shown). These data are illustrated in Fig. 2 and Table 1. The addition of the 1649A3C1C2 and 1690A3C1C2 subunits, rA3 domain (residues 1690–2019) (75 nM), each similarly inhibited plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336), with an ~55% decrease in inactivation rate. Furthermore, the addition of a higher concentration (250 nM) of these A3C1C2 forms showed ~90% decreases in inactivation rates. In contrast, in the presence of 1722A3C1C2, the inactivation rates were reduced by ~30 and ~55% at both concentrations, respectively, and the inhibitory effects were less than those of the other three A3C1C2 forms. The presence of rC2 domain (residues 2174–2332) had little effect. Taken together, these findings indicated that the 1690–1721 region of the A3 domain contributed to plasmin-catalyzed inactivation of factor VIIIa through Lys356 cleavage.

Effects of the A3C1C2 Forms on Plasmin-catalyzed Cleavage at Lys356 within the A1 Domain—To evaluate visually the effect of the various A3C1C2 forms on cleavage by plasmin at Lys356 in

TABLE 1
Rates of plasmin-catalyzed inactivation of factor VIIIa forms reconstituted with various A1 and A3C1C2 forms

| A1 form | A11–336 | A137–336 |
|---------|---------|---------|
| 1649A3C1C2 | 7.8 ± 0.2 | 0.2 ± 0.04 |
| 1690A3C1C2 | 7.9 ± 0.4 | 0.2 ± 0.05 |
| 1722A3C1C2 | 3.4 ± 0.2 | 0.2 ± 0.03 |

Inactivation rates were determined by the rate obtained using the straight line fitting to evaluate the rate of plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336) mediated by cleavage at Lys356.
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FIGURE 2. Effects of the addition of A3C1C2 forms on plasmin-catalyzed inactivation of factor VIIIa forms with A1-1–336. Reconstituted A1-(1–336)/1649A3C1C2 dimer (100 nM) reacted with the A2 subunit (300 nM) and phospholipid vesicles (10 μM). Factor VIIIa inactivation was then monitored over time in the presence of various A3C1C2 forms (75 nM) and plasmin (1 nM) using a factor Xa generation assay. The symbols used are as follows: open circles, no A3C1C2; closed circles, 1649A3C1C2; open squares, 1690A3C1C2; closed squares, 1722A3C1C2; open triangles, A3; closed triangles, C2. Plasmin-catalyzed inactivation values were corrected by subtracting the corresponding values for factor VIIIa decay observed in the absence of plasmin. Solid lines were drawn from the linear regression fitting to evaluate the rate of plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336).

TABLE 2
Rates of plasmin-catalyzed inactivation of factor VIIIa with A1-1–336 by the addition of exogenous A3C1C2 forms

| Amounts (nM) | 1649A3C1C2 | 1690A3C1C2 | 1722A3C1C2 | RA3 | rC2 |
|-------------|-------------|-------------|-------------|-----|-----|
| 0           | 8.0 ± 0.4   | 8.0 ± 0.4   | 8.0 ± 0.4   | 8.0 ± 0.4 | 8.0 ± 0.4 |
| 75          | 3.7 ± 0.2   | 3.6 ± 0.2   | 5.8 ± 0.2   | 3.7 ± 0.1 | 8.1 ± 0.2 |
| 250         | 0.7 ± 0.2   | 0.8 ± 0.2   | 0.3 ± 0.3   | 0.6 ± 0.2 | 7.9 ± 0.3 |

Inactivation of factor VIIIa forms was estimated by the rate obtained using the straight line fitting by the first several points (within 5 min) of the data shown in Fig. 2. Data points represent mean ± S.D. values of at least three separate experiments.

Our experiments utilizing the factor VIIIa with various A3C1C2 forms as substrate suggested that the N terminus of the A3 domain contains the predominant region contributing to plasmin-catalyzed cleavage at Lys36. To confirm that the inhibition of plasmin cleavage at Lys36 was mediated by the interaction between the dimer and plasmin, binding experiments with A1/A3C1C2 dimers were further examined. Both of the A1 dimers with 1649A3C1C2 and 1690A3C1C2 bound to Ah-plasmin with similar affinities (Kd, 26.9 and 32.9 nM, respectively), and these affinities were ~2.5-fold higher than those with either form of A3C1C2 alone. This somewhat higher affinity may have been derived from a synergistic effect of the two binding domains and/or a conformational change resulting from interaction with the two domains. However, the A1/1722A3C1C2 dimer bound with an ~4-fold weaker affinity (Kd, 124 nM) than with the other dimers, and the isolated A1 alone bound very poorly (Kd, ~200 nM). The findings therefore suggest that residues 1690–1721 are indeed involved in a plasmin-binding site for cleavage at Lys36.
We further evaluated the interaction between the A3C1C2 subunit and plasmin using a solid-phase binding assay in which Ah-plasmin was immobilized onto microtiter wells. For these experiments, varying amounts of the A3C1C2 subunits were reacted with 200 nM immobilized Ah-plasmin. Bound factor VIII was detected using biotinylated anti-C2 NMC-VIII/5 mAb. Control experiments confirmed that this mAb did not affect the reaction between plasmin and the light chain (data not shown). Results are presented in Fig. 4B. Reactions between the A3C1C2 forms and Ah-plasmin yielded saturable binding curves, well fitted using a single-binding site model. This method is not based on a true equilibrium binding assay, however, and the $K_d$ values obtained represent an apparent $K_d$ value for the interactions. The results obtained for the $^{1649}$A3C1C2 and the $^{1690}$A3C1C2 subunits binding to Ah-plasmin were $97 \pm 10$ and $89 \pm 9$ nM, respectively, similar to those obtained in the SPR-based assays. However, the binding affinity ($265 \pm 21$ nM) for the $^{1722}$A3C1C2 subunit was $3$-fold lower than that for the two other forms. Again, the C2 domain failed to bind. Overall, the affinities determined using the ELISA-based assays were in good agreement with those obtained in the SPR-based analyses, and the findings were mutually supportive.

**Effect of Factor IXa on A3C1C2 Form Binding to Ah-plasmin**—Our solid-phase binding assays demonstrated that the $^{1722}$A3C1C2 subunit bound to Ah-plasmin, albeit with relatively weak affinity. These data led us to speculate on the presence of another plasmin-interactive site(s) in the A3 domain. We have recently shown that factor IXa inhibited plasmin-catalyzed inactivation of factor VIIIa, and we identified overlapping binding sites for plasmin and factor IXa in the A2 domain of factor VIII(a) (21). It is known that residues 1804–1818 in the A3 domain of factor VIII interact with factor IXa on phospholipid surfaces (30), and we therefore investigated the inhibitory effect of factor IXa on the light chain binding to Ah-plasmin in our ELISA method. The $^{1649}$A3C1C2 subunit (120 nM) was mixed with varying amounts of active site-modified EGR-factor IXa for 1 h prior to incubation with Ah-plasmin (200 nM) immobilized onto microtiter wells. Bound $^{1649}$A3C1C2 was detected using biotinylated anti-C2 mAb. EGR-factor IXa blocked $^{1649}$A3C1C2 subunit binding to Ah-plasmin by $40\%$ at the maximum concentrations employed (500 nM), and this effect was dose-dependent (Fig. 5). The apparent $K_i$ value for factor IXa obtained from curve fitting was $160 \pm 51$ nM. The association between the factor VIII light chain and factor IXa is surface-dependent, however (25), and hence the effects of factor IXa in our current binding studies were also examined in the presence of phospholipid. EGR-factor IXa blocked binding in a dose-dependent manner, and the inhibitory effect ($\sim60\%$) was...
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Figure 4. Direct binding of the A3C1C2 forms to Ah-plasmin. A, SPR-based assay. Various concentrations of 1649A3C1C2 (panel a), 1722A3C1C2 (panel b), and rA3 (panel c) were added to Ah-plasmin (7 ng/mm²) immobilized on the sensor chip for 4 min, followed by a change of running buffer for 4 min as described under “Materials and Methods.” Lines 1–4 show response curves for 1649A3C1C2 (50, 100, 150, and 180 nM, respectively); lines 5–8 show curves for 1722A3C1C2 (50, 100, 200, and 300 nM, respectively); and lines 9–11 show curves for rA3 (60, 120, and 240 nM, respectively). B, ELISA-based assay. Various concentrations of 1649A3C1C2 (open circles), 1690A3C1C2 (closed circles), 1722A3C1C2 (open squares), and rC2 (closed squares) were incubated with Ah-plasmin (200 nM) that had bound to microtiter wells. Bound A3C1C2 forms were detected using biotinylated anti-C2 (NMC-VIII/5)mAb IgG. Absorbance values were plotted as a function of the concentration of the A3C1C2 form, and the data were fitted using Equation 1 according to the single site binding model described under “Materials and Methods.”

Table 3

| Ligands | kₐ | Kd | Kd* |
|---------|----|----|-----|
| Factor VIII | 26.3 ± 0.8 | 0.8 ± 0.1 | 3.1 |
| 1649A3C1C2 | 2.9 ± 0.5 | 2.0 ± 0.7 | 68.2 |
| 1690A3C1C2 | 4.9 ± 1.3 | 2.9 ± 0.5 | 60.3 |
| 1721A3C1C2 | 4.5 ± 2.3 | 7.9 ± 2.2 | 176 |
| rA3 | 3.9 ± 0.3 | 1.7 ± 0.3 | 44.2 |
| C2 | ND | ND | |
| A1/1649A3C1C2 | 7.8 ± 2.4 | 2.1 ± 0.6 | 26.9 |
| A1/1690A3C1C2 | 7.0 ± 1.6 | 2.3 ± 0.5 | 32.9 |
| A1/1722A3C1C2 | 4.9 ± 1.1 | 6.1 ± 1.2 | 124 |
| A1 | 5.2 ± 0.4 | 10.9 ± 0.9 | 208 |

* Values were calculated as kₐ/kₐ*

ND indicates not determined.

greater than that in the absence of phospholipid. Furthermore, the Kₐ value (10.5 ± 1.5 nM) obtained from curve fitting was ~15-fold lower in the presence of phospholipid than that in its absence. These values were similar to the Kₐ values for light chain and factor IXa association in the presence and absence of phospholipid determined in a fluid-phase model (25).

It was evident, however, that inhibition of the interaction between 1649A3C1C2 and plasmin mediated by EGR-factor IXa was only partial (~60%), and to exclude the possibility that the 1690–1721 region in A3 contributed to the binding reaction in these experiments, we repeated the assays using the 1722A3C1C2 subunit (240 nM), instead of the nontruncated A3C1C2. EGR-factor IXa blocked the binding of 1722A3C1C2 subunit to Ah-plasmin by ~95 and ~80% in the presence and absence of phospholipid, respectively, at the maximum concentrations employed (Fig. 5). The calculated Kₐ values for factor IXa obtained from curve fitting were 9.8 ± 1.1 and 179 ± 23 nM, respectively. This significant competitive reaction between factor IXa and 1722A3C1C2 for binding to plasmin therefore suggested that an alternative plasmin-interactive site in the factor VIII light chain, within residues 1804–1818 in the A3 domain, might overlap or juxtapose the factor IXa-binding site.

Binding of the 1690–1705 and 1804–1818 Peptides to Ah-plasmin—Further experiments focused on two distinct regions, residues 1690–1721 and 1804–1818, in the A3 domain, responsible for plasmin docking. We have recently demonstrated, in binding-inhibition assays using the plasmin-specific competitor 6-aminohexanoic acid, which directly binds to LBS (21), that the association between plasmin and the factor VIII light chain is mediated by an LBS-dependent mechanism. The known amino acid sequence of the A3 domain indicates that the clustered lysine residues are located in residues 1690–1705 (Lys1693 and Lys1694) and 1804–1818 (Lys1804, Lys1808, Lys1813, and Lys1818), and these two regions are highly conserved in other species (Fig. 6). Therefore, to confirm that these lysine residues confer interactive sites for plasmin, two synthetic peptides derived from sequences 1690–1705 and 1804–1818 were prepared and examined with Ah-plasmin in competitive inhibitory ELISA.

The 1649A3C1C2 subunit (120 nM) was incubated with immobilized Ah-plasmin (200 nM) in the presence of increasing concentrations of A3 peptides as described under “Materials and Methods.” The results are shown in Fig. 7A. Both the 1690–
1705 and 1804–1818 peptides blocked the binding of \(^{1649}A3C1C2\) to Ah-plasmin by \(~55\%\) and \(~40\%\), respectively, at the maximum concentration employed (500 \(\mu M\)). The \(K_i\) values obtained from curve fitting for the 1690–1705 and 1804–1818 peptides were similar (18.5 ± 3.2 and 20.6 ± 1.5 \(\mu M\), respectively). Furthermore, an equimolar mixture of both peptides yielded an enhanced inhibitory effect (by \(~65\%\)) compared with the individual peptides alone, suggesting an additive effect of the two peptides. Similar experiments using the \(^{1722}A3C1C2\) subunit (240 \(\mu M\)) demonstrated that the 1804–1818 peptide blocked the binding of \(^{1722}A3C1C2\) to Ah-plasmin by \(~75\%\) with a \(K_i\) of 17.3 ± 2.1 \(\mu M\) (Fig. 7B). As expected, the 1690–1705 peptide had little inhibitory effect on this binding.

To further investigate the importance of the lysine residues and/or structural alignments in the two sequences (1690–1705 and 1804–1818), we prepared peptides with scrambled sequences of the same composition and synthesized peptides where the lysine residues were replaced by alanine (1690–1705Ala and 1804–1818Ala, respectively). An equimolar mixture of the scrambled peptide (200 \(\mu M\)) did not inhibit the \(^{1649}A3C1C2\) binding to Ah-plasmin (data not shown). Furthermore, \(1690–1705\) Ala and 1804–1818Ala did not significantly inhibit the binding of \(^{1649}A3C1C2\) or \(^{1722}A3C1C2\) subunits to Ah-plasmin, respectively (Fig. 7B, inset). These results indicated that the lysine residues in the A3 domain, within sequences 1804–1818 and 1690–1705, contribute to the plasmin-interactive sites in the light chain.

**Effects of A3 Peptides on Plasmin-catalyzed Cleavage at Lys\(^{36}\) in A1** —To further confirm the functional role of residues 1690–1705 and 1804–1818 in plasmin binding, we examined the effects of the A3 peptides on Lys\(^{36}\) cleavage by plasmin. The A1-(1–336)/\(^{1690}A3C1C2\) dimer (100 \(\mu M\)) was reconstituted with the A2 subunit (300 \(\mu M\)), followed by the addition of plasmin (4 \(nM\)) and phospholipid vesicles (10 \(\mu M\)) in the presence of A3 peptides (150 \(\mu M\)). Lys\(^{36}\) cleavage in A1-(1–336), representing the disappearance of A1-(1–336), fragment, was analyzed by Western blotting using an anti-A1 (58.12) IgG in a timed course reaction (Fig. 8A). Change of band density, was evaluated by scanning densitometry (Fig. 8B). Compared with the absence of A3 peptide (Fig. 8A, panel a), both individual 1690–1705 and 1804–1818 peptide slightly delayed the disappearance of A1-(1–336) (panels b and c, respectively). Furthermore, in the presence of equimolar amounts of mixture of both peptides, the disappearance of A1-(1–336) was markedly slow, and the band could be observed by \(~50\%\) even at 15 min after adding of plasmin, supportive of significant inhibition of plasmin-induced Lys\(^{36}\) cleavage.
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![Image](49x394 to 407x733)

**FIGURE 8.** Effects of A3 peptides on plasmin-catalyzed cleavage at Lys\(^{36}\) of factor VIIIa with A1\(^{1–336}\). (A), reconstituted A1\(^{-}(1–336)/1649A3C1C2\) dimer (100 nM) reacted with the A2 subunit (300 nM) and phospholipid vesicles (10 μM). Factor VIIIa with A1\(^{-}(1–336)\) was incubated with plasmin (4 nM) in the presence of 150 μM A3 peptide (panel a; no peptide, panel b; 1690–1705 peptide, panel c; 1804–1818 peptide, panel d; equimolar mixture of 1690–1705 and 1804–1818 peptides) for the indicated times. Samples were analyzed on 8% gels from blotting data obtained from A. Band density of A1\(^{-}(1–336)\) at time 0 was regarded as 100%. The symbols used are as follows: open circles, no peptide; closed circles, 1690–1705 peptide; open squares, 1804–1818 peptide; closed squares, both peptides. C shows the effect of 1690–1705/1804–1818 peptide on plasmin-catalyzed cleavage at Lys\(^{36}\). Various concentrations of 1690–1705/1804–1818 peptide (open circles) or 1690–1705-Ala/1804–1818Ala (closed circles) were mixed with factor VIIIa with A1\(^{-}(1–336)\), followed by incubation with plasmin (4 nM) for 8 min. Samples were analyzed by Western blotting as above experiments. Density of A1\(^{-}(1–336)\) before or after addition of plasmin in the absence of peptide was regarded as 100 or 0%, respectively.

Furthermore, the inhibitory effect of the mixture of 1690–1705 and 1804–1818 peptides showed the dose-dependent manner, and the inhibition effect was ~80% at maximum concentration employed (300 μM) (Fig. 8C). A control experiment using mixture of 1690–1705-Ala and 1804–1811-Ala peptides showed no significant inhibition (by ~10%). These findings were in keeping with the concept that both the 1690–1705 and 1804–1818 regions in A3 were essential for plasmin docking during factor VIIIa inactivation induced by Lys\(^{36}\) cleavage, although each region interacted separately with the protease.

**DISCUSSION**

Plasmin inactivates factor VIIIa by proteolysis at specific sites within the heavy and light chains of the activated molecule. We have recently demonstrated that Arg\(^{184}\) in the A2 domain of factor VIII significantly contributes to plasmin docking for proteolytic cleavage at Arg\(^{336}\) in the A1 subunit during enzyme-catalyzed factor VIIIa inactivation (21). Our present study further revealed that proteolytic cleavage at Lys\(^{36}\) in the A1 domain is supported by plasmin-interactive sites located in the A3 domain of the light chain. This conclusion is based on several novel findings using well-established models. (i) The rate for plasmin-catalyzed inactivation of factor VIIIa reconstituted with A1-(1–336) and 1721A3C1C2, reflecting cleavage at Lys\(^{36}\), was reduced by ~55% compared with those with 1649A3C1C2 and 1690A3C1C2. Furthermore, plasmin-catalyzed inactivation of factor VIIIa with A1-(1–336) was significantly inhibited by the addition of exogenous 1690A3C1C2 and rA3, but to a much lesser extent by 1721A3C1C2. (ii) Plasmin cleavage at Lys\(^{36}\) in factor VIIIa with A1\(^{-}/1722A3C1C2\) dimer was significantly slower than that with A1/1690A3C1C2. (iii) The 1721A3C1C2 subunit bound to Ah-plasmin with an ~3-fold weaker affinity than the 1649A3C1C2 or 1690A3C1C2. The rA3 domain bound to Ah-plasmin with similar affinity as 1649A3C1C2, whereas the rC2 domain failed to bind, although a contributory role for the C1 domain in this plasmin binding remains to be completely excluded. (iv) Factor IXa (that binds to the 1804–1818 region) or the A3 peptides (residues 1690–1705 and 1804–1818) competed for light chain binding to Ah-plasmin. (v) The presence of both A3 peptides inhibited plasmin-catalyzed Lys\(^{36}\) cleavage of factor VIIIa with A1-(1–336) by ~80%. These identified amino acid residues 1690–1705 and 1804–1818 within the A3 domain as essential to plasmin docking for proteolytic cleavage at Lys\(^{36}\).

We observed the partial inactivation of reconstituted factor VIIIa activity with A1-(1–336) or A1-(37–336) by plasmin in this study. Factor Xa generation assay showed that the reconstituted factor VIIIa with truncated A1 forms (A1-(1–336) and A1-(37–336)) retained significant activity, whereas the one-stage clotting assay completely lost these activities (13, 18). This discrepancy can be explained as we described in an earlier report (13). In the factor Xa generation, a Km value for factor X using factor Xase with native factor VIIIa is ~40 nM, whereas this value is increased 5-fold (Km ~200 nM) for that with factor VIIIa with A1-(1–336) or A1-(37–336). Because the typical factor Xa generation assay uses concentrations (400 nM) of substrate that yield near V\(_{\text{max}}\) reaction rates, the rates are inde-
Loss of activity resulting from cleavage at Lys36 is associated with Arg336 and Lys36 in the A1 domain (33). An earlier study stage clotting assay. A1-(37–336)/A2/A3C1C2 loses the factor VIIIa activity in one-stage clotting assay, and consequently the A1-(1–336)/A2/A3C1C2 or truncated A1 forms) markedly depresses the rate of factor Xase catalyzed cleavage at Lys36, however, is governed by the factor VIII light chain-Ah-plasmin interaction within the A3 domain.

Both factor Xa and plasmin inactivate factor VIIIa by cleavage at Arg336 and Lys36 in the A1 domain (33). An earlier study by Nogami et al. (13, 33) revealed that proteolysis by factor Xa at these sites in A1 correlated with inactivation of cofactor function. Close analysis of A1-(1–336) and A1-(37–336) sub-units demonstrated inactivation of native factor VIIIa activity by ~30 and ~60%, respectively, in factor Xa generation assay. Loss of activity resulting from cleavage at Lys36 is associated with an altered molecular conformation that markedly affects the affinity of the A1 subunit for A2 (13). Similarly, factor Xa maximal generation with A1-(1–336) ~55 and ~25 nm/min, respectively, supporting that the N terminus of the A3 domain is unlikely related to associate with the A1 domain. The cleavage rate by factor Xa at Lys36 was >10-fold lower than that at Arg336 (33). Factor Xa-catalyzed cleavage at Lys36, however, is governed by the mid-section containing Lys1693, Lys1808, Lys1813, and Lys1818.

We have now identified plasmin-interactive sites in these two regions by competition experiments using synthetic peptides composed of residues 1690–1705 and 1804–1818. Random peptides with scrambled sequences and peptides in which lysine was substituted with alanine failed to compete in the light chain-Ah-plasmin interactions. Our results therefore suggested that lysine residues and structural arrangements within both regions contributed significantly to plasmin docking in the A3 domain.

As noted above, both individual A3 peptides (1690–1705 and 1804–1818) partially inhibited (by ~50%) the binding of 1649A3C1C2 to plasmin, and these effects appeared to be additive. In contrast, however, only mixtures of both peptides significantly inhibited plasmin-catalyzed cleavage of functional factor VIIIa at the Lys35 site. These findings were similar to those observed using functional and binding experiments with the A1-(337–372) region, in particular by Asp361–363 residues (34), and proteolysis at Arg336 might interfere with factor Xa docking to the 337–372 region. Cleavage at the identical site by plasmin is regulated by the A3 domain, not by A1. Therefore, we can speculate that Lys36 cleavage by plasmin may be faster than that by factor Xa, although the kinetics on cleavages at both sites by its protease remains to be determined.

The LBS in plasmin consists basically of a cationic center (Lys35 and Arg71), an anionic center (Asp35 and Asp71), and a hydrophobic core (Trp62, Phe64, Trp72, and Tyr74) (35). The LBS facilitates interaction with substrates and proteins by hydrogen bond and/or ion pair interaction with the cationic or anionic center and van der Waals electronic interaction with the hydrophobic core (36). Recently, we reported that 6-aminohexinoic acid, a specific inhibitor of LBS, blocked interaction between plasmin and the factor VIII light chain by ~90% (21), strongly suggesting that this mechanism is LBS-dependent. Two lysine-rich regions reside in the A3 domain at the N terminus containing Lys1804 and Lys1808, and the Lys1818.

FIGURE 9. Three-dimensional presentation of putative plasmin binding regions in the factor VIII A3 domain based on the ceruloplasmin-based triple A domain model. The A domains (A1-A2-A3 domain) are shown in ribbon format with α-helix in red and β-sheets in yellow. The cleavage sites at Lys36 (magenta) and Arg336 (gray) are shown in space-filling format. The A domains residues (Lys1693, Lys1804, Lys1808, Lys1813, and Lys1818) within 1690–1705 and 1804–1818 regions that participate in plasmin interaction within the A3 domain are shown in space-filling format (light blue). The A2 residues (Lys377, Lys466, Arg471, and Arg484) that contribute to plasmin interaction within the A2 domain (21) are shown in space-filling format (green).
Plasmin-interactive Sites in the Factor VIII Light Chain

The factor VIII domain model based on homology with ceruloplasmin (37) describes putative lysine residues, Lys1693, Lys1694, Lys1803, Lys1808, Lys1813, and Lys1818, within residues 1690–1705 and 1804–1818 of the A3 domain, arranged spatially adjacent and exposed on the A3 surface (Fig. 9). This provides an extended surface for plasmin binding, but it is far removed from the cleavage site of Lys36 within the A1 domain. Glu-plasminogen contains five kringle domains and a catalytic domain in a closed form with a radius of gyration of ~40 Å. Conversion to Lys-plasmin by plasminogen activator, however, induces a marked conformational change, termed an open form, with greater flexibility and increased radius of gyration (~60 Å). The dramatic structural alteration enhances high affinity enzyme interactions with protein ligands (38, 39). Molecular mechanisms of this nature could explain the functional relationship between the remote plasmin-interactive sites in A3 and the proteolytic cleavage site at Lys36. In addition, structural modification of the factor VIII light chain bound to plasmin might preferentially support catalysis at Lys36. Interestingly, comparison of amino acid sequences among human, porcine, murine, and canine factor VIII molecules reveals that the two A3 regions are well conserved, supporting the concept that both binding domains are fundamental for protein interactions.

Our study indicated that these functionally essential subunits, involving residues 1690–1705 and 1804–1818 in the A3 domain of factor VIII, constitute a highly basic spacer region exposed on the surface contributing to interaction with plasmin. The 1804–1818 sequence is known to participate in the interaction with at least three proteins, including factor IXa (30), alloantibody inhibitors from multiply transfused hemophilia A patients (40), and low density lipoprotein receptor-related protein that mediates clearance of factor VIII from the circulation (41). Therefore, it is clear that these particular residues play a significant role in the modulation of coagulation reactions by up- and down-regulation of factor VIIIa cofactor function. The 1690–1705 sequence does not seem to have been reported to be involved in other protein interactions.

EGR-factor IXa inhibited factor VIII light chain binding to Ah-plasmin in a solid-phase assay. Furthermore, the presence of phospholipid enhanced the inhibitory effect of EGR-factor IXa and resulted in an ~15-fold decrease of the $K_i$ value for EGR-factor IXa binding. These $K_i$ values were consistent with the $K_i$ values obtained for the light chain-factor IXa interactions observed using a steady-state fluorescence energy transfer fluid assay (25). However, binding stoichiometry for A3C1C2 form and EGR-factor IXa in the presence of phospholipid was unexpectedly different in competition assays. Several possibilities for this reason may be raised. A solid-phase ELISA is a limited assay and is not based on an equilibrium binding.

Because the binding affinity for the A3C1C2 of Ah-plasmin is much lower (~15-fold) than that of factor IXa, this difference may affect the competitive inhibition. Furthermore, the A3C1C2 itself bound to factor IXa on the phospholipid membranes may partially affect competitive inhibition, for instance the influence of plasmin binding because of conformational alteration, etc. In addition, the possibility of steric hindrance because of the EGR molecule of its competitor cannot be excluded. However, the precise reason is unclear at present.

A plasmin-interactive site has been recently identified in the A2 domain within and/or close to a factor IXa-interactive site (21), and in our studies, peptide 1804–1818, representing a factor IXa-interactive site, similarly inhibited the interaction between light chain and Ah-plasmin. Moreover, EGR-factor IXa blocked plasmin-catalyzed inactivation of factor VIIIa in a clotting assay. Therefore, overall the data support that factor IXa, bound to factor VIIIa on the phospholipid surface, might restrict plasmin-induced inactivation of factor VIIIa by occupying the plasmin-interactive sites in the A2 and A3 domains. This mechanism by which factor IXa protects factor VIIIa from plasmin-catalyzed inactivation appears to be similar to that observed with APC-catalyzed inactivation of factor VIIIa (26).

In conclusion, the extended surface of the factor VIII A3 domain, centered on lysine residues in both the 1690–1705 and 1804–1818 regions, contributes to a unique plasmin-interactive site that facilitates plasmin docking during cofactor inactivation by cleavage at Lys36. Our present results further suggest that factor VIII cofactor function is regulated by more complex mechanisms than previously anticipated.

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