Differential Interaction of Coagulation Factor VIII and Factor V with Protein Chaperones Calnexin and Calreticulin*

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Factor VIII (FVIII) and factor V (FV) are homologous coagulation cofactors sharing a similar domain organization (A1-A2-B3-C1-C2) and are both extensively glycosylated within their B-domains. In mammalian cell expression systems, compared with FV, the FVIII primary translation product is inefficiently transported out of the endoplasmic reticulum. Here we show that FVIII is degraded within the cell by a lactacystin-inhibitable pathway, implicating the cytosolic 20 S proteasome machinery. Protein chaperones calnexin (CNX) and calreticulin (CRT) preferentially interact with glycoproteins containing monoglucosylated N-linked oligosaccharides and are proposed to traffic proteins through degradative and/or secretory pathways. Utilizing co-immunoprecipitation assays, intracellular FVIII was detected in association with CNX maximally within 30 min to 1 h following synthesis, whereas FV could not be detected in association with CNX. In contrast, both FVIII and FV displayed interaction with CRT during transit through the secretory pathway. B-domain deleted FVIII significantly reduced the CNX and CRT interaction, indicating the B-domain may represent a primary CNX and CRT interaction site. In the presence of inhibitors of glucose trimming, the interactions of FVIII with CNX, and of FVIII and FV with CRT, were significantly reduced whereas the secretion of FVIII, and not FV, was inhibited. In addition, transfection in a glucosidase I-deficient Chinese hamster ovary cell line (Lec23) demonstrated that both degradation and secretion of FVIII were inhibited, with little effect on the secretion of FV. These results support that CNX and CRT binding, mediated at least in part by the B-domain of FVIII, is required for efficient FVIII degradation and secretion. In contrast, FV does not require CNX interaction for efficient secretion. The results suggest a unique requirement for carbohydrate processing and molecular chaperone interactions that may limit the productive secretion of FVIII.

Factor VIII (FVIII)† and factor V (FV) are homologous glycoproteins that function as essential cofactors for proteolytic activation of factor X and prothrombin, respectively. Elucidation of the primary structure of factors V (1, 2) and VIII (3, 4) demonstrated that they share amino acid identity and have a conserved domain organization of A1-A2-B3-C1-C2. The A-domains of factors V and VIII share approximately 35% identity to each other and to the A-domains of ceruloplasmin, a copper-binding plasma protein, suggesting a role in metal-ion binding. The C-domains share approximately 35% identity to each other and to phospholipid-binding proteins, such as milk fat globule protein, suggesting a role in phospholipid interaction (5). In contrast, there is negligible amino acid homology between the FV and FVIII B-domains (1). However, both B-domains contain a large number of asparagine (Asn)-linked oligosaccharides, where FV has 25 and FVIII has 19 potential N-linked glycosylation sites. Within the genome, the FVIII and FV B-domains reside on unusually large single exons (6, 7), suggesting their B-domains originated from a single exon and may function in similar roles in regulating the expression and/or activity of FV and FVIII. Deletion of the B-domain in either FVIII or FV results in molecules that are secreted in a functional form (8, 9).

In vivo, FV is expressed in the hepatocyte and megakaryocytes (10, 11). Although most evidence supports that FVIII is expressed at least in hepatocytes (12–15), the major physiological source for the in vivo expression of FVIII is unknown. Whereas HepG2 cells express FV (10), there are no known established or primary cell lines that express FVIII. Thus, our knowledge of FVIII biosynthesis is derived from interpretation of results from expression of the FVIII cDNA using expression vectors in transfected mammalian cells. Expression of FVIII in these transfection systems is 2–3 orders of magnitude lower than that observed with other genes using similar vectors and approaches. Studies have identified at least three reasons for the low level of expression: 1) the FVIII mRNA is inefficiently expressed (16–19); 2) high levels of von Willebrand factor, a protein that binds and stabilizes FVIII in plasma, are required in the conditioned medium to promote stable accumulation of FVIII (16, 20); and 3) the primary translation product is inefficiently transported from the endoplasmic reticulum (ER) to the Golgi apparatus (21). In contrast to FVIII, FV is efficiently transported from the ER and secreted into the medium (22). The reason for the differences in the relative efficiency of secretion of these homologous proteins may be related to their relative efficiency of folding and their differential interaction with ER chaperones. In support of this hypothesis, FVIII displays extensive interaction with the protein chaperone immunoglobulin-binding protein (BiP), where FV does not detectably bind BiP (21, 22).

The ER is a major site for the folding and assembly of secretory and integral membrane proteins. Proteins destined for the cell surface are co-translationally translocated into the
lumen of the ER. Newly synthesized proteins fold and assemble into tertiary structures within the ER and these reactions are facilitated by enzymes and molecular chaperones. Many protein chaperones interact with folding intermediates of polypeptides that are eventually destined for the cell surface. The mechanism that ensures only transport of completely folded proteins has been termed “quality control.” Two homologous protein chaperones that prevent transport of unfolded glycoproteins are calnexin (CNX), a highly conserved integral ER membrane protein (23), and calreticulin (CRT), an ER luminal protein (24), that share 42–78% identity. The sequence similarity between CNX and CRT suggests that these distinct ER proteins may have common functions. CNX and CRT associate transiently and selectively with newly synthesized glycoprotein folding intermediates, thereby preventing their transit through the secretory compartment (25–29). Prolonged association with CNX or CRT is observed when proteins are misfolded or unable to oligomerize (30–34). The CNX and CRT recognition motifs within newly synthesized proteins is at least partly determined by the structures of asparagine-linked oligosaccharides. Upon translocation into the lumen of the ER, a core unit of 14 saccharides (GlcNAc3Man9Glc3) is added to selective asparagine residues. Immediately after, trimming of the three terminal glucose residues occurs by sequential action of glucosidase I that cleaves the terminal α(1,2)-glucose and glucosidase II that cleaves the two internal α(1,3)-glucose residues on the core oligosaccharide structure. Binding to either CNX or CRT requires monoglucosylated asparagine-linked oligosaccharides and likely does not monitor the folding status of the glycoprotein (35–38). Release from CNX and/or CRT correlates with removal of the last glucose residue, mediated by glucosidase II. A UDP-glucose:glycoprotein glucosyltransferase can re-glucosylate high-mannose containing side chains (39, 40) and the activity of the glucosyltransferase is stimulated by unfolded protein (41, 42). It is proposed CNX and CRT specificity for binding and retention of unfolded proteins is due to the selective re-glucosylation of unfolded protein by the UDP-glucose:glycoprotein glucosyltransferase (42). These monoglucosylated products then bind CNX and/or CRT and are retained in the ER. In this way, a cycle of de- and re-glucosylation acts to regulate the association of glycoproteins with CNX and CRT (43, 44). After proper folding, terminal glucose residues are removed and the protein is released from CNX and/or CRT to transit to the Golgi apparatus.

We have studied the intracellular trafficking and interaction of FVIII and FV with CNX and CRT in Chinese hamster ovary (CHO) cells. Whereas intracellular FVIII is degraded by a lactacystin-inhibitable process, FV is efficiently secreted. The results demonstrate that FVIII interacts with CNX but FV does not, whereas both FVIII and FV interact with CRT. Interaction of FVIII with CNX and CRT is mediated in part by the B-domain. Analysis of the secretion of FVIII and FV in the absence of glucose trimming demonstrated that FVIII secretion displays a unique requirement for glucose trimming, whereas secretion of FV does not. Comparison of FV and FVIII intracellular trafficking provides a unique system to study the role for CNX and CRT in protein folding and may provide insight into novel ways to improve the efficiency of FVIII expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit anti-FV polyclonal antibody was purchased from The Binding Site Inc. (San Diego, CA). Anti-CNX and anti-CRT polyclonal antibodies were obtained from Affinity Bioreagents (Golden, CO). Anti-FVIII heavy chain monoclonal antibody F-8 coupled to Sepharose CL-4B was kindly provided by Debra Pittman, Genetics Institute Inc. (Cambridge, MA) (45). FVIII-deficient plasma and normal pooled human plasma were purchased from George King Biomedical (Overland, KS). Activated partial thromboplastin (APTT reagent) and CaCl2 were purchased from General Diagnostics Organon Teknika Corp. (Durham, NC). Fetal bovine serum, Dulbecco’s modified Eagle's medium, methionine-free Dulbecco’s modified Eagle’s medium, and Opti-MEM were purchased from Life Technologies, Inc. (St. Louis, MO). Soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, and aprotonin were purchased from Boehringer Mannheim. Castanospermine (CST), deoxynojirimycin (DNJ), and methionine were purchased from Sigma.

**RESULTS**

**Relative Efficiency of Secretion of FVIII, FV, and ΔB-VIII**—The secretion of FVIII, FV, and ΔB-VIII were compared in stably transfected CHO cells. Cells were pulse-labeled with [35S]methionine and chased for 4 h in medium containing excess unlabeled methionine and 0.2 mg/ml aprotonin. Cells were subcultured 18 h prior to labeling at a density of 33% and were approximately 80% confluent at the time of labeling. Cell extracts were harvested as described previously (47, 48). Conditioned medium was harvested with the addition of 1 mg/ml soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride. FVIII was quantitatively immunoprecipitated with an anti-FVIII monoclonal antibody F-8 coupled to Sepharose CL-4B. FV, CNX, and CRT were immunoprecipitated with rabbit polyclonal antibodies and recovered with protein A-Sepharose beads. The antibodies were tested prior to the experiments to determine the amount of antibody required for quantitative immunoprecipitation. In all cases excess antibody was used. The immunoprecipitates were washed three times in Triton X-100 as described (47).

**DNA Transfection and Analysis**—Plasmid DNA was transfected into CHO and Lec23 cells by LipofectAMINE (Life Technologies, Inc., Gaithersburg, MD). Plasmid DNA was transfected into COS-1 cells by the DEAE-dextran procedure as described (47). Conditioned medium was harvested 48 h post-transfection in the presence of 10% fetal bovine serum. FVIII activity was measured by a chromogenic assay based on factor Xa generation (49). Antigen measurements were performed by sandwich enzyme-linked immunosorbent assay using antibodies for FVIII described previously (50) and anti-FV antibodies from Dako (Carpenteria, CA) and The Binding Site Inc. Purified FV was obtained from Hematological Technologies (Essex Junction, VT).

**RESULTS**

**Relative Efficiency of Secretion of FVIII, FV, and ΔB-VIII**—The secretion of FVIII, FV, and ΔB-VIII were compared in stably transfected CHO cells. Cells were pulse-labeled with [35S]methionine and chased for 4 h in medium containing excess unlabeled methionine. Immunoprecipitation and SDS-PAGE analysis of the pulse-labeled cell extracts detected the FVIII, FV, and ΔB-VIII primary translation products (Fig. 1, lanes 1, 3, 7, and 9). FVIII is synthesized in CHO cells as a 280-kDa single chain polypeptide, whereas ΔB-VIII is synthesized as a 170-kDa polypeptide. Prior to secretion, FVIII is processed within the Golgi compartment by cleavage after residue 1648 within the B-domain to form a heterodimer composed of a 200-kDa amino-terminal derived heavy chain associated with an 80-kDa carboxyl-terminal derived light chain. ΔB-VIII, however, despite the absence of the B-domain, still undergoes proteolytic processing prior to secretion. This processing is important in resulting in the appearance of a ΔB-VIII single chain polypeptide of approximately 170 kDa, and a ΔB-VIII HC of 90 kDa in association with an 80-kDa light chain polypeptide. The FVIII single chain primary translation product was detected within the cell extract migrating at 280 kDa and was synthesized at a slightly lower rate than FV, detected migrating at 330 kDa. In contrast, the rate of ΔB-VIII synthesis was markedly greater than FVIII. Since an equal number of cells were analyzed, these differences reflect the different synthetic
rates of these proteins in the respective cell lines. After the 4-h chase period, 73% of the FVIII single chain was lost from the cell extract, whereas over 99% of the FV primary translation product was chased out over a similar period (Fig. 1, lanes 2 and 4). Under the same conditions only 34% of the synthesized ΔB-VIII, detected as a 170-kDa single chain, was lost from the cell extract over 4 h (Fig. 1, lanes 8 and 10). If all of the protein detected in the cell extract is secreted into the medium, then an equal amount of labeled protein should be recovered in the conditioned medium. FV was recovered efficiently from the conditioned medium and detected as a single chain polypeptide (Fig. 1, lane 6). However, very little FVIII was observed in the conditioned medium, detected as a 200-kDa heavy chain and an 80-kDa light chain that are processed from the single chain precursor (Fig. 1, lanes 5 and 11). Compared with wild-type FVIII, although a reduced proportion of the ΔB-VIII primary translation product was lost from the cell extract over 4 h, there was a marked increased recovery of protein within the conditioned medium (Fig. 1, lane 12). Analysis of autoradiograph band intensities indicates that the amount of pulse-labeled ΔB-VIII lost from the cell extract over 4 h correlates with recovery of ΔB-VIII secreted into the conditioned medium. These results show that FV is efficiently secreted into the medium, the ΔB-VIII shows intermediate secretion efficiency, whereas wild-type FVIII displays a low secretion efficiency. We hypothesized that the low recovery of wild-type FVIII in the conditioned medium compared with ΔB-VIII and FV was due to defective trafficking of FVIII within the cell, with a significant proportion of the primary translation product also being targeted to degradation machinery.

Inhibition of ER-associated Degradation with the Proteasome Inhibitor, Lactacystin, Stabilizes FVIII within the Cell Extract—We utilized lactacystin, a specific inhibitor of the 20 S proteasome (51) to determine if any of the observed loss of FVIII- and FV-labeled primary translation products from the cell extracts could be attributed to targeting toward this degradation machinery. FVIII wild-type was observed to chase out of the cell extract over a 4-h period, but in the presence of lactacystin, 60% of the primary translation product was retained (Fig. 2). In contrast, FV did not accumulate in the presence of lactacystin, indicating that the FV lost from the cell extract over the chase period is not targeted toward proteasomal degradation. Although FVIII degradation was inhibited, secretion of FVIII into the conditioned medium was not increased in the presence of lactacystin (data not shown), suggesting that the protein targeted to degradation under these conditions could not be rescued toward secretion. Thus, despite their extensive homology and similar post-translational modifications, FV and FVIII exhibit significant differences in their ER trafficking, degradation, and secretion. Since these differences may be related to differential interactions with ER chaperones, the following experiments were designed to characterize FVIII and FV interactions with CNX and CRT.

CNX and CRT Display Different Preferences for FVIII and FV Interaction—To study the CNX and CRT interaction of FVIII and FV within the secretory pathway, co-immunoprecipitation analysis was performed. CHO cells that express FVIII or FV were labeled with [35S]methionine for 15 min, chased for 30 min in excess unlabeled methionine, and cell extracts prepared for immunoprecipitation. Anti-FVIII antibody detected the FVIII primary translation product of 280 kDa in the cell extract (Fig. 3, lane 2). Immunoprecipitation of the same amount of cell extract with anti-CNX antibody precipitated CNX, detected migrating at 90 kDa, but also co-immunoprecipitated a significant amount of FVIII (Fig. 3, lane 6). Similar analysis of FV expressing CHO cells demonstrated the FV primary translation product as a polypeptide migrating at 330 kDa upon immunoprecipitation with anti-FV antibody (Fig. 3, lane 4). However, immunoprecipitation with anti-CNX antibody did not detect FV in a complex with CNX (Fig. 3, lane 8). This co-immunoprecipitation experiment was repeated with additional chase points of 15 min, 1 h, and 2 h, demonstrating the greatest proportion of CNX-FVIII complexes at 30 min, but did not detect any significant CNX-FV complexes at any point within the 2-h chase period (data not shown). These results indicate that these two large homologous heavily N-linked glycosylated proteins exhibit distinctively different interactions with CNX. Anti-CRT antibody precipitated CRT, detected migrating at 60 kDa, and co-immunoprecipitated the majority of the FVIII and a significant proportion of the FV pulse-labeled proteins (Fig. 3, lanes 10 and 12). Thus, in respect to these glycoproteins, FVIII and FV, CNX and CRT have different substrate specificities. Ternary complexes between FVIII, BiP, and CNX were not detected, suggesting that FVIII is either bound to BiP or to CNX. A 78-kDa protein (indicated by *) that was previously identified as BiP (21), co-immunoprecipitated with CRT and FVIII but not with CRT and FV, suggesting the presence of CRT-FVIII-BiP ternary complexes (Fig. 3, lane 10 versus 12). The absence of detectable CRT-FV-BiP ternary complex suggests that the CRT association with BiP is a specific interaction within the CRT-FVIII-BiP complex.

**Fig. 1.** Secretion of FVIII, FV, and ΔB-VIII in mammalian cells. Equal numbers of stably transfected CHO cells (approximately 6 × 10⁶) expressing FVIII, FV (Panel A), and ΔB-VIII (Panel B) were pulse (P)-labeled with [35S]methionine for 30 min and chased (C) for 4 h in medium containing excess unlabeled methionine. Cell extracts were harvested, one-half of the harvested cell extract was then immunoprecipitated with anti-FVIII and anti-FV specific antibodies, and separated by SDS-PAGE. Molecular weight markers are indicated in the left margin of each panel. SC, single chain; HC, heavy chain; LC, light chain. * represents the primary translation product.

**Fig. 2.** Pulse-chase analysis of FVIII and FV in the presence of proteasomal inhibition. Transiently transfected COS-1 cells expressing FVIII (lanes 1–3) and FV (lanes 4–6) were pulse (P)-labeled with [35S]methionine for 15 min, then chased (C) for 4 h in either the presence (+) or absence (−) of the proteasomal inhibitor, lactacystin (50 μM), added to the conditioned medium. Equal proportions of cell extracts were immunoprecipitated with either anti-FVIII or anti-FV antibodies and separated by SDS-PAGE.
FVIII B-Domain Is a Primary Determinant for Interaction with CNX and CRT—Since CNX is known to specifically bind monoglucosylated oligosaccharide structures on glycoproteins, and the B-domain of FVIII is the most extensively glycosylated region of the molecule, we tested whether the B-domain mediated the CNX interaction. Immunoprecipitation analysis of the [35S]methionine-labeled extracts from cells expressing ΔB-VIII demonstrated that deletion of the B-domain yielded a molecule, detected migrating at 170 kDa, that complexed with CNX in a proportion much reduced from that of FVIII (Fig. 3, compare lanes 2 and 6 versus 3 and 7). Similar results were observed with recovery of ΔB-VIII-CRT complexes. These results suggest that a primary determinant for CNX and CRT binding was contained within the FVIII B-domain. That ΔB-VIII retains some residual interaction with CNX and CRT is consistent with the presence of two N-linked glycosylation sites within the A1-domain and two within the 80-kDa light chain (20).

Interaction with CNX and CRT Requires Glucose Trimming—Nascent glycoproteins achieve a monoglucosylated form of their oligosaccharide core through the sequential actions of the ER glucosidases I and II. Cells stably expressing FVIII and FV were incubated with CST, an inhibitor of glucosidases I and II, prior to pulse labeling. Inhibition of glucose trimming was monitored by the reduced mobility of the primary translation products within the CST-treated cell extracts, consistent with the persistence of the 3 glucose residues on the oligosaccharide core structures (Fig. 4, lanes 2–5). Although FVIII-CNIX complexes were observed in the untreated cells (Fig. 4A, lane 6), CST treatment effectively reduced FVIII association with CNX (Fig. 4A, lane 7). This is consistent with FVIII being dependent upon achieving a monoglucosylated form of its oligosaccharide core structures for interaction with CNX. Under the same experimental conditions, CST treatment significantly reduced, but did not completely eliminate either the FVIII or FV association with CRT (Fig. 4B, lanes 13 and 17). This suggests that the CRT association may also involve protein-protein interactions that are independent of the state of the oligosaccharide structure or the efficiency of the glucose trimming.

Efficient Secretion of FVIII Requires Glucose Trimming—

The fundamental question concerning the significance of the observed CNX and CRT interactions is whether this facilitates or inhibits secretion. The efficiency of FVIII and FV secretion was studied in the presence and absence of glucose trimming. First, cells were treated with DNJ, another inhibitor of glucosidases I and II. The secretion of FVIII, reflected in activity recovered from the conditioned medium of expressing cells, was significantly reduced in the presence of DNJ compared with the amount of FV, measured by enzyme-linked immunosorbent assay, secreted under similar conditions (Fig. 5A). The requirement for glucose trimming by glucosidase I in FVIII, ΔB-VIII, and FV secretion was also studied by transient transfection of glucosidase I-deficient CHO cells (Lee23) that were previously isolated by lectin resistance (52). Wild-type CHO and Lee23 cells were transfected with FVIII, the ΔB-VIII, or FV expression vectors and the activity in the conditioned medium was analyzed. Whereas FVIII activity was significantly reduced in the Lee23 cells compared with the wild-type CHO cells, the secretion of ΔB-VIII was partially reduced and the amount of FV secreted was only slightly reduced (Fig. 5B). Quantitation of FVIII protein antigen by enzyme-linked immunosorbent assay indicated that the amount of FVIII protein secreted was reduced with no change in the specific activity (data not shown).

The efficiency of the transient transfection of the CHO and Lee23 cells was examined by pulse labeling. Equal numbers of cells were present at the time of labeling, and equal proportions of the labeled cell extracts were immunoprecipitated and loaded for SDS-PAGE analysis. The intensity of the pulse-labeled primary translation products from Lee23 cells expressing FVIII, ΔB-VIII, and FV were significantly reduced compared with CHO cells (Fig. 6, lanes 3, 5, 7, 9, and 11). Thus, the reduced activity recovered from the transfected Lee23 cells can in part be attributed to a reduced rate of synthesis. To determine the contribution of glucose trimming to the rate of secretion, pulse-chase analysis was performed. Cell extracts were harvested after a 4-h chase in medium containing excess unlabeled methionine. All of the pulse-labeled primary translation products isolated from the Lee23 cells migrated more slowly upon SDS-PAGE compared with those from CHO cells, consistent with inhibition of glucose trimming in the Lee23 cells (Fig. 6, lanes 3, 7, and 11 versus 1, 5, and 9). The majority
of FVIII disappeared after the 4-h time point within the cell extract of wild-type CHO cells (Fig. 6, lane 2). As previously shown, this disappearance reflects secretion as well as intracellular degradation. In contrast, in the Lec23 cells, FVIII did not disappear after the 4-h chase compared with the amount of pulse-labeled product, consistent with defective trafficking and transport out of the cell (Fig. 6, lane 4). A similar observation was made for the ΔB-VIII-expressing CHO and Lec23 cells (Fig. 6, lanes 6 and 8). In contrast, despite a reduced rate of synthesis of FV within the Lec23 cells, FV was chased out of the cell extract efficiently over 4 h, similar to CHO cells (Fig. 6, lanes 10 and 12). Thus, the reduced FVIII activity in conditioned medium from Lec23 cells is attributed to both a decreased rate of synthesis as well as a block to secretion in the absence of glucose trimming. The block to FVIII secretion by the absence of glucosidase I activity is specific for the trafficking of FVIII, with no effect on FV. In addition, this trafficking defect could not be entirely corrected by deletion of the B-domain.

To confirm that the observed secretion defect in the Lec23 cells was due to glucosidase I deficiency, we co-transfected FVIII and ΔB-FVIII with a human glucosidase I cDNA expression vector (53). Co-transfection of glucosidase I increased the amount of ΔB-VIII activity secreted from Lec23 cells to a level close to that observed secreted from CHO cells, and partially increased the amount wild-type FVIII activity secreted from Lec23 cells (Fig. 5C). These results support that the FVIII secretion defect is due to the absence of glucosidase I, and thus the inability to deglucosylate the oligosaccharide core structures. The FVIII activity recovered in the presence of glucosidase I co-expression in the Lec23 cells, although reduced compared with that in wild-type CHO cells, is thus reflective of the reduced rate of synthesis in the Lec23 cells. These results show a selective inhibition of FVIII transport compared with FV in the absence of glucose trimming and suggest that CNX interaction is required for secretion of wild-type FVIII but not for FV.

**FIG. 6.** Deficiency of glucosidase I prevents both secretion and degradation of FVIII. Transiently transfected CHO cells expressing FVIII (lanes 1 and 2), ΔB-FVIII (lanes 5 and 6), or FV (lanes 9 and 10) and Lec23 cells expressing FVIII (lanes 3 and 4), ΔB-FVIII (lanes 7 and 8), or FV (lanes 11 and 12) were pulse-labeled (0 h) with [35S]methionine for 1 h then chased for 4 h in medium containing excess unlabeled methionine. Equal proportions of cell extracts were immunoprecipitated with either anti-FVIII or anti-FV antibodies. The immunoprecipitated proteins were separated by SDS-PAGE. Molecular weight markers are indicated in the right margin.

**FIG. 5.** Glucose trimming is required for FVIII secretion. Panel A, transiently transfected CHO cells expressing FVIII or FV were treated with 2.5 mM DNJ overnight. FVIII activity in the conditioned medium was measured by a chromogenic assay. FV in the conditioned medium was measured by enzyme-linked immunosorbent assay. Panel B, conditioned medium from transiently transfected CHO and Lec23 cells expressing FVIII or ΔB-FVIII were measured for FVIII activity by chromogenic assays. Error bars represent standard error from multiple determinations of cells transfected in duplicate. Panel C, CHO cells were transiently cotransfected at a 1:1 ratio with (a) FVIII or ΔB-FVIII and a control vector (pEDΔC). Lec23 cell were transiently cotransfected at a 1:1 ratio with either (a) FVIII or ΔB-FVIII and pEDΔC or (b) FVIII or ΔB-FVIII and the human glucosidase I cDNA in pEDΔC. FVIII activity was measured by chromogenic assay. Error bars represent the standard error from multiple determinations of cells transfected in duplicate.

Co-immunoprecipitation and cross-linking experiments demonstrating associations between various ER chaperones have suggested that the ER functions as an “affinity chromatography matrix” transiently adsorbing incompletely folded and assembled proteins, preventing aggregation, while glucose trimming and re-glycosylation of oligosaccharide glycans drives an on-off cycle of chaperone-glycoprotein substrate interactions (54). CNX and CRT represent members of a family of endogenous lectin molecules within the ER matrix that display substrate specificity for glycoproteins containing partially glucosylated N-linked core oligosaccharides (35, 55). Glucosidases I
and II are responsible for trimming the glucose residues from the core oligosaccharides to a monoglucosylated form, an important process for CRT and CNX association with glycoproteins (56, 57). However, the precise role of CNX and CRT in the secretion of newly synthesized proteins in the ER remains unclear. Most early hypotheses speculated that they share redundant functions by interacting with individual newly synthesized proteins and facilitating folding within the ER before transport to the Golgi compartment. Recent dissection of the ER secretary and quality control machinery both in vitro and within different protein expression systems has highlighted both the similarities and differences between these chaperones. Particular insight can be gained by comparing different glycoproteins within the same system. For that reason, we have compared CRT and CNX interaction with the homologous cofactors V and VIII. Since monoglucosylated glycanas can be generated in the ER via the glucose trimming pathway (glucosidases I and II) as well as the re-glucosylation pathway (UDG-glucose:glycoprotein glucosyltransferase), CRT and CNX may differentially associate with monoglucosylated glycoproteins derived by either of these two pathways (58). In support of this, blockade of glucosidase activity by castanospermine treatment stabilized CRT/T-cell receptor-α protein interactions, but not CNX/T-cell receptor-α protein complexes. This suggests that removal of glucose residues from nascent glycan chains is an important step in the disassembly of T-cell receptor-α proteins from CRT, but not from CNX. This difference may be attributed to differential access of the N-glycans to glucosidase II. Since CNX is a transmembrane protein and CRT is a luminal protein, it was proposed that CNX holds the newly translated protein in the ER, whereas CRT associates and dissociates frequently from the protein (57). Alternatively, CNX may interact with domains on nascent proteins associated with the ER membrane, while the soluble CRT may interact domains not associated with the ER membrane. The finding that FVIII and FV, both similar proteins that do not interact with the ER membrane display different interactions with CNX does not support the generality of this latter hypothesis. Ternary complexes have also been observed in molecular chaperone interactions with nascent glycoproteins (54), giving rise to the possibility that some of the CRT or CNX interactions detected may be indirect. The finding that BiP was only present in complexes of FVIII with CRT and not in complexes of FV with CRT, support that the ternary complexes we have detected are specific to FVIII.

Previous work with FVIII and FV interaction with ER chaperones has been confined to the immunoglobulin-binding protein (BiP), also known as the glucose-regulated protein of 78 kDa (59) within the lumen of the ER (21, 60). BiP is a member of the 70-kDa heat-shock protein (hsp70) family that exhibits a peptide-dependent ATPase activity (61) and for which expression is induced by the presence of unfolded protein or unassembled protein subunits within the ER (62, 63). In previous work, inefficient secretion of FVIII compared with FV correlated with FVIII interaction with BiP. FVIII release from BiP and transport out of the ER required high levels of intracellular ATP (64). In contrast, FV did not detectably associate with BiP and did not require high levels of ATP for secretion (22). Replacement of a 110-amino acid segment in the FVIII A1-domain for homologous FV sequences improved secretion by 10-fold and likely reduced BiP interaction (65). More recent studies suggest that a primary BiP-binding site likely resides in the FVIII A1-domain and demonstrated the ability to modify an ER chaperone interaction to improve the efficiency of FVIII expression (66).

This study builds upon these findings by detecting CNX interaction with FVIII but not FV and CRT interaction with both FVIII and FV. Whereas only a fraction of FVIII was in a complex with CNX, the majority of intracellular FVIII was in a complex with CRT. In these studies, very little FVIII was detected in association with CNX after a 15-min pulse label whereas after a 30-min chase period, FVIII association with CNX was maximal. In contrast, FVIII binding to BiP in previous studies was maximal immediately after translation (21), similar to the kinetics for FVIII interaction with CRT.2 These results suggest that FVIII first binds BiP and CRT, and then is transferred to CNX, similar to observations with vesicular stomatitis virus G glycoprotein (67) and thyroglobulin (31). The CNX and CRT interactions with FVIII and FV were largely dependent on glucosidase activity. Treatment with glucosidase inhibitors, CST and DNJ, significantly blocked CNX interaction with FVIII. This complements reports that show that CNX association is specific for monoglucosylated core glycans (35, 36, 56). CRT association in the presence of these inhibitors was reduced but not as significantly as CNX association. This is consistent with the observations with T-cell receptor-α protein (58) and suggests that FVIII may also require glucose trimming to dissociate from CRT. Alternatively, there may be peptide interactions or ternary complexes that maintain a detectable association in the absence of glucose trimming. In support of the latter hypothesis, we observed that the CRT associated with FVIII was also in a complex with BiP, whereas, CNX was not detected in this complex.

The difference in CNX association between FVIII and FV and the unique requirement for glucose trimming for secretion of FVIII, and not of FV, suggests possible unique binding regions in FVIII that may be functionally important for chaperone interaction and secretion. Results from these studies suggest that FVIII interacts with CNX, likely through oligosaccharide structures within the heavily glycosylated B-domain, and this interaction is required for FVIII secretion. In contrast, secretion of FV, a molecule that also contains a heavily glycosylated B-domain but did not interact with CNX, was unaffected by inhibition of glucosidase activity. Deletion of the B-domain in FVIII reduced its CNX interaction and also reduced its dependence on glucose trimming for secretion. It should be noted that B-domain-deleted FVIII is expressed at a greater level than wild-type FVIII in a number of different cell culture systems studied (8, 9, 46, 68–70), and this is one reason that most gene therapy studies for hemophilia A have utilized the B-domain deletion molecule. B-domain-deleted FVIII is expressed at a greater level because of a large increase in its mRNA level and consequently, protein translation (9). However, there is a much larger percentage of newly synthesized, B-domain-deleted FVIII left in the cell extract after 4 h compared to either FVIII or FV. This leads to two conclusions regarding the trafficking of B-domain deleted FVIII: first, the B-domain-deleted FVIII is inefficiently transported through the secretory pathway despite a significantly higher rate of synthesis, and second, the B-domain-deleted FVIII primary translation product is not degraded by the proteasome as rapidly as wild-type FVIII. This resistance to degradation observed for the B-domain-deleted FVIII suggests that certain chaperone interactions may also be required to target FVIII away from the secretory pathway and toward the degradative pathway as part of ER quality control.

The observation that CNX and CRT interact differentially with FV and FVIII and that inhibition of glucose trimming selectively prevented secretion of FVIII, supports the hypoth-
esis that CNX and CRT perform distinct molecular functions in the folding, assembly, and degradation of these newly synthesized homologous proteins within the ER. Although FVIII interaction with CNX was not detectable in the presence of glucosidase inhibitors, a significant proportion of CRT was still associated with FVIII. However, the persistent CRT interaction with FVIII was not sufficient to promote FVIII secretion, suggesting that CRT may play little role in promoting efficient FVIII secretion. Interestingly, intracellular FVIII degradation was not observed in the Lec23 cells, although FVIII degradation did continue in the presence of glucosidase inhibitors. We suggest that complete inhibition of glucosidases I and II may not occur upon treatment with DNJ or CST and this may account for the different effects of drug intervention and genetic deficiency to prevent glucose trimming on FVIII intracellular degradation.

A growing amount of data supports that a significant number of proteins localized to the ER are degraded by the cytosolic proteasomal machinery (71–74). To date, there are examples of transmembrane proteins (75–79) and a couple of secretory proteins (74, 80), where dislocation through the Sec61 protein channel is likely mediating their trafficking to the proteasome. In the case of a secretory protein, it is difficult to envision how an ER luminal glycosylated and disulfide-bonded protein is directed to the cytosolic proteasomal machinery. In one example, the mutant Z allele of α1-antitrypsin is proposed to associate with CNX and subsequently CNX is ubiquitinated and acts as a signal to deliver mutant α1-antitrypsin degradation to the proteasome (81). However, the general requirement for CNX in the degradative process is unknown. To date, reported observations suggest both positive and negative roles for CNX in mediating degradation of ER luminal proteins (25, 72, 81, 82). We have presented evidence, using the specific proteasome inhibitor lactacystin, that FVIII, a luminal protein that transits the ER, is degraded by the proteasomal machinery by a mechanism that requires glucose trimming, suggesting a role for CNX in this process.

In conclusion, we propose that FVIII secretion is dependent upon the productive interaction with two protein chaperones, BiP and CNX, with both cycling between FVIII bound and unbound states. Upon translocation of FVIII into the lumen of the ER, FVIII immediately interacts with BiP through a primary binding site within the A1-domain. In reactions that require ATP hydrolysis for BiP release and glucosidases I and II for glucose trimming, FVIII molecules containing monoglycosylated structures interact with CNX and CRT. Studies utilizing glucosidase inhibition suggest that the CNX interaction plays a positive role for FVIII secretion, possibly by promoting release of FVIII from BiP. Interaction with CRT is either coincidental with the BiP interaction or else insufficient to compensate for a deficient CNX interaction. Glucosidase II would subsequently remove glucose and promote release of FVIII from CNX and CRT. However, if FVIII is improperly folded it will activate the UDP-glucose:glycoprotein glucosyltransferase to re-glycosylate oligosaccharide cores, primarily within the B-domain and subsequently promote another round of CNX interaction. In this manner, only fully folded FVIII may escape the chaperone-mediated retention mechanisms and finally be transported to the Golgi apparatus and out of the cell. Those FVIII polypeptides that are persistently retained within the ER through chaperone interactions are eventually targeted to the cytosol for degradation by the proteasome. In contrast to FVIII, FV has evolved a pathway of protein folding that is very efficient and does not rely on BiP and CNX to prevent the secretion of unfolded intermediates or to assist its secretion.
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