Communication

Mutagenesis of a Potential Immunoglobulin-binding Protein-binding Site Enhances Secretion of Coagulation Factor VIII*

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Coagulation factor VIII (FVIII) and factor V are homologous glycoproteins that have a domain structure of A1-A2-B-A3-C1-C2. FVIII is a heterodimer of the heavy chain (domains A1-A2-B) and the light chain (domains A3-C1-C2) in a metal ion-dependent association between the A1- and A3-domains. Previous studies identified a 110-amino acid region within the FVIII A1-domain that inhibits its secretion and contains multiple short peptide sequences that have potential to bind immunoglobulin-binding protein (BiP). FVIII secretion requires high levels of intracellular ATP, consistent with an ATP-dependent release from BiP. Site-directed mutagenesis was used to elucidate the importance of the potential BiP-binding sites in FVIII secretion. Mutation of Phe at position 309 to Ser or Ala enhanced the secretion of functional FVIII and reduced its ATP dependence. The F309S FVIII had a specific activity, thrombin activation profile, and heat inactivation properties similar to those of wild-type FVIII. However, F309S FVIII displayed increased sensitivity to EDTA-mediated inactivation that is known to occur through metal ion chelation-induced dissociation of the heavy and light chains of FVIII. The results support that Phe309 is important in high affinity heavy and light chain interaction, and this correlates with a high affinity BiP-binding site. Introduction of the F309S mutation into other secretion defective FVIII mutants rescued their secretion, demonstrating the ability of the this mutation to improve secretion of mutant FVIII proteins retained in the cell.

Two structurally related plasma glycoproteins, coagulation factor VIII (FVIII) and factor V (FV), are essential cofactors for the proteolytic activation of factor X and prothrombin, respectively. FVIII and FV have similar domain structures of A1-A2-B-A3-C1-C2 (1–4). The A-domains exhibit 35–40% amino acid identity to each other and to the A-domains of the copper-binding protein ceruloplasmin. Upon transit through the secretory compartment, FVIII is processed to a heterodimer consisting of a carboxyl-terminal derived light chain of 80 kDa (domains A3-C1-C2) in a metal ion-dependent association with an amino-terminal derived 200-kDa heavy chain (domains A1-A2-B) (5). FVIII is inefficiently secreted, and this correlates with its interaction with the protein chaperone immunoglobulin-binding protein (BiP), also known as the glucose-regulated protein of 78 kDa (GRP78) (6, 7). BiP is a peptide-dependent ATPase that interacts with unfolded, mutant, or unassembled protein subunits within the ER (8). FVIII release from BiP requires high intracellular concentrations of ATP. Depletion of intracellular ATP by treatment with low concentrations of the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) specifically inhibited FVIII secretion, whereas secretion of FV was not affected (9, 10). Further studies demonstrated that FVIII secretion requires not only high intracellular ATP level but also the ATPase activity of BiP (11).

Previous studies using chimeric FVIII and FV cDNA molecules identified a 110-amino acid region within the FVIII A1-domain that either actively retains FVIII in the ER or alters protein folding to reduce FVIII secretion (12). Exchange of this region with the homologous residues in FV yielded an efficiently secreted FVIII/FV chimeric protein that was not active and for which the heavy and light chains were dissociated. Based on the ability of 7-mer peptides displayed in filamentous phage to bind BiP, Gething and co-workers devised a statistical method to predict the BiP binding potential of a particular 7-mer peptide (13). Within the 110-amino acid region that inhibits FVIII secretion a hydrophobic cluster of residues from Ile291 to Phe309 contains multiple 7-mer peptides having a high probability of binding BiP (12). We tested the role of these residues in FVIII secretion by their mutagenesis to the respective amino acid present in FV. Mutation of a single amino acid Phe309 was sufficient to increase the secretion efficiency of biologically active FVIII by severalfold and reduce its ATP dependence for transport out of the cell.

EXPERIMENTAL PROCEDURES

Plasmid Mutagenesis—Site-directed mutagenesis was performed by oligonucleotide overlap extension polymerase chain reaction (PCR). Partially complementary primers that contained the mutation were utilized with two primers directed at the MluI sites at 226 and 336 in the FV/FV chimeric cDNA V227–336 previously described (12) to amplify two overlapping products that contain the directed mutation. Then these two fragments were isolated and fused together by PCR using the two MluI site containing primers. The resultant MluI fragment was then subcloned into the MluI-digested FVIII/FV 226–336 chimera within the expression vector pEDAC (14). All mutations were confirmed by DNA sequencing over the PCR-amplified region using the Sequenase kit (U. S. Biochemical Corp.). For every mutation analyzed, two independently isolated mutant clones were analyzed in transfection experiments.

DNA Transfection and Analysis—Expression vectors encoding the indicated mutants were transfected into COS-1 cells by the DEAE-dextran procedure (15). Conditioned medium was harvested 60 h post-transfection in the presence of 10% heat-inactivated fetal bovine serum for FVIII assay. Protein synthesis and secretion were analyzed at 60 h post-transfection by pulse-chase labeling of cells (16). Proteins were subjected to SDS-PAGE under reducing conditions and visualized by autoradiography after treatment with En3Hance (DuPont Corp.,

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Mutation of BiP Binding Site Improves Factor VIII Secretion

We then tested whether insertion of additional mutations may further increase Factor VIII activity detected in the conditioned medium. Because the optimal peptide size for BiP binding is a 7-mer (11), we additionally mutated each of the residues in the hydrophobic pocket adjacent to F309S. The amount of secreted activity for each double mutant was close to that obtained with the F309S mutation alone (Table I). The only double mutant that yielded a slight increase over the F309S mutation alone was the F309S/L303E double mutation (3.0-fold greater than wild type in three separate experiments), which corresponds to a doubly modified 7-mer peptide.

Finally, we tested whether introduction of the mutations that improve Factor VIII secretion could rescue the secretion of other Factor VIII mutations that destroyed activity recovered in the conditioned medium. Addition of the F309S/L303E double mutation to either the L294T or the L300V mutation increased their secretion close to the Factor VIII wild-type level (Table I). In contrast, addition of the F309S/L303E mutations into the F293S mutant did not improve secretion. These results show that the F309S/L303E mutations can increase secretion of some but not all secretion defective mutants of Factor VIII.

Metabolic pulse-chase labeling was performed to characterize whether the increased activity correlated with an increase in synthesis, secretion, or specific activity. Analysis of the pulse-labeled and chase cell extract samples indicated that Factor VIII wild-type and all mutants were synthesized at the same rate and disappeared from the cell extract at similar rates (Fig. 1A). However, analysis of the conditioned medium demonstrated that the F309S mutation increased the recovery of Factor VIII in the conditioned medium (Fig. 1B). Immunoprecipitation with the anti-heavy chain monoclonal antibody co-precipitated the light chain for all the mutants, demonstrating an association between the heavy and light chains. Additionally,
ular weight markers are shown on the left of the gel in the order of increasing activity obtained in the conditioned medium. SC, HC, and LC represent pulse and chase for cell extracts. Molecular weight markers are shown on the left. Samples were loaded onto the gel in the order of increasing activity obtained in the conditioned medium. The amount of F309S mutant FVIII recovered in the conditioned medium was similar to the amount of the FVIII-FV hybrid V(227–336) (Fig. 1B, lane 1). In contrast, secretion of F306W mutant FVIII was not different from the wild-type FVIII. The relative amount of secreted FVIII protein observed correlated with the amount of activity recovered in the conditioned medium (compare Table I with Fig. 1B). Mutation of L294T inhibited FVIII secretion, in proportion to the reduced activity (Table I), and additional mutation of L303E and F309S improved secretion of the mutant FVIII to a level similar to that of wild type (Fig. 1C). Importantly, because wild type and all mutants were synthesized at the same rate, differences in transfection efficiency or expression cannot account for the differences in the amount of secreted protein recovered in the conditioned medium. These results demonstrate that the increased activity for all FVIII molecules harboring the F309S mutation resulted from increased secretion compared with wild-type FVIII. The increased secretion was almost equal to that of the FVIII-FV hybrid molecule V(227–336).

**BiP Interaction and Secretion of Mutant F309S FVIII**—To characterize the secretion of the F309s mutant FVIII in more detail, stably transfected CHO cell lines were derived that expressed the hybrid FVIII-FV V(227–336) protein or the F309S mutant FVIII. Of 35 original transfected CHO cell clones selected for dihydrofolate reductase expression, we obtained five clones that express significant levels of F309S FVIII (greater than 1 unit/ml/10^6 cells/day). Two of these clones express greater levels of FVIII compared with the original 10A1 cell line that was obtained by screening over 1000 original transfected cell clones (5). Thus, at this initial stage of selection in low concentrations of methotrexate, the F309S mutation permits high level FVIII expression to be obtained more readily. Pulse-chase labeling with [35S]methionine and immunoprecipitation with anti-FVIII antibody demonstrated that FVIII wild type, V(227–336), and F309S mutant FVIII molecules were synthesized and secreted from CHO cells at similar rates with no significant difference in BiP association (data not shown).

To further characterize the BiP interaction we analyzed the effect of ATP depletion on the transport of FVIII wild type, V(227–336), and F309S mutant FVIII from CHO cells. Cells were pulse-labeled with [35S]methionine and chased for 6 h in the presence of increasing concentrations of CCCP. Cell extracts were harvested and immunoprecipitated with anti-FVIII or anti-F antibody for analysis by SDS-PAGE. Whereas transport of the majority of wild-type FVIII was inhibited by 10 μM CCCP, FV required higher concentrations to significantly inhibit transport. The V(227–336) hybrid molecule also required high levels of CCCP (250 μM) to inhibit its transport (Fig. 2). The single point mutant F309S displayed an ATP dependence that was intermediate between wild-type FVIII and FV. These results are consistent with the interpretation that the F309S mutant FVIII binds BiP but with an apparent lesser affinity and that this interaction does not require high levels of ATP for release.

**F309s Mutant FVIII Displays Increased Sensitivity to EDTA Inactivation**—The properties of the F309s mutant FVIII were studied by purification of this mutant from conditioned medium from CHO cells. Wild-type FVIII was purified in parallel for control. The specific activity determined by the factor Xa generation assay and enzyme-linked immunosorbant assay of wild-type and F309s FVIII were not significantly different (approximately 2500 units/mg). F309s mutant FVIII displayed a thrombin activation very similar to that of wild-type FVIII (Fig. 3A). The thermal stabilities of wild-type and F309s FVIII, tested by treating the purified proteins at 50 °C for increasing periods of time, were similar (Fig. 3B). However, F309s FVIII was inactivated by EDTA treatment at an approximately 10-fold greater rate than wild-type FVIII (Fig. 3C). These results show that F309s mutation did not alter FVIII specific activity, thrombin activation, or thermal inactivation. However, this mutation did significantly increase the sensitivity to EDTA-induced inactivation.
proposed based on the recently elucidated structure of cerulo-
ependent A1- and A3-domain interaction.
intriquing that F309S is adjacent to a ligand in the type I
two homologous molecules may be distinctively different. It is
that the requirements for folding and final structures of these
sequences that have the potential to bind BiP. Mutagenesis of
(12). This region is also required for heavy and light chain
meric proteins demonstrated that sequences within the carbox-
EDTA inactivation (19), thermal inactivation at 50 °C (20),
alyzed for thrombin activation (A),
reduction of intracellular ATP levels by treatment of cells with
the protonophore CCCP. We interpret these findings in either
one of two ways. First, the Phe309 mutant FVIII may exhibit a
weaker interaction for BiP, and its release may therefore
require fewer molecules of ATP. Alternatively, the mutant mol-
eucule may exhibit improved folding properties so that it more
quickly attains its final conformation required for transport
from the cell and therefore is bound to BiP for a lesser period of
time. Dramatically, introduction of the F309S mutation into
FVIII molecules that were defective for secretion was able to
rescue their secretion defect. This suggests that hemophilia A
associated with mutations that reduce FVIII secretion may be
improved by introduction of the F309S mutation into the FVIII
gene in these patients.

The F309S mutant FVIII was characterized to exhibit throm-
bin activation and thermal lability indistinguishable from wild-
type FVIII. However, the molecule did exhibit a 10-fold in-
creased sensitivity to EDTA-induced inactivation. EDTA is
known to inactivate FVIII through chelation of a metal ion that
is required for association of the A1-domain in the heavy chain
with the A3-domain in the light chain. FVIII contains 1 mol of
copper/mole of protein, and copper ion is only detected when the
heavy chain is associated with the light chain (21). Mutation of
C310S in wild-type FVIII produced an inactive molecule in
which the heavy and light chains were dissociated (22). The
requirement for Cys310 in FVIII and its absence in FV indicate
that the requirements for folding and final structures of these
two homologous molecules may be distinctively different. It is
intriguing that F309S is adjacent to a ligand in the type I
copper-binding site that may be essential for copper ion-de-
pendent A1- and A3-domain interaction.

Recently, a structural model of the FVIII A-domains was
proposed based on the recently elucidated structure of cerulo-
plasmin (23, 24). The proposed structure places the phenyl side
chain of Phe309 3.7 Å from Phe308 in the A2-domain, suggesting
a potential hydrophobic interaction between the A1- and A2-
domains. If this model is correct, mutation of F309S should
weaken the A1- and A2-domain interaction. Our experimental
data on the characterization of the F309S mutant FVIII show
that thrombin inactivation kinetics are similar for wild-type
and F309S mutant FVIII. Because inactivation of thrombin-
activated FVIII occurs as a consequence of A2-domain disso-
ciation (25), our experimental evidence does not suggest a weak-
ened interaction between the A1- and A2-domains. Further
experimental evidence should elucidate the validity of the
structural model.

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FIG. 3. F309S FVIII displays increased sensitivity to EDTA inactivation. Wild type (solid line) and F309S mutant (dotted line) FVIII were purified from CHO cell conditioned medium and analyzed for thrombin activation (A), thermal inactivation at 50 °C (B), and EDTA inactivation (C) as described under "Experimental Procedures."