Factor VIII A1 Domain Residues 97−105 Represent a Light Chain-Interactive Site†

Charles Ansong, Stephen M. Miles, and Philip J. Fay*

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, New York 14642

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ABSTRACT: Results from a recent study on subunit association in factor VIIIa indicated that the A1 and A3C1C2 domains contribute approximately 90% of the interchain binding energy in factor VIII and that A3 domain residues 1954−1961 participate in the interaction with A1 domain (Ansong, C., and Fay, P. J. (2005) *Biochemistry 44*, 8850−8857). Enhanced trypsin-accessibility at four sites within residues 89−142 in free A1 compared with that of A3C1C2-bound A1, as determined by mass spectrometry, suggested that residues within this region are interactive with the A3C1C2 domains. A synthetic peptide to A1 domain residues 97−105, predicted to be A3 domain-interactive from molecular modeling, inhibited the formation of a functional A1/A3C1C2 dimer (apparent $K_i = 0.64 \pm 0.21$ μM) and reduced the efficiency of energy transfer between a fluorescein-labeled A1 subunit and an acrylodan-labeled A3C1C2 subunit. B-domainless factor VIII point mutants, His99Ala, Val101Ala, and Gly102Ser, exhibited reduced specific activity (32%, 51%, and 45%, respectively) compared with that of factor VIII wild type. Furthermore, the activity of factor VIII His99Ala was less stable ($t_{1/2} = 2.3 \pm 0.2$ min) compared with that of factor VIII wild type ($t_{1/2} = 6.2 \pm 0.7$ min) following heat denaturation analysis. This reduced stability appeared to result from an ∼40% increase in the dissociation rate for the mutant factor VIII heterodimer as judged by solid-phase binding assays. These results indicate that residues within segment 97−105 of the A1 domain interact with residues within the A3C1C2 domains of the light chain and contribute to the interface in the factor VIII heterodimer.

The hereditary bleeding disorder hemophilia A is caused by a defect or deficiency in the plasma protein factor VIII. Factor VIII functions as a cofactor for the serine protease factor IXa in the phospholipid surface-dependent conversion of zymogen factor X to serine protease factor Xa, a reaction critical for the propagation phase of the coagulation cascade (*1*−*4*).

Factor VIII is synthesized as an ∼300 kDa single chain protein with the following domain structure: NH_2-A1-A2-B-A3-C1-C2-COOH. Factor VIII is processed into a series of heterodimers by cleavage at the B-A3 junction generating a heavy chain (HC[1]), represented by the A1-A2-B domains, and a light chain (LC) comprising the A3-C1-C2 domains (*5*, *6*). The HC is minimally represented by the A1 and A2 domains and associates with the LC via metal-ion-dependent and -independent interactions (*7*−*9*). A single copper ion has been identified in factor VIII and is suggested to contribute to this linkage (*10*, *11*). The circulating heterodimeric form of factor VIII represents the inactive procofactor form of the protein. Cleavage by thrombin at Arg 372 at the A1-A2 junction, Arg 740 at the A2-B junction and Arg 1689 near the *N*-terminus of the A3 domain generates the active cofactor form of factor VIII (*12*). Thus, activated factor VIII (factor VIIIa) is a heterotrimeric structure in which the A1/A3C1C2 dimer retains the metal-ion-dependent and -independent linkages, whereas the A2 subunit is in a weak electrostatic interaction with this dimer (*13*, *14*).

Intersubunit interactions in factor VIII remain poorly characterized. Previous studies suggested that both electrostatic and hydrophobic interactions mediate the reconstitution of factor VIII from isolated HC and LC (*15*). More recent studies indicated that hydrophobic interactions appear to be important for A1/A3C1C2 association and are not required for the interaction between A2 and the A1/A3C1C2 dimer in factor VIIIa (*16*). In a recent study (*17*), we evaluated subunit association in factor VIIIa. Affinity parameter values suggested that the A1 and A3C1C2 domains contribute at least 90% of the intersubunit binding energy in factor VIII with the A2 domain making little if any contribution. Studies characterizing the A1/A3C1C2 interface including peptide and site directed mutant analyses suggested that residues within the segment 1954−1961 of the A3C1C2 subunit contributed to the interaction with the A1 subunit in factor VIII(a).

In this study, we continue to characterize the A1/A3C1C2 intersubunit interaction with respect to contributions of the

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* To whom correspondence should be addressed. Phone: 585-275-6576. Fax: 585-473-4314. E-mail: Philip_Fay@urmc.rochester.edu.

[1] Abbreviations: MALDI, matrix assisted laser desorption ionization; TOF, time of flight; MS, mass spectrometry; CHCA, α-cyano-4-hydroxycinnamic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; MES, 4-morpholinethanesulfonic acid; BSA, bovine serum albumin; HC, factor VIII heavy chain; LC, factor VIII light chain; Ac-A3C1C2, acrylodan-labeled A3C1C2 subunit; Fl-A1, fluorescein-5-maleimide-labeled A1 subunit; FRET, fluorescence resonance energy transfer; BHK, baby hamster kidney.

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A1 domain. Binding of the A3C1C2 subunit to the A1 subunit decreased trypsin accessibility to residues within the A1 segment 89−142 suggesting that this region represents an A3C1C2 interactive site. Subsequent results from peptide inhibition studies and the analysis of selected site-directed mutants expressed as B-domainless factor VIII localize an A3C1C2-interactive site to residues within A1 segment 97−105.

MATERIALS AND METHODS

Reagents. Recombinant factor VIII (Kogenate) was a gift from Dr. Lisa Regan of Bayer Corp. (Berkeley, CA). Mass spectrometry grade trypsin (Promega, Madison, WI) and CHCA (Sigma Aldrich, St. Louis, MO) were purchased from the indicated vendors. The synthetic peptides representing A1 subunit residues 97−105 (SLHAVGVSY) and the scrambled sequence version (YVSGVSHLA) were obtained from BioSource, Camarillo, CA. Peptides were >90% pure as judged by HPLC. Phospholipid vesicles containing 20% PS, 40% PC, and 40% PE (Sigma, St. Louis, MO) were prepared using *N*-octylglucoside (*18*). Factor VIII-deficient plasma was prepared as previously described (*19*). Activated partial thromboplastin reagent was purchased from General Diagnostics Organon Teknika. The anti-factor VIII monoclonal antibody R8B12 (*14*), which recognizes a discontinuous epitope in the A2 domain (*20*), was obtained from Green Mountain Antibodies. The anti-factor VIII monoclonal antibody ESH-8, which recognizes the C2 domain of the light chain, was obtained from American Diagnostica, Inc. The B-domainless factor VIII expression vector (RENeo factor VIII) and Bluescript cloning vector (pBS factor VIII) were kindly provided by Dr. Pete Lollar and John Healey. All of the reagents used for the BHK cell culture were obtained from Invitrogen. The reagents α-thrombin, factor IXa, factor X, and factor Xa (Enzyme Research Laboratories, South Bend, IN), hirudin (VWR), and the chromogenic Xa substrate S-2765 (*N*-α-benzoyloxycarbonyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide-dihydrochloride; DiaPharm Group, Westchester, OH) were purchased from the indicated vendors. Fluorescein-5-maleimide and acrylodan were obtained from Molecular Probes (Eugene, OR).

Isolation of Factor VIIIa Subunits. Factor VIIIa subunits (A1, A2, and A3C1C2) and the A1/A3C1C2 dimer were isolated as previously described (*17*).

Limited Proteolysis. Time-limited proteolysis of factor VIII A1 subunit (1 μM final concentration) and factor VIII A1/A3C1C2 dimer (1 μM final concentration) was initiated by the addition of 5 μL of 100 ng/μL mass spectrometry grade trypsin in 50 mM NH_4HCO_3 buffer. The proteins were digested at 37 °C and stopped at the indicated times by the addition of an equal volume of a 50% acetonitrile and 1% TFA solution to the samples.

Mass Spectrometry. One microliter of sample was spotted to a stainless steel MALDI plate along with 1 μL of matrix solution (10 mg/mL CHCA in 1:1 acetonitrile/1% TFA). MALDI-TOF spectra were recorded in reflector mode (positive ion) on a Voyager-DE STR mass spectrometer. Mass spectra were externally calibrated using a set of five MS peptide standards (New England Biolabs, Ipswich, MA). Identification of the MALDI peptide masses was performed using the UCSF Protein Prospector suite of proteomic tools (http://prospector.ucsf.edu) and the MASCOT program (http://www.matrixscience.com) to match the experimental masses to the factor VIII sequence (accession number: P00451). For each time point in the time-limited proteolysis, 2−4 total mass spectra were acquired, from two different sample spots. The intensity values for the monoisotopic peaks were used to determine the relative abundance of the tryptic fragments generated by MALDI.

Assays. Factor VIII cofactor activity was determined using a factor Xa generation assay (*21*). A1 and A3C1C2 were mixed at the indicated concentrations in 20 mM HEPES at pH 7.2, 300 mM NaCl, 25 mM $CaCl_2$, and 0.01% Tween 20 at 4 °C overnight. The mixture was diluted 10-fold, and factor VIIIa was formed by the addition of the indicated amounts of A2 subunit in 20 mM HEPES at pH 7.2, 5 mM $CaCl_2$, 50 mM NaCl, 0.01% Tween 20, 100 μg/mL BSA, and 10 μM PSPCPE vesicles. Then, 50 nM factor IXa was added to the resultant factor VIIIa, and time-course reactions were initiated with the addition of 300 nM factor X. Aliquots were removed at appropriate times to assess initial rates of product formation and added to tubes containing EDTA (50 mM final concentration) to stop the reaction. Rates of factor Xa generation were determined by monitoring the cleavage of the chromogenic substrate, S-2765 (0.46 mM final concentration). Reactions were read at 405 nm using a Vmax microtiter plate reader (Molecular Devices, Sunnyvale. CA). All reactions were run at room temperature. Data points represent the mean of three separate determinations.

Factor VIII activity measured in a one-stage clotting assay was performed using substrate plasma chemically depleted of factor VIII as previously described (*22*).

Factor VIII Mutagenesis, Expression, and Purification. The point mutations, His99Ala, Val101Ala, and Gly102Ser, were constructed and transfected into BHK cells, and B-domainless factor VIII protein was expressed and purified as described previously (*17*). Active fractions were detected using a one-stage clotting assay. Factor VIII protein was measured by sandwich ELISA using the LC antibody ESH-8 (10 μg/mL) as a capture antibody and the HC antibody biotinylated R8B12 (10 μg/mL) as a detection antibody as previously described (*22*). Resultant factor VIII preparations were typically >80% pure as judged by SDS−PAGE with albumin representing the major contaminant. Factor VIII samples were stored at −80 °C.

Labeling of Subunits. Labeling of factor VIII A1 and A3C1C2 subunits with fluorescein 5-maleimide and acrylodan, respectively, was performed as described previously (*17*). Levels of probe incorporation were ∼0.8 mol acrylodan/mol of A3C1C2 subunit and ∼0.9−1.0 mol fluorescein-5-maleimide/mol of A1 subunit.

Fluorescence Resonance Energy Transfer. Fifty nanomolar A3C1C2 (or Ac-A3C1C2) and 0−300 nM A1 (or Fl-A1) were reconstituted in the presence of peptide overnight at 4 °C in 20 mM HEPES at pH 7.2, 25 mM $CaCl_2$, 300 mM NaCl, 0.01% Tween 20, and 200 μg/mL BSA. Fluorescence measurements were performed using an Aminco-Bowman Series 2 spectrometer at room temperature at 395 nm excitation wavelength and 4 nm bandwidth. Emission fluorescence was measured at 420−550 nm and all spectra corrected for background. The percentage of donor fluorescence quenching as a result of subunit reassociation was calculated from integrated fluorescence intensities at λ =

460−490 nm. The relative fluorescence was calculated as follows:

$$F(\%) = ([F_{DA} - F_A]/F_D) \times 100 \quad (1)$$

where F_{DA} is the fluorescence intensity of the labeled donor plus the labeled acceptor (Ac-A3C1C2/Fl-A1), F_A is the fluorescence intensity of the unlabeled donor plus the labeled acceptor (A3C1C2/Fl-A1), and F_D is the fluorescence intensity of the labeled donor plus the unlabeled acceptor (Ac-A3C1C2/A1). Triplicate values were obtained for each sample, and two independent analyses were performed.

Functional Stability Assay. The stability of factor VIII activity for wild-type or mutant proteins (20 nM) was assessed by factor Xa generation assay as described previously (*17*).

ELISA-Based Stability Assay. The intramolecular stability of factor VIII wild type or mutant (50 nM) was assessed as described previously (*17*) with the following modifications. Samples were incubated at 55 °C, and aliquots were removed at the indicated time points and applied to a sandwich ELISA assay to quantitate the amount of factor VIII heterodimer. Biotinylated R8B12 (10 μg/mL) and biotinylated 10104 (0.5 μg/mL) were used to detect the bound factor VIII heavy chain and light chain, respectively.

Data Analysis. Analysis of the interaction between A1 and A3C1C2 subunits using fluorescence resonance energy transfer was performed using the following equation by nonlinear least-squares regression:

$$F = 100 - k(L_0 + H_0 + K_d) - \sqrt{((L_0 + H_0 + K_d)^2 - 4L_0H_0)} \quad (2)$$

where F represents relative fluorescence (%), L_0 is the A3C1C2 concentration, H_0 is the A1 concentration, K_d represents the dissociation constant, and k is a constant.

An analysis of the inhibition of the interaction between A1 and A3C1C2 subunits by synthetic peptide using the factor Xa generation assay was performed using the following equation by nonlinear least-squares regression:

$$Y = C \times L_0/((1 + (I_0/K_i)) \times K_d) + L_0 \quad (3)$$

where Y represents activity (% of initial), L_0 is the A3C1C2 concentration, K_i is the dissociation constant for the A3C1C2-peptide complex, K_d is the dissociation constant for the A1/A3C1C2 complex, I_0 represents inhibitor concentration, and C is a constant. Nonlinear least-squares regression analysis was performed using the above equation. Analysis of the intramolecular stability of the various forms of expressed factor VIII molecules using the functional assay was performed using the following equations.

$$A = A_0 \times \exp[(-10^{-C}) \times t] \quad (4)$$

where A represents activity (% of initial), A_0 is the activity at initial time point, t is time, and C is $-\log k$, where k is the rate constant.

$$t_{1/2} = 10^C \times \ln(2) \quad (5)$$

where $t_{1/2}$ represents the half-life of exponential decay, and C is defined as in eq 4. Parameter values (A and C) and their standard deviations were estimated by nonlinear least-squares regression analysis using eq 4. Half-life ($t_{1/2}$) values were obtained using eq 5. The Student's t-test was performed on the average value for C.

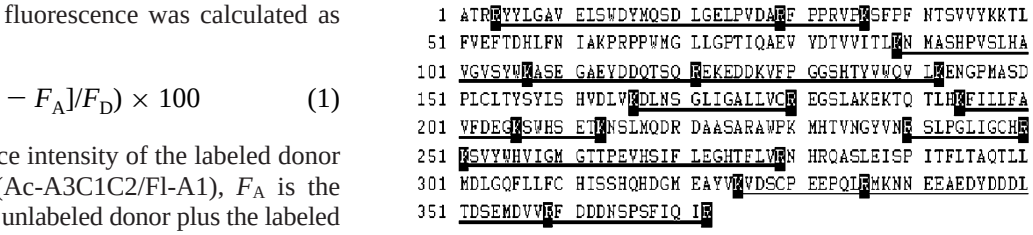

FIGURE 1: Sequence coverage of the A1 subunit following tryptic digest. Peptides observed by MALDI-TOF mass spectrometry are represented by underlined sequences. Highlighted Lys (K) and Arg (R) residues indicate cleavage sites that were analyzed for trypsin accessibility. The peptide map generated covers ~54% of the A1 sequence.

RESULTS

Tryptic Peptide Mapping of A1 Subunit by MALDI-MS. Cleavage of the A1 subunit with trypsin under nondenaturing conditions produced a set of peptide fragments that covered ~54% of the A1 sequence (Figure 1). Digestion times were limited in order to probe the more surface accessible cleavage sites. The peptides identified by MALDI included a number of regions of documented structural and functional importance including fragment 90−107, which contains a high concentration of apolar residues suggested to contribute to hydrophobic interactions mediating interchain association in factor VIII (*16*). This fragment also includes His99, that may potentially participate in a type 2 copper site, bridging the heavy and light chains (*23*). Also included in this peptide set are fragments 108−121 and 122−142, which contain a Ca^{2+} binding site in factor VIII (residues 110−126) (*24*) necessary for its active conformation, and fragments 326−336 and 360−372, which contain the P1 residues for cleavage by activated protein C (Arg336) (*25*) and thrombin (Arg372), respectively (*26*). This latter fragment has also been identified as an interactive site for factor X and Xa binding (*27*). Similar trypsin treatment of the isolated A3C1C2 subunit yielded a more restrictive set of peptides representing ~42% coverage of this subunit (data not shown).

Selective Protection of A1Domain Regions by A3C1C2. MALDI-MS analysis of the time-limited proteolysis experiments (Figure 2) were performed to assess sites in the A1 subunit that may be protected by association with the A3C1C2 subunit. Figure 2A and B represent the mass spectra for a tryptic digest of the A1 subunit following 15 and 30 min of incubations with trypsin, respectively. Figure 2C and D represents the mass spectra for a tryptic digest of the A1/A3C1C2 dimer following similar incubation times with trypsin. The above spectra are representative and typical of the several spectra acquired for each set of digest conditions. A comparison of the two time points for each reaction showed a general increase in the intensity of minor peaks, consistent with a time dependence for reaction rates. A comparison of the MALDI spectra for the reactions using the free A1 subunit (Figure 2A and B) relative to the spectra for the reactions using the A1/A3C1C2 dimer (Figure 2C and D) shows significant reductions in the relative ion abundance for the tryptic fragments at $m/z = 1556$ (corre-

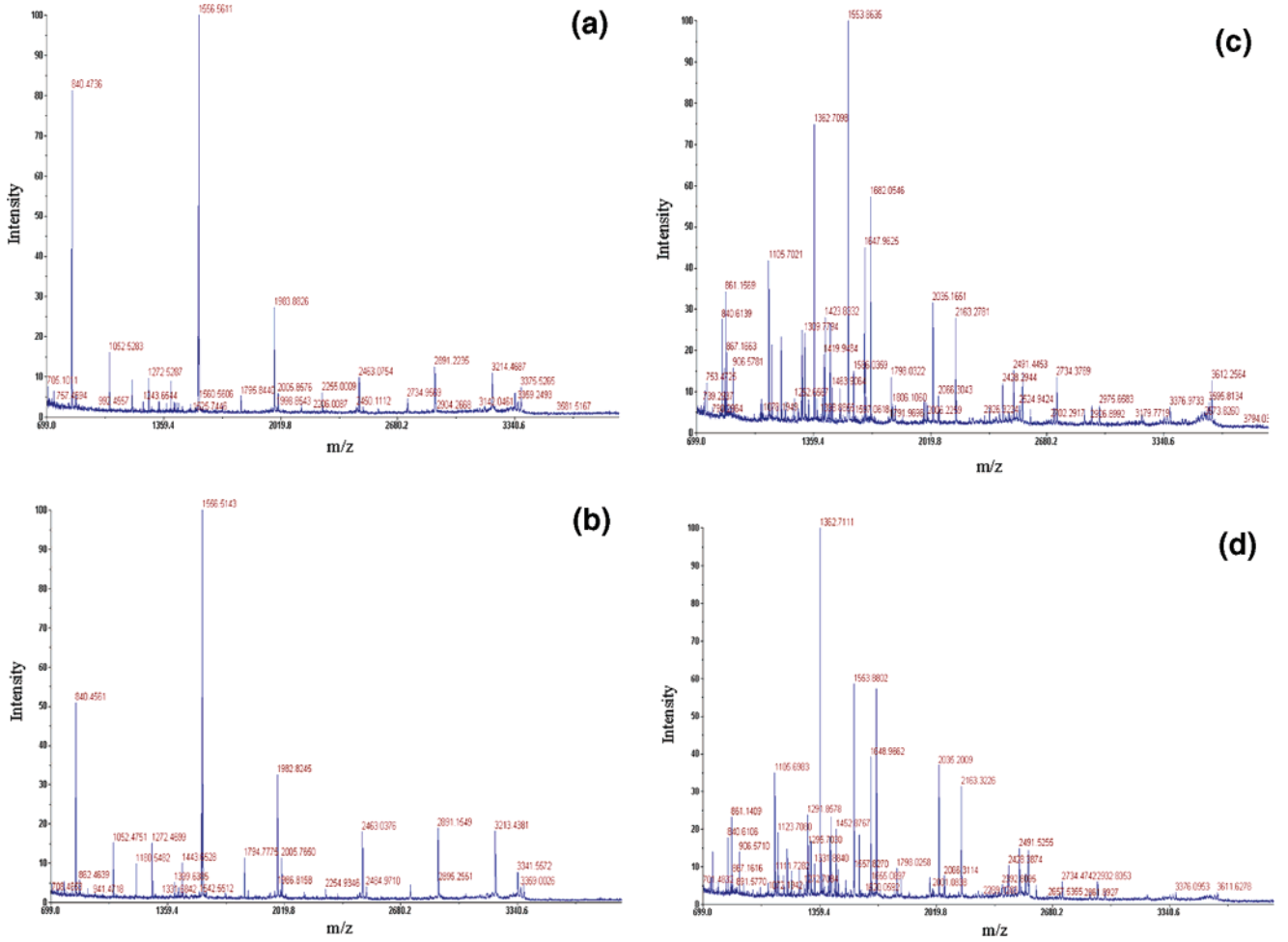

FIGURE 2: Change in relative ion abundance over the time course of limited proteolysis. Panels (a) and (b) represent MALDI-TOF mass spectra for a 15 min tryptic digest and 30 min tryptic digest of the A1 subunit, respectively. Panels (c) and (d) represent MALDI-TOF mass spectra for a 15 min tryptic digest and 30 min tryptic digest of the A1/A3C1C2 dimer, respectively.

Table 1: Match of Observed A1 Subunit Peptide Sequences to Theoretically Predicted A1 Subunit Peptide Sequences[a]

MALDI-tofmasses		position	
observed	theoretical	start	end
840.5166	840.5096	30	36
1052.5754	1052.5675	241	250
1180.6866	1180.6625	241	251
1272.5892	1272.5894	326	336
1398.7647	1398.7673	195	206
1443.8046	1443.7994	167	180
1553.7318	1553.7236	360	372
1556.6462	1556.6465	108	121
1982.9902	1982.9911	90	107
2254.1767	2254.1548	195	213
2462.2417	2462.2356	122	142
2733.0947	2733.1144	337	359
2890.4162	2890.3609	5	29
3213.7881	3212.6355	252	279
3341.9275	3340.7305	251	279

[a] The matching of observed *m*/*z* values to theoretically predicted *m*/*z* values was performed using the University of California, San Francisco (UCSF) Protein Prospector suite of proteomics tools (http://prospector.ucsf.edu) and the MASCOT program (http://www.matrixscience.com).

sponding to residues Ala108−Arg121, see Table 1), 1983 (residues Asn90−Lys107), and 2463 (residues Glu122−Lys142), indicating a reduced rate of proteolysis at the P1 residues Lys89, Lys107, Arg121, and Lys142 required to generate these fragments. However, the tryptic fragment at *m*/*z* = 840 (residues Phe30−Lys36), which yields a consistently strong signal, exhibits an ~2-fold variation in intensity, independent of the A3C1C2, suggesting that this segment is not contained within the A1/A3C1C2 interface. These observations suggest association of A1 subunit with the A3C1C2 subunit blocks the above P1 sites reducing accessibility to the protease. Conversely, we observed a significant increase in the relative abundance of the tryptic fragments at *m*/*z* = 1553 (Phe360−Arg372) and 2734 (Met337−Arg359) in the time-limited proteolysis of the A1 subunit in the A1/A3C1C2 dimer (Figure 2C and D) relative to that of the free A1 subunit. An increase in intensity in these peptides, which comprise the *C*-terminal region of the A1 subunit, suggests that the binding of this subunit to A3C1C2 induces a conformational change in the A1 subunit, enhancing the exposure of Arg336 and Arg359. Figure 3 shows a graphical representation of the summary of trypsin accessibility to the A1 subunit in the absence and presence of A3C1C2. Results from this Figure suggest that when values are averaged over multiple runs, discrete sites in the A1 subunit are identified that show consistent protection from (residues 90−142) or exposure to (residues 336−372) trypsin cleavage upon binding A3C1C2.

Reciprocal experiments were performed to assess sites in the A3C1C2 subunit that may be protected by association with the A1 subunit (data not shown). However, these

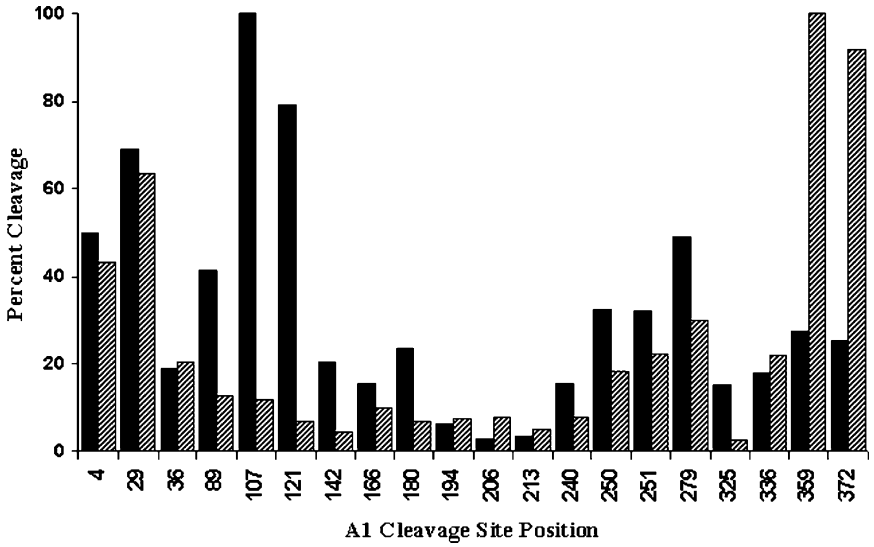

FIGURE 3: Trypsin accessibility to the A1 subunit in the absence and presence of A3C1C2. A summary of trypsin accessibility to cleavage sites (Lys/Arg) in free A1 (black bars) or A3C1C2-bound A1 (hatched bars) averaged over multiple runs for each digest is presented, showing an overall protection of discrete regions in A1 from tryptic cleavage in the presence of A3C1C2.

experiments attempting to demonstrate selective protection of sites in A3C1C2 in the A1/A3C1C2 dimer failed to yield substantive information largely due to the limited coverage (~40%) observed for this subunit.

Effect of the A1 Domain Peptide 97−105 on A1/A3C1C2 Interaction. In a previous report, we showed that A3 domain residues 1954−1961 were interactive with the A1 domain in forming the HC−LC interface in factor VIII (*17*). An examination of the ceruloplasmin-based factor VIII homology model of Pemberton and colleagues (*25*) predicts that this segment in A3 may be interactive with A1 domain residues contained in the sequence 97−105. This information, coupled with the direct observation that this sequence was contained within the region of A1 protected from trypsin proteolysis, provided the rationale for a more thorough evaluation of this A1 segment as an A3C1C2 subunit interactive site.

A synthetic peptide corresponding to the sequence 97−105 (SLHAVGVSY) was prepared, and its effect on the reconstitution of factor VIIIa activity was assessed. The peptide was titrated into a solution containing A1 (100 nM) and A3C1C2 (100 nM) subunits, and the mixtures were incubated overnight at 4 °C. Factor VIIIa was subsequently reconstituted by the addition of 50 nM A2 subunit, and cofactor activity was determined as described under Materials and Methods. The results are shown in Figure 4. The A1 domain peptide 97−105 inhibited the regeneration of cofactor activity by >50% with an apparent K_i value of 0.64 ± 0.21 μM. A scrambled sequence peptide (YVSGVSHLA) failed to inhibit factor VIIIa activity regeneration, suggesting that the inhibition observed was sequence specific.

To ensure that the inhibition observed was specific for the interaction between A1 and A3C1C2 subunits and did not result from perturbing other interactions involved in factor Xa generation, we evaluated the effects of the peptide using an intact A1/A3C1C2 dimer purified from factor VIIIa, which shows high stability over an extended time course (*28*). Increasing concentrations of the 97−105 peptide added to reactions containing 25 nM A1/A3C1C2 dimer and 100 nM A2 subunit failed to inhibit cofactor activity regeneration as judged by factor Xa generation (Figure 4). This result indicated that the peptide specifically blocked the A1/A3C1C2 interaction and did not affect either the A2 subunit interaction with the dimer or the subsequent interactions involving factor VIIIa with factor IXa or factor Xase with factor X. Overall, these results are consistent with residues 97−105 representing a portion of an A3 interactive site in the A1 domain.

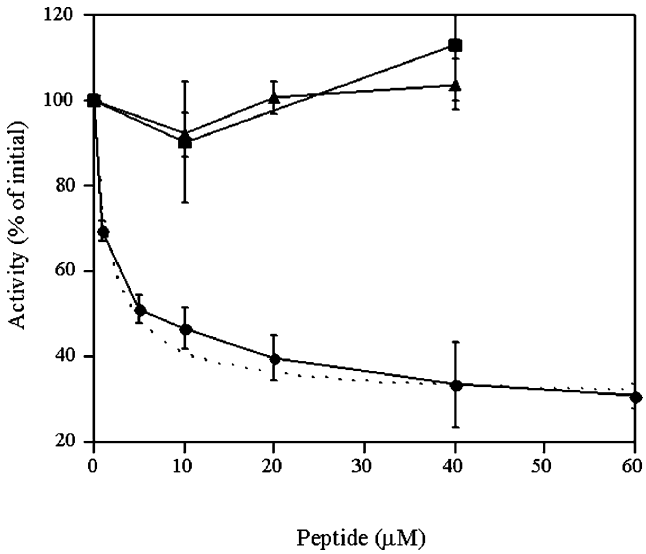

FIGURE 4: Effect of synthetic peptide on factor VIIIa activity reconstitution. Indicated amounts of the synthetic peptide to the A1 domain sequence 97−105 (●) or the scrambled sequence version (■) were titrated into a reconstitution mixture containing A1 and A3C1C2 subunits (100 nM each). The 97−105 peptide was also reacted with a preformed A1/A3C1C2 dimer (25 nM; (▲)). The A2 subunit was added, and cofactor activity of the reconstituted factor VIIIa was measured in a factor Xa generation assay as described in Materials and Methods. The dashed line represents the curve fit for the titration with peptide 97−105. Each point represents the mean and standard deviation for three individual measurements.

Effect of the A1 Domain Peptide on A1/A3C1C2 Association. Fluorescein-5-maleimide-labeled A1 and acrylodan-labeled A3C1C2 subunits were prepared as described under Materials and Methods. The activity of the labeled subunits

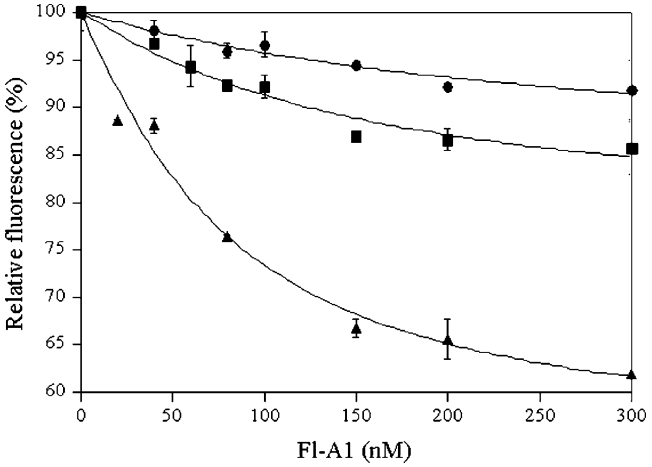

FIGURE 5: Effect of synthetic peptide on subunit reassociation measured as a function of relative fluorescence. Ac-A3C1C2 (50 nM) and varying levels of the Fl-A1 subunit in the absence of peptide (▲); (*17*)) or in the presence of 50 μM (■) or 100 μM (●) peptide were reacted overnight at 4 °C. The fluorescence of Ac-A3C1C2 was measured at λ = 460−490 nm. The relative fluorescence (%) represents the ratio of Ac-A3C1C2 fluorescence intensity in the presence of Fl-A1 to that of Ac-A3C1C2 alone and was plotted as a function of Fl-A1 concentration and fitted by nonlinear least-squares regression to the formula shown in Materials and Methods.

was determined following the reconstitution of factor VIIIa from Fl-A1, Ac-A3C1C2 subunits, and saturating amounts of unlabeled A2 subunit. Factor VIIIa reconstituted from Fl-A1 and Ac-A3C1C2 possessed >60% of the activity of factor VIIIa prepared from unlabeled subunits as judged by factor Xa generation (data not shown), indicating that the subunits retained significant activity upon fluorophore incorporation.

The effect of the A1 domain peptide on the affinity of Fl-A1 for Ac-A3C1C2 was measured using fluorescence resonance energy transfer. The extent of donor (acrylodan) fluorescence quenching was used as an indicator of Ac-A3C1C2 and Fl-A1 binding, with appropriate unlabeled controls. We recently determined the affinity for the A1/A3C1C2 interaction (K_d = 51.6 ± 16.6 nM) in the presence of 25 mM Ca^{2+} (*17*). This value was equivalent to the K_d value obtained in that study using a factor Xa generation assay, indicating that modification of the subunits with the fluorescence probes did not perturb subunit binding. Association of Fl-A1 and Ac-A3C1C2 subunits under similar conditions, in the presence of 50 and 100 μM A1 domain peptides resulted in a dose-dependent decrease in the intersubunit affinity as judged by K_d values of 125 ± 48 and 237 ± 128 nM, respectively (Figure 5), consistent with the peptide partially blocking the association of the two subunits. The failure of high concentrations of the peptide to completely abrogate this interaction suggested that sites other than those involving the A1 sequence 97−105 participate in the interchain affinity. The ∼4-fold increase in intersubunit K_d observed in the presence of high concentrations of peptide is reflective of a loss of ∼1 kcal mol^{-1} (∼10% of the total binding energy) in the thermal stability for this interaction. We also observed a peptide-dependent increase in the relative fluorescence (RF) value. The earlier value observed for this parameter 61 ± 1% (*17*) was markedly increased to 86 ± 3% and 92 ± 3% in the presence of 50 and 100 μM peptide, respectively (Figure 5). These results suggest a decrease in the efficiency of fluorescence energy transfer in the presence of the A1 domain peptide, possibly the result of an increased interfluorophore separation. Taken together, the results from the peptide analyses suggest that residues 97−105 form a portion of an A3 domain-interactive site in the A1 domain.

Factor VIII A1 Domain Site-Directed Mutant Analysis. To further investigate the role of specific residues within the 97−105 sequence in contributing to an A3-interactive site, factor VIII point mutations His99Ala, Val101Ala, and Gly102Ser were constructed, and protein was expressed as B-domainless factor VIII in BHK cells. The selection of residues to mutate and the amino acid substitutions to employ was based on the hemophilia A mutation database and proximity to the A3 domain as judged by the factor VIII homology model. His99, Val101, and Gly102 all appear to be in close proximity to the A3 domain according to the model (Figure 6). Furthermore, His99 is suggested to participate in a type 2 copper coordination site that may contribute to bridging the A1 and A3C1C2 subunits (*23*). Val101Ala and Gly102Ser are point mutations listed in the hemophilia A mutation database (*29*), whereas His99 is not listed in the database and was, thus, converted to Ala. Specific activity values for the stably expressed factor VIII forms were determined by measuring factor VIII activity and protein levels by one-stage clotting and ELISA assays, respectively. His99Ala, Val101Ala, and Gly102Ser demonstrated specific activity values of ∼32%, ∼51%, and ∼45% of the wild type, respectively. All factor VIII forms were similarly cleaved by thrombin as judged by SDS−PAGE analysis (data not shown), indicating that the mutations did not affect interactions with this activating proteinase.

To determine whether the reduction in the specific activity of the mutants derived from a decreased stability, the effects of temperature on the wild-type and mutant factor VIII forms were assessed. The factor VIII preparations (20 nM) were incubated at 55 °C, and at the indicated times, aliquots were assayed for factor Xa generation activity as described under Materials and Methods. Results are shown in Figure 7a and Table 2. Factor VIII mutant His99Ala exhibited an ∼3-fold increase in the rate of the loss of activity compared to that of the wild type, suggesting a reduction in the stability of this factor VIII form. However, factor VIII mutants Val101Ala and Gly102Ser exhibited statistically significant decreased rates of loss of activity compared to that of the wild type, suggesting somewhat increased stabilities of these factor VIII forms. The reason for this positive effect is not clear, but these results suggested that the reduced specific activity values observed for these mutant factor VIII forms did not result from diminished interchain stability.

To determine whether the temperature-dependent parameter changes observed in the mutants derived from altered interactions at the HC−LC interface, the stability of this interaction was directly assessed in a solid phase assay. Factor VIII (50 nM) was incubated at 55 °C, and at the indicated times, an aliquot was assayed using a sandwich ELISA, as described under Materials and Methods, to measure the amount of the remaining intact heterodimer. Results are shown in Figure 7b and Table 2. In all cases, we observed a time-dependent dissociation of the heterodimer as judged by the loss of HC from immobilized LC. Compared with the wild type, the His99Ala mutant exhibited an ∼40% decrease in interchain stability, whereas the Val101Ala mutant showed no significant difference, and the Gly102Ser

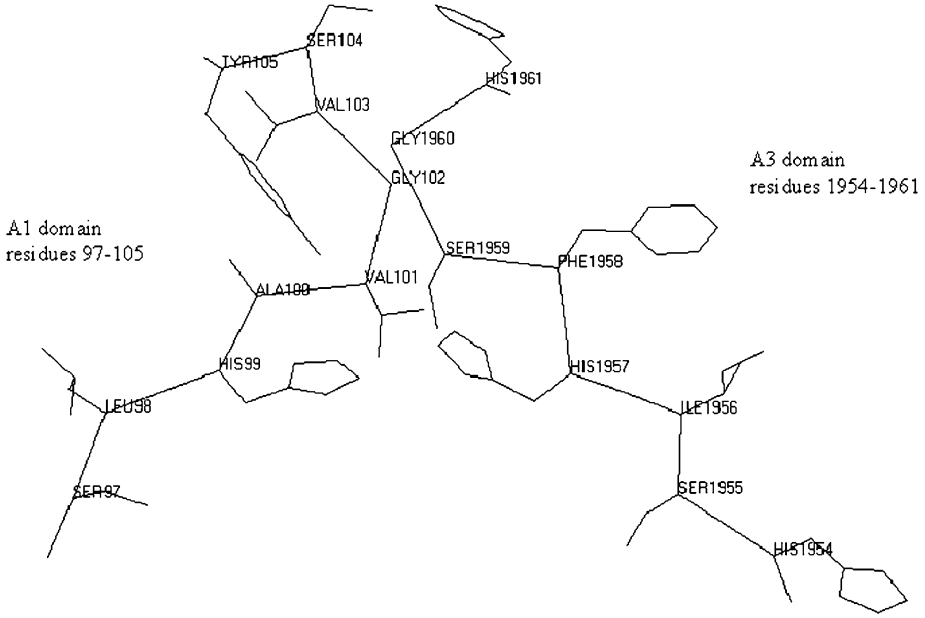

FIGURE 6: Interface between the A1 and A3 domains. The factor VIII homology model of Pemberton et al. (*23*) is used to visualize a portion of the factor VIII heterodimer interface involving A1 domain residues 97−105 and A3 domain residues 1954−1961.

mutant showed a somewhat increased stability. These results parallel those observed in the activity-based assays, suggesting that the discrepancies (∼2−3-fold) observed for $t_{1/2}$ values obtained with the functional and physical assays reflect differences in the assays that are not presently clear. Control experiments in which a LC antibody that recognizes an epitope distinct from the LC capture antibody showed no significant loss of the capture antibody-bound LC over the course of this experiment (data not shown). Taken together, these data suggest that the A1 domain residue His99 likely makes a positive contribution to interchain affinity, whereas others residues within this segment also appear to affect the interface.

DISCUSSION

In this study, we demonstrate that a segment of the A1 domain (residues 97−105) represents a region of factor VIII HC interactive with the LC (A3C1C2 domains). The rationale for examining this fragment was based upon the observations that (i) this sequence was contained within a segment of A1 protected from tryptic cleavage in the presence of the A3C1C2 subunit, (ii) this region showed proposed complementarity to a site in the A3 domain (residues 1954−1961) predicted by the ceruloplasmin-based homology model (*23*) and demonstrated experimentally to be A1 domain interactive (*17*), and (iii) mutations within this sequence alter factor VIII activity (*29*). MALDI-TOF mass spectrometry analysis demonstrated that a segment of the A1 domain showed significant reduction in the generation of fragments 90−107, 108−121, and 122−142, suggesting that accessibility to the P1 residues Lys89, Lys107, Arg121, and Lys142 was impaired in the A1/A3C1C2 dimer. This observation is consistent with the homology model, which suggests that Lys107 and Lys142 may be partially buried following binding to the A3C1C2 domains. Conversely, the reciprocal experiments (not shown) attempting to demonstrate the selective protection of sites in the A3C1C2 in the dimer failed to yield substantive information. The reason for this result is not clear but may be due in part to the lower observed

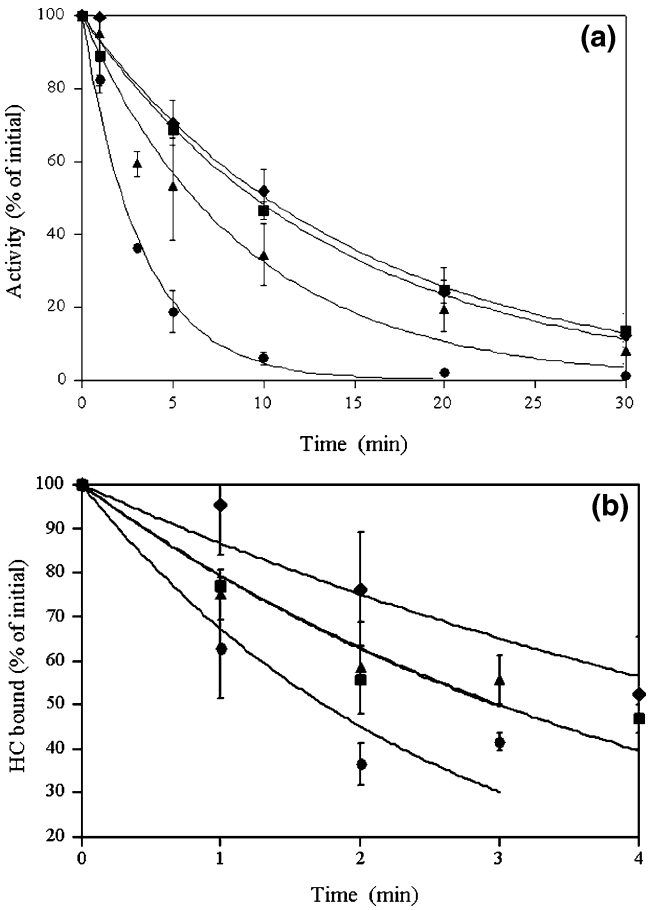

FIGURE 7: Effects of A1 domain mutations on factor VIII stability. Factor VIII wild type (▲), His99Ala (●), Val101Ala (■), and Gly102Ser (♦) were incubated at 55 °C. (Panel a) Aliquots removed at the indicated times and assayed for activity as described in Materials and Methods. (Panel b) aliquots removed at the indicated times and applied to an ELISA assay to measure the amount of heterodimer as described in Materials and Methods. The LC antibody ESH-8 (10 μg/mL) was used as the capture antibody and the HC antibody biotinylated R8B12 (10 μg/mL) used as the detection antibody. Each point represents the mean and standard deviation of at least three individual measurements.

Table 2: Intramolecular Stability Parameters for Factor VIII Wild Type and Mutants[a]

	functional assay[b]		solid-phase binding assay[c]	
FVIII	$t_{1/2}$ (min)	p-value[d]	$t_{1/2}$ (min)	p-value[d]
FVIII wild type	6.2 ± 0.7		2.97 ± 0.30	
His99Ala	2.3 ± 0.2	$p < 0.005$	1.74 ± 0.26	$p < 0.05$
Val101Ala	9.5 ± 0.4	$p < 0.005$	3.00 ± 0.36	$p > 0.5$
Gly102Ser	10.2 ± 0.5	$p < 0.005$	4.85 ± 0.67	$p < 0.05$

[a] The reactions were performed as described in Materials and Methods. Parameter values and standard deviations were estimated by nonlinear least-square regression from data shown in Figure 7a and Figure 7b using the formula described in Materials and Methods. [b] Data shown in Figure 7a. [c] Data shown in Figure 7b. [d] Data is compared with that of the FVIII wild type.

coverage of tryptic products from this subunit by MALDI-TOF (42% vs 54% for the A1 subunit). We did observe a fragment consistent with trypsin cleavage at Arg1941 and Arg1966 representing residues 1942−1966 (observed and theoretical masses of 2932.7 and 2931.4, respectively). However, there was little difference in observed peak abundance in the absence or presence of the A1 subunit, suggesting that these P1 residues were not encumbered in the dimer. An examination of the homology model suggests these two Arg residues are surface exposed in the factor VIII heterodimer, consistent with their observed accessibility independent of the presence of A1.

Synthetic peptide and mutagenesis studies support the A1 domain sequence S^{97}LHAVGVSY^{105} as contributing to the interchain interface. The former studies showed selective inhibition of the reconstitution of factor VIIIa activity from A1 and A3C1C2 subunits by the synthetic peptide, as well as inhibition of subunit association as measured by FRET. The presence of peptide yielded an approximate 4-fold reduction in the copper-independent intersubunit affinity and suggested an altered factor VIII conformation as judged by a potential increase in the interfluorophore spatial separation, in addition to the significant loss in cofactor activity following association of the A2 subunit. Interestingly, mutagenesis at three sites within this factor VIII sequence yielded altered cofactor activity as well as interchain stability values relative to those of the wild-type protein.

Recapitulation of two mutations listed in the Hemophilia A database (*23*), Val101Ala and Gly102Ser, showed apparent greater stability than the wild-type factor VIII, suggesting that a reduction in specific volume at residue 101 or a bulkier, more polar side chain at residue 102 actually favored residue packing at the interface (Figure 6). It is not clear whether these residue replacements resulted in improved direct contacts with factor VIII LC or more favorably affected interactions at neighboring sites. However, given the observation that either mutation yields a hemophilic phenotype indicates that these alterations are detrimental to the conformation of the active cofactor.

However, the reduction in specific activity of the His99Ala mutant may correlate directly to a reduction in interchain stability. Our observations are consistent with earlier results from Tagliavacca et al. (*11*) that used a transient expression system to show that a His99Ala mutant possessed a marked reduction in activity and was defective in chain association. His99 has been suggested to contribute to a putative type 2 copper site along with H1957 in the A3 domain (*23*). This site would bridge the A1 and A3 domains and thus would be expected to make a contribution to the interchain affinity and subsequent stability. In the current report, we observed an ∼3-fold reduction in the stability of cofactor activity with this mutation. However, in a recent study, we showed a more marginal effect of the mutation at His1957 that when replaced with Ala, yielded an ∼30% reduction in the stability of cofactor activity (*17*). Although the site of copper coordination in factor VIII remains controversial (*30*), recent observations indicate the contribution of copper to the affinity of A1 and A3C1C2 subunits (*17*) or HC and LC (*31*) is similar and represents an ∼5-fold enhancement that is derived in part from an ∼3-fold reduction in the dissociation rate constant and <2-fold increase in the association rate constant (*31*). Because the levels of expression of the recombinant factor VIII proteins preclude isolation of the separated chains for more thorough affinity determinations, we are not able to make strong conclusions regarding the occupancy of the putative type 2 copper site. However, given the overall small contribution of copper binding to interchain stability (∼1 kcal mol^{-1} comparing K_d values of ∼4 and ∼20 nM obtained for HC and LC reconstitution in the presence and absence of copper, respectively; (*31*)), the observation that mutation at either His99 or His1957 yields a factor VIII showing reduced stability could support this hypothesis. Furthermore, the observed greater relative effect of mutation at His99 on stability parameters may be an indicator that other, non-copper-dependent interactions are also contributed by this residue.

The mechanisms for subunit association in factor VIII are poorly understood. Recently, we showed that the A2 domain makes little or no contribution to the affinity of factor HC and LC (*17*), suggesting that the A1 and A3C1C2 subunits mediate the bulk of intersubunit affinity in the factor VIII heterodimer. In that study, we focused upon evaluation of the sequence 1954−1961 (HSIHFSGH) in the A3 domain and demonstrated that this region was A1 subunit-interactive using approaches of peptide inhibition and site-directed mutagenesis similar to those used in the present report. The rationale for the earlier study was largely based upon the prediction from the ceruloplasmin-based homology that the segment is A1-interactive. Although the homology model predicts that the A1 domain segment 97−105 evaluated in the current study contains residues that are complementary to those contained within 1954−1961, we have no experimental evidence to support or refute this hypothesis. Experimentally determined interactions between the A1 and A3C1C2 subunits shows dependence upon electrostatic and hydrophobic interactions based upon ionic strength dependence and effects of an apolar probe (*16*), pH dependence (*31*) suggesting a role for the protonation state of His residues, and a role for copper ion coordination (*23*). Specific residues meeting these requisite critieria are contained within these sequences (Figure 6).

In studying the effects of the A3C1C2 subunit on trypsin accessibility within the A1 subunit, we observed that the *C*-terminal region of the A1 subunit appeared more exposed to the protease in the dimer compared with that of the free subunit. This observation was based upon enhanced cleavage rates at Arg336 and Arg359 to yield fragments 337−359 and 360−372. The acidic residue rich *C*-terminal region of

A1 (residues 337−372) has been implicated in several studies to be functionally important for factor VIIIa cofactor activity. Earlier work has shown that this segment is important in the orientation of the A2 subunit for optimal interaction with factor IXa in the factor Xase enzyme complex (*32*). This site also contains a factor X interactive site (*33*), and the truncation of factor VIIIa by cleavage at Arg336 yields a 5-fold increase in the *K*m for factor X (*34*). More recent mutagenesis studies have identified a cluster of acidic residues, Asp361/Asp362/Asp363, as contributing to a factor X binding site (*27*). We speculate that regions in this functionally important site in the A1 subunit may be somewhat masked in the free domain and become more ordered and/or exposed upon association of this subunit with the LC-derived subunit of factor VIII.

In summary, we have used a combination of mass spectrometry, peptide, and site-directed mutational analysis to demonstrate that residues within the 97−105 segment of the A1 subunit represent an interactive site for A3C1C2. Taken together with a previous study implicating a role for A3 residues 1954−1961 as A1 interactive (*17*), these results are consistent with an interactive surface as predicted by the A domain homology model (*23*). Although questions concerning regions of complementarity and the role of this region in a copper-ion-dependent interaction remain to be experimentally verified, the results in the present study provide experimental evidence supporting a role for this A1 domain sequence in contributing to the interfactor VIII chain interaction.

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