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Identification and Functional Requirement of Cu(I) and Its Ligands within Coagulation Factor VIII*

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Coagulation factor VIII (FVIII) is a heterodimer consisting of a light chain of 80 kDa (domains A3-C1-C2) in a metal ion-dependent association with a 220-kDa heavy chain (domains A1-A2-B). The nature of the metal ion-dependent association between the heavy and light chains was investigated using atomic absorption spectroscopy, electron paramagnetic resonance spectroscopy (EPR), and site-directed mutagenesis and expression of the FVIII cDNA. Whereas copper ion was not detected in intact recombinant FVIII, EDTA dissociation of the chains yielded an EPR signal consistent with 1 mol of Cu(I)/mol of active protein, supporting the hypothesis that a single molecule of reduced copper ion is buried within intact FVIII and is released and oxidized upon treatment with EDTA. Cu(I), and not Cu(II), was able to reconstitute FVIII activity from dissociated chains, demonstrating a requirement for Cu(I) in FVIII function. Three potential copper ion binding sites exist within FVIII: one type-2 site and two type-1 sites. The importance of these potential copper ion ligands was tested by studying the effect of site-directed mutants. Of the two histidines that compose the type-2 binding site, the His-1957 → Ala mutant displayed secretion, light and heavy chain assembly, and activity similar to wild-type FVIII, while mutant His-99 → Ala was partially defective for secretion and had low levels of heavy and light chain association and activity. In contrast, FVIII having the mutation Cys-310 → Ser within the type-1 copper binding site in the A1 domain was inactive and partially defective for secretion from the cell, and the heavy and light chains of the secreted protein were not associated. Mutant Cys-2000 → Ser within the A3 domain displayed secretion, assembly, and activity similar to that for wild-type FVIII. These results support the hypothesis that Cu(I) is buried within the type-1 copper binding site within the A1 domain and is required for FVIII chain association and activity.

Factor VIII (FVIII)[1] is the X-chromosome-linked gene product that is deficient or defective in the bleeding disorder hemophilia A. FVIII functions in the blood-clotting cascade as the cofactor for factor IXa proteolytic activation of factor X. FVIII has a domain organization of A1-A2-B-A3-C1-C2 and is synthesized as a single chain polypeptide of 2351 amino acids, from which a 19-amino acid signal peptide is cleaved upon translocation into the lumen of the endoplasmic reticulum (ER) (1, 2). Upon secretion from the cell, FVIII is further processed by cleavage after residue 1648 to yield a heterodimer consisting of a 220-kDa amino-terminal-derived heavy chain (domains A1-A2-B) associated in a metal ion-dependent manner with a carboxyl-terminal-derived light chain (domains A3-C1-C2) (3).

The FVIII heterodimer circulates in plasma in a complex with von Willebrand factor (vWF) in an inactive form that requires proteolytic cleavage by thrombin or factor Xa for release from vWF and generation of coagulant activity. Cleavage within the heavy chain after Arg-740 generates a 90-kDa fragment that is further cleaved after Arg-372 to yield fragments of 50 and 43 kDa (4, 5). The 80-kDa light chain is cleaved after Arg-1689 to generate a 73-kDa polypeptide. Activated FVIII (FVIIIa) is a heterotrimer of A1, A2, and A3/C1/C2 subunits. The A1 and A3/C1/C2 subunits interact through a stable metal ion-dependent association between the A1 and A3 domains, while the A2 domain is associated with the A1 domain through a weak electrostatic interaction (6).

The triplicated A domains of FVIII exhibit 35–40% amino acid identity to each other and to the triplicated A domains in coagulation factor V (FV) and in the copper-binding protein ceruloplasmin (7–9). Each of the A domains exhibits conserved disulfide bridges and can be subdivided into two subdomains (subdomains d1–d6 for the three A domains in FVIII, FV, and ceruloplasmin). Each d subdomain resembles the β-barrel folded structure of the ancient copper ion-binding protein cupredoxin (10).

The blue copper-containing proteins and proteins related to them form a heterogeneous group that extend from the small blue proteins in bacteria and plants, to the blue oxidases in plants and mitochondria, and to the coagulation factors V and VIII in vertebrate plasma (11). These proteins contain copper ions of three types that are defined by the amino acid ligands that constitute the binding site. The blue copper-containing proteins can bind from 1 to 9 copper atoms, where the blue color is attributed to the presence of a type-1 copper binding site with characteristic spectral and redox properties (12). The blue oxidases and related proteins, which include the plant proteins laccase, ascorbate oxidase, and the plasma protein ceruloplasmin, contain an intensely blue type-1 Cu(II) ion with absorption in the visible region, a trinuclear cluster consisting of a pair of magnetically coupled type-3 copper ions characterized by a strong absorption in the near-ultraviolet region and the absence of an EPR signal, and a magnetically isolated type-2 Cu(II) ion having an undetectable absorption. The type-1 and type-2 Cu(II) ions have unique EPR spectra that disappear and absorption properties that change upon reduction of the copper ion to Cu(I) (13). Laccase and ascorbate oxidase perform oxida-

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[1] The abbreviations used are: FVIII, factor VIII; FV, factor V; vWF, von Willebrand factor; EPR, electron paramagnetic resonance; WT, wild-type; PAGE, polyacrylamide gel electrophoresis; ALLN, *N*-α-Leu-Leu-norleucinal; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay.

tion reactions by transferring four electrons from a reducing substrate to a molecule of oxygen that is subsequently reduced to water. Sequence alignments and three-dimensional structures support the hypotheses that the small blue proteins and the blue oxidases are homologous (14) and evolved from a common origin through gene duplication and divergence (11, 15). The evolutionary and structural conservation of the A domains between the coagulation factors and the blue oxidases suggests a role for copper ion binding in the coagulation factors. Indeed, approximately 1 mol of copper ion/mol of protein was detected directly in FVIII by atomic absorption spectroscopy (16) and in FV by atomic absorption and atomic emission spectroscopy (17). At present, there is no information regarding the oxidation state or the ligands that coordinate copper ion in FVIII or FV.

Based on the homologies to ancient copper-binding proteins and the known structures for the ancient copper-binding proteins, it is hypothesized that copper ion may be important for the metal ion-dependent interaction between the A1 and A3 domains of FVIII. The nature of the metal ion-dependent bridge between the FVIII heavy and light chains was investigated previously using different strategies. Incubation of the plasma-derived FVIII with a chelating agent, such as EDTA, dissociated the heavy and light chains and destroyed coagulant activity (18). Reconstitution of the FVIII heavy and light chain subunits with different divalent cations showed specific ion requirements and ionic strength dependence. Under these *in vitro* conditions, Mn(II) was the most effective cation in regenerating FVIII activity, yielding 30% reconstitution, whereas Ca(II) yielded 18% reconstitution. In contrast, copper ion, in the form of cupric solution, did not promote reconstitution (19, 20). Atomic absorption spectrometry analysis of FVIII heavy and light chain heterodimers and thrombin-activated FVIII complexes detected copper ion only when the chains were associated (16). In contrast, neither magnesium or manganese were detected.

At present it is unknown what amino acids within FVIII are responsible for coordinating the copper ion. The predicted amino acid sequence of FVIII contains two consensus type-1 copper ion binding sites, each defined by the canonical ligands His-Cys-His-Met that are located in the A1 and A3 domains, respectively (15). However, to date, no direct experimental evidence supports the hypothesis that these potential copper binding sites are occupied by copper ion. An initial structural prediction of the arrangement of the FVIII A domains was made based on the homology with the copper ion-binding protein nitrite reductase (21). More recently, this model was refined based on the 3-Å structure of ceruloplasmin (22, 23). In these models, a type-2 copper ion binding site composed of histidine residues at positions 99 and 1957 is proposed to bridge the A1 and A3 domains of FVIII. To elucidate the nature of the copper ion binding site in FVIII, we performed chemical analysis, functional assays, and site-directed mutagenesis to identify the copper ion content and its ligands within FVIII.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant FVIII, anti-heavy chain factor VIII monoclonal antibody (F-8), and F-8 conjugated to CL-4B Sepharose were obtained from Genetics Institute Inc. (Cambridge, MA). Centriplus columns were purchased from Amicon Inc. (Beverly, MA). Light chain factor VIII monoclonal antibodies ESH-4 and ESH-8 were purchased from American Diagnostica Inc. (Greenwich, CT). Coamatic chromogenic factor VIII activity assay kit was purchased from Pharmacia Hepar (Franklin, OH). Factor VIII-deficient plasma was obtained from George B. King Biomedical Inc. (Overland Park, KS). Activated partial thromboplastin reagent and $CaCl_2$ were purchased from General Diagnostics Organ Technique Corp. (Durham, NC). Human α-thrombin was from Hemtological Technology Inc. (Burlington VT). Soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and aprotinin were purchased from Boehringer Mannheim. *N*-α-Leu-Leu-norleucinal (ALLN) was purchased from Sigma. [^{35}S]Methionine (>1000 Ci/mmol) was purchased from Amersham Life Science Inc. EN^{3}HANCE was purchased from DuPont Corp. Dulbecco's modified Eagle's medium and methionine-free Dulbecco's modified Eagle's medium were obtained from Life Technologies, Inc. Chelex-100 resin was purchased from Bio-Rad.

Atomic Absorption Analysis—Atomic absorption measurements were made on a Perkin Elmer 3300. Recombinant FVIII protein (350 μl) was diluted 1/1 in 1% HNO_3 (by volume), injected in the pyrolitic graphite tube heated by joule effect with the following program: 120 K for 50 s, 1000 K for 30 s, and 2300 K for 5 s. The calibration curve was obtained by dilution of the standard copper solution in 0.5% HNO_3. Each value was the average of two measurements.

Spectroscopy—EPR measurements were made using a Varian Century line X-band (9 GHz) EPR spectrometer equipped with a cryogenic Dewar system. The conditions for the detection of the cupric ions were as follows: microwave power, 10 milliwatts; microwave frequency, 9.17 GHz; modulation frequency, 100 kHz; amplitude, 0.1 millitesla; temperature, 130K. For each spectrum, the analog output was recorded digitally on the computer via a data acquisition board made by ComputerBoard Inc. (Mansfield, MA).

Activation of FVIII—Human recombinant FVIII in 50 mM Tris-HCl (pH 7.2), 0.4 mol/liter NaCl, 5 mM $CaCl_2$, and 0.1% Tween 80 was incubated at 37 °C with human α-thrombin. At short intervals, aliquots were removed and assayed for FVIII clotting activity by the one-stage clotting assay using FVIII-deficient plasma.

Dissociation of FVIII Subunits and Reconstitution of FVIII Activity—Highly purified recombinant FVIII was treated with 50 mM EDTA, pH 8.0, for 16 h at room temperature. The rate of dissociation was monitored by loss of activity measured by the one-stage clotting assay. The reaction was dialyzed against a metal ion-free buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 2.5 mM $CaCl_2$, 5% glycerol. Metal-free water, filtered through a Chelex-100 resin, was used throughout the experiment. The reconstitution of FVIII activity was performed by adding solutions of cupric chloride or cuprous chloride to the dissociated FVIII protein (35 μg/ml) as indicated. Reactions were performed at room temperature, and aliquots were removed at the indicated times for measure of activity using the one-stage clotting assay.

Plasmid Mutagenesis—The FVIII expression vector pMT2-VIII MluI-1648 has been described previously (24). Prior studies demonstrated that the introduction of the *Mlu*I restriction site (yielding a Thr-Arg at amino acid residues 1647–1648) did not alter the synthesis, secretion, or functional activity of the molecule. Site-directed oligonucleotide-mediated mutagenesis was performed by polymerase chain reaction procedures (25). Codon 99 was mutated from CAT to GCT predicting an amino acid change from histidine to alanine (His-99 → Ala), codon 310 was mutated from TGT to AGT predicting an amino acid change from cysteine to serine (Cys-310 → Ser), codon 1957 was mutated from CAT to GCT predicting an amino acid change from histidine to alanine (His-1957 → Ala), and codon 2000 was mutated from TGC to TCC predicting an amino acid change from cysteine to serine (Cys-2000 → Ser). All mutations were confirmed by DNA sequencing over the mutagenized region and extensive mapping with restriction endonuclease enzymes.

DNA Transfection and Analysis—Plasmid DNA was transfected into COS-1 monkey cells by the diethylaminoethyl (DEAE)-dextran procedure (26). Conditioned medium was harvested 60 h after transfection in the presence of 10% heat-inactivated fetal bovine serum for FVIII assay. Primary translation products were analyzed by pulse-labeling cells for 30 min with [^{35}S]methionine (250 μCi/ml in methionine-free medium) and preparing cell extracts in a Nonidet P-40 lysis buffer (27). Protein secretion was monitored by metabolic pulse-labeling cells for 30 min with [^{35}S]methionine (250 μCi/ml) and chasing for the indicated period of time in medium containing an excess of unlabeled methionine as described (28). The FVIII was immunoprecipitated from the cell extract and conditioned medium with an anti-heavy chain factor VIII monoclonal antibody F-8 (27) coupled to CL-4B Sepharose. The immunoprecipitates were washed as described (27) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using a low bisacrylamide containing 8% polyacrylamide gel. Proteins were visualized by autoradiography after fluography by treatment with EN^{3}HANCE. The band intensities were quantitated by scanning the lanes using an LKB UltroScan XL laser densitometer (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Chain association was measured by co-immunoprecipitation experiments. After immunoprecipitation with an anti-heavy chain antibody F-8, the supernatant was subsequently immunoprecipitated with the anti-light chain monoclonal antibody ESH-4 coupled to Affi-Gel. Immu-

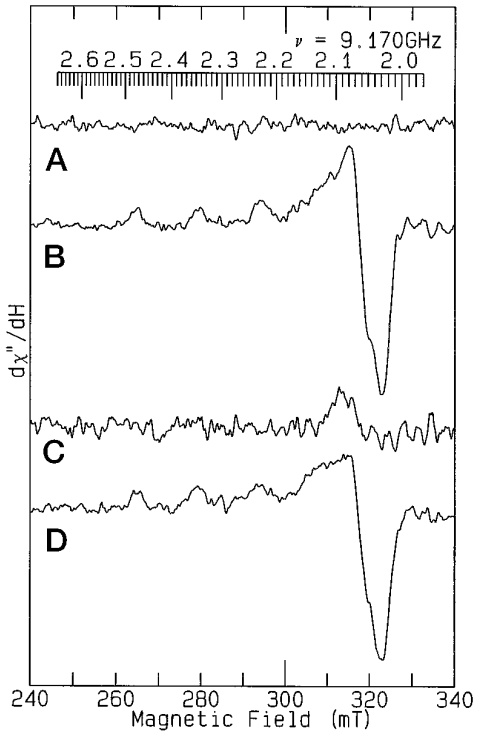

FIG. 1. **EPR spectra of FVIII.** EPR spectra were obtained as described under "Experimental Procedures." *A*, recombinant FVIII in 50 mM Tris-HCl, pH 7.2, 0.4 M NaCl, 2.5 mM $CaCl_2$, 0.1% Tween 80; *B*, FVIII incubated in 50 mM EDTA; *C*, FVIII digested with human thrombin; *D*, FVIII digested with thrombin and subsequently incubated with 50 mM EDTA.

noprecipitated proteins were analyzed by SDS-PAGE as described above.

FVIII Assay—Factor VIII activity was measured by the coamatic chromogenic assay (Coamate) according to the manufacturer or by the one-stage activated partial thromboplastin time clotting assay using FVIII-deficient plasma. The antigen level was measured by an enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibodies ESH-4 and ESH-8 that recognize the FVIII light chain (29). For highly purified FVIII, the protein concentration was measured by the Bradford method (30).

RESULTS

Atomic Absorption and EPR Detection of Copper Ion in FVIII—Homogeneous recombinant FVIII, purified from Chinese hamster ovary cells was analyzed by atomic absorption to determine the copper ion content and to correlate the amount of copper ion/mol of active protein. The specific activity of the recombinant FVIII was 1250 units/mg, as measured by the one-stage clotting assay and Bradford protein assay. Atomic absorption spectroscopy detected 484 nM copper ion/500 nM active protein, consistent with 1 mol of copper ion/mol of active FVIII protein, and is consistent with a previous report (16). For EPR analysis, FVIII was concentrated through a Centriplus column and the antigen was measured by ELISA using anti-light chain antibodies. The concentrated protein (55 μM) was analyzed by EPR to determine the copper ion content. EPR spectroscopy of frozen FVIII solution revealed no EPR detectable copper ion (Fig. 1*A*). In addition, the presence of other paramagnetic metal ions, including Mn^{2+}, was not detected. Since atomic absorption spectrometry identified 1 mol of copper ion/mol of active FVIII protein and copper ion would be silent by EPR if it were protected within the protein in a reduced form, we tested whether heavy and light chain dissociation by metal ion chelation would release and oxidize protected copper ion. The FVIII heavy and light chains were separated by incubation with EDTA as previously reported (18), and the dissociation was monitored by inactivation of FVIII activity measured in the one-stage clotting assay. After overnight incubation with 60 mM EDTA, the amount of coagulant FVIII activity decreased to less than 1% of the initial activity. EPR spectroscopy of the dissociated FVIII detected a significant copper ion signal corresponding to 17 μM, supposedly as an EDTA-chelated complex (Fig. 1*B*). EPR analysis of the EDTA solution excluded contamination of copper ion. In addition, an EPR signal for copper ion was not detected in the cavity. These data demonstrate that native recombinant FVIII contains copper ion in a reduced form Cu(I) that is EPR silent. EDTA treatment dissociated the FVIII heavy and light chains, destroyed coagulant activity, and fully oxidized Cu(I) to Cu(II), thus allowing the recovery and detection of copper ion in the oxidized form.

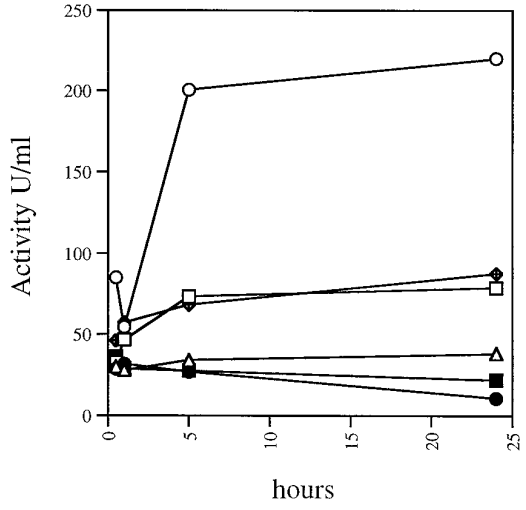

FIG. 2. ***In vitro* reconstitution of FVIII activity.** Purified EDTA-dissociated FVIII at 35 μg/ml was incubated at room temperature alone (△), or in the presence of 10 μM Cu(I) (○), 100 μM Cu(I) (●), 10 μM Cu (II) (□), 100 μM Cu(II) (■), or 10 μM Cu(I)-GSH (♦). Portions of the reactions were taken at 1.5 and 24 h for FVIII activity determination by the one-stage clotting assay.

We next tested whether thrombin cleavage and activation may release bound copper ion. After incubation with thrombin, we observed a rapid increase in the coagulant activity, followed by a first-order decay in activity that is known to be accompanied by dissociation of the A2 domain subunit (31, 32). EPR analysis was performed 3 h after thrombin cleavage, at a time when most of FVIII activity had decayed. The analysis did not detect copper ion (Fig. 1*C*). Incubation of the thrombin-treated sample with 50 mM EDTA for 30 min promptly yielded a detectable Cu-EDTA complex (Fig. 1*D*). These results demonstrate that thrombin cleavage and activation are not accompanied by oxidation of bound copper ion and that thrombin-activated FVIII still retained Cu(I) in an EDTA-releasable state. The observation that Cu(I) is present in native FVIII provided the rationale to test whether Cu(I) could promote *in vitro* reconstitution of FVIII from dissociated heavy and light chains.

Cu(I), but Not Cu(II), Promotes Reconstitution of FVIII Activity—Reconstitution of FVIII activity from EDTA-dissociated subunits was performed using different copper ion containing solutions in the reduced or oxidized forms. Cuprous chloride was most effective in promoting FVIII activity (Fig. 2). Complete reconstitution (where the reconstituted FVIII exhibited the same specific activity as the starting material) occurred with 10 μM Cu(I), which represents an approximate ratio of 30–50 mol of Cu(I)/mol of FVIII. Cuprous chloride at 100 μM inhibited reconstitution of FVIII activity, probably due to nonspecific binding of the metal ion to the subunits. The reconsti-

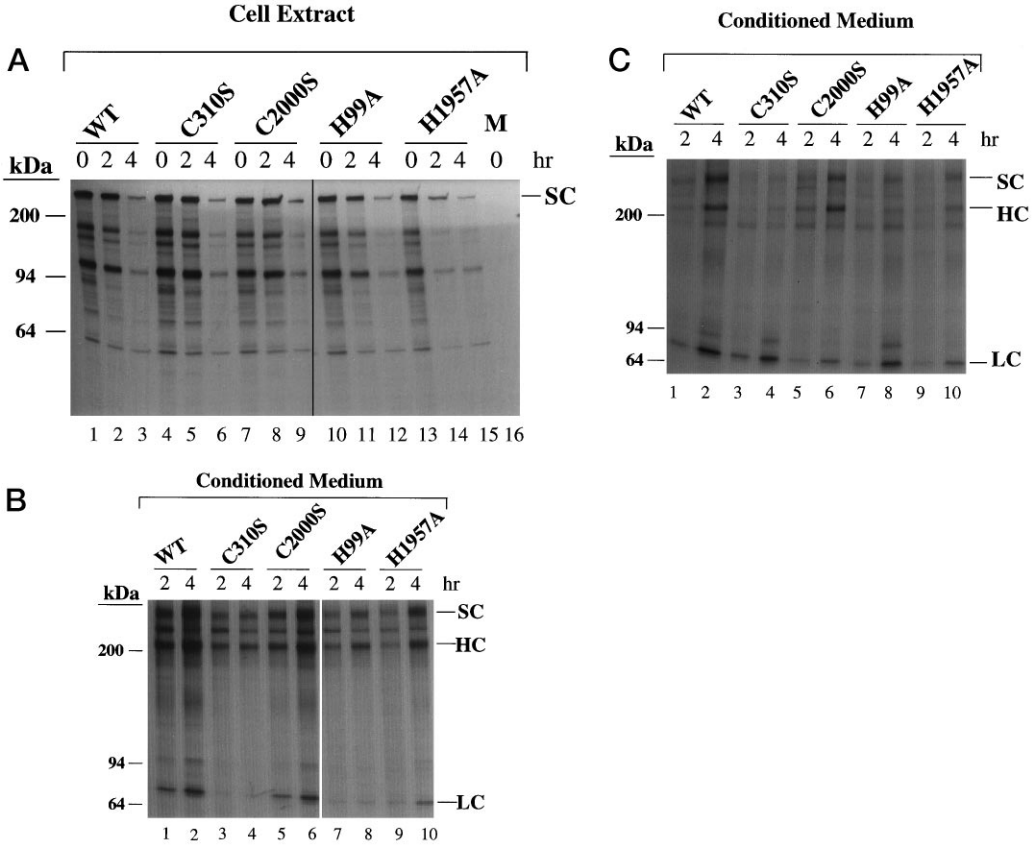

FIG. 3. **Expression of FVIII WT and mutants in COS-1 cells.** WT and mutant FVIII expression plasmids were transfected into COS-1 monkey cells. At 60 h post-transfection, cells were pulse-labeled with [^{35}S]methionine for 30 min and the cell extracts were harvested and immunoprecipitated with anti-FVIII heavy chain antibody (*A*). Duplicate plates were labeled for 30 min and chased for 2 h or 4 h in medium containing excess unlabeled methionine, and then cell extracts and conditioned medium were harvested. Equal volumes were immunoprecipitated with anti-FVIII heavy chain antibody and were analyzed by SDS-PAGE (*B*). The supernatants from the conditioned medium samples were subsequently immunoprecipitated with an anti FVIII light-chain antibody (*C*). The migration of the FVIII in the cell extract is detected as a single chain (*SC*). FVIII in the conditioned medium is detected as single chain (*SC*), heavy chain (*HC*), and light chain (*LC*) species. *M* indicates mock cells that did not receive any plasmid DNA.

tution of FVIII activity reached a plateau after 5 h at room temperature and maintained the activity for at least 24 h at room temperature indicating a stable interaction and assembly of the heavy and light chain subunits in the presence of reduced copper ion. By contrast, the reconstitution of FVIII activity from dissociated heavy and light chain subunits in the absence of metal ion (control) gave only background activity. Cupric solutions, at the same molar ratio as the cuprous solutions, were evaluated for their ability to promote reconstitution. A solution of 10 μM Cu(II) yielded a 2-fold increase of FVIII activity, while 100 μM Cu(II) was ineffective.

Glutathione (GSH) is the most abundant ligand for copper ion within the mammalian secretory pathway. *In vitro* reconstitution of the copper-containing proteins ceruloplasmin (33), Cu,Zn-superoxide dismutase (34), apohemocyanin (35), and thioneins (36) occurs by means of the copper ion associated in a glutathione complex as Cu(I)-GSH. Under our conditions of reconstitution, Cu(I)-GSH was poorly effective in reconstituting FVIII activity (Fig. 2).

Site-directed Mutagenesis at the Proposed Type-1 Copper Binding Sites—Because copper ion within FVIII is detectable only after chelation with EDTA, it is not possible to elucidate the coordination properties of copper ion in FVIII by EPR analysis. Therefore, we tested the significance of the potential copper ion ligands within FVIII by site-directed mutagenesis. FVIII contains two consensus type-1 copper ion binding sites that are located in the A1 domain (composed of residues His-265, Cys-310, His-315, and Met-320) and A3 domain (composed of residues His-1954, Cys-2000, His-2005, and Met-2010). Because the Cys residue is an essential component of the type-1 copper ion binding site (37), we mutated each of the two Cys residues Cys-310 and Cys-2000 to the conserved residue Ser to determine their requirement in FVIII chain association and activity. Expression vectors containing the wild-type FVIII cDNA or mutants bearing either of the Cys to Ser substitutions were transfected into COS-1 monkey cells. At 60 h post-transfection, FVIII synthesis and secretion were evaluated by [^{35}S]methionine metabolic pulse labeling and chase analysis. Samples of cell extracts and conditioned medium were analyzed by immunoprecipitation with anti-FVIII heavy chain antibody and SDS-PAGE. Analysis of the cell extracts demonstrated that wild-type FVIII and the mutants were synthesized as single chain polypeptides migrating at approximately 280 kDa (Fig. 3*A*). Quantitation of the band intensities demonstrated similar amounts of radiolabel incorporation into FVIII protein for all the constructs indicating similar translation rates. In addition, analysis of the 2- and 4-h chase time points demonstrated that the wild-type and both mutants disappeared from the cell extract at similar rates, suggesting similar rates of intracellular degradation and/or secretion.

The FVIII secreted into the conditioned medium over the 2–4-h chase period was collected and subjected to immunoprecipitation analysis. FVIII chain association was analyzed by immunoprecipitation of the conditioned medium with anti-FVIII heavy chain antibody. The supernatant was subsequently immunoprecipitated with anti-FVIII light chain anti-

TABLE I
Specific activity of FVIII WT and mutants secreted into conditioned medium

Plasmid DNA was transfected into COS-1 cells. The cells were fed fresh medium after 40 h. After 24 h the conditioned medium was collected for FVIII activity analysis by the chromogenic assay and ELISA for quantitation of antigen. Cells were then labeled with [^{35}S] methionine and analyzed as described in Fig. 3. The activity data are the average of three independent transfection experiments.

Plasmid DNA	Coatest assay	Antigen	Specific activity
	% WT ± S.D.	*% WT ± S.D.*	*% WT*
WT	100	100	100
Cys-310 → Ser	1.2 ± 3.4	72 ± 1.5	2.2
Cys-2000 → Ser	51 ± 19	35 ± 7.7	128
His-99 → Ala	19 ± 3.3	78 ± 6.1	27
His-1957 → Ala	68 ± 40	60 ± 7.7	122

body. Since the anti-heavy chain antibody does not recognize the light chain and the anti-light chain antibody does not recognize the heavy chain, analysis of the amount of co-immunoprecipitation indicates the extent of chain association. After immunoprecipitation with the anti-heavy chain antibody, wild-type FVIII is detected in the conditioned medium as a single chain of 300 kDa, a heavy chain of 220 kDa, and an associated light chain of 80 kDa. FVIII wild-type and mutants appeared in the conditioned medium after the 2-h chase time point, although the amount of single chain and heavy chain detected for the Cys-310 → Ser mutant were slightly less than the respective species for the wild-type protein (73% of wild-type) (Fig. 3*B*). The amount of heavy chain for wild-type and Cys-2000 → Ser mutant FVIII increased 1.5-fold between the 2-h and 4-h chase time points. In contrast, the amount of Cys-310 → Ser mutant secreted into the conditioned medium did not significantly increase during this period. After the 4-h chase time point, the amount of the secreted heavy chain for the Cys-310 → Ser mutant was about 50% of the wild-type. Significantly, compared with wild-type, the heavy and light chain were not associated for the Cys-310 → Ser mutant, as a very low level of light chain was co-immunoprecipitated with the anti-heavy chain antibody (Fig. 3*B*, compare *lanes 1* and *2* with *lanes 3* and *4*).

Immunoprecipitation with the anti-light chain FVIII antibody demonstrated that the amount of light chain present in the conditioned medium at the 2- and 4-h chase time points for the Cys-310 → Ser mutant was similar to the wild-type (Fig. 3*C*; compare *lanes 1* and *2* with *lanes 3* and *4*). In the wild-type FVIII, there was a considerable amount of heavy chain that co-immunoprecipitated with the anti-light chain antibody. This again indicates a complex between the heavy chain and the light chain and that the initial immunoprecipitation with anti-heavy chain antibody was not quantitative under these conditions. In contrast, heavy chain was not detected upon immunoprecipitation of the Cys-310 → Ser mutant with the anti-light chain antibody, supporting the absence of a heavy and light chain complex. Analysis of the FVIII activity in the conditioned medium demonstrated that activity for the Cys-310 → Ser mutant was at least 100-fold reduced compared with the wild-type or the Cys-2000 → Ser mutant and was not detectable above the background (Table I). These results show that the Cys-310 → Ser mutant was properly synthesized, transported from the ER to the Golgi compartment where the protein was cleaved to its mature heavy and light chains, and secreted as dissociated chains. However, the heavy chain did not accumulate in the conditioned medium. At present, we do not know if the heavy and light chain are unassociated in the Golgi compartment or are rapidly dissociated upon secretion into the conditioned medium.

In contrast to the Cys-310 → Ser mutant, the Cys-2000 → Ser mutant displayed a secretion rate similar to wild-type, and the heavy and light chains were associated as detected by co-immunoprecipitation with the anti-FVIII heavy chain antibody (Fig. 3*B*, *lanes 5* and *6*). The activity of the secreted protein was 50% of wild-type FVIII, while the specific activity was actually higher than the wild-type. The higher specific activity may be due to a conformational change in the epitope recognized by the light chain antibodies used to measure the antigen level by ELISA (Table I). This is supported by the observation that immunoprecipitation with anti-heavy chain antibody detected similar amounts of protein for the Cys-2000 → Ser mutant and wild-type. Thus, similar amounts of FVIII were detected by the immunoprecipitation analysis, whereas the ELISA using anti-light chain antibodies detected less of the Cys-2000 → Ser mutant.

Site-directed Mutagenesis at the Proposed Type-2 Copper Binding Site—A structural model of the FVIII A domains based on the crystal structure of ceruloplasmin predicts a type-2 copper ion binding site that coordinates the interaction between the A1 and A3 domains. The two histidines proposed to coordinate the copper ion are His-99 within the A1 domain and His-1957 within the A3 domain (21). Expression of FVIII with the His-1957 → Ala substitution in COS-1 cells produced 60% activity of the wild-type and proportionally a lesser amount of protein secreted into the conditioned medium. Immunoprecipitation of the conditioned medium with either the anti-heavy or anti-light chain antibodies demonstrated that the heavy and light chain for the His-1957 → Ala mutant were associated to a similar extent as wild-type (Fig. 3*B*, *lanes 9* and *10*). These results indicate that mutation of His-1957 → Ala results in a FVIII protein that has its heavy chain associated with the light chain. The reduced activity for the His-1957 → Ala mutant in the conditioned medium is attributable to less efficient secretion. However, as for the Cys-2000 → Ser mutant, the specific activity of the His-1957 → Ala mutant was higher than wild-type (Table I), and possibly reflects weaker interaction with the antibody used in the ELISA. These results indicate that His-1957 does not play an essential role in heavy and light chain association and/or cofactor activity.

We also studied mutation of His-99 within the A1 domain. The His-99 → Ala mutant yielded a FVIII protein that was synthesized at a rate similar to wild-type FVIII (Fig. 3*A*, *lanes 10–12*). Although the protein disappeared from the cell extract at a rate similar to wild-type, it was less efficiently recovered in the conditioned medium (Fig. 3*B*, *lanes 7* and *8*). Immunoprecipitation with anti-heavy chain antibody demonstrated that the His-99 → Ala heavy chain was less efficiently secreted than wild-type (70% of the wild-type) and displayed less light chain association. The activity of the secreted protein was approximately 20% of the wild-type (Table I).

To quantitate the extent of chain association for the Cys-310 → Ser and His-99 → Ala mutants that were poorly secreted and difficult to detect, we immunoprecipitated equal numbers of counts of conditioned medium with the anti-heavy chain antibody and subjected the samples to SDS-PAGE. PhosphoImage quantitation demonstrated that, compared with wild-type, the Cys-310 → Ser mutant displayed 7% chain association and the His-99 → Ala mutant displayed 16% chain association. The results show that the Cys-310 → Ser mutant is severely defective in chain association while the His-99 → Ala mutant is partially defective in chain association.

Cys-310 → Ser and His-99 → Ala FVIII Are Not Targeted to Intracellular Degradation—The mutants Cys-310 → Ser and His-99 → Ala mutant FVIII molecules were properly synthesized and chased out of the cell but were poorly recovered in the conditioned medium. To investigate whether intracellular deg-

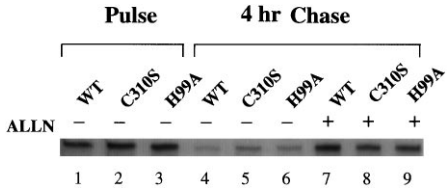

FIG. 4. **Cys-310 → Ser and His-99 → Ala FVIII mutants do not preferentially accumulate within the cell in the presence of ALLN.** WT and mutant FVIII expression vectors were transfected into COS-1 monkey cells. At 60 h post-transfection, the cells were labeled with [^{35}S]methionine for 30 min and chased for 4 h in the absence (*lanes 4–6*) or in the presence (*lanes 7–9*) of 250 μM of ALLN. The cell extracts were harvested, and equal volumes were immunoprecipitated with anti-FVIII heavy chain antibody for analysis by SDS-PAGE. Bands represent the single chain form of FVIII detected in the cell extracts.

radation was responsible for the reduced recovery of these mutants in the conditioned medium, we studied the effect of intracellular protease inhibition. ALLN is a cysteine protease inhibitor that prevents the degradation of FVIII retained within the secretory pathway (38). At 60 h post-transfection, cells were metabolically pulse-labeled for 30 min and chased for 4 h in medium containing ALLN. Equal amounts of cell extract and conditioned medium were collected, immunoprecipitated with the anti-FVIII heavy chain specific antibody, and analyzed by SDS-PAGE. Treatment with ALLN showed a similar increase in FVIII in the cell extract for the wild-type FVIII as for the mutant FVIII (Fig. 4). In addition, there was no increase in FVIII protein detected in the conditioned medium (data not shown). These results show that inhibition of intracellular degradation did not promote accumulation of mutant protein in the cell extract. In addition, it was not possible to rescue the secretion of either the Cys-310 → Ser or the His-99 → Ala mutant FVIII into the conditioned medium by cysteine protease inhibition. We conclude that the reduced amount of Cys-310 → Ser and His-99 → Ala FVIII protein detected in the conditioned medium is probably not due to degradation within the ER.

DISCUSSION

A structural model of the A domains of FVIII would be helpful in understanding its interaction with the other proteins in the coagulation cascade. The large size, heterogeneity, and extensive post-translational modifications of FVIII make it difficult to obtain a crystallographic structure. Most of the present information concerning FVIII structure are obtained from the interpretation of results from *in vitro* transfection studies of the wild-type or mutant FVIII molecules bearing specific amino acid changes. Although computer modeling of FVIII based on homology with the copper-binding proteins nitrite reductase (21) or ceruloplasmin (23) was attempted, there is no direct experimental evidence to support models for the interaction of the FVIII A1 and A3 domains.

Of critical importance to understanding FVIII structure is the question of where and what nature is the metal ion bridge that tethers the A1 domain to the A3 domain. Our atomic absorption spectroscopy demonstrated that recombinant FVIII contained copper ion at 1 mol/mol of active protein. EPR analysis detected copper ion in FVIII only after EDTA-induced release and oxidation, supporting that Cu(I) is buried within the molecule and protected from the oxidizing environment. EPR analysis did not detect copper ion in thrombin-inactivated FVIII, unless it was also EDTA-treated, suggesting that copper ion is bound and protected within the A1-A3/C1/C2 heterodimer. The detection of EDTA-releasable Cu(I) within FVIII before and after thrombin cleavage, indicates that FVIII activation is not associated with Cu(I) oxidation and excludes an electron transfer function for the copper ion in the activation process. Taken together, the results suggest that the metal ion within FVIII provides primarily a structural role. Finally, cuprous chloride, and not cupric chloride, promoted *in vitro* reconstitution of FVIII activity from dissociated heavy and light chain subunits, confirming the Cu(I) requirement for FVIII function.

Amino acid sequence analysis of FVIII predicts two type-1 mononuclear copper binding sites in the A1 and A3 domains that are conserved as free cysteines in the plasma copper-binding protein ceruloplasmin, where they coordinate a mononuclear copper ion with redox function (22). Our mutagenesis experiments support the importance of Cys-310 within the A1 domain in coordinating the type-1 copper binding site and rule out the importance of the type-1 copper binding site within the A3 domain of FVIII.

By computer modeling, His-1957 and His-99 were proposed to form a pocket of positive charge capable of coordinating a type-2 copper ion molecule (23). However, mutation of His-1957 to Ala did not affect the folding, secretion, or activity of the molecule, indicating either that the His ligand is substituted by a molecule of water in that mutant or that His-1957 does not coordinate copper ion. FVIII with His-99 mutated to Ala exhibited a moderate effect on activity and did not completely impair chain association of the secreted protein. Analysis of FVIII protein by means of the fluorescent, apolar probe bisanilinonaphthalsulfonic acid (bis-ANS), that specifically binds to hydrophobic sites detected two regions within the A1 and A3 domains that are exposed only after dissociation of the A1-A3/C1/C2 heterodimer (39). One of these regions contains nonpolar residues adjacent to His-99. Our mutagenesis data support a positive role for His-99 in promoting chain association. We propose that Cys-310 is an essential component of the type-1 copper binding site in the A1 domain that coordinates Cu(I) and provides an essential role for A1 domain folding so that the A1 domain can interact with the A3 domain, and that His-99 is also essential for the A1 and A3 domain interaction but is not a ligand for copper ion.

Our results support a requirement for Cu(I) interaction within FVIII for proper assembly of the heavy and light chains. Of crucial importance is where within the biosynthetic pathway the copper ion is required for FVIII assembly. It may be required within the ER, the Golgi compartment, or upon secretion of FVIII from the cell. The region adjacent to the proposed type-1 copper binding site within the A1 domain of FVIII contains a hydrophobic cluster of amino acids predicted to be a potential binding site for the ER protein chaperone BiP (40). Compared with FV, FVIII displays a reduced secretion efficiency that correlates with BiP interaction within the ER. In contrast, FV does not interact with BiP (41, 42). Exchange of residues containing this hydrophobic cluster can confer the secretion properties of FV onto FVIII, although the efficiently secreted molecule is not active, and the heavy and light chains are not associated (40). Within this cluster, Phe-309, adjacent to Cys-310, is the most responsible for BiP interaction and/or inefficient folding (43). FV does not have the typical sequence requirements for the proposed type-1 copper binding site, having Ser at the position corresponding to Cys-310 within FVIII (44). Substitution of Cys at position 310 with Ser in the A1 domain of FVIII generated a molecule in which the heavy and light chains were not associated and the protein was not active. These data provide strong support for the hypothesis that the copper ion in FVIII is liganded to different residues than the copper ion in FV. It is interesting that a potential high affinity BiP binding site is adjacent to the Cys-310 ligand in the type-1 copper ion binding site that is required for heavy and light

chain association. Bip interaction adjacent to Cys-310 may facilitate CU(1) binding, possibly by preventing oxidation of the cysteine residue. We propose that the different secretion efficiencies between FV and FVIII may be a consequence of their different requirements for copper ion binding within the A1 domain.

If the assembly of FVIII heavy and light chains with reduced copper ion occurs in the oxidizing environment of the secretory pathway, then there must be a mechanism to prevent oxidization of Cu(I) to Cu(II). This observation leads to the hypothesis that another protein may be involved in delivering Cu(I) to FVIII within the secretory pathway. Although the mechanism of copper transport and trafficking within the cell is poorly understood, at least two genes are known to be involved in copper homeostasis in humans. Defects in either of the two genes lead to Wilson's or Menkes' disease (45–48). The protein products of these genes reside in the Golgi compartment, are members of the P-type ATPase family, and are responsible for the translocation of copper ions across the intracellular membrane within the secretory pathway (49). While 95% of the patients with Wilson's disease have ceruloplasmin deficiency (50), there are no reports of FV/FVIII deficiency in Wilson's or Menkes' patients. Analysis of the plasma FVIII level in 10 patients affected by Wilson's disease and ceruloplasmin deficiency demonstrated their levels to be normal.[2] This indicates that, although similar, the three proteins exhibit different requirements for copper ion in protein folding within the secretory pathway.

It is also possible that assembly of the FVIII heavy and light chains with Cu(I) occurs upon secretion from the cell. Pulse-chase analysis of the secreted FVIII mutant Cys-310 → Ser demonstrated that the amount of light chain secreted into the conditioned medium was similar to the wild-type and remained stable during the time course. Although the amount of the Cys-310 → Ser heavy chain detected at 2 h of chase was similar to the wild-type, it did not accumulate during a 4-h chase. This suggests that the heavy chain is unstable in the absence of light chain association. Taken together, these experiments support the conclusion that the Cys-310 → Ser mutant is expressed and secreted with the same efficiency as wild-type FVIII, however the heavy and light chains are not associated and the heavy chain is unstable in the conditioned medium. This phenotype is reminiscent of the observation that FVIII synthesized in the absence of vWF in the conditioned medium is secreted as separate heavy and light chains, of which the heavy chain is subsequently degraded (3). In addition, vWF can promote *in vitro* reconstitution of FVIII activity from dissociated subunits (51). These observations suggest that vWF may promote the association of the FVIII heavy and light chains upon secretion from the cell. The extremely rich cysteine content of the D domains of vWF is similar to that of metallothioneins (52) which are copper-binding proteins. It is possible that vWF may promote FVIII heavy and light chain association by delivering a copper ion to FVIII upon secretion from the cell.

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