Contribution of Factor VIIIa A2 and A3-C1-C2 Subunits to the Affinity for Factor IXa in Factor Xase†

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ABSTRACT: Contributions of factor (F) VIIIa subunits to cofactor association with FIXa were evaluated. Steady-state fluorescence resonance energy transfer using an acrylodan-labeled A3-C1-C2 subunit and fluorescein-Phe-Phe-Arg-FIXa yielded K_d values of 52 ± 10 and 197 ± 55 nM in the presence and absence of phospholipid vesicles, respectively. A3-C1-C2 was an effective competitor of FVIIIa binding to FIXa as judged by inhibition of FXa generation performed in the absence of vesicles ($K_i \approx 1.6K_d$ for FVIIIa−FIXa). However, the capacity for A3-C1-C2 to inhibit FVIIIa-dependent FXa generation in the presence of phospholipid was poor with a K_i values (∼400 nM) that were ∼100-fold greater than the K_d for FVIIIa−FIXa interaction (4.2 ± 0.6 nM). These results indicated that a significant component of the interprotein affinity is contributed by FVIIIa subunits other than A3-C1-C2 in the membrane-dependent complex. The isolated A2 subunit of FVIIIa interacts weakly with FIXa, and recent modeling studies have implicated a number of residues that potentially contact the FIXa protease domain (Bajaj et al. (2001) *J. Biol. Chem. 276*, 16302−16309). Site-directed mutagenesis of candidate residues in the A2 domain was performed, and recombinant proteins were stably expressed and purified. Functional affinity determinations demonstrated that one mutant, FVIII/Asp712Ala exhibited an 8-fold increased K_d (35 ± 1.5 nM) relative to wild-type suggesting a contribution by this residue of ∼10% of the FVIIIa−FIXa binding energy. Thus both A2 and A3-C1-C2 subunits contribute to the affinity of FVIIIa for FIXa in the membrane-dependent FXase.

The plasma glycoproteins factor VIII (FVIII)[1] and factor IX (FIX) are crucial components in the normal hemostatic pathway, deficiencies in the proteins giving rise to the bleeding diatheses hemophilia A and hemophilia B, respectively. FVIII in its activated form, FVIIIa, acts as a cofactor to serine protease FIXa in the conversion of FX to FXa, increasing the catalytic efficiency of FIXa by several orders of magnitude. This association of enzyme and cofactor occurs on an anionic phospholipid membrane in the presence of Ca^{2+} ions, the components forming a complex designated as the intrinsic FXase.

FVIII circulates in plasma as a heterodimer composed of a heavy chain in the domain arrangement A1-A2-B, and a light chain in the arrangement A3-C1-C2 linked in a metal ion-dependent interaction (*1*−*3*). FVIII is activated by thrombin-catalyzed cleavage at Arg372 near the A1-A2 junction, Arg740 at the A2-B junction, and Arg1689 near the N-terminus of the A3 domain (*4*). The resulting FVIIIa molecule is a noncovalently linked trimer, with the metal ion-dependent interaction retained between the A1 and A3-C1-C2 subunits, and a weak electrostatic interaction linking the A2 subunit with the A1 subunit (*5*, *6*). FIXa, a vitamin K-dependent serine protease, is a two-chain protein linked by a disulfide bond (*7*). The light chain comprises the N-terminus containing the γ-carboxyglutamic acid (Gla)-rich domain and two EGF domains, whereas the heavy chain comprises the serine protease domain.

FVIIIa binds the phospholipid membrane via the C2 domain of the A3-C1-C2 subunit (*8*) with high affinity ($K_d \approx 10^{-9}-10^{-11}$ M depending on membrane composition; *9*). The binding of FIXa to phosphatidylserine-containing vesicles occurs with a somewhat lower affinity ($K_d \approx 15$ nM) (*10*). Binding to the phospholipid membrane has the effect of limiting the interaction to two dimensions and reduces both the K_d for FVIIIa−FIXa interaction and the K_m for substrate FX (*11*). Two subunits of the FVIIIa trimer contain FIXa interactive sites. The isolated A2 subunit has been shown to interact with the serine protease domain of FIXa, but with

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[1] Abbreviations: Ac-A3-C1-C2, acrylodan-labeled A3-C1-C2; FVIIIa, factor VIIIa; B-FVIII, B-domainless factor VIII (defined as corresponding to human factor VIII residues 1−745 and 1640−2332); FIXa, factor IXa; FXa, factor Xa; Fl-FFR-FIXa, factor IXa modified at its active site with fluorescein-Phe-Phe-Arg-chloromethyl ketone; EGF, epidermal growth factor; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; BHK, baby hamster kidney; FBS, fetal bovine serum; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 4-morpholinethanesulfonic acid.

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low affinity ($K_d \approx 300$ nM; *12*) as compared to intact FVIIIa ($K_d = 2-20$ nM; *13*, *14*). However, in contrast to the other isolated subunits that have no discernible FIXa stimulating activity, the A2 subunit stimulates the k_{cat} for FIXa-catalyzed conversion of FX by as much as 100-fold (*12*). Mutations associated with hemophilia A within the FIXa-interactive 558−565 region of FVIII have been shown to affect the k_{cat} of FXase but do not appreciably alter the affinity of FVIIIa for FIXa, demonstrating the importance of this region in promoting cofactor activity (*15*). On the other hand, a high-affinity interaction between FVIII light chain and FIXa ($K_d \approx 15$ nM) (*16*) was obtained using a solid-phase nonequilibrium binding assay. This site was localized within the A3 domain, occurring between residues 1811−1818 (*17*). Based on the differing affinities of FVIIIa and its subunits for FIXa, a model has been proposed in which FVIIIa binds FIXa via the high-affinity interaction between the A3 domain of the FVIII light chain and the FIXa light chain, permitting the A2 subunit to modulate the active site region of the proteinase (*18*).

In this study, we examine the affinity of A3-C1-C2 subunit for FIXa by a steady-state fluorescence approach in the absence and presence of phospholipid vesicles. Competition analyses using a functional assay reveal a disparate capacity for this subunit to inhibit the FVIIIa−FIXa interaction dependent upon the presence of vesicles and suggest a significant contribution by A2 subunit to the binding energy in the membrane-formed FXase. This role for A2 is directly confirmed following assessment of site-directed mutations in A2 affecting the interprotein affinity.

MATERIALS AND METHODS

Reagents. Recombinant FVIII preparations were a kind gift of the Bayer Corporation. The reagents α-thrombin, FIXaβ, FX, FXa (Enzyme Research Laboratories), fluorescein-Phe-Phe-Arg-factor IXa (Fl-FFR-IXa) (Molecular Innovations), and hirudin, PE, PC, and PS (Sigma) were purchased from the indicated vendors. Phospholipid vesicles composed of 40% PE, 20% PS, and 40% PC were prepared using octyl glucoside as previously described (*19*). FVIII-deficient plasma was prepared as previously described (*20*). Activated partial thromboplastin was purchased from General Diagnostics Organon Teknika. The anti-FVIII monoclonal antibody R8B12 (*21*), which recognizes the COOH-terminal region of the A2 domain, was obtained from Green Mountain Antibodies. The anti-FVIII monoclonal antibody ESH-8, which recognizes the C2 portion of the light chain, was purchased from American Diagnostica Inc. The B-domainless FVIII (B-FVIII) expression construct HSQ-MSAB-NotI-RENeo was a gift kindly provided by Dr. Pete Lollar and John Healey. FBS was obtained from Gemini Bioproducts. All other reagents used for BHK cell culture were obtained from Gibco BRL. The synthetic peptides corresponding to FVIII residues 431−447 and 708−717 were obtained from Quality Controlled Biochemicals Inc.

Subunit Preparation. FVIII light chain and heavy chains were isolated following treatment with EDTA and subsequent chromatography using SP-Sepharose and Q-Sepharose as previously described (*22*). The A2 subunit was prepared by cleavage of heavy chain as previously described (*22*). The A3-C1-C2 subunit was prepared following cleavage of FVIII light chain by thrombin (molar ratio 400:1) in 0.2 M NaCl−buffer A (20 mM Hepes (pH 7.2), 10 mM $CaCl_2$, 0.01% Tween 20) at 37 °C for 30 min. The reaction was stopped by the addition of a molar excess over thrombin of D-Phe-Pro-Arg chloromethyl ketone. A3-C1-C2 was dialyzed into 0.4 M NaCl, 20 mM MES, 10 mM $CaCl_2$, 0.01% Tween 20 (pH 6.0). The concentration of NaCl was adjusted to 0.2 M prior to application to SP-Sepharose (10 mL). The column was washed with 10 column volumes of 0.1 M NaCl−buffer A, and A3-C1-C2 was eluted by the addition of 0.6 M NaCl−buffer A. FVIII light chain labeled with acrylodan (Ac) was prepared as previously described (*23*). Ac-A3-C1-C2 was prepared by thrombin cleavage of Ac-light chain and purified as described above.

Assays. The rate of FXa generation was measured in a purified system either in the presence or in the absence of phospholipid vesicles as previously described (*15*). FVIII was activated in the presence of FIXa by the addition of 10 nM thrombin, and after 1 min, thrombin was inhibited by the addition of hirudin (2.5 u/ml). Alternatively, isolated A2 subunit was used in place of FVIIIa. The conversion of substrate FX to FXa was initiated by addition of FX (1 μM). Aliquots were removed after an appropriate time to assess initial rates of FXa production and added to tubes containing EDTA (80 mM final concentration). Rates of FXa generation were measured by the addition of the chromogenic substrate S-2765 (0.46 mM final concentration), and the reactions were read at 405 nm using a Vmax microtiter plate reader (Molecular Devices).

FVIII activity measured in a one-stage clotting assay was performed using substrate plasma chemically depleted of FVIII. FVIII antigen (Ag) was measured by sandwich ELISA using the antibody ESH-8 (10 μg/mL) as a capture antibody and biotinylated R8B12 (10 μg/mL) as a detection antibody as described (*15*).

FVIII Mutagenesis, Expression and Partial Purification. Alanine substitutions for residues Met567, Glu633, Asp712, and Lys713 were separately constructed in the FVIII expression construct HSQ-MSAB-NotI-RENeo as previously described (*15*). FVIII expression constructs were mutated by site-directed mutagenesis and transfected into BHK cells by liposome-mediated transfection. Stably expressing clones were selected by resistance to the neomycin analogue G418. For comparisons of FVIII activity and antigen, the FVIII-expressing BHK cells were plated and grown to near confluence in serum-supplemented DMEM/F12 medium. The medium was then replaced with AIM-V/PFHMII (1:1, v/v), and the plates were placed in a 30 °C/5% CO_2 incubator for 48 h, at which time the medium was collected. FVIII activity and antigen in the conditioned media were measured by one-stage clotting assay and ELISA as previously described (*15*). To partially purify native or mutant FVIIIHSQ, FVIII-expressing BHK cells were grown in three roller bottles, and 100 mL of AIM-V/PFHSMII (1:1) expression media was harvested daily. The conditioned media from daily collections was centrifuged, filtered through a 0.45 μm Nalgene filter, and buffered to pH 6.0 using 20 mM MES. This material was applied to a column of SP-Sepharose (5 mL) equilibrated with 20 mM MES (pH 6.0), 0.1 M NaCl, and 5 mM $CaCl_2$ at 4 °C. The column was washed with 10 volumes of 0.2 M NaCl−buffer A, and FVIII was eluted using 0.6 M

NaCl−buffer A. Protein concentrations were determined by Coomassie dye binding (*24*). FVIII was concentrated using a MicroCon concentrator (10-kDa cutoff) (Millipore, Billerica, MA) and dialyzed in either 50 mM NaCl− or 0.1 M NaCl−buffer A, 20 mM Hepes (pH 7.2), and 5 mM $CaCl_2$. The FVIII was approximately 70% pure based upon visualization of the stained gel. The primary contaminant was albumin.

Fluorescence Spectroscopy. Fluorescence energy transfer was used to measure the interaction of Ac-A3-C1-C2 with Fl-FFR-IXa. Reactions contained 100 nM Ac-A3-C1-C2 (or A3-C1-C2) and varying amounts of Fl-FFR-IXa (or FIXa) in the presence or absence of 50 μM PS/PC/PE vesicles. Reactions were performed at room temperature in 0.1 M NaCl−buffer A. Fluorescence measurements were performed using an Aminco-Bowman series 2 luminescence spectrometer (Thermo-Spectronic, Rochester, NY). Samples were excited at 395 nm (bandwidth = 4 nm), and fluorescence emission was monitored between 420 and 540 nm. All spectra were corrected for background. Corrected fluorescence values were obtained by integrating fluorescence emission between 450 and 480 nm. Duplicate values were obtained for each sample, and four separate samples were analyzed.

Electrophoresis. SDS−PAGE was performed by the method of Laemmli (*25*) using a Bio-Rad minigel system. Electrophoresis was performed at 100 V for 60 min, and protein bands were visualized using Coomassie blue staining.

Data Analysis. Functional K_d values were determined from data fitted using a single-site ligand-binding model given by the following equation using the Marquart algorithm and computed using Kaleidagraph software.

$$B = \frac{[\text{Free}]B_{max}}{[\text{Free}] + K_d} \quad (1)$$

Because the concentration of FVIIIa was much greater than the concentration of FIXa for all FVIIIa levels, the value for free FVIIIa used the total FVIIIa concentration; therefore, the K_d determined is an apparent K_d. Values for k_{cat} were calculated using the capacity term of eq 1. Competitive K_i values were obtained using the eq 2, where values for B_{max} and K_d are derived from eq 1.

$$B = \frac{[\text{Free}]B_{max}}{\left(1 + \frac{I}{K_i}\right)K_d + [\text{Free}]} \quad (2)$$

The relative fluorescence values (F) were determined using the equation

$$F = \frac{F_{DA} - F_A}{F_D} \quad (3)$$

where F_{DA} is the fluorescence intensity of labeled donor plus labeled acceptor (Ac-A3-C1-C2/Fl-FFR-IXa), F_D is the fluorescence intensity of labeled donor plus unlabeled acceptor (Ac-A3-C1-C2/FIXa), and F_A is the fluorescence intensity of unlabeled donor plus labeled acceptor (A3-C1-C2/Fl-FFR-FIXa). The values obtained were plotted as a function of Fl-FFR-IXa, and data from all samples were fitted

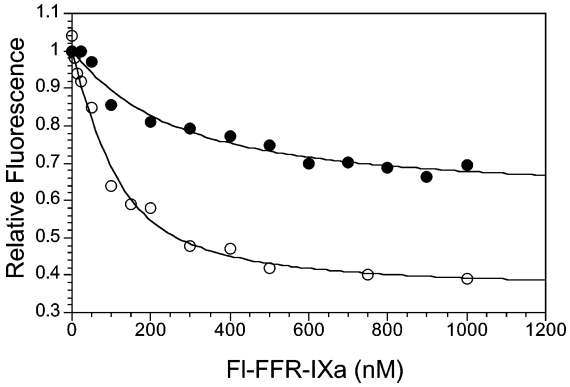

FIGURE 1: Relative fluorescence of Ac-A3-C1-C2 in the presence of variable Fl-FFR-IXa. The relative fluorescence emission of 100 nM Ac-A3-C1-C2 between 450 and 480 nm was measured in the presence of varying amounts of Fl-FFR-IXa. Reactions were performed in the presence of 50 μM PS/PC/PE vesicles (○) and absence of vesicles (●). Values shown were determined after measurement of appropriate controls and calculated as described in Materials and Methods. Curves were drawn from the fitted data using the equilibrium equation described in Materials and Methods. Data points represent the mean of at least three separate determinations.

using the previously described quadratic equation (*23*),

$$F = 1 - k(A_0 + [\text{FIXa}]_0 + K_d - \sqrt{(A_0 + [\text{FIXa}]_0 + K_d)^2 - 4A_0[\text{FIXa}]_0}) \quad (4)$$

where F is the relative fluorescence obtained from eq 3, A_0 is the A3-C1-C2 concentration, $[\text{FIXa}]_0$ is the concentration of FIXa, K_d is the dissociation constant, and k is a constant.

RESULTS

Preparation of A3-C1-C2 and Ac-A3-C1-C2. To assess the contribution made by the A3-C1-C2 subunit to FIXa interaction, as well as the capacity for this subunit to inhibit the FVIIIa−FIXa interaction, purified A3-C1-C2 and the acrylodan-labeled subunit were prepared as described in Materials and Methods. Activity of the labeled and unlabeled subunits was determined following reconstitution of FVIIIa with saturating amounts of A1 and A2 subunits. FVIIIa reconstituted from the fluorophore-labeled A3-C1-C2 possessed ~50% the activity of FVIIIa prepared from the unmodified subunit as judged by FXa generation assays and approached the activity observed for native FVIIIa (data not shown), indicating the subunit preparations retained significant activity that was not markedly affected by incorporation of the fluorophore.

Determination of Affinity of A3-C1-C2 for FIXa. The affinity of the isolated A3-C1-C2 for FIXa was measured by fluorescence energy transfer. Fluorescence emission of 100 nM Ac-A3-C1-C2 (fluorescence donor) following interaction with increasing concentrations Fl-FFR-IXa (fluorescence acceptor) was measured with appropriate unlabeled controls (Figure 1). Increasing concentrations of Fl-FFR-IXa yielded a saturable decrease in Ac-A3-C1-C2 emission (Table 1), the value of which was dependent upon the presence of phospholipid vesicles. This observation suggested an altered spatial separation of fluorophores; hence, a differing orientation of components in the membrane-bound compared with soluble complex.

Table 1: Affinity and Fluorescence Quenching Parameters Following Interaction of A3-C1-C2 and FIXa

reactions[a]	K_d[b] Ac-A3-C1−C2 (nM)	RF[c] max
plus PS/PC/PE	52 ± 10	0.31 ± 0.03
minus PS/PC/PE	197 ± 55	0.58 ± 0.03

[a] Reactions were performed at room temperature as described in Materials and Methods. [b] K_d values were obtained from eq 4. [c] Relative fluorescence of Ac-A3-C1-C2 at saturating levels of Fl-FFR-IXa.

Affinity values were determined by use of the equation described in Materials and Methods. The affinity value determined for the A3-C1-C2−FIXa interaction (K_d = 52 ± 10 nM) in the presence of phospholipid was somewhat greater, though of a similar magnitude to previously described affinity of A1/A3-C1-C2 dimer for FIXa as determined by steady-state fluorescence anisotropy (11.6 ± 6.7 nM) (*26*), indicating only a small contribution of A1 subunit to the binding energy for this interaction. In the absence of PS/PC/PE vesicles, the interprotein affinity was decreased ~4-fold. Taken together, the results from energy transfer indicate that inclusion of membrane makes a small contribution to the affinity of A3-C1-C2 for FIXa, whereas the membrane appears to alter the conformation or orientation of the bound complex or both.

Competitive Inhibition of FVIIIa Interaction with FIXa. The above results indicated the FVIIIa A3-C1-C2 subunit participates in a high-affinity interaction with FIXa. To assess this interaction relative to that of intact FVIIIa for FIXa, a series of competition experiments were performed. FXa generation reactions were performed to determine K_i values for A3-C1-C2 interaction with FIXa in the presence of varying concentrations of FVIIIa (Figure 2a,b). The observed inhibition of FXa generation reflects A3-C1-C2 competition with FVIIIa for FIXa binding since this FVIIIa subunit shows no affinity for the FX substrate (*27*). Reactions were run in the absence and presence of two concentrations of A3-C1-C2 (250 and 500 nM) and further performed both in the presence and in the absence of PS/PC/PE vesicles. As shown in Table 2, reactions run in the absence of the competitor yielded functional K_d values of 4 and ~350 nM for the interprotein interaction in the presence and absence of vesicles, respectively. Comparison of these data with the data derived from the energy transfer analysis indicated that in the absence of vesicles, the affinity value for the FVIIIa−FIXa interaction was similar for that of A3-C1-C2 with FIXa, consistent with the latter interaction providing essentially all of the binding energy derived from the cofactor. However, inclusion of vesicles resulted in a 10-fold increase in the affinity for FVIIIa ($K_d \approx 4$ nM, Table 2) compared with A3-C1-C2 ($K_d \approx 52$ nM, Table 1). This result was consistent with a membrane-dependent contribution from other FVIIIa subunit(s) to the interaction with FIXa.

While the K_i values determined from the functional inhibition of FVIIIa−FIXa by the A3-C1-C2 inhibitor (in the absence of vesicles) were within a factor of 2 of the K_d value determined, the A3-C1-C2 subunit was observed to be a poor inhibitor of FXase formed on the membrane surface (Table 2). The K_i values determined in this situation were 2 orders of magnitude greater than the affinity of the FVIIIa−FIXa interaction, supporting the contribution of additional FVIIIa subunits to the membrane-bound FXase. Since an earlier observation indicated little contribution of the A1 subunit to this interaction, as judged by similar affinity of A1/A3-C1-C2 (*26*) to that of A3-C1-C2 for binding FIXa determined in this study, we focused our attention on the role of the A2 subunit in this association.

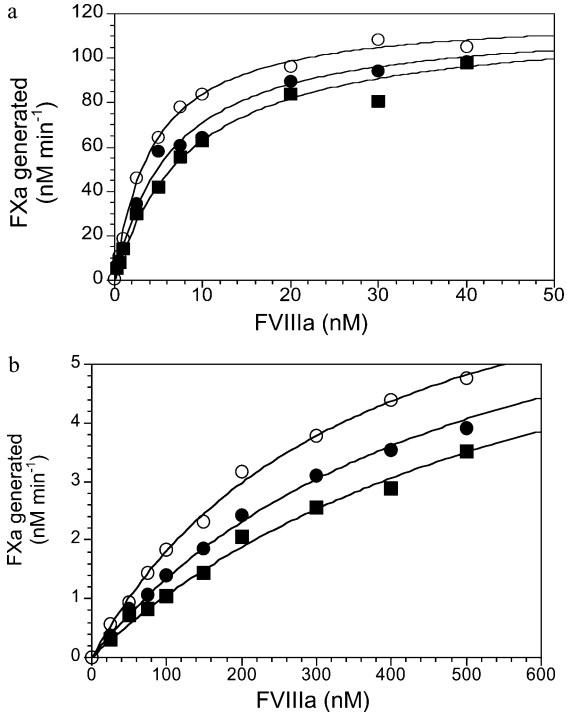

FIGURE 2: Effects of A3-C1-C2 on FIXa-mediated FXa generation in the presence of FVIIIa. FXa generation reactions were performed in the absence of A3-C1-C2 (○) and presence of 250 nM A3-C1-C2 (●) and 500 nM A3-C1-C2 (■) and contained indicated amounts of FVIIIa. In panel a, reactions were performed in the presence of 10 μM PS/PC/PE vesicles and 1 nM FIXa. In panel b, reactions were run in the absence of vesicles and contained 5 nM FIXa. All reactions contained 1 μM FX. Curves were fitted using the equilibrium equations described in Materials and Methods. Data points represent the mean of three to five separate determinations.

Table 2: Affinity Determinations for the FVIIIa−FIXa Interaction and Effects of A3-C1-C2 Subunit

reaction[a]	K_d[b] FVIIIa	K_i[b] 250 nM A3-C1-C2	K_i[b] 500 nM A3-C1-C2
plus PS/PC/PE	4.0 ± 0.3	404 ± 60	432 ± 48
minus PS/PC/PE	351 ± 29	553 ± 124	546 ± 104

[a] Reactions were performed as described in Materials and Methods. [b] K_i values and range were determined using eq 2 where K_d ± standard deviations were obtained from reactions run in the absence of A3-C1-C2 using eq 1 as described in Materials and Methods.

Effects of Synthetic Peptides on FXa Generation. Prior results have shown that peptides containing the A2 sequence 558−565 block the functional interaction of FVIIIa (or isolated A2 subunit) with FIXa (*26*, *28*). Recent modeling studies suggest an extended interactive surface for the A2−FIXa interface (*29*). This model includes the 558−565 region, as well as spatially adjacent residues contained within regions 431−447 and 708−717. Peptides to these latter segments were synthesized as defined by the sequence with the exception that Cys710 was replaced with serine to eliminate peptide dimerization. The effects of these peptides

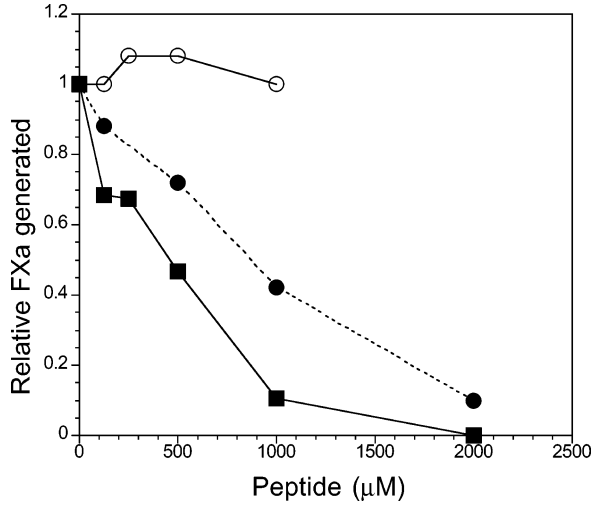

FIGURE 3: Effects of synthetic peptides on A2- and FVIIIa-mediated FXa generation. The indicated concentrations of peptide 708−717 were added to reactions containing 200 nM A2 and 5 nM FIXa (●) and reactions containing 1 nM FVIIIa and 5 nM FIXa (■). Reactions containing the indicated amount of peptide 431−447 with 200 nM A2 subunit and 5 nM FIXa are shown in open circles. All reactions were run in the presence of 10 μM PS/PC/PE vesicles. Data points represent the mean of at least three separate determinations.

on rates of A2 subunit-dependent FXa generation were determined. Increasing concentrations of peptide 431−447 in FXa reactions containing 200 nM A2 and 5 nM FIXa run in the presence of 10 μM PS/PC/PE vesicles showed no effect on FXa generation rates (Figure 3). In contrast, increasing concentrations of peptide 708−717 resulted in inhibition of FXa generation in a dose-dependent manner ($IC_{50} \approx 830$ μM). This effect was also observed in reactions replacing the isolated A2 subunit with 1 nM FVIIIa ($IC_{50} \approx$ 450 μM) (Figure 3). The inhibitory effect of the peptide 708−717 on both the A2- and FVIIIa-dependent stimulation of FIXa-mediated generation of FXa suggested that residues within this region contribute to the interaction with FIXa.

FVIII A2 Domain Site-Directed Mutant Analyses. In a recent study, we evaluated point mutations in the 558 loop of A2 and found little if any contribution of these residues to the inter-FVIIIa−FIXa affinity with the possible exception of Asp560 (*15*). The above results using synthetic peptides suggested the potential for residues within the 708−717 segment of A2 being FIXa-interactive. Charged residues within this segment predicted by the interface model to be interactive with FIXa (Asp712 and Lys713) were individually mutated to alanine. These and other selected alanine-substituted point mutations at proposed FIXa-interactive residues in A2 (*29*) were constructed, and protein was expressed as B-domainless FVIII (FVIIIHSQ) in BHK cells. The point mutations evaluated are shown in Figure 4, which indicates the relative activity of the stably expressed FVIII forms as compared to antigen. Native FVIIIHSQ had the predicted activity-to-antigen ratio of near unity (1.1). Of the mutant FVIII forms assessed, FVIII/Asp712Ala showed a markedly reduced activity to antigen (0.2, $p < 0.01$), whereas the value for FVIII/Lys713Ala was only marginally altered (0.7, $p < 0.01$). Ratio values for the other mutants (Met567Ala and Glu663Ala) were >0.8 and thus deemed wild-type-like and not subjected to further evaluation.

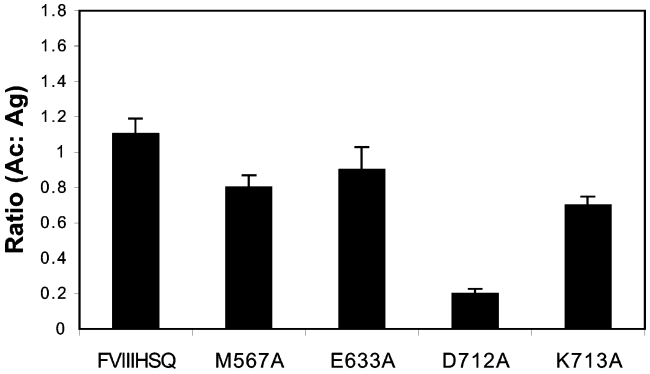

FIGURE 4: Activity/antigen ratios of FVIIIHSQ and FVIII mutants. The FVIII activity and antigen levels of expressed FVIIIHSQ and mutants were measured by clotting activity and ELISA, respectively. The ratio of activity to antigen was determined on the basis that 300 ng of FVIII corresponds to 1 unit. Values shown are the mean and standard deviation of six individual measurements.

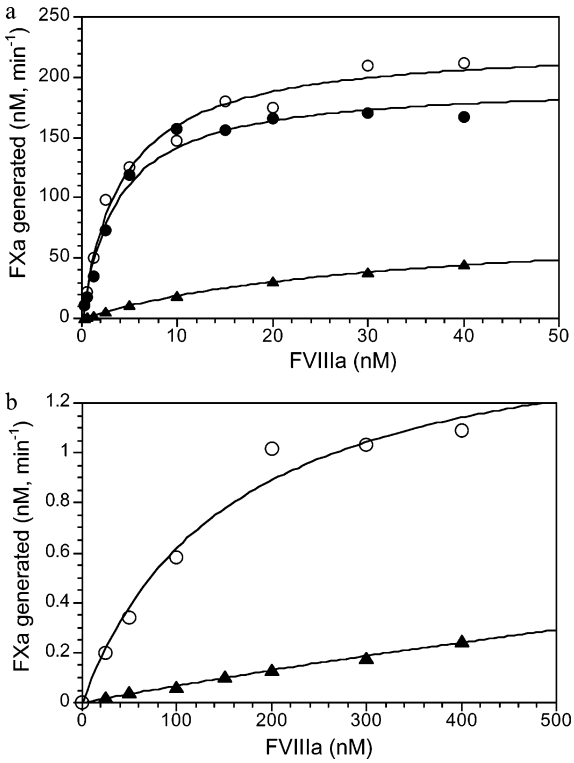

FIGURE 5: Effects of A2 domain mutations on FIXa-catalyzed FXa generation. In panel a, reactions were performed using 1 nM FIXa, 10 μM PS/PC/PE vesicles, and variable amounts of FVIIIa prepared from FVIIIHSQ (○), FVIII/D712A (▲), and FVIII/K713A (●). In panel b, reactions were performed in the absence of vesicles for FVIIIHSQ (○) and FVIII/D712A (▲) containing 1 nM FIXa. Data points represent the mean of at least three separate determinations.

The mutant proteins FVIII/D712A and FVIII/K713A and wild-type FVIIIHSQ were partially purified from conditioned media. Cofactor activities and functional affinities of the mutant proteins for FIXa were assessed by FXa generation assays in a purified system in the presence (Figure 5a) and absence (Figure 5b) of PS/PC/PE vesicles. While the k_{cat} obtained with FVIII/K713A in the presence of vesicles was not appreciably affected, substitution of Asp712 to alanine reduced this parameter by ~3-fold (Table 3). This result suggested Asp712 contributed to modulating the active site region of FIXa. FXase comprised of saturating levels of the mutant FVIII forms in the presence of vesicles showed

Table 3: Kinetic and Binding Parameters for Expressed FVIIIHSQ and Mutant Proteins[a]

FVIII	K_d (nM)	K_m (nM)	k_{cat}	K_d (−PS/PC/PE)	$k_{cat(app)}$ (−PS/PC/PE)
FVIIIHSQ	4.2 ± 0.6	45 ± 5	227 ± 8	173 ± 30	1.6 ± 0.1
Asp712Ala	35 ± 1.5	30 ± 9	83 ± 2	757 ± 152	0.6 ± 0.1
Lys713Ala	3.7 ± 0.6	60 ± 5	195 ± 8	*b*	*b*

[a] Reactions were run in the absence or presence of 10 μM PS/PC/PE vesicles as described in Materials and Methods. Parameter values and standard deviations were derived from curve fitting. Values for k_{cat} obtained in the absence of vesicles are apparent values due to the high K_m (>1 μM) for FX. K_m values were determined from FX titrations performed in the presence of 10 μM PS/PC/PE vesicles. [b] Not determined.

modest effects on the K_m as determined by FX titrations (Table 3), suggesting interactions with substrate FX were not dramatically altered.

Functional affinity parameters determined by FXa generation revealed that FVIII/D712A showed a marked decrease (~8-fold) in affinity for FIXa in comparison to native FVIIIHSQ or the K713A mutant when assayed in the presence of PS/PC/PE vesicles. In the absence of vesicles, this mutation yielded a 4-fold reduction in affinity. Taken together, these data suggest alteration of Asp712 disrupts the interprotein interaction. The reduction in thermodynamic stability attributed to the Asp712Ala mutation observed in the presence of vesicles (1.2 kcal mol^{-1}; 10.2 kcal mol^{-1} versus 11.4 kcal mol^{-1} for mutant and wild-type, respectively) was similar to that observed in their absence (1.1 kcal mol^{-1}; 8.1 kcal mol^{-1} versus 9.2 kcal mol^{-1}). Thus the reduced specific activity for this FVIII form, as judged by the activity/antigen ratio, likely resulted from a combination of reduced affinity for FIXa and impaired capacity to modulate the FIXa active site.

DISCUSSION

The mechanisms by which FVIIIa binds and subsequently modulates FIXa activity remain controversial and poorly understood. Both FVIII and FVIIIa have been shown to interact with FIXa, though cleavage of the FVIII heterodimer to FVIIIa by thrombin is required to increase the catalytic efficiency of FIXa (see refs *30* and *31* for reviews). While the FVIII light chain has been proposed to account for essentially all of the interprotein binding energy contributed by FVIIIa, these conclusions arise from data employing nonequilibrium binding assays and competition assays using FVIII and FVIII light chain (*16*, *17*) rather than FVIIIa and the A3-C1-C2 subunit. Results presented in this report assess A3-C1-C2 affinity for FIXa using both physical (steady-state fluorescence resonance energy transfer) and functional (competition with FVIIIa-dependent rates of FXa generation) assays.

The affinity of A3-C1-C2 for FIXa determined by fluorescence energy transfer yielded similar values (within a factor of ~4) independent of the presence of a membrane surface. Furthermore, the competition-based functional assay performed in the absence of membrane revealed similar values for the K_i for the subunit and K_d for the FVIIIa−FIXa interaction, and these values were equivalent to the K_d value obtained for A3-C1-A2 and FIXa. Taken together, these data suggest that the A3-C1-C2 subunit contributes most if not all of the thermodynamic stability within the soluble complex. However, the observation that A3-C1-C2 was a poor competitor to the FVIIIa−FIXa interaction in the presence of vesicles, based on the observation that K_i was ~100-fold greater than K_d, suggested that interactions contributed by other FVIIIa subunits within the membrane-bound, physiological FXase add to the interprotein binding.

The isolated A2 subunit of FVIIIa has been shown to stimulate the k_{cat} of FIXa by ~100-fold in the presence of a membrane (*12*), whereas limited stimulation is observed in its absence (*32*) as a result of the inability to saturate the response. With use of this stimulation of FXa generation to estimate binding affinity, values of ~300 nM (*12*) and >5 μM (*32*) for the affinity of A2 subunit for FIXa have been reported for the membrane-dependent and -independent reactions, respectively. The reason for these disparate affinity values is not clear, since A2 shows no capacity to directly interact with membrane. Alternatives include the membrane-bound FIXa assuming a conformation more amenable to binding A2 or an increase in the probability for productive collisions in the presence of membrane or both. This speculation is consistent with specific mutations in FIXa where replacement of the EGF1 domain with that of FVII increased the affinity for FVIIIa by ~10-fold (*33*). Furthermore, elongation of the spacer region between EGF1 and EGF2 resulted in reduced levels of stimulation by FVIIIa or the isolated A2 subunit without altering affinity of the latter (*34*). Thus FIXa conformation is critical for interactions with FVIIIa (subunits) and subsequent stimulation of catalytic activity.

Based upon data suggesting that the 558 loop region in A2 binds the 330 helix in the protease domain of FIXa, Bajaj et al. (*29*) proposed a model for this interface that comprised the 558 loop as well as spatially adjacent regions. In a recent study, mutagenesis of several of the individual 558 loop residues to alanine was accomplished, and resultant FVIII forms were expressed, purified, and evaluated for FIXa binding (*15*). Results of that study showed that while several residues appeared critical in contributing to the stimulation of FIXa activity, only Asp560 appeared to make a contribution, albeit marginal, to binding FIXa. More recently, we determined that a cluster of basic residues within the A2 domain comprised of Arg489, Arg490, and Lys493 together contributed to cofactor activity based upon alanine-scanning mutagenesis studies while individually made little if any contribution to affecting FIXa activity (*35*). Similar to many of the 558 loop residues, these basic residues did not appear to play a role in the interprotein affinity.

In the present study, inhibition of the A2−FIXa interaction by synthetic peptides comprising regions of A2 proposed to localize at the interprotein interface was used in combination with the interface model to assess the role of these regions. The observation that the 708−717 peptide inhibited both FVIIIa- and isolated A2-dependent stimulation of FIXa-catalyzed generation of FXa, coupled with the prediction that Asp712 and Lys713 lie at the interface (*29*), supported a study assessing mutagenesis at these sites. These latter studies revealed that Asp712 contributed to the binding of FVIIIa to FIXa based upon a similar reduction in thermodynamic stability of the complex measured in the absence or presence of membrane following replacement of this residue with

alanine. While these results add general support to the interface model, a lack of effect of several other A2 subunit residues postulated to contribute to forming the interface suggests that some refinement of the model is necessary.

The observation that A2 subunit contributes to the FVIIIa−FIXa interactive surface and thermodynamic stability explains the failure of isolated A3-C1-C2 to effectively inhibit the interaction of cofactor with the proteinase in the physiological FXase. However, this contribution of A2 appears to be dependent upon the presence of a membrane. Thus we speculate that in the presence of membrane, A2 subunit is correctly positioned via interactions with A1 subunit (*36*) and possibly A3-C1-C2 to properly align with the protease domain of FIXa thereby allowing formation of intermolecular contacts as predicted in part by the interface model (*29*). These contacts supplement those formed between A3-C1-C2 and other regions in FIX, which also appear dependent upon the membrane surface for proper orientation based upon differences in donor quenching values observed in the absence and presence of vesicles. In total, these interactions have been shown to stabilize the labile FVIIIa structure (*37*, *38*) by a mechanism consistent with a reduction in the A2 dissociation rate constant (*39*). Thus preservation of a relatively stable cofactor structure bound to FIXa via contacts with two FVIIIa subunits precludes facile inhibition by isolated A3-C1-C2.

In the absence of membrane, this FIXa-dependent stabilizing effect on the FVIIIa heterotrimer is lost (*37*) and A2 subunit affinity for the A1/A3-C1-C2 dimer is reduced (*38*). This effect was observed by fluorescence analysis showing saturable quenching of Ac-A2 subunit by Fl-A1/A3-C1-C2 in the presence of FIXa and PS/PC/PE vesicles, whereas saturation of donor quenching was not obtained in the absence of the membrane component (*32*). Thus the weakened interaction of A2 subunit with the A1/A3-C1-C2 dimer as well as with FIXa, the latter brought about the absence of membrane, is consistent with a lack of significant contribution of the subunit to altering the inhibition kinetics of A3-C1-C2. However, a similar contribution of Asp712 to the thermodynamic stability of the FVIIIa−FIXa interaction, independent of membrane, suggested that this contact is maintained in the absence of phospholipid while other yet to be identified contacts are lost.

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