The C-terminal acidic region in the A1 domain of factor VIII facilitates thrombin-catalyzed activation and cleavage at Arg^{372}

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Essentials

- Factor (F)VIII is activated by thrombin-catalyzed cleavage at three Arg sites.
- Thrombin-interactive sites responsible for cleavage at Arg^{372} in A1 residues remain unknown.
- Residues 346-349 provided a thrombin-interactive site responsible for cleavage at Arg^{372}.
- A hirugen-hybrid A1 mutant was more susceptible to thrombin cleavage at Arg^{372} than wild type.

Summary

***Background*:** Factor (F)VIII is activated by thrombin-catalyzed cleavage at three sites. Previous reports indicated that the A2 domain contained thrombin-interactive sites responsible for cleavage at Arg^{372}. We have also found that the A1 domain of FVIII bound to the anion-binding exosite I of thrombin. The present study focused, therefore, on thrombin interaction with A1 residues 337-372 containing clustered acidic and hirugen-like sequences. **Aim:** To identify specific thrombin-interactive site(s) within the A1 acidic region of FVIII. ***Methods and Results*:** The synthetic peptide of residues 337-353 with sulfated Tyr^{346} (337-353S) significantly blocked thrombin-catalyzed FVIII activation and cleavage at Arg^{372}, whilst a corresponding peptide of residues 354-372 had no significant effect. Treatment with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to cross-link thrombin and 340-350S suggested that the 344-349 clustered acidic region was involved in thrombin interaction. Alanine-substituted FVIII mutants, Y346A and D347A/D348A/D349A, depressed thrombin-catalyzed activation and cleavage at Arg^{372}, with peak activation at ~50% and cleavage rates of ~10-20% compared to wild type (WT). The peak level of thrombin-catalyzed activation and the cleavage rate at Arg^{372} using FVIII mutants with 337-346 residues substituted with hirugen-sequences (MKNNEEAEDY337-346GDFEEIPEEY) were ~1.5- and ~2.5-fold of WT, respectively. Surface plasmon resonance-based analysis demonstrated that the K_d for active-site modified thrombin interactions using Y346A and D347A/D348A/D349A mutants was ~3-6-fold higher than that of WT, and that the hirugen-hybrid mutant facilitated association kinetics ~1.8-fold of WT. ***Conclusion*:** Residues 346-349 with sulfated Tyr provided a thrombin-interactive site responsible for activation and cleavage at Arg^{372}. A hirugen-hybrid A1 mutant showed more efficient thrombin-catalyzed cleavage at Arg^{372}.

Key words: Factor VIII, Thrombin, Mutant Protein, Hirudin, Protein-Protein Interaction Domain

Introduction

Factor (F)VIII, a plasma protein deficient or defective in the severe inherited bleeding disorder, hemophilia A, functions as a procofactor for the serine protease, FIXa, in phospholipid surface-dependent conversion of FX to FXa [1]. FVIII is synthesized as a multi-domain, single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa [2,3]. FVIII is processed during secretion by furin cleavage, generating a variably proteolyzed sized heavy chain (HCh) consisting of the A1-A2-B domains, linked to a light chain (LCh) consisting of the A3-C1-C2 domains [2-4].

Thrombin functions as a positive feedback amplifier in the coagulation cascade by specific cleavage of FVIII [5]. The pro-cofactor is converted to the active-cofactor, FVIIIa, by limited proteolysis. Proteolysis occurs in the HCh at Arg^{372} separating the A1-A2 domains, and at Arg^{740} at the A2-B domain junction to generate the 50-kDa A1 and 40-kDa A2 subunits. The LCh is cleaved at the N-terminal Arg^{1689} to release a 1649-1689 residue-fragment from the 80-kDa subunit generating 70-kDa polypeptide [5]. Cleavage at Arg^{372} site exposes a functional FIXa-interactive site within the A2 subunit that is cryptic in the unactivated molecule [6]. Cleavage at Arg^{1689} liberates the cofactor from its carrier protein, von Willebrand factor [7], and contributes to overall specific activity of the cofactor [8,9].

The potential pro-thrombotic activity of thrombin is controlled by regulatory surface loops and by two anion-binding exosites (ABE-I and ABE-II). These ABEs are characterized by a high density of solvent-exposed basic residues, and play crucial roles in regulating substrate specificity. ABE-I binds to fibrinogen [10], and involves the acidic residue-rich tail (residues 54-65, termed hirugen) associated with hirudin interactions [11,12]. ABE-II binds to heparin [13] and serpins [14]. Both ABEs are also involved in the activation and cleavage of FV and FVIII [15]. Studies using 53 thrombin mutants with ABE residues substituted with alanine, indicated that the ABE-I binding sites responsible for FVIII activation involved cleavages at Arg^{372} and Arg^{1689}, whilst ABE-II appeared to be essential for regulating proteolysis at Arg^{372} and Arg^{740} [16].

Less complete information is available, however, on thrombin-interactive sites in FVIII. We have previously identified some thrombin-interactive sites in the different domains of FVIII. The C2 domain was shown to govern thrombin cleavage at Arg^{1689} during pro-cofactor activation [17]. The acidic region comprising residues 389-394 in A2 interacted with thrombin via ABE-II and

supported cleavage at Arg^{740} [18]. The clustered basic residues within the 484-509 region in A2 contributed to thrombin-catalyzed cleavage at Arg^{372}, ABE-independently [19]. In addition, Fay and colleague reported that the clustered acid residues within the 720-725 region at the C-terminus of A2 seemed to participate in thrombin-catalyzed cleavage at Arg^{372} [20]. However, further interactions involving cleavage at Arg^{372} remained to be fully identified.

Hirudin is a 65-residue polypeptide inhibitor of α-thrombin derived from the leech *Hirudo medicinails* [21]. Hirudin binds to α-thrombin with exceedingly high affinity [21], and residues 54-65 (GDFEEIPEEYLQ) in the C-terminus of hirudin, termed hirugen, participate in this binding significantly [22]. Sulfation at Tyr^{63} in hirudin contributes to its interaction with thrombin by increasing affinity ~10-fold [23]. We previously reported that hirudin competitively blocked the interaction of the FVIII A1 domain with thrombin dose-dependently, strongly indicating that thrombin might interact with this subunit in an ABE-I-related mechanism [18]. Notably, the amino acid sequence of A1 acidic region appears to be similar to that of hirugen. In the present study, therefore, we focused on the N-terminus region (residues 337-353; MKNNEEAEDYDDDLTDS) within the acidic region of A1, involving both the clustered acidic residues and the hirugen-like sequence, including sulfation at Tyr^{346}, to characterize the thrombin-interactive region responsible for activation and cleavage at Arg^{372}.

Materials and Methods

Reagents - Purified recombinant FVIII preparations were generous gifts from Bayer Corp. Japan (Osaka, Japan). Human α-thrombin, FXa, Phe-Pro-Arg-chloromethylketone (PPA-ck), and recombinant hirudin (Calbiochem, San Diego, CA), peroxidase-conjugated streptavidin (Sigma-Aldrich, St Louis, MO), and EDC (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride, Pierce; Rockford, IL) were purchased from the indicated vendors. A mAbJR8 recognizing the C-terminus (residues 563-740) in A2 was obtained from JR Scientific Inc. (Woodlamd, CA) [24,25]. The synthetic peptides corresponding to residues 337-353 and 354-372, and that corresponding to 337-353 with sulfated Tyr^{346} (337-353S), and biotinylated and non-biotinylated peptide with residues 340-350 with sulfated Tyr^{346} (340-350S) were prepared by Biosynthesis (Lewisville, TX). All synthetic peptides demonstrated >95% purity.

Mutagenesis, expression, and purification of mutated FVIII - B domain-deleted FVIII (lacking Q744-S1637), rFVIII wild type (WT), mutated products Lys338Glu (K338D), Asn340Glu

(N340E), Glu342Ile (E342I), Asn340Glu/Glu342Ile (N340E/E342I), Glu344Ala/Asp345Ala (E344A/D345A), Tyr346Ala (Y346A), Asp347Ala/Asp348Ala/Asp349Ala (D347A/D348A/D349A), and the FVIII hybrid containing the hirugen sequence (MKNNEEAEDY337-346GDFEEIPEEY) were stably expressed in baby hamster kidney cells and purified [26]. The "legacy" numbering for FVIII residues was used for consistency with the earlier study. Resultant FVIII forms were typically >90% pure as judged by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and staining with GelCode® Blue-Stain Reagent (Pierce), with albumin representing the major contaminant. FVIII concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) using two anti-FVIII mAbs. Samples were quick-frozen and stored at −80 °C.

Preparation of active site-modified PPA-thrombin - Thrombin (15 μM) was incubated overnight at 4 °C with a 10-fold molar excess of PPA-ck in 50 mM HEPES, pH 7.2, 0.1 M NaCl, and 0.01% Tween 20 (HBS-buffer) containing 5 mM $CaCl_2$. Unbound PPA-ck was removed by extensive dialysis at 4 °C in 20 mM HEPES, pH 7.2, and 0.1 M NaCl. Chromogenic assays using S-2238 (Chromogenix, Diapharma Inc., West Chester, OH) demonstrated less than 0.1% residual thrombin activity (data not shown).

Clotting assays of FVIII activity - FVIII coagulation activity was measured in one-stage clotting assays using commercial FVIII-deficient plasma (Sysmex, Kobe, Japan) with a STart4 Hemostasis Analyzer□ (Diagnostica Stago, Asnieres, France). All reactions were performed at 37 °C. FVIII (10 nM) was activated by the addition of thrombin (0.4 nM) in HBS buffer containing 5 mM $CaCl_2$ and 0.01% bovine serum albumin. Aliquots were removed from the mixtures, and thrombin was rapidly inactivated by the addition of hirudin (1 U/ml) and 2,500-fold dilution [19]. FVIII activity in FVIII-WT and mutants was also determined using a chromogenic, FXa generation assay (Chromogenix). The presence of thrombin and hirudin in the diluted samples did not affect FVIII activity in these assays (data not shown).

FVIII cleavage by thrombin - Thrombin (2 nM) was added to FVIII (50 nM) at a 1:25 molar ratio in HBS-buffer at 37 °C. Samples were obtained at the indicated times, and the reactions were immediately terminated and prepared for SDS-PAGE by adding SDS under reducing conditions and boiling for 3 min.

Electrophoresis and Western blotting - SDS-PAGE was performed at 150 V for 1 hr, using 8 % gels followed by Western blotting using a Bio-Rad mini-transblot apparatus (BIO-RAD, Hercules, CA) at 100 V for 1 hr. Protein bands were probed using an anti-A2 mAbJR8, followed by goat anti-mouse peroxidase-linked secondary antibody. Signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA). Densitometric scans were quantitated using Fusion Solo S™ (Vilber Lourmat, Collegien, France).

EDC cross-linking assay - Zero-length cross-linking reactions with EDC were examined in HBS buffer containing 1 mM $CaCl_2$ at 37 °C [19]. Thrombin (300 nM) was incubated with various amounts of biotinylated 340-350S for 1 hr. EDC (500 μM) was added to the mixtures, followed by a further 45 min-incubation. After the addition of sample buffer under reducing conditions and boiling, the reactant mixtures were subjected to electrophoresis on 15% gels. Proteins were transferred to PVDF membranes, and were probed using peroxidase-conjugated streptavidin. Regarding the N-terminal sequence of cross-linked product, thrombin (1 μM) was incubated with 340-350S (1 mM) in the presence of EDC (5 mM). The reactant mixtures prepared under reducing conditions were subjected to SDS-PAGE, followed by transferring to membrane. The blots of cross-linked products were stained with 0.2% Coomassie brilliant blue in 40% methanol and 10% acetic acid for 10 min. The samples from individual bands were loaded into the cartridge of ABI492 Prociss HT protein sequencer, rinsed with ethyl acetate, dried, and the N-terminal sequence of cross-linked product was analyzed commercially by Bio-Synthesis Inc. (Lewisville, TX).

Surface-plasmon resonance (SPR)-based assay - The kinetics of FVIII and thrombin interaction were determined by SPR based assays at 37 °C using Biacore T200™ instrument (Cytiva, Sheffield, UK). PPA-thrombin was covalently coupled to the CM5 sensor chip at a coupling density of 1,770 response units (RU). Ligand binding was monitored in running buffer (20 mM HEPES, 1 mM $CaCl_2$, 0.005% polysorbate20) for 2 min at a flow rate 10 μl/min. The dissociation of bound ligand was recorded over a 2-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 surface on the sensor chip was regenerated by washing 50 mM NaOH. The rate constants for association (k_{asso}) and dissociation (k_{diss}) were determined by nonlinear regression analysis using evaluation software provided by Biacore AB. Dissociation constants (K_d) were calculated as $k_{diss}/k_{asso.}$ The residuals of the fitted data (Chi-square test; χ^2) as

a function of time were used to assess possible systematic deviation.

Data analysis - All experiments were performed on two or three separate occasions, and the average and standard deviation values are shown. Nonlinear least squares regression analyses were performed using Kaleidagraph (Synergy Reading, PA). The rate constants (k) for cleavage at Arg^{372} by thrombin were calculated using the equation $A_t = A_0(1 - e^{-kt})$ (Equation 1); where A_0 is the initial concentration of HCh form in nM, A_t is the concentration at time point (t) of A2 subunit, k is the rate constant in $minutes^{-1}$, and t is the time in minutes.

Results

Impact of 337-353S peptide on thrombin-catalyzed FVIII activation - The N-terminal region within the acidic region in the A1 domain (residues 337-372) consists of clustered acidic residues and a sulfated Tyr. This sequence is highly conserved among species and is similar to hirugen (**Figure 1A and B**). We speculated, therefore, that this region might contribute to thrombin interaction. Synthetic peptides were prepared, therefore, corresponding to residues 337-353 and 354-372, encompassing the 337-372 region, together with a 337-353 peptide containing sulfated Tyr^{346} (337-353S). The impact of these peptides was initially examined on thrombin-catalyzed FVIII activation (**Figure 2**). FVIII (10 nM) together with peptide (200 μM) was incubated with thrombin (0.4 nM). Both the 337-353 peptide and the 354-372 peptide modestly depressed peak levels of thrombin-catalyzed FVIII activation, although the inhibitory effect of the former peptide appeared to be greater (**Figure 2A-a**). As expected, the inhibitory effect of the 337-353S peptide was more pronounced compared to the 337-353 peptide. FVIII (10 nM) was also mixed with various amounts of these peptides prior to incubation with thrombin (0.4 nM), and activation was assessed by measuring FVIIIa activity at 1 min. All peptides inhibited thrombin-catalyzed FVIII activation dose-dependently. The IC_{50} values of 337-353, 354-372, and 337-353S peptides were ~80, 400<, and <25 μM, respectively (**Figure 2A-b**). In addition, the 337-353S peptide again exhibited a significantly greater inhibitory effect on thrombin-catalyzed activation, suggesting that the 337-353 region including a sulfated Tyr predominantly contributed to thrombin interactions.

Inhibition of thrombin cleavage of the HCh at Arg^{372} by the 337-353S peptide - Mixtures of FVIII (50 nM) and synthetic peptides (200 μM) were incubated with thrombin (2 nM), prior to SDS-PAGE and Western blotting analysis as described in Methods. **Figure 2B** illustrates the time-

course of HCh cleavage reactions determined by Western blotting using an anti-A2 mAJR8. The disappearance of A1-A2 fragments and generation of A2 fragments reflects cleavage at Arg^{372}. The 354-372 peptide did not affect thrombin cleavage of the HCh. The 337-353 peptide modestly delayed the disappearance of A1-A2 fragments and the generation of A2 fragments compared to that in its absence. In contrast, the inhibitory effect of the 337-353S peptide was markedly greater than that of the 337-353 peptide. These results indicated that residues 337-353 with sulfated Tyr^{346} in the A1 acidic region contributed to the HCh cleavage at Arg^{372}.

N-terminal sequence analysis of zero-length cross-linked products of A1 acidic peptides and thrombin - The involvement of A1 residues 337-353 in thrombin interactions was further examined by direct binding using EDC-mediated cross-linking experiments [19]. The 337-353S peptide failed biotinylation, however, and a 340-350S peptide was utilized for these purposes. Thrombin (300 nM) was incubated with increasing amounts of biotinylated 340-350S and EDC (500 μM). The formation of cross-linked products of ~34 kDa was demonstrated by an increasing intensity of band staining at various concentrations of peptide (**Supplemental Figure 1A**). Thrombin consists of an A chain (~5 kDa) and B chain (~32 kDa) linked by a disulfide bond. Since the samples on SDS-PAGE analysis were prepared under reducing conditions, the cross-linked B chain is observed. The mass of the combined product (~34 kDa) was consistent with a 1:1 stoichiometry of the peptide (~1.5 kDa) and the B chain of thrombin (~32 kDa). No cross-linked products were evident with peptides using BSA in control experiments (data not shown). To confirm the specificity of interaction between the biotinylated 340-350S and thrombin, various amounts of unlabeled 340-350S peptides were combined with the fixed concentrations of thrombin (300 nM) and biotinylated peptide (0.8 μM), and the mixtures were reacted with EDC (500 μM). Unlabeled 340-350S significantly inhibited the cross-linking formation between biotinylated peptide and thrombin in a dose-dependent manner, supporting the specificity of this interaction (**Supplemental Figure 1B**).

N-terminal sequence analyses of the peptide-thrombin adducts were performed in order to identify the specific residue(s) in 340-350S that cross-linked with thrombin. The peptide-thrombin compound contained two N-termini, and each cycle of sequencing cleaved a residue from each chain. The residues identified by these analyses matched the predicted residues for the N-termini of the peptide and thrombin (**Table 1**), confirming a 1:1 stoichiometry of peptide and thrombin. Sequence analysis of the native peptide demonstrated that residues 344-349 were evident from

the 5th to 10th cycles (data not shown). These residues were not detected, however, during sequencing of the cross-linked adduct. In contrast, the expected yields of all residues in the B-chain chain were detectable in all thrombin cycles, other than the 5th, 9th and 11th. Residues involved in covalent cross-links of this type might not be detected in sequence analyses, however, resulting in an apparent gap in the sequences of that chain, and in these circumstances, interpretation of the data was limited. Nevertheless, the data tentatively confirmed that the acidic residues 344-349 (EDYDDD) in the A1 peptide formed a bridge with a yet unidentified carboxylic basic residue in the B-chain of thrombin. The sequence of the A-chain was not detected, confirming the participation of the B-chain of thrombin between cross-linked adducts.

Thrombin-catalyzed activation and cleavage within 344-349 residues of FVIII mutants - The residues responsible for thrombin interaction within 344-349 sequences were directly identified using a series of FVIII mutants prepared with Ala substitution using a BHK cell system; one double mutant E344A/D345A, one single mutant Y346A, and one triple mutant D347A/D348A/D349A. Specific activities were determined by a one-stage clotting assay and a FXa-generation assay (**Table 2**). The specific activities of E344A/D345A, Y346A, and D347A/D348A/D349A were 56 and 85%, 61 and 124%, and 28 and 51% of WT respectively in the two assays, indicating lower specific activities measured by one-stage assay compared to FXa generation assays. These results were consistent with the characteristics of hemophilia A patients with *F8* mutations associated with thrombin cleavage- or adjacent site(s) [27,28]. The Y346A and D347A/D348A/D349A mutations appeared reflect a greater influence than the E344A/D345A mutant.

These FVIII mutants (10 nM) were further examined for activation by thrombin (0.4 nM). Time-course activation and inactivation curves are shown in **Figure 3A**. Activation of the E344A/D345A mutant was slightly decreased to ~80% of WT. Thrombin-catalyzed activation of Y346A and D347A/D348A/D349A mutants was diminished, however, with peak activity of ~50% of WT (**Table 3**). We also investigated thrombin-catalyzed proteolytic cleavage of the HCh at Arg^{372}. **Figure 3B-a** shows the time-course patterns as described above. In addition, since the ratio of A2/A2+A1A2 fragments reflects thrombin-catalyzed cleavage at Arg^{372}, cleavage rates were calculated using quantitative densitometry (**Figure 3B-b, Table 3**). The cleavage rate of E344A/D345A appeared to be ~90% of the WT, in keeping with the similar cleavage pattern of WT FVIII. The cleavage rates of the Y346A and D347A/D348A/D349A mutants were decreased,

however, to ~10% and ~20% of WT. These results demonstrated that the 346-349 clustered acidic region, including sulfated Tyr, governed thrombin activation of FVIII through cleavage at Arg^{372}.

Thrombin-catalyzed activation and cleavage of FVIII A1 acidic mutants replaced by the hirugen sequence - The sequence 337-346 (MKNNEEAEDY) in A1 appears to be similar to the hirugen sequence (**Figure 1B**). It seemed possible therefore, that FVIII mutants containing residues of the hirugen sequence within 337-346 could be more efficiently activated and proteolyzed by thrombin than WT. On this basis, four FVIII mutants were prepared with one or two amino acid residues substituted with corresponding hirugen residues (K338D, N340E, E342I, and N340E/E342I), together with a FVIII mutant comprising full hirugen sequence (MKNNEEAEDY337-346GDFEEIPEEY; a1-hirugen hybrid mutant). The specific FVIII activities of these mutants determined by one-stage clotting assays were 35-45% and were lower than those obtained by FXa generation assays, consistent with a possible association between thrombin and these mutated residues.

Figure 4A illustrates that peak levels of activation of the four mutants (K338D, N340E, E342I, and N340E/E342I) by thrombin were decreased by 50-60% compared to WT. In contrast, thrombin-catalyzed activation of a1-hirugen hybrid mutant was increased by ~1.5-fold of WT (**Table 3**). In addition, time course analysis of thrombin cleavage at Arg^{372} of these FVIII mutants was repeated (**Figure 4B-a**), and suggested that, except with E342I and the a1-hirugen hybrid, the cleavage rate was 35-55% slower than that of WT. There was no significant difference in the cleavage rate of E342I compared to WT. Conversely, the rate of thrombin cleavage at Arg^{372} in a1-hirugen hybrid mutant exhibited an ~2.5-fold increase compared to WT (**Figure 4B-b,c, Table 3**). These results demonstrated that FVIII A1 mutant containing the full hirugen sequence facilitated thrombin-catalyzed activation and cleavage at Arg^{372}.

Direct binding of Y346A, D347A/D348A/D349A, and a1-hirugen hybrid mutant to PPA-thrombin - The direct binding of Y346A, D347A/D348A/D349A, and a1-hirugen hybrid mutants to thrombin, was investigated using a fluid-phase SPR-based assay. Various concentrations of FVIII mutants were added to active-site modified PPA-thrombin immobilized onto the CM5 sensor chip as described in Methods, and **Figure 5** illustrates representative binding curves. The data was comparatively fitted by non-linear regression using a 1:1 binding model with a drifting baseline. The binding affinities of Y346A (k_{ass} = 0.90 ± 0.10×10^5 $M^{-1}s^{-1}$, k_{diss} = 22.7 ± 7.3×10^{-4} s^{-1},

K_d ; 25.3 nM; $\chi^2 = 2.3$) and D347A/D348A/D349A ($k_{ass} = 1.3 \pm 0.17 \times 10^5$ $M^{-1}s^{-1}$, $k_{diss} = 61.3 \pm 9.9 \times 10^{-4}$ s^{-1}, K_d ; 47.4 nM; $\chi^2 = 8.7$) mutants for PPA-thrombin were reduced by ~3-fold and ~5.5-fold compared to WT ($k_{ass} = 1.0 \pm 0.11 \times 10^5$ $M^{-1}s^{-1}$, $k_{diss} = 8.6 \pm 3.8 \times 10^{-4}$ s^{-1}, K_d ; 8.5 nM; $\chi^2 = 17.2$), confirming that residues 346-349 played a direct and important role in thrombin interactions. Alternatively, the association kinetics of the a1-hirugen hybrid mutant ($k_{ass} = 1.8 \pm 0.24 \times 10^5$ $M^{-1}s^{-1}$, $k_{diss} = 26.0 \pm 1.9 \times 10^{-4}$ s^{-1}, K_d ; 14.6 nM; $\chi^2 = 36.7$) to PPA-thrombin was enhanced by ~1.8-fold. However, since the dissociation kinetics was reduced by ~3-fold of WT, the binding affinity (K_d) for this interaction was not enhanced.

Discussion

Thrombin and FXa activation of FVIII by limited proteolysis at Arg^{372}, Arg^{740}, and Arg^{1689} is essential for normal blood coagulation. In this context, cleavage at Arg^{372} and Arg^{1689} is especially important for generating FVIIIa cofactor activity on the FXase complex [9]. Our earlier study demonstrated that the 337-372 acidic region in A1 domain of FVIII, in particular the clustered acidic residues D361/D362/D363, contained a FXa-interactive site [29-31]. We had previously shown that the A1 subunit bound directly to an active-site modified Phe-Pro-Arg-thrombin and to mutated recombinant thrombin (Ser205Ala) [19]. This interaction was competitively blocked by the addition of hirudin, indicating an ionic-strength-dependent interaction involving the ABE-1 of thrombin [19]. We have now extended these studies to further characterize the thrombin-interactive site(s) in the A1 acidic region (residues 337-372). Our results demonstrated for the first time that the Y346/D347/D348/D349 residues in A1 directly participate in the thrombin interactions responsible for proteolytic cleavage at Arg^{372}. In addition, the a1-hirugen hybrid mutant was associated with more efficient thrombin-catalyzed activation and cleavage.

Detailed peptide experiments indicated that the N-terminus residues 337-353 in the A1 acidic region contributed to thrombin-catalyzed activation and cleavage at Arg^{372}. Furthermore, our data confirmed that sulfation of Tyr^{346} could be essential for thrombin association. Earlier reports demonstrated that tyrosine sulfation of FVIII is required for full FVIIIa cofactor activity [32,33], indicating the importance of tyrosine sulfation in this mechanism. In addition, hemophilia A patients identified with Tyr346His, Tyr346Cys and Tyr346* mutations reported in the F8 gene variant database (http://www.factorviii-db.eahad.org) exhibited a mild-severe hemophilic phenotype, consistent with a functional role for this residue.

Our results utilizing FVIII mutants where acidic residues in the 344-349 segment were replaced with Ala showed reductions in specific activity. In particular, the D347A/D348A/D349A mutation severely affected specific activity. Thrombin activation and cleavage rates with the E344A/D345A mutant were not significantly different from WT. In contrast, Y346A and D347A/D348A/D349A mutants appeared to be linked to reduced rates for thrombin-catalyzed activation and cleavage at Arg^{372}. Moreover, SPR-based analysis showed that Y346A and D347A/D348A/D349A mutants possessed an ~3-5-fold lower affinity for active-site modified thrombin relative to WT. These results suggested that the sulfated residue (Tyr^{346}) and the clustered 347-349 acidic region played an important role in direct thrombin interaction via ABE-I. The findings were consistent with those of Michnick *et al.* [33], emphasizing that sulfation at Tyr^{346} increased the efficiency of thrombin activation and cleavage.

Alternative studies have suggested that Ala mutations of the acidic residues in the A2 domain, E720A, D721A, E724A, and D725A, also impaired thrombin activation and cleavage rates at Arg^{372} [20]. Our observations paralleled those results, and showed that the acidic residue flanking tyrosine sulfation in FVIII significantly contributed to thrombin-catalyzed activation and cleavage. Binding of thrombin to the 484-509 region in A2 also appeared to govern cleavage at Arg^{372} [19]. These series of investigations on thrombin interactions have suggested, therefore, that thrombin cleavage at Arg^{372} is likely regulated by a range of binding mechanisms involving; i) the clustered basic 484-509 region independent of ABE, ii) the clustered acidic 346-349 region dependent on ABE-I, and iii) the clustered acidic 720-725 region [18-20].

Some of the FVIII A1 mutants containing one or two amino acid residue(s) of the hirugen sequence (K338D, N340E, E342I, and N340E/E342I) reduced the catalytic efficiency of thrombin-catalyzed reactions compared to WT. The cleavage rate of E342I by thrombin was not significantly different from that of WT, however. The reason for this remains unclear, but it may be that this residue is not crucial for thrombin cleavage. Notably, however, results from the time-course experiments of thrombin-catalyzed activation and cleavage at Arg^{372} using the a1-hirugen hybrid mutant demonstrated an ~1.5-fold and ~2.5-fold increase, respectively, compared to those with WT. These data indicate that this novel FVIII mutant might confer more efficient thrombin cleavage at Arg^{372} in FVIII.

We speculated that the full hirugen sequence might contribute to higher affinity of thrombin

interactions, possibly resulting in more efficient thrombin activation and cleavage. However, SPR-based assays designed to examine the interactions between the hybrid mutant and PPA-thrombin failed to reveal any significant enhancement of affinity in thrombin binding compared to WT. The kinetic determinants for both thrombin association (k_{ass}) and dissociation (k_{diss}) with the a1-hirugen hybrid mutant were greater than those in WT. Thrombin appeared, therefore, to bind rapidly and dissociate from this mutant easily. It may be that the full hirugen sequence itself inserted in the A1 domain may have increased binding potency but induced an unfavorable conformational change for fully effective thrombin interactions. A previous study reported that P3-P3' residues flanking Arg^{740} and Arg^{1689} were associated with more potent thrombin cleavage than those flanking Arg^{372} [34]. In addition, Newell-Caito *et al.* [35] suggested that FVIII mutants composed of residues flanking the Arg^{372} site exhibited more rapid rates of thrombin-catalyzed cleavage and pro-cofactor activation than WT, indicating that optimum thrombin interactions may be influenced by this sequence.

Our present study provided new insights into novel thrombin-interactive site in the A1 acidic region of FVIII. Furthermore, the full hirugen sequence in the mutated A1 acidic region (residues 337-346) appeared to be more susceptible to thrombin cleavage at Arg^{372} than WT. However, the binding mechanism(s) responsible for these findings remain to be determined and do not exclude possible allosteric effects in the FVIII mutants away from the A1 acidic region and thrombin exosite.

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Authorship

Contribution: YN performed the experiments, analyzed the data, made the figure, wrote the paper; KN designed the research, interpreted the data, wrote the paper, edit the manuscript and approved the final version to be published.

Conflict of interests

The authors declare that they have no conflict of interests.

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Figure Legends

Figure 1. Sequence alignment of residues 337-353 in human and other mammalian FVIII proteins and of residues 53-65 of hirugen - *Panel* (**A**) shows that the acidic residues within residues 337-353 in FVIII are highly conserved across species. *Panel* (**B**) shows the sequence of hirugen compared to that of human FVIII. Acidic residues and tyrosine residues are illustrated in

bold and in *italic bold*, respectively.

Figure 2. Effects of synthetic peptides of the A1 acidic region on activation and cleavage at Arg^{372} of FVIII by thrombin - *Panel* (**A-a**); *Time course reaction*: FVIII (10 nM) was mixed with A1 peptide (200 μM; *open circles*; 337-353 peptide, *closed circles*; 354-372 peptide, *closed squares*; 337-353S peptide, *open triangles*; no peptide), and was incubated with thrombin (0.4 nM). The reactions were terminated at the indicated times prior to measuring FVIIIa activity in a one-stage clotting assay as described in Methods. Initial FVIII levels were ~9.9 IU/ml. FVIIIa activities were expressed as fold of initial, and were plotted as a function of incubation time. *Panel* (**A-b**); *Dose-dependent effect*: FVIII (10 nM) was mixed with various concentrations of A1 peptide (*open circles*; 337-353 peptide, *closed circles*; 354-372 peptide, *closed squares*; 337-353S peptide) prior to incubation with thrombin (0.4 nM) for 1 min. FVIIIa activity at 1 min without peptide was ~180 IU/ml and this level was regarded as initial. FVIIIa activity was plotted as a function of peptide concentration. *Panel* (**B**); *Time course of cleavage*. FVIII (50 nM) was incubated with thrombin (2 nM) for the indicated times in the absence or presence of A1 peptide (200 μM; 337-353 peptide, 354-372 peptide, and 337-353S peptide). Samples were analyzed by 8% SDS-PAGE, followed by Western blotting using an anti-A2 mAJR8 as described in Methods. Experiments in (**A**) and (**B**) were performed three separate times. Average and standard deviation values in (**A**) and representative data in (**B**) are shown.

Figure 3. Thrombin-catalyzed activation and cleavage at Arg^{372} of FVIII mutated in the 344-349 sequence - *Panel* (**A**); *Time course of activation*: FVIII WT or mutants (10 nM) were incubated with thrombin (0.4 nM) prior to measuring FVIIIa activity at the indicated times in a one-stage clotting assay as described in Methods. The initial FVIII activities of WT (*closed circles*), E344A/D345A (*open circles*), Y346A (*closed squares*), D347A/D348A/D349A (*open squares*), were ~8.2, 4.6, 5.0, and 2.3 IU/ml at t=0, respectively. FVIIIa activity was expressed as fold of initial, and was plotted as a function of incubation time. *Panel* (**B**); *Time course of cleavage*: WT or mutants (E344A/D345A, Y346A, and D347A/D348A/D349A) of FVIII (50 nM) were incubated with thrombin (2 nM) for the indicated times. Samples were run on 8% gels, followed by Western blotting using an anti-A2 mAbJR8 (*panel* **a**) as described in Methods. *Panel* (**b**) shows change in the band density of HCh, representing cleavage at Arg^{372} assessed by quantitative band densitometry in *panel* (**a**). The ratios of A2/A2+A1A2 band of the FVIII forms in *panel* (**a**) measured by quantitative densitometry are shown in *panel* (**b**). The symbols used are:

closed circles; WT, *open circles*; E344A/D345A, *closed squares*; Y346A, *open squares*; D347A/D348A/D349A. Experiments in (**A**) and (**B**) were performed three separate times and twice, respectively, and average and standard deviation calculations are shown. HCh; heavy chain

Figure 4. Thrombin-catalyzed activation and cleavage at Arg^{372} for FVIII mutants with hirugen-sequence substitution in the 337-346 region - *Panel* (**A**); *Time course of activation*: The WT or mutants (K338D, N340E, E342I, N340E/E342I, and a1-hirugen hybrid) of FVIII (10 nM) were incubated with thrombin (0.4 nM) prior to measuring FVIIIa activity at the indicated times in a one-stage clotting assay as described in Methods. The initial FVIII activities of WT (*closed circles*), K338D (*closed squares*), N340E (*open squares*), E342I (*closed triangles*), N340E/E342I (*open triangles*), and a1 hirugen (*open circles*) were ~8.2, 3.8, 3.8, 3.1, 3.0, and 2.8 IU/ml at t=0, respectively. FVIIIa activity was expressed as fold of initial. *Panel* (**B**); *Time course of cleavage*. The WT or mutants (K338D, N340E, E342I, N340E/E342I, and a1-hirugen hybrid substitution) of FVIII (50 nM) were incubated with thrombin (2 nM) for the indicated times. Samples were run on 8% gels, followed by Western blotting using an anti-A2 mAbJR8 (*panel* **a**) as described in Methods. *Panel* (**b**) shows change in the band density of HCh, representing cleavage at Arg^{372} assessed by quantitative band densitometry in *panel* (**a**). The ratios of A2/A2+A1A2 band of FVIII forms in *panel* (**a**) measured by quantitative densitometry are shown in *panel* (**b**). The *panel* (**b**) and *panel* (**c**) show the time-course data for 30 min and an enlargement of the scale for 3 min, respectively. The symbols used are: *closed circles*; WT, *closed squares*; K338D, *open squares*; N340E, *closed triangles*; E342I, *open triangles*; N340E/E342I, and *open circles*; a1 hirugen. Experiments in (**A**) and (**B**) were performed three separate times and twice, respectively, and average and standard deviation calculations are shown. HCh; heavy chain, a1 hirugen; MKNNEEAEDY337-346GDFEEIPEEY

Figure 5. Fluid-phase SPR-based binding assay of Y346A, D347A/D348A/D349A, and a1-hirugen hybrid FVIII mutants to immobilized PPA-modified thrombin - Various concentrations of FVIII WT (*panel* **A**), Y346A (**B**), D347A/D348A/D349A (**C**), and a1-hirugen hybrid (**D**) mutants were incubated for 2 min with PPA-modified thrombin immobilized on a CM5 sensor chip, prior to a change of running buffer for 2 min as described in Methods. The *lines* 1-5 show representative association and dissociation curves of FVIII mutants at various concentrations (5, 10, 25, 50, and 100 nM, respectively), and the fitted curves using the one-site binding model are shown (*solid thin line*). The K_d between PPA-modified thrombin and FVIII

mutants is shown on each panel. a1 hirugen; MKNNEEAEDY337-346GDFEEIPEEY

Supplemental Figure 1.

EDC cross-linking between 340-350S peptide and thrombin - *Panel* (**A**); Thrombin (300 nM) was incubated with various concentrations of biotinylated 340-350S in the presence of EDC (500 μM), prior to SDS-PAGE using 15% gels and immunoblotting by using streptavidin for detection as described in Methods. *Panel* (**B**); Thrombin (300 nM) and biotinylated 340-350S (0.8 μM) were incubated with EDC (500 μM) together with various concentrations of unlabeled 340-350S and were immunoblotted. Experiments were performed at three separate times, and representative data is shown.

Table 1. N-terminal sequence of cross-linked products of 340-350S peptide and thrombin

Cross-linked materials were obtained following incubation of the 340-350S peptide (1 mM) with the B chain of thrombin (1 μM) and EDC (5 mM). Cycles refer to the Edman's degradation number and results of sequence analyzes of the cross-linked product are presented as subtraction chromatograms where the yield (in pmol) for a given residue (except the initial residue) was deducted from the value for that residue in the previous cycle. E, D, Y, D, D and D were not identified at cycles 5-10 in the 340-350S sequences. The parentheses around amino acid residue show that these residues could not be identified from the analysis of N-terminal sequence.

		Cycle										
	Sequence	1	2	3	4	5	6	7	8	9	10	11
Peptide	340-350	N	E	E	A	(E)	(D)	(Y)	(D)	(D)	(D)	L
	(pmol)	3.08	2.54	5.31	1.94	—	—	—	—	—	—	1.42
Thrombin	1-11	I	V	E	G	(S)	D	A	E	(I)	G	(M)
	(pmol)	6.51	6.30	5.31	3.56	—	2.76	3.08	2.45	—	2.21	—

Table 2. Specific activity of FVIII mutated within the A1 acidic region

The FVIII activities in wild type (WT) and mutants of FVIII were measured by a one-stage clotting assay (OS) and a FXa generation assay (FXa-G). Specific activities of the FVIII mutants are shown as a fold of WT. The ratios of specific activity using the two assays were expressed as OS/FXa-G (ratio). *; a1 hirugen (MKNNEEAEDY337-346GDFEEIPEEY)

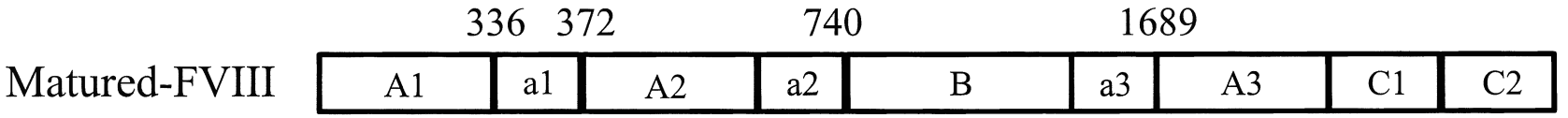
Specific activity of rFVIII			
FVIII mutant	OS / XaG	FVIII mutant	OS / XaG
	fold of WT (OS/XaG ratio)		*fold of WT* (OS/XaG ratio)
WT	1	WT	1
E344A/D345A	0.56 / 0.85 (0.66)	K338D	0.46 / 1.09 (0.42)
Y346A	0.61 / 1.24 (0.49)	N340E	0.46 / 0.84 (0.55)
D347A/D348A/D349A	0.28 / 0.51 (0.55)	E342I	0.38 / 0.60 (0.63)
		N340E/E342I	0.37 / 0.94 (0.39)
		a1-hirugen hybrid*	0.34 / 0.47 (0.72)

Table 3. Kinetic parameters characterizing thrombin-catalyzed FVIII activation and cleavage at Arg^{372} for FVIII mutated within the A1 acidic region

All values of activation and cleavage at Arg^{372} in wild type (WT) and mutants of FVIII were calculated from the data shown in Figures 3 and 4 using the formula described in Methods. Activation and cleavage values of the FVIII mutants are shown as a fold of WT. *; blot detection using JR8. OS: one-stage clotting assay, **; a1 hirugen (MKNNEEAEDY337-346GDFEEIPEEY)

	Activation	Arg^{372} cleavage*		Activation	Arg^{372} cleavage*
FVIII mutant 344-349	Peak activity (OS)	Rate constant (*k*)	FVIII mutant 337-345	Peak activity (OS)	Rate constant (*k*)
	fold of initial (fold of WT)	*min^{-1} (fold of WT)*		*fold of initial (fold of WT)*	*min^{-1} (fold of WT)*
WT	14.1 ± 0.52 (1)	0.65 ± 0.19 (1)	WT	14.1 ± 0.52 (1)	0.65 ± 0.19 (1)
E344A/D345A	11.5 ± 0.37 (0.82)	0.60 ± 0.049 (0.92)	K338D	9.2 ± 0.13 (0.65)	0.42 ± 0.026 (0.65)
Y346A	7.0 ± 0.92 (0.50)	0.064 ± 0.014 (0.10)	N340E	8.6 ± 0.41 (0.61)	0.44 ± 0.083 (0.68)
D347A/D348A/D349A	6.1 ± 0.43 (0.43)	0.12 ± 0.015 (0.18)	E342I	9.4 ± 1.1 (0.67)	0.68 ± 0.027 (1.05)
			N340E/E342I	6.7 ± 0.53 (0.48)	0.29 ± 0.033 (0.45)
			a1-hirugen hybrid**	19.9 ± 0.21 (1.41)	1.60 ± 0.17 (2.46)

(A)

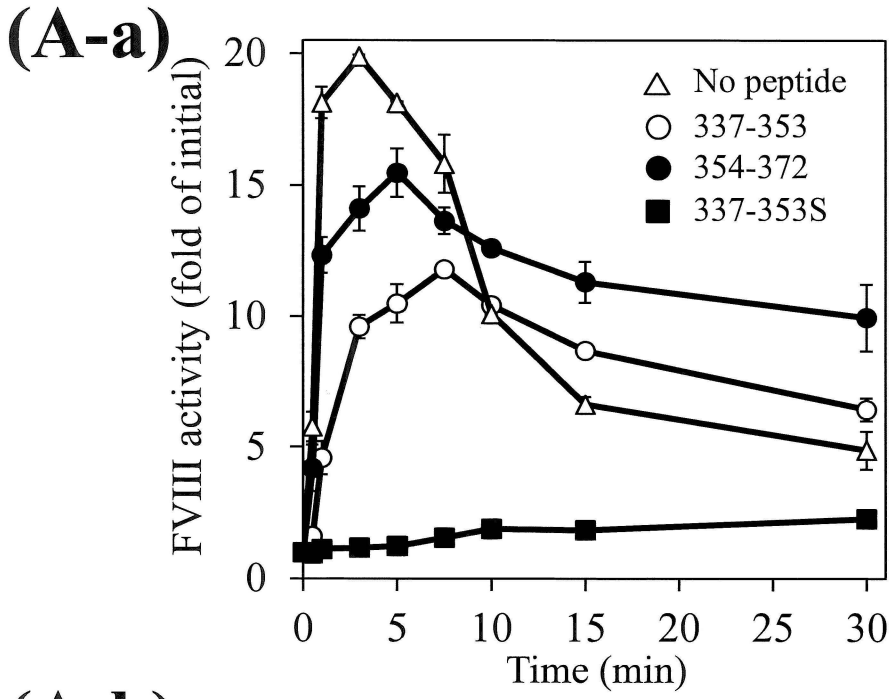


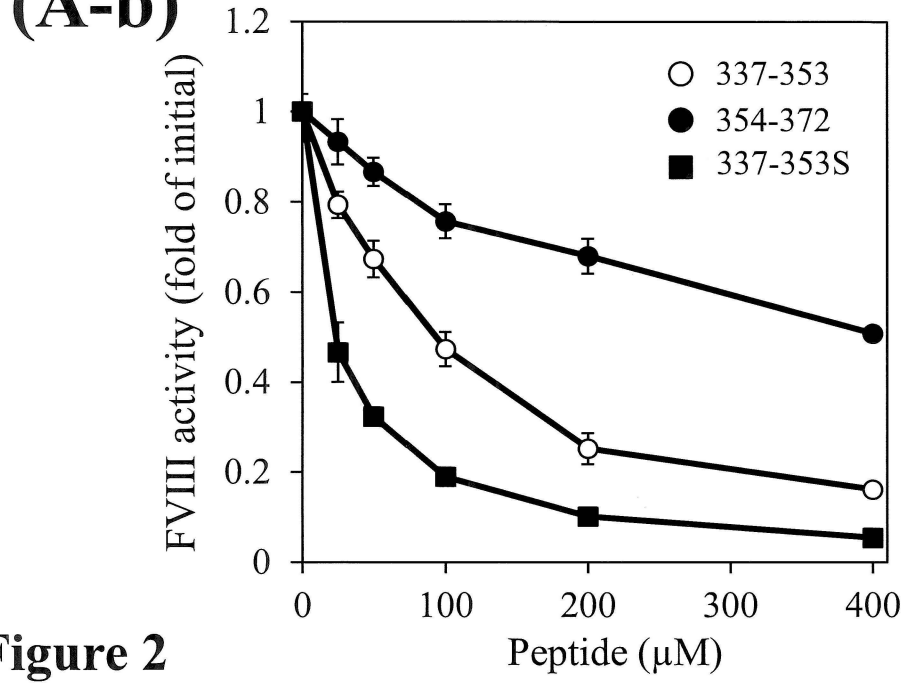
337			340					345					350				
M	K	N	N	**E**	**E**	A	**E**	**D**	***Y***	**D**	**D**	**D**	L	T	D	S	Human
R	K	A	D	**E**	**E**	–	**E**	**D**	***Y***	**D**	**D**	N	L	Y	D	S	Porcine
K	K	N	N	**E**	**E**	M	**E**	**D**	***Y***	**D**	**D**	**D**	L	Y	S	E	Murine
M	K	N	N	**E**	**D**	–	K	**D**	***Y***	**D**	**D**	G	L	Y	D	S	Canine

(B)

337				340					345					
R	M	K	N	N	**E**	**E**	A	**E**	**D**	***Y***	**D**	**D**	**D**	Human FVIII
N	G	**D**	F	**E**	**E**	I	P	**E**	**E**	***Y***	L	Q		Hirugen (53-65)

Figure 1


(A-a)
FVIII activity (fold of initial)
20
15
10
5
0
0
5
10
15
20
25
30
Time (min)
No peptide
337-353
354-372
337-353S


(A-b)
FVIII activity (fold of initial)
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
Peptide (μM)
337-353
354-372
337-353S

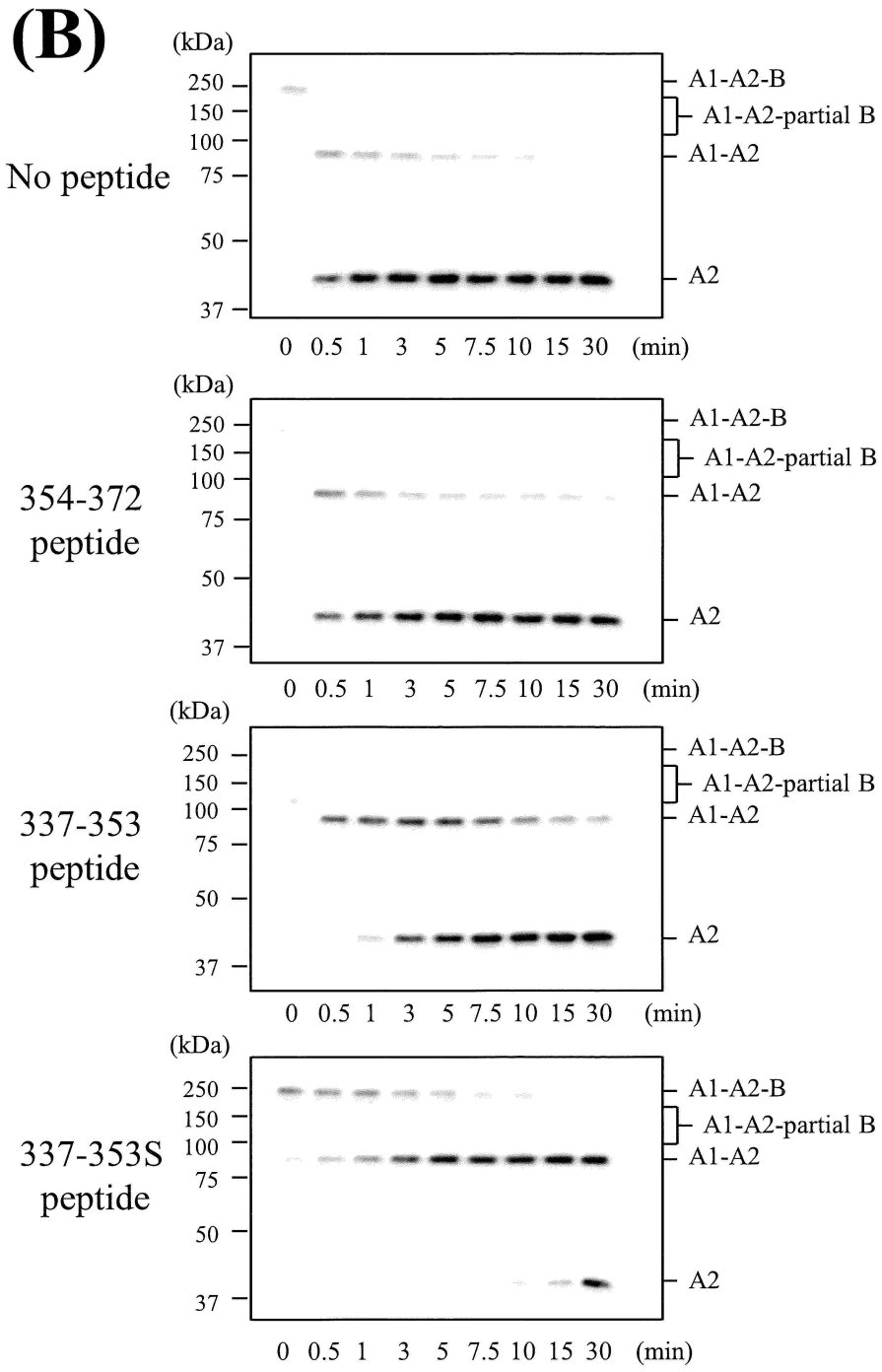
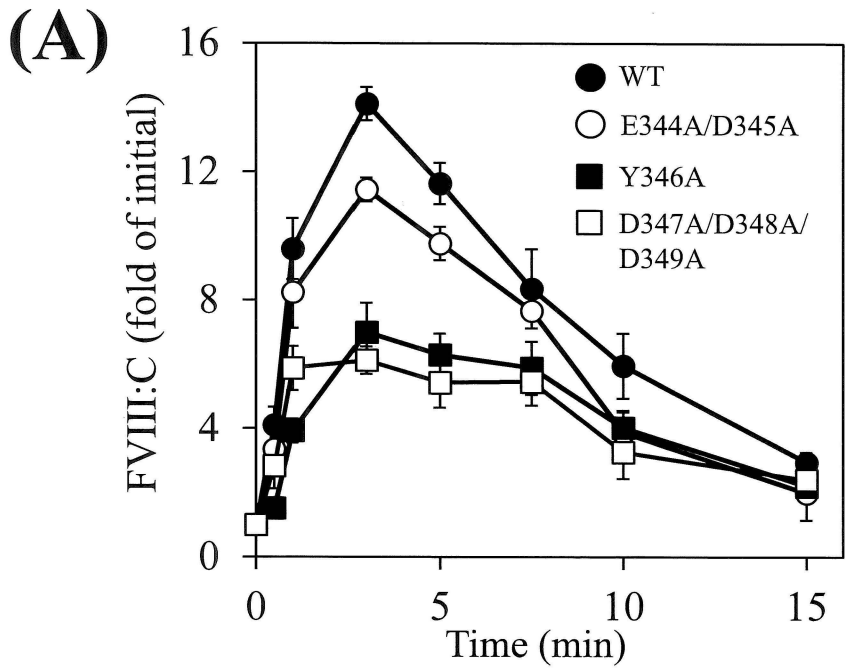
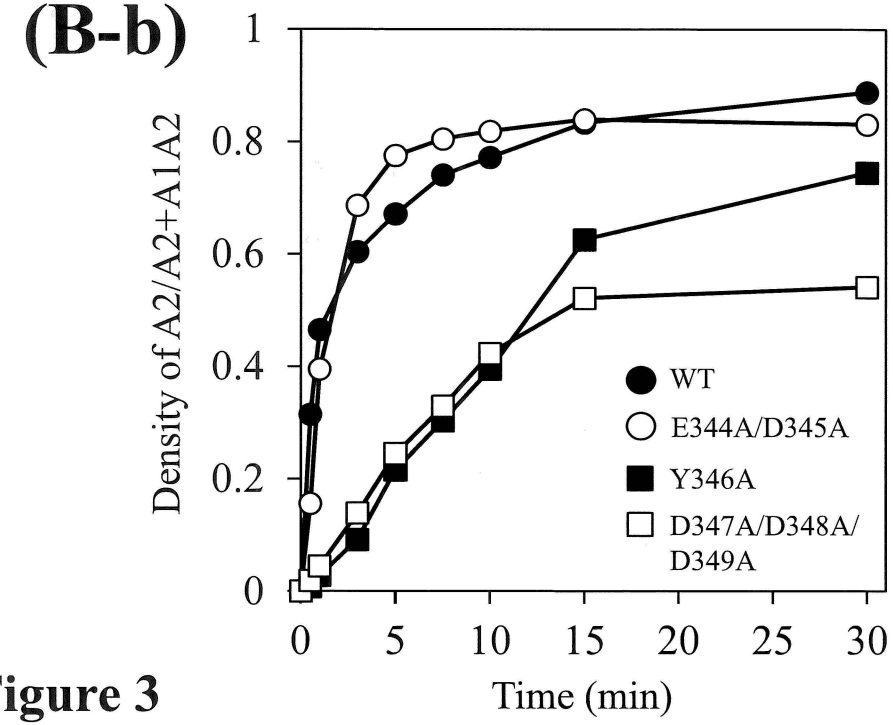

(B)
No peptide
354-372 peptide
337-353 peptide
337-353S peptide
(kDa)
250
150
100
75
50
37
A1-A2-B
A1-A2-partial B
A1-A2
A2
0 0.5 1 3 5 7.5 10 15 30 (min)

Figure 2


(A)
FVIII:C (fold of initial)
16
12
8
4
0
0
5
10
15
Time (min)
WT
E344A/D345A
Y346A
D347A/D348A/
D349A


(B-b)
Density of A2/A2+A1A2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Time (min)
WT
E344A/D345A
Y346A
D347A/D348A/
D349A

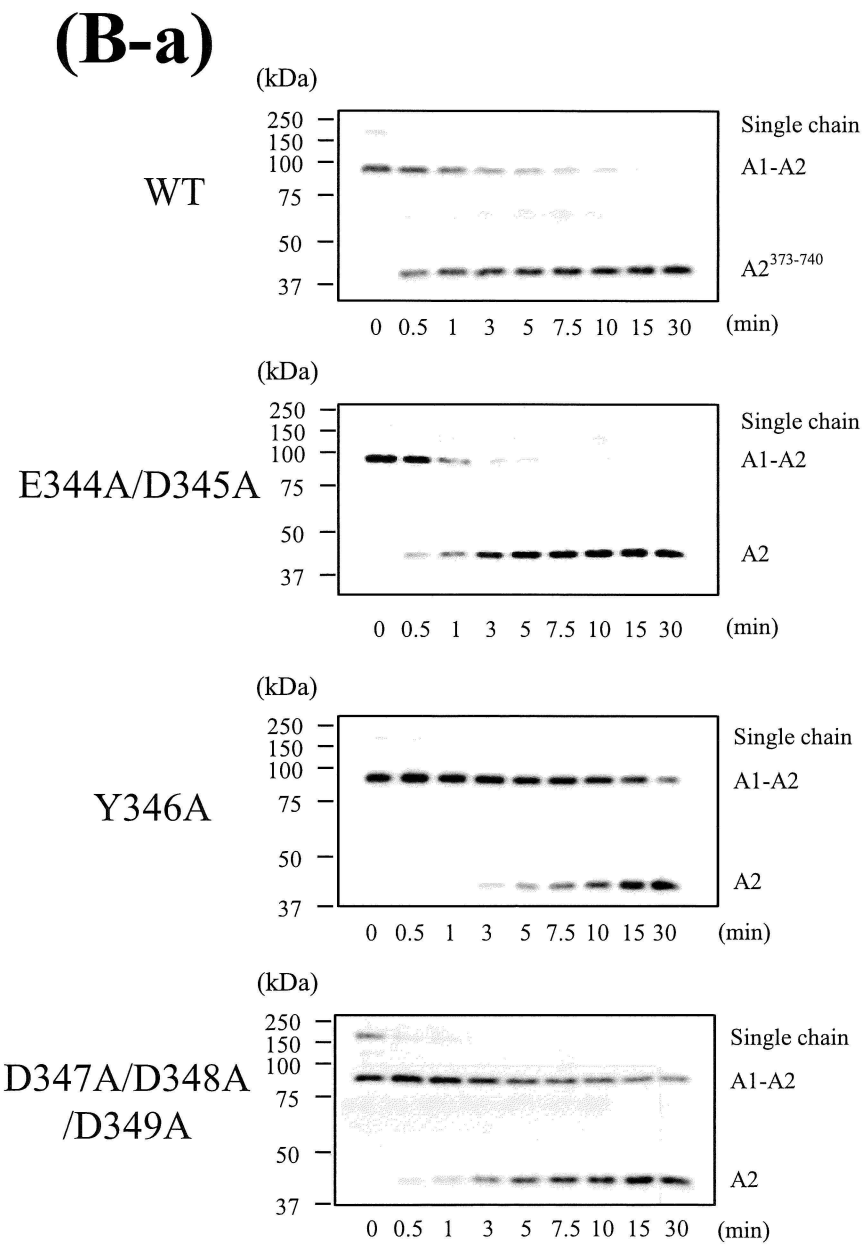

(B-a)
WT
E344A/D345A
Y346A
D347A/D348A
/D349A
(kDa)
250
150
100
75
50
37
Single chain
A1-A2
A2373-740
A2
0 0.5 1 3 5 7.5 10 15 30
(min)

Figure 3

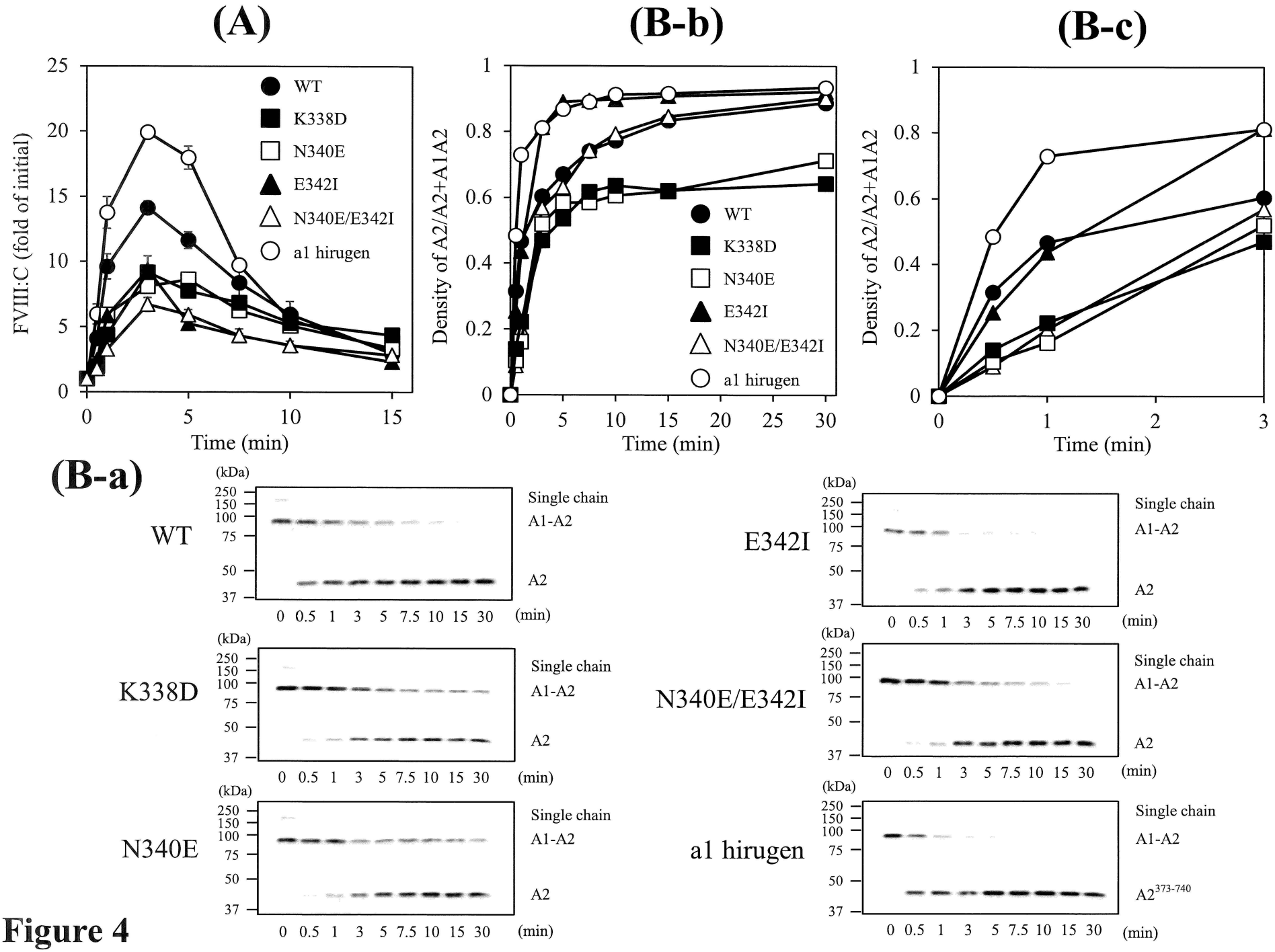

(A)
(B-b)
(B-c)
(B-a)
FVIII:C (fold of initial)
Time (min)
Density of A2/A2+A1A2
WT
K338D
N340E
E342I
N340E/E342I
a1 hirugen
(kDa)
250
150
100
75
50
37
Single chain
A1-A2
A2
A2373-740
0 0.5 1 3 5 7.5 10 15 30
(min)

Figure 4

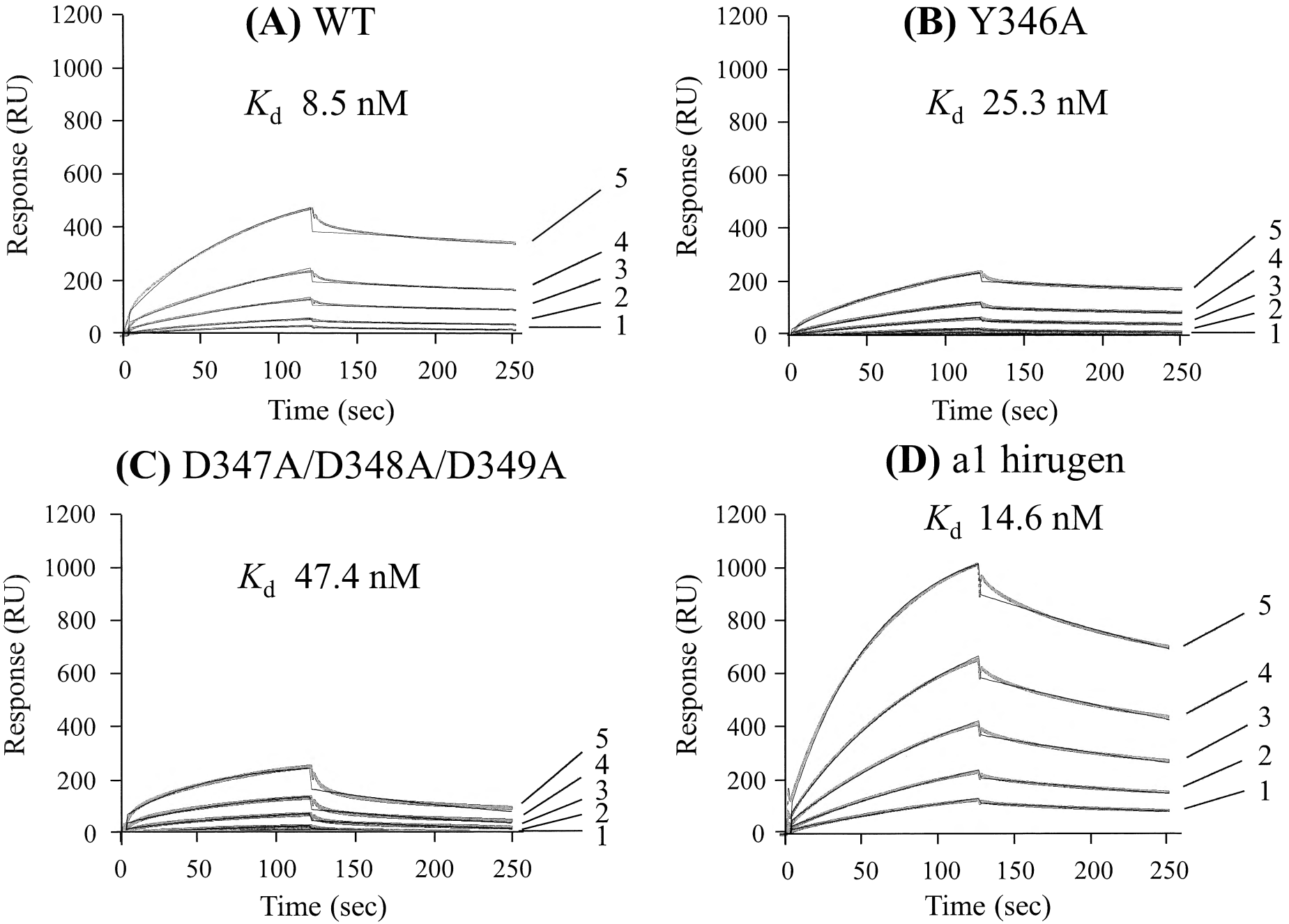
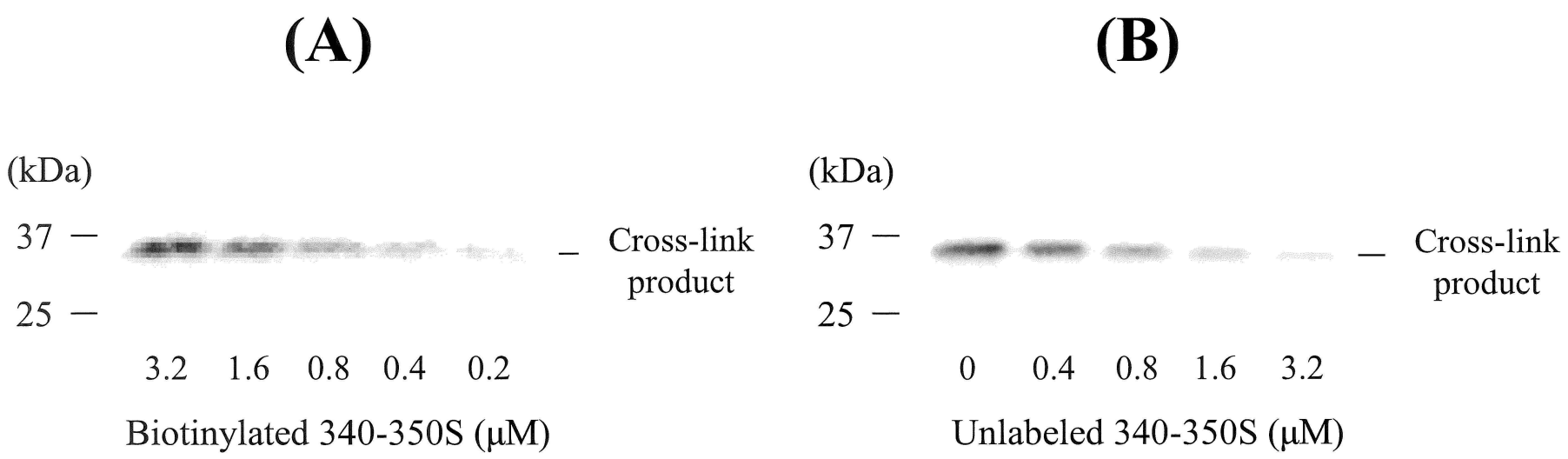

(A) WT
K_d 8.5 nM
(B) Y346A
K_d 25.3 nM
(C) D347A/D348A/D349A
K_d 47.4 nM
(D) a1 hirugen
K_d 14.6 nM
Response (RU)
Time (sec)
0
200
400
600
800
1000
1200
0
50
100
150
200
250
1
2
3
4
5

Figure 5



Supplemental Figure 1