ATP-Dependent Dissociation of Non-Disulfide-Linked Aggregates of Coagulation Factor VIII Is a Rate-Limiting Step for Secretion[†]

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ABSTRACT: Deficiency in coagulation factor VIII leads to the bleeding disorder hemophilia A. Previous studies demonstrated that factor VIII secretion is limited due to an ATP-requiring step early in the secretory pathway. In this report, we identified that this ATP-dependent rate-limiting step involves the dissociation of non-disulfide-linked aggregates within the endoplasmic reticulum (ER). In contrast to the numerous examples of interchain disulfide-linked aggregates, factor VIII is the first protein characterized to form non-disulfide-linked high molecular weight aggregates within the ER. Approximately a third of newly synthesized factor VIII was detected in high molecular weight aggregates. These aggregates disappeared over time as functional factor VIII appeared in the medium. The aggregated complexes did not require proteasomal degradation for clearance. Aggregate formation was enhanced by ATP depletion, and upon restoration of metabolic energy, these aggregates were dissociated and secreted. With the coexpression of von Willebrand factor (vWF), a small portion of vWF coaggregated with factor VIII. However, vWF dissociated from the aggregates more rapidly than factor VIII, supporting that these aggregates are dynamic. An increase in the factor VIII expression level elicited a corresponding increase in the fraction of factor VIII that was aggregated. In addition, a 110 amino acid sequence containing a hydrophobic β-sheet within factor VIII was identified that may predispose factor VIII to aggregation. These data show that formation and ATP-dependent dissolution of nondisulfide-linked factor VIII aggregates is a dynamic, rate-limiting step during the folding process in the early secretory pathway. In summary, we have identified an unprecedented requirement for protein transport out of the ER that involves an ATP-dependent dissociation of non-disulfide-linked aggregates within the ER.

The[1] primary amino acid sequence of a protein contains all the information required to fold into a stable, functional tertiary and quaternary structure (*1*). However, protein folding and maturation within the cell is a highly assisted process. For secretory and integral membrane proteins, folding begins as the nascent chain is cotranslationally translocated into the lumen of the endoplasmic reticulum (ER) and is accompanied by a significant reduction in free energy (*2*−*6*). Nascent polypeptides associated with polysomes are less likely to interact with homotypic adjacent nascent chains to form aggregates until they are released (*3*), although this may not always be true (*7*). Once released from a polysome, a protein is likely to aggregate if one or more of its domains are only partially folded. Only properly folded proteins exit the ER for transport through the secretory pathway to reach their final destination. Multiple cellular mechanisms exist that retain incompletely folded and misfolded proteins in the ER and collectively are called a "quality control" system (*8*). The lumen of the ER provides a unique oxidizing environment to promote disulfide bond formation. In numerous cases, retained proteins exist as high molecular weight aggregates that contain interchain aberrant disulfide bonds (*4*−*6*, *9*−*14*). Asparagine-(N-) linked glycosylation plays a central role during the initial folding of newly synthesized glycoproteins. Disruption of N-linked glycosylation frequently results in aggregation of the nonglycosylated proteins that accumulate and do not transit the ER. The accumulated nonglycosylated intermediates also form interchain disulfide-linked aggregates (*4*, *5*). The dissolution of these disulfide-linked aggregates requires disulfide bond exchange activities. In contrast, high molecular weight aggregates within the ER that do not contain interchain disulfide bonds have not been described.

Some aggregated proteins have been detected in association with the ER resident chaperones known as the immunoglobulin binding protein (BiP), calnexin, and/or calreticulin that may retain aggregates within the ER (*4*, *15*−*18*). Recent evidence suggests that aggregation of misfolded protein not only provides a mechanism that prevents exit from the ER but also may actually represent a transient intermediate in the productive folding process (*4*, *5*, *9*, *14*, *16*). Thyroglobulin, influenza hemagglutinin, MHC class II antigens, and procollagen transiently aggregate immediately after synthesis

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[1] Abbreviations: vWF, von Willebrand factor; BiP, immunoglobulin binding protein; ER, endoplasmic reticulum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; CHO, Chinese hamster ovary; GRP, glucose-regulated protein.

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and the aggregates disappear over time without concomitant protein degradation, supporting the hypothesis that aggregation is a kinetically reversible process. Although it is presumed that protein chaperones facilitate correct protein folding, it is not known if they affect protein aggregation in vivo.

Factor VIII is the plasma coagulation factor deficient in the X-chromosome linked genetic bleeding disorder hemophilia A. Factor VIII is a large glycoprotein having the domain structure A1-A2-B-A3-C1-C2 and contains 25 potential N-linked glycosylation sites, 18 of which are clustered in the B domain (*19*, *20*). Upon secretion from the cell, factor VIII is cleaved to an amino-terminal 220 kDa heavy chain fragment that is in a Cu^{+} ion-dependent association with an 80 kDa carboxy-terminal-derived light chain fragment. In plasma, factor VIII circulates in a noncovalent complex with von Willebrand factor (vWF) (*21*). The recommended treatment for patients afflicted with hemophilia A is the repetitive infusion of recombinant-derived factor VIII preparations produced in mammalian cells. However, availability of recombinant-derived factor VIII is limited due to a low level of production. Therefore, studies have focused on identifying the factors that limit factor VIII expression.

To date there are no known established cell lines that naturally express factor VIII; thus synthesis and secretion have been studied by expression of factor VIII cDNA in transfected mammalian cells. Factor VIII secretion in these systems is 5−10-fold less efficient than that of most other proteins, including the homologous coagulation factor, factor V. The rate-limiting step in factor VIII secretion is the transport from the ER to the Golgi apparatus (*22*). In the ER, factor VIII associates with protein chaperones BiP, calnexin, and calreticulin (*23*, *24*). In addition, factor VIII requires ATP for release from BiP and secretion (*25*). In this study we demonstrate that factor VIII transiently forms aggregates immediately after its synthesis. We have studied the properties of this aggregation to conclude that aggregate formation increases with the factor VIII expression level and is reversible. More importantly, the aggregates do not contain interchain disulfide bonds, although they do require ATP for disaggregation. Finally, a hydrophobic region within factor VIII (amino acid residues 227−336) was identified that may predispose factor VIII to aggregation. When this region was replaced by the homologous residues from coagulation factor V, the extent of aggregation was significantly reduced.

EXPERIMENTAL PROCEDURES

Materials. Anti-factor VIII heavy chain monoclonal antibody F-8 coupled to Sepharose CL-4B and purified recombinant factor VIII were provided by D. Pittman (Genetics Institute Inc., Cambridge, MA). Anti-GRP78 polyclonal antibody was obtained from Affinity Bioreagents (Golden, CO). Anti-calnexin polyclonal antibody SPA-860 was obtained from StressGen Biotechnologies Corp, (Victoria, BC, Canada). Anti-vWF polyclonal antibody was purchased from Dako Inc. (Carpinteria, CA). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), glucose-free DMEM were purchased from Life Technologies Inc. (St. Louis, MO). Soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, and aprotinin were purchased from Boehringer Mannheim (Indianapolis, IN). Tunicamycin, 2-deoxy-D-glucose, *N*-ethylmaleimide (NEM), and sodium azide were purchased from Sigma (St. Louis, MO). [^{35}S]-Methionine (>1000 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). EN^{3}HANCE was purchased from Du Pont (Boston, MA). Lactacystin was obtained from E. J. Corey, Harvard University, Cambridge, MA. The derivation of Chinese hamster ovary (CHO) cell lines that express factor VIII [10A1 (*26*) and H9 (*27*)] and the cell line that stably coexpresses factor VIII with vWF [10A1C6 (*26*)] was previously described.

Metabolic Labeling. Cells were subcultured 18 h prior to labeling in 100 mm dishes (approximately 10^{6} cells/plate) and were 80% confluent at the time of labeling. Cells were labeled in 1 mL of methionine-free medium containing 250 mCi/mL [^{35}S]methionine for 20 min and chased as indicated with conditioned medium containing excess unlabeled methionine (*28*, *29*). At the end of the chase period, cells were rinsed in phosphate-buffered saline containing 20 mM NEM and harvested in 0.5 mL of lysis buffer [20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.2% (w/v) Triton X-100] containing 20 mM NEM, 1 mg/mL soybean trypsin inhibitor, 1 mM phenylmethanesulfonyl fluoride, and 0.2 mg/mL aprotinin. For depletion of intracellular ATP, cells were pulse-labeled for 20 min with [^{35}S]methionine in regular DMEM and chased for 2 h in the presence of the ATP-depleting medium (glucose-free DMEM containing 20 mM 2-deoxy-D-glucose and 10 mM sodium azide) (*30*). Cycloheximide at a final concentration of 10 mg/mL was added to the medium. The cells were further incubated with regular medium containing excess unlabeled methionine for 4 h to recover from ATP depletion (*28*) or lysed in ice-cold HEPES buffer containing 20 mM NEM and protease inhibitors as above. Cell extracts were analyzed by sucrose gradient fractionation. For tunicamycin treatment, cells were pretreated with tunicamycin (10 mg/mL) for 2 h prior to labeling as described above. Tunicamycin was also maintained throughout the label and the chase period. For sodium butyrate treatment, cells were treated with sodium butyrate (5 mM) for 24 h before labeling with [^{35}S]methionine.

Sucrose Gradient Analysis. Cell lysates were centrifuged at 10000*g* for 10 min at 4 °C and the supernatant was applied to a 10 mL linear 5−20% (w/v) sucrose gradient. Immunoprecipitation of factor VIII demonstrated that less than 10% of the total cellular factor VIII was present in the postnuclear pellet. For analysis of the calnexin-bound material, cells were harvested in digitonin lysis buffer and immunoprecipitated with the anti-calnexin antibody. Bound material was eluted with 1% Triton X-100 as described (*14*) and applied to a sucrose gradient. Gradients were centrifuged for 16 h at 4 °C in a SW41 rotor (Beckman Instruments) at 39 000 rpm. Fractions were collected from the bottom of the tube and immunoprecipitated with an anti-factor VIII heavy chain monoclonal antibody F-8 coupled to Sepharose CL-4B (*31*). Immunoprecipitates were washed three times with phosphate-buffered saline containing Triton X-100 as described (*28*). Proteins were separated by electrophoresis on a sodium dodecyl sulfate−8% polyacrylamide gel (SDS−PAGE) and visualized by autoradiography after treatment with EN^{3}-HANCE (*29*). The autoradiograph band intensities were quantitated by NIH Image software (public domain). Estimates of the molecular weight aggregates were obtained as

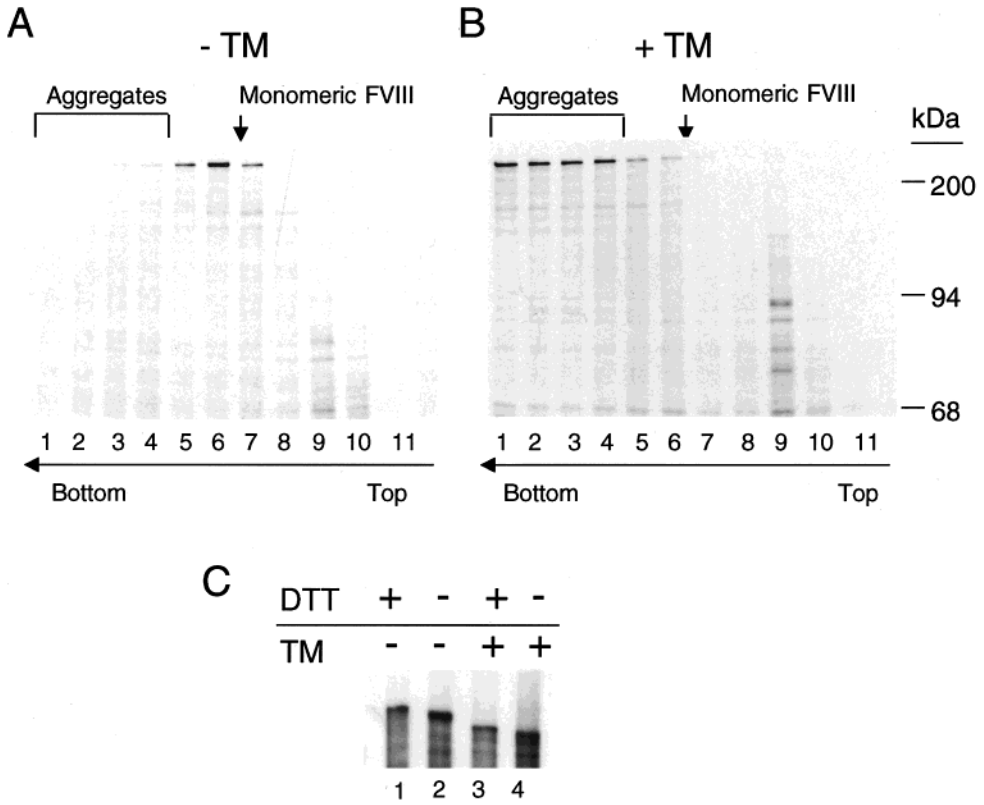

FIGURE 1: Inhibition of N-linked glycosylation induces factor VIII aggregation. Stably transfected CHO cells expressing factor VIII (clone 10A1) were pulse-labeled with [^{35}S]methionine for 20 min and chased for 4 h in medium containing excess unlabeled methionine (panel A). Duplicate plates were pretreated with 10 mg/mL tunicamycin (TM) for 2 h before labeling, and tunicamycin was maintained during the label and chase period (panel B). Detergent lysates were prepared and fractionated by sedimentation on sucrose density gradients. Gradient fractions were collected from the bottom of the tube and factor VIII was immunoprecipitated with anti-heavy chain factor VIII specific antibody. Immunoprecipitates were analyzed by SDS−PAGE and autoradiography. Fraction numbers from bottom to top are indicated. The sedimentation of functional factor VIII is indicated by an arrow at the top. Factor VIII that was immunoprecipitated from the cell extracts prepared above was analyzed by SDS−PAGE under reducing (+DTT, 10 mM) and nonreducing (−DTT) conditions (panel C).

described (*32*). Purified recombinant factor VIII (Genetic Institute, Cambridge, MA) was added to CHO cell extract and analyzed by sucrose fractionation as a molecular weight standard. Aliquots from each fraction were assayed for factor VIII activity by the one-stage activated partial thromboplastin time clotting assay using factor VIII-deficient plasma (*33*). The protein in each sucrose fraction was precipitated with 10% trichloroacetic acid, analyzed by SDS−PAGE, and visualized by Coomassie blue staining.

RESULTS

Nonglycosylated Factor VIII Forms Non-Disulfide-Linked Aggregates in the ER. Tunicamycin inhibits N-linked glycosylation by blocking the synthesis of the dolichol-linked oligosaccharide core that is transferred onto nascent polypeptides as they are translocated into the lumen of the ER. The absence of N-linked oligosaccharides promotes the formation of interchain disulfide-linked aggregates within the ER (*4*, *5*). To characterize whether unglycosylated factor VIII forms aggregates, cells were metabolically pulse-labeled with [^{35}S]-methionine in the presence and absence of tunicamycin and chased in the presence of excess unlabeled methionine. Cell extracts were prepared, fractionated by sucrose gradient centrifugation, and then analyzed by immunoprecipitation with an anti-factor VIII antibody and SDS−PAGE under reducing conditions. After a pulse label and 4 h chase, the factor VIII primary translation product detected in the extract from untreated cells sedimented in the middle of the sucrose gradient as a 230 kDa species (Figure 1A, fractions 6 and 7). This sedimentation overlapped that of purified monomeric functional factor VIII that was detected in fractions 6 and 7 (see Figure 2). In tunicamycin-treated cells, factor VIII was present as heterogeneous aggregates with the highest molecular weight complexes migrating at the bottom of the gradient (Figure 1B, fractions 1−4). The estimated molecular mass of the largest aggregates was at least 2×10^6 Da. The primary [^{35}S]-labeled protein detected in these aggregates was factor VIII. However, it is possible that the aggregates contain a mixture of additional ER resident proteins that have a long half-life and a slow rate of synthesis. These data show that, upon inhibition of N-linked glycosylation, factor VIII forms stable aggregates that are retained in the ER and validate the sucrose fractionation assay for intracellular aggregation studies on factor VIII.

To evaluate the presence of interchain disulfide bonds within the factor VIII aggregates, factor VIII from cell extracts was immunoprecipitated and equivalent portions were analyzed by SDS−PAGE under reducing and nonreducing conditions. Upon reduction, factor VIII migrated slightly slower, consistent with reduction of intrachain disulfide bonds (Figure 1C, lanes 1 and 2). Factor VIII produced in the presence of tunicamycin migrated faster, consistent with the absence of N-linked oligosaccharides (Figure 1C, lane 4). Upon reduction, the nonglycosylated

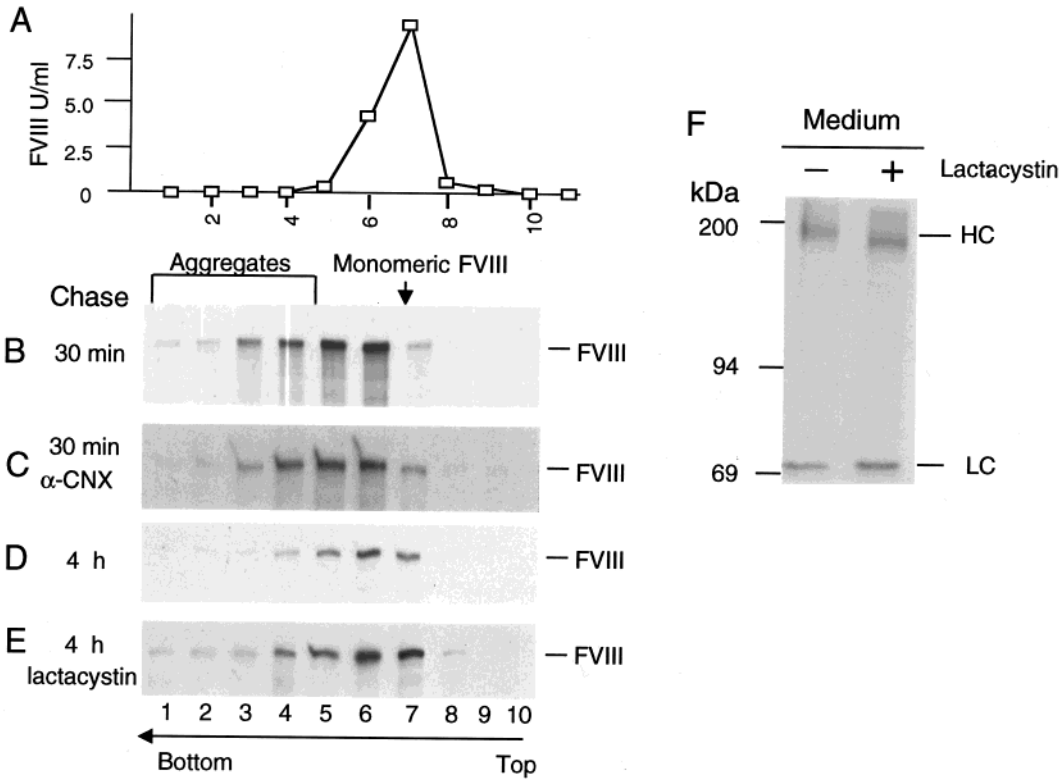

FIGURE 2: A portion of the newly synthesized factor VIII transiently aggregates. Purified recombinant factor VIII was added to CHO cell lysate fractionated by sucrose gradient centrifugation. The concentration of factor VIII activity in each fraction is shown (panel A). CHO cells that express factor VIII (clone 10A1) were pulse-labeled for 20 min with [^{35}S]methionine and chased with excess unlabeled methionine for 30 min (panels B and C) or 4 h (panels D−F). Chase was performed in the presence (panels E and F) and absence (panels B−D and F) of lactacystin (50 mM). Lysates were prepared and fractionated on sucrose density gradients. For analysis of calnexin association, 10A1 cells were metabolically labeled and cell extracts were prepared in buffer containing 1% (w/v) digitonin. Extracts were immunoprecipitated with anti-calnexin antibody. The precipitated polypeptides were eluted in 1% Triton X-100 at 25 °C. Eluted material was fractionated by sucrose density gradient centrifugation (panel C). Factor VIII was immunoprecipitated from the fractions and analyzed by SDS−PAGE under reducing conditions (panels B−E). Factor VIII was immunoprecipitated from 4 h chase-conditioned medium and analyzed by SDS−PAGE under reducing conditions (panel F). The heavy chain (HC) and light chain (LC) of factor VIII are indicated.

factor VIII also migrated slightly slower than the nonreduced and nonglycosylated factor VIII, consistent with reduction of intrachain disulfide bonds (Figure 1B, lane 3). The detection of similar amounts of factor VIII before and after reduction supports the absence of interchain disulfide bonds within the nonglycosylated aggregates.

Factor VIII Transiently Aggregates Early after Synthesis. Pulse−chase experiments suggested that even in the absence of tunicamycin a portion of factor VIII was aggregated (data not shown). Therefore, the formation and fate of factor VIII aggregates in the absence of tunicamycin was studied. Factor VIII aggregation was analyzed after a 20 min pulse label and a 30 min chase time point in order to complete synthesis of nascent chains. Cells were harvested and analyzed by sucrose gradient centrifugation. Immunoprecipitation of factor VIII from each fraction and SDS−PAGE analysis detected factor VIII migrating at 230 kDa. Analysis of the fractions indicated that factor VIII sedimented throughout the sucrose gradient, with the greatest amount in fractions 5 and 6 (Figure 2B). However, approximately 30% of the total factor VIII protein was detected in fractions 1−4, indicative of high molecular weight aggregates (Figure 2B). The factor VIII high molecular weight complexes were stable upon isolation and resedimentation in a second sucrose gradient (data not shown). Purified recombinant factor VIII protein that was added to CHO cell extract and size-fractionated under the same conditions was detected in fractions 6 and 7 (Figure 2A). These results support that the heterogeneous sedimentation of intracellular factor VIII was specific and not due to factor VIII binding to cellular protein complexes. After a 4 h chase time point, 70% of the factor VIII disappeared from the cell extract due to secretion as well as intracellular degradation (Figure 2D) (*24*). At this 4 h time point, most of the high molecular weight factor VIII aggregates disappeared from the cell extract and the majority of factor VIII was detected in fractions 6 and 7, similar to intact functional factor VIII.

A portion of factor VIII in the ER is degraded by the cytosolic 26S proteasomal pathway (*24*). The high molecular weight aggregates observed shortly after synthesis could represent a dead-end pathway for factor VIII that is retained and subsequently degraded, or alternatively a productive folding intermediate. To help distinguish between these two possibilities, lactacystin, a specific inhibitor of the 26S proteasome, was used to prevent factor VIII degradation. Quantification of factor VIII immunoprecipitated from the sucrose gradient fractions demonstrated that after a 4 h chase in the presence of lactacystin, 60% of the factor VIII primary translation product was still retained within the cell. Therefore, a portion (approximately 30%) of the newly synthesized factor VIII was targeted for degradation (Figure 2E). The remaining 40% was recovered in the medium (Figure 2F). The sedimentation profile for factor VIII produced in the presence of lactacystin was very similar to that in the absence

of lactacystin, except the former had approximately twice the amount of labeled factor VIII (Figure 2, compare panels D and E). Compared to the 30 min chase time point, the factor VIII in both 4 h chase time point samples shifted from higher molecular weight forms to lower molecular weight forms sedimenting in fractions 4−7. However, lactacystin treatment did not increase the amount of factor VIII secreted into the medium (Figure 2F), indicating that products destined for degradation follow a distinctly separate pathway from secretion. Our assay cannot distinguish whether the high molecular weight forms of factor VIII disaggregated to form lower molecular weight complexes that are retained and eventually degraded in the cell or to yield factor VIII that is eventually secreted. The results demonstrate that a portion of factor VIII is transiently aggregated in high molecular weight complexes shortly after synthesis; however, factor VIII is targeted to proteasomal degradation regardless of the aggregation state. The high molecular weight factor VIII aggregates disappear over the time and do not preferentially accumulate after inhibition of proteasomal degradation, indicating that proteasome function is not required for their disaggregation.

Protein chaperones recognize aggregated proteins and are proposed to facilitate their disaggregation and subsequent refolding or degradation. Factor VIII was previously detected in a complex with the molecular chaperone calnexin, with maximal interaction occurring 30 min after synthesis (*24*). To address whether this chaperone may play a role in disaggregating the factor VIII complexes, the association of calnexin with factor VIII was studied. Cells were pulse-labeled followed by a 30 min chase, and extracts were prepared for immunoprecipitation with anti-calnexin antibody. These complexes were then released from calnexin by incubation with Triton X-100 and analyzed by sucrose gradient centrifugation (*14*). Fractions were immunoprecipitated with anti-factor VIII antibody and analyzed by SDS−PAGE. The results demonstrated that calnexin binds to the entire spectrum of factor VIII species and does not preferentially associate with the high molecular weight aggregates (Figure 2B,C). Similar observations were made for MHC class II proteins (*14*, *34*).

ATP Is Required for Disaggregation of Factor VIII Complexes. To study the ATP requirement for factor VIII folding, cells were pulse-labeled in complete medium and then chase was performed in ATP-depletion medium (glucose-free medium containing 2-deoxy D-glucose and sodium azide). This treatment rapidly and reversibly depletes intracellular ATP levels (*30*). Cells were harvested at the end of the 2 h chase time point and analyzed by sucrose gradient fractionation. Upon depletion of intracellular ATP, factor VIII aggregates accumulated (Figure 3A, upper gel). Under these conditions factor VIII was not secreted (data not shown) as previously described (*25*). To test whether the aggregated factor VIII protein could be rescued by addition of metabolic energy, cells were pulse-labeled and chased for 2 h in ATP-depletion medium as above and then incubated 4 h in regular medium to regenerate ATP. About 50% of factor VIII was rescued from the aggregated complexes (Figure 3A, lower gel). Significantly, disappearance of the high molecular weight aggregates correlated with the appearance of low molecular weight forms and/or factor VIII monomeric protein in fractions 6 and 7 and with secretion of factor VIII into

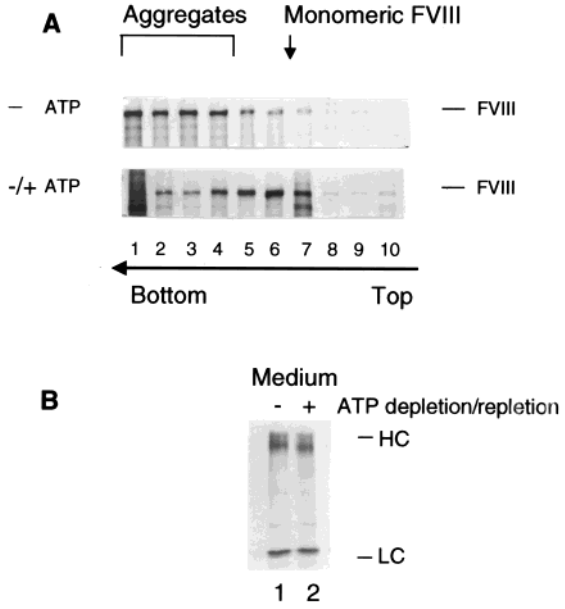

FIGURE 3: ATP is required for factor VIII folding, disaggregation, and refolding. CHO cells that express factor VIII (10A1*)* were pulse-labeled for 20 min with [^{35}S]methionine and chased for 2 h with ATP-depletion medium (glucose-free DMEM with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide) (panel A, upper gel). A duplicate plate of metabolically labeled and 2 h ATP-depleted cells was given fresh complete medium and incubated for an additional 4 h at 37 °C (panel A, −/+ ATP, lower gel). Cell extracts were prepared for sucrose gradient fractionation (panel A, lower gel). Factor VIII was immunoprecipitated from the fractions and analyzed by SDS−PAGE and autoradiography. The conditioned medium from control untreated (lane 1) and ATP-depleted and then ATP-repleted (lane 2) cells was collected, immunoprecipitated with the anti-heavy chain factor VIII antibody, and analyzed by SDS−PAGE (panel B). The heavy chain (HC) and light chain (LC) of factor VIII are indicated.

the conditioned medium (Figure 3B, lane 2). The same amount of factor VIII was secreted from cells that were not ATP-depleted (Figure 3B, lane 1) as from cells that were ATP-depleted and then ATP-repleted (Figure 3B, lane 2). SDS−PAGE analysis in the presence and absence of reducing agents demonstrated that the factor VIII aggregates generated in the absence of ATP did not contain aberrant interchain disulfide bonds (data not shown). These results support that insufficient intracellular levels of ATP may be one cause for factor VIII aggregation within the ER.

vWF Coaggregates with Factor VIII. Can polypeptides synthesized from different polysomes aggregate together? Von Willebrand factor (vWF) is synthesized as a 300 kDa precursor glycoprotein that binds and stabilizes factor VIII in the circulation. Previous studies demonstrated that vWF coexpression with factor VIII in CHO cells improves the recovery of factor VIII secreted into the medium (*26*). Within the coexpressing cells, the two glycoproteins significantly differed in their transit through the secretory pathway. In contrast to factor VIII, vWF did not significantly interact with BiP and was efficiently secreted in a manner that did not require high levels of intracellular ATP (*25*, *35*). However, vWF and factor VIII complexes were not detected within the cell. This was attributed to a requirement for vWF propolypeptide cleavage for interaction with factor VIII and for sulfation at Tyr1680 in factor VIII for high-affinity interaction with vWF (*36*, *37*). Both of these posttranslational modifications occur late in the secretory pathway and are

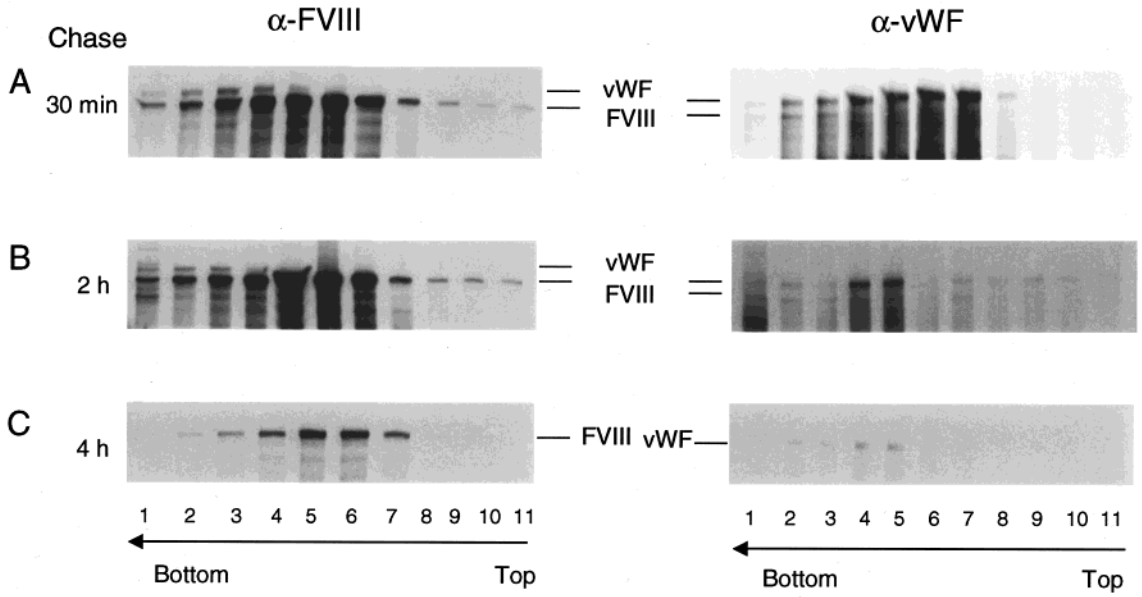

FIGURE 4: A portion of vWF can coaggregate with factor VIII. CHO cells (10A1C6) that express factor VIII and vWF were pulse-labeled for 20 min with [^{35}S]methionine and chased for 30 min (panel A), 2 h (panel B), or 4 h (panel C) with medium containing excess unlabeled methionine. Cells extracts were prepared and fractionated on sucrose density gradients. Fractions were immunoprecipitated either with anti-factor VIII antibody (left panels) or with anti-vWF antibody (right panels) and analyzed by SDS−PAGE and autoradiography. Migration of factor VIII and vWF are indicated.

not detected on the intracellular forms of factor VIII and vWF that accumulate early in the secretory pathway.

To elucidate whether vWF can coaggregate with factor VIII, cells that coexpress factor VIII and vWF were pulse-labeled. After chase in unlabeled medium for increasing periods of time, cell extracts were prepared and analyzed by sucrose gradient fractionation and immunoprecipitation with either anti-factor VIII or anti-vWF antibodies. After a 30 min chase, factor VIII was recovered throughout the sucrose gradient and a fraction of the newly synthesized factor VIII was detected in aggregated forms as previously described (Figure 4A, left). Analysis of vWF immunoprecipitated from the same fractions demonstrated the majority of vWF did not form aggregates, although a small portion of vWF was present in the high molecular weight fractions of the gradient (Figure 4A, right). Although the majority of the newly synthesized vWF did not coimmunoprecipitate with factor VIII, a small portion of vWF was detected in the high molecular weight factor VIII aggregates (Figure 4A, left, fractions 1−5). Significantly, vWF was not detected in the factor VIII immunoprecipitate from the slower sedimenting fractions (fractions 6 and 7), although these fractions contained the majority of vWF. This demonstrates specificity for the coaggregates of factor VIII and vWF detected in fractions 1−5. In addition, a portion of factor VIII was detected in the vWF immunoprecipitation that migrated at the bottom of the sucrose gradient (Figure 4A, right). After a 2 h chase the sucrose gradient profile for factor VIII remained unchanged, indicating that little factor VIII was chased out of the cell (Figure 4B, left). However, less vWF was detected in association with the aggregated forms of factor VIII, particularly in fractions 3−5 (Figure 4B, left). Immunoprecipitation of vWF detected only a small amount of vWF in the cell after the 2 h chase (Figure 4B, right). After the 4 h chase time point approximately 30% of the initial factor VIII was retained in the cell in fractions 4−7. These results demonstrate the ability for a portion of vWF to aggregate with factor VIII within the cell. Although factor VIII and vWF coaggregate, vWF was chased out at a greater rate than factor VIII. Therefore, these two proteins have different requirements for disaggregation.

Induction of Factor VIII Synthesis Increases Aggregate Formation. Previously, a CHO cell line was derived in which, upon addition of sodium butyrate, the transcription of the amplified transfected factor VIII gene is induced. In this system, sodium butyrate increases the synthesis of factor VIII mRNA and protein, although the majority of the induced factor VIII protein is not secreted from the cell and is detected in a complex with BiP (*27*). The effect of increased factor VIII synthesis on its aggregation was studied by metabolic labeling after a 24 h treatment with sodium butyrate. Analysis of protein synthesis in the total cell extract of pulse-labeled cells demonstrated a 4-fold increase in factor VIII synthesis upon sodium butyrate treatment (Figure 5A, lanes 1 and 2). Sucrose gradient fractionation of cell lysates prepared from pulse-labeled and 30 min chased cells demonstrated the induced factor VIII was detected primarily in aggregated forms (Figure 5B). Similar sodium butyrate treatment and analysis of CHO cells expressing factor VIII constitutively, i.e., not inducible by sodium butyrate, demonstrated that sodium butyrate itself did not induce factor VIII aggregation. Rather, aggregation resulted from the increased factor VIII expression. Reduction of lysate from the sodium butyrate-treated cells with dithiothreitol prior to fractionation did not alter the sedimentation of the factor VIII aggregates (Figure 5C). These results support that these aggregates formed as a result of increased factor VIII synthesis are not interchain disulfide-linked.

A Region within the A1 Domain Predisposes Factor VIII to Aggregation. Coagulation factor V is a glycoprotein that is structurally and functionally homologous to factor VIII. Previous studies demonstrated that factor V does not interact with BiP or calnexin and is efficiently secreted (*24*, *38*). In contrast to factor VIII, factor V secretion did not require

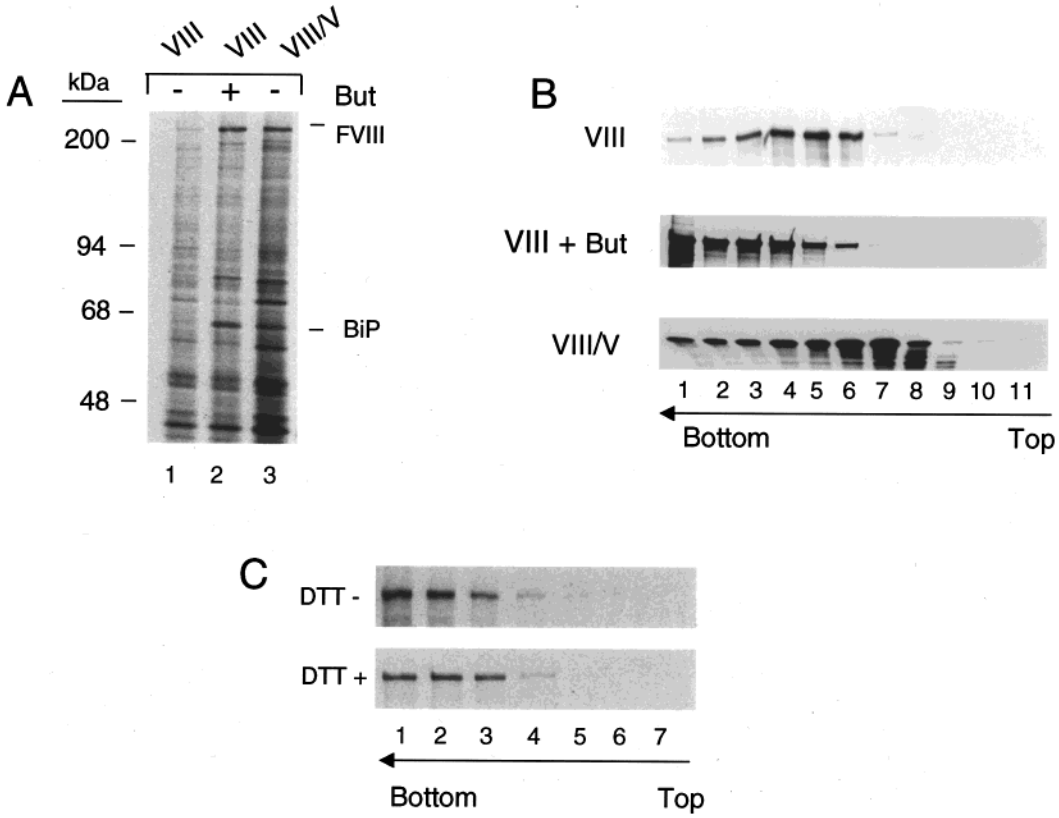

FIGURE 5: Induction of factor VIII synthesis enhances aggregation. CHO cells that inducibly express factor VIII (clone H9) (VIII) were either treated (+But) or not treated (−But) with 5 mM sodium butyrate for 24 h. Then cells were pulse-labeled with [^{35}S]methionine and chased in medium containing excess unlabeled methionine for 30 min. In parallel, a CHO cell line expressing a factor VIII(227−336)/factor V chimera (*46*) was labeled without sodium butyrate treatment (VIII/V). Cell extracts were prepared and aliquots were directly analyzed by SDS−PAGE under reducing conditions and autoradiography (panel A). The remainder of the lysate was fractionated by sucrose gradient centrifugation. Factor VIII was immunoprecipitated from each fraction and analyzed by SDS−PAGE under reducing conditions (panel B). Cell extracts from H9 cells treated with sodium butyrate were incubated in the absence (DTT−) or presence (DTT+) of 10 mM DTT for 2 h prior to fractionation on a sucrose gradient. Similar results were obtained with 100 mM DTT treatment (data not shown). Factor VIII was immunoprecipitated from the bottom 7 fractions (from 11 total) and the immunoprecipitates were analyzed by SDS−PAGE under reducing conditions (panel C).

high levels of intracellular ATP. Analysis of chimeric factor VIII and factor V proteins identified a 110 amino acid region within the A1 domain of factor VIII, amino acid residues 227−336, that when exchanged for homologous sequences in factor V increased its secretion efficiency. The secretion of the chimeric factor VIII and factor V protein displayed a lower requirement for ATP (*39*). This protein was expressed at a 4-fold greater level than wild-type factor VIII (Figure 5A, lanes 1 and 3). Upon induction of wild-type factor VIII synthesis with sodium butyrate, their levels of synthesis were similar (Figure 5A, lanes 2 and 3). The aggregation status of these two proteins was compared by sucrose gradient fractionation. The results demonstrated a significant difference in the amount of aggregated factor VIII. Where the majority of wild-type factor VIII migrated in fractions 1−4, the majority of VIII/V migrated in fractions 5−8 (Figure 5B; compare VIII + But with VIII/V). Replacement of the 110 amino acid region within factor VIII with homologous sequences from factor V significantly reduced aggregation status at a similar level of expression.

DISCUSSION

Defining the energy requirements for protein folding within the oxidizing environment of the ER remains a long-standing objective in cell biology. Most frequently, the rate-limiting step for protein secretion is transport from the ER to the Golgi compartment (*40*). This rate is dependent upon the time required for proteins to attain their final folded 3-dimensional structure. Recent studies have identified specific mechanisms that mediate retention of unfolded proteins within the ER. One mechanism employs the specific interaction with ER-localized protein chaperones such as BiP, the glucose-regulated protein of 94 kDa (GRP94), calnexin, and calreticulin, thereby preventing their export. It has also been suggested that protein aggregation may provide a specific retention role (*3*−*5*, *9*, *14*, *16*). Proteins that are incompletely or improperly folded would expose hydrophobic patches that should predispose them to aggregate and thereby prevent their recruitment into COPII coated vesicles. Dissecting the factors that influence protein aggregation and disaggregation and that facilitate protein folding within the ER has been difficult because the aggregates previously characterized contained interchain disulfide bonds (*4*−*6*, *9*−*14*). For example, upon depletion of intracellular ATP levels, protein misfolding occurs that results in the formation of interchain disulfide-linked aggregates of influenza hemagglutinin (*30*). Under these conditions, it is not known whether ATP is required to facilitate disassembly of disulfide-linked aggregates or whether it provides another function to promote protein disaggregation. Although ATP is not

known to be directly involved in the process of disulfide bond formation, it may be required to maintain the oxidizing potential of the ER, a necessity for disulfide bond exchange and proper protein folding.

It was surprising that the factor VIII aggregates characterized here did not contain interchain disulfide bonds, despite the presence of 23 cysteine residues within the factor VIII polypeptide. The lack of interchain disulfide bonds suggests that the physical interactions that stabilize the factor VIII aggregates are less complex and distinctively different from those previously detected. However, our assay cannot distinguish whether new and/or different intrachain disulfide bonds are present in the aggregated protein. In addition, we do not know if a single type of aggregate or multiple types of aggregates exist. In this study we have identified factors that influence factor VIII aggregation and disaggregation within the lumen of the ER. The state of factor VIII aggregation was influenced by N-linked glycosylation, the level of factor VIII synthesis, the primary sequence of factor VIII, and intracellular levels of ATP. Aggregation following inhibition of N-linked glycosylation is likely due to exposure of hydrophobic patches within the polypeptide that reduces its solubility. These nonglycosylated aggregates did not contain interchain disulfide bonds and did not dissociate over the time course of our experiments (6 h), consistent with the irreversible glycosylation block imposed by tunicamycin. An increase in the factor VIII expression level also increased aggregate formation, indicating a correlation between aggregation level and protein concentration in the ER. Inhibition of the cytosolic 26S proteasomal degradation pathway did not significantly affect either the aggregation or disaggregation of factor VIII, supporting that this degradative pathway is not required to form or remove the aggregates. A portion of factor VIII was detected in an aggregated state immediately after its synthesis and subsequently disappeared. Depletion of intracellular ATP stimulated the accumulation of nondisulfide cross-linked aggregates that were for the most part disassociated and secreted once metabolic energy was made available. These findings support the hypothesis that protein disaggregation and refolding requires energy and aggregate formation can be a reversible process to yield secretion-competent protein.

These results support that an ATP-dependent step is required for disaggregation of non-disulfide-linked aggregates within the ER and implicate ATPases in this function. Several ATPases that are localized to the ER are BiP, GRP94, and GRP170 (*41*−*43*). In addition, *Saccharomyces cerevisiae* contains a cytosolic heat shock protein, Hsp104, that is known to disaggregate irreversibly aggregated protein complexes in an ATP-dependent manner (*44*). Factor VIII was previously detected in association with BiP, calnexin, and calreticulin (*23*, *24*). Of these chaperones, only BiP is known to exhibit ATPase activity. Therefore, it is attractive to propose that BiP may provide this ATPase function. Indeed, upon modest depletion of intracellular ATP levels, factor VIII was trapped in a stable complex with the protein chaperone BiP within the ER and was not secreted (*25*). These findings suggested that factor VIII displays a unique requirement for ATP secretion, possibly a consequence of an ATP-dependent release from BiP. We have now shown that, under conditions of ATP depletion, factor VIII forms non-disulfide-linked aggregates. Therefore, we suggest that the rate-limiting and ATP-requiring step in factor VIII secretion is the dissociation of factor VIII aggregates that may involve the ATPase activity of BiP.

Previously, a region within the A1 domain of factor VIII, from amino acid residues 227 to 336, was identified that reduced the secretion efficiency of factor VIII and increased its ATP requirement for secretion. A chimeric protein that contained factor V homologous sequences for residues 227−336 in factor VIII was secreted severalfold more efficiently. In addition, the secretion of this chimeric protein was more resistant to ATP depletion than wild-type factor VIII (*39*). Our results now show that this chimeric factor VIII/factor V protein is more soluble than wild-type factor VIII at a similar level of expression in CHO cells. This region contains a β-sheet (residues 298−309) where seven residues are Leu or Phe. Because peptides enriched in Leu or Phe were previously shown to bind BiP with high affinity (*45*), it was proposed that this β-sheet may be a primary BiP-binding site (*46*). However, on the basis of the results of this study, it is possible that this hydrophobic region predisposes factor VIII to aggregation and as a consequence increases its ATP dependence for secretion. This hydrophobic pocket may only indirectly affect BiP interaction.

One important question is where do the factor VIII aggregates go? Unfolded proteins within the ER are degraded by a process of retrotranslocation through the Sec61 channel to the cytosol for targeting to the cytosolic 26S proteasome (*47*, *48*). However, before retrotranslocation, aggregated proteins require disaggregation in order to fit into the translocon channel (40−60 Å). Inhibition of proteasomal degradation by means of the specific proteasome inhibitor lactacystin did not result in accumulation of the high molecular weight aggregated forms of factor VIII within the cell. In contrast, the majority of the aggregates disappeared under these conditions. This indicates that the active proteasome is not required for removal of the aggregates and suggests that these transiently formed aggregates may be rescued for secretion.

vWF is a large glycoprotein that binds factor VIII through noncovalent interactions in the plasma and prevents its degradation. Patients with severe von Willebrand disease also have a factor VIII deficiency. The factor VIII deficiency may be attributed to the absence of the stabilizing influence of vWF on factor VIII in these patients. It was also shown that vWF can promote release of factor VIII from the cell surface and thereby promote its stable accumulation in the conditioned medium of cultured cells (*26*). This provides an alternate mechanism for vWF to increase plasma levels of factor VIII. To express factor VIII at high levels for therapeutic use in hemophilia A, cell lines were derived that coexpress vWF with factor VIII. Surprisingly, upon coexpression of vWF, the secretion efficiency and accumulation of factor VIII in the medium increased by approximately 10-fold compared to the level obtained by supplying excess vWF to the culture medium (*26*). We hypothesize that coexpressed vWF may in some manner facilitate factor VIII transport through the secretory pathway. Our studies now show that a portion of vWF coaggregates with factor VIII and that the vWF was chased out of the coaggregates, while the majority of factor VIII remained. These results suggest that the presence of vWF may destabilize aggregates that form and may as a consequence improve factor VIII

secretion. Alternatively vWF may compete with another ER resident protein directly involved in factor VIII aggregate formation. Although factor VIII is primarily synthesized in hepatocytes, and vWF is primarily synthesized in endothelial cells and megakaryocytes, it is possible that factor VIII and vWF are coexpressed in some, yet unknown cell type in vivo, where this mechanism may be important. The possibility that vWF and factor VIII coexpression occurs in vivo was previously discussed (*49*). Further studies are required to test the validity of this hypothesis.

Our studies have identified an ATP requirement to minimize factor VIII protein aggregation and/or promote its folding within the ER. The ATP expenditure for transient reversible aggregation of a protein within the ER may provide a mechanism to reduce the concentration of soluble protein in the ER, to promote its productive folding. Evidence does support that ATP exists in the ER, although its concentration in that compartment is unknown. An ADP/ATP transporter exists in the ER membrane (*50*). When firefly luciferase expression was directed into the lumen of the ER, upon addition of luciferin, light was emitted (*51*). This decarboxylation reaction of luciferin requires ATP. It remains to be tested whether an increase in the ER intralumenal concentration of ATP would increase the protein folding efficiency and secretion for proteins, such as factor VIII, that may be limited by transient aggregate formation.

REFERENCES

1. Anfinsen, C. B. (1973) *Science 181*, 223−230.
2. Bergman, L. W., and Kuehl, W. M. (1979) *J. Biol. Chem. 254*, 8869−8876.
3. Hurtley, S. N., Bole, D. G., Hoover-Litty, A., Helenius, A. and Copeland, C. S. (1989) *J. Cell Biol. 108*, 2117−2126.
4. Kim, P. S., Bole, D., and Arvan, P. (1992) *J. Cell Biol. 18*, 541−549.
5. Marquardt, T. and Helenius, A. (1992) *J. Cell Biol. 117*, 505−513.
6. Haynes, R. L., Zheng, T. and Nicchitta, C. V. (1997) *J. Biol. Chem. 272*, 17126−17133.
7. Redick, S. D. and Schwarzbauer, J. E. (1995) *J. Cell Sci. 108*, 1761−1769.
8. Hurtley, S. M. and Helenius, A. (1989) *Annu. Rev. Cell Biol. 5*, 277−307.
9. Kellokumpu, S., Suokas, M., Risteli, L. and Myllyla, R. (1997) *J. Biol. Chem. 272*, 2770−2777.
10. Maddox, B. K., Keene, D. R., Sakai, L. Y., Charbonneau, N. L., Morris, N. P., Ridgway, C. C., Boswell, B. A., Sussman, M. D., Horton, W. A., Bachinger, H. P. and Hecht, J. T. (1997) *J. Biol. Chem. 272*, 30993−30997.
11. Ishii, S., Kase, R., Okumiya, T., Sakuraba, H. and Suzuki, Y. (1996) *Biochem. Biophys. Res. Commun. 220*, 812−815.
12. Mirazimi, A. and Svensson, L. (1998) *J. Virol. 72*, 3887−3892.
13. Kim, P. S., Kim, K. R. and Arvan, P. (1993) *Am. J. Physiol. 265*, C704−C711.
14. Marks, M. S., Germain, R. N. and Bonifacino, J. S. (1995) *J. Biol. Chem. 270*, 10475−10481.
15. Bonnerot, C., Marks, M. S., Cosson, P., Robertson, E. J., Bikoff, E. K., Germain, R. N. and Bonifacino, J. S. (1994) *EMBO J. 13*, 934−944.
16. Cotner, T. and Pious, D. (1995) *J. Biol. Chem. 270*, 2379−2386.
17. Hebert, D. N., Foellmer, B. and Helenius, A. (1996) *EMBO J. 15*, 2961−2968.
18. Zhang, J. X., Braakman, I., Matlack, K. E. and Helenius, A. (1997) *Mol. Biol. Cell 8*, 1943−1954.
19. Vehar, G. A., Keyt, B., Eaton, D., Rodriguez, H., O'Brien, D. P., Rotblat, F., Oppermann, H., Keck, R., Wood, W. I., Harkins, R. N., Tuddenham, E. G. D., Lawn, R. M. and Capon, D. J. (1984) *Nature 312*, 337−342.
20. Toole, J. J., Knopf, J. L., Wozney, J. M., Sultzman, L. A., Buecker, J. L., Pittman, D. D., Kaufman, R. J., Brown, E., Shoemaker, C. and Orr, E. C. (1984) *Nature 312*, 342−347.
21. Sadler, J. E. (1991) *J. Biol. Chem. 266*, 22777−22780.
22. Kaufman, R. J., Pipe, S. W., Tagliavacca, L. and Swaroop, M. (1997) *Blood Coagul. Fibrin 8*, S3-S14.
23. Dorner, A. J., Bole, D. G. and Kaufman, R. J. (1987) *J. Cell Biol. 105*, 2665−2674.
24. Pipe, S. W., Morris, J. A., Shah, J. and Kaufman, R. J. (1998) *J. Biol. Chem. 273*, 8537−8544.
25. Dorner, A. J., Wasley, L. C. and Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7429−7432.
26. Kaufman, R. J., Davies, M. V., Pathak, V. K. and Hershey, J. W. (1989) *Mol. Cell. Biol. 9*, 946−958.
27. Dorner, A. J., Wasley, L. C. and Kaufman, R. J. (1989) *J. Biol. Chem. 264*, 20602−20607.
28. Pittman, D. D. and Kaufman, R. J. 1993) *Methods Enzymol. 222*, 236−260.
29. Dorner, A. J. and Kaufman, R. J. (1990) *Methods Enzymol. 185*, 577−596.
30. Braakman, I., Helenius, J. and Helenius, A. (1992) *Nature 356*, 260−262.
31. Pittman, D. D., Millenssen, M., Bauer, K., Kaufman, R. J. and Marquette, K. (1992) *Blood 79*, 389−397.
32. Martin, R. J. and Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372−1379.
33. Proctor, R. R. and Rapaport, S. I. (1961) *Am. J. Clin. Pathol. 36*, 212−213.
34. Anderson, K. S. and Cresswell, P. (1994) *EMBO J. 13*, 675−682.
35. Kaufman, R. J., Wasley, L. C., Davies, M. V., Wise, R. J. and Israel, D. I. (1989) *Mol. Cell. Biol. 9*, 1233−1242.
36. Bendetowicz, A. V., Morris, J. A., Wise, R. J., Gilbert, G. E., and Kaufman, R. J. (1998) *Blood 92*, 529−538.
37. Michnick, D. A., Pittman, D. D., Wise, R. J. and Kaufman, R. J. (1994) *J. Biol. Chem. 269*, 20095−20102.
38. Pittman, D. D., Tomkinson, K. N. and Kaufman, R. J. (1994) *J. Biol. Chem. 269*, 17329−17337.
39. Marquette, K. A., Pittman, D. D. and Kaufman, R. J. (1995) *J. Biol. Chem. 270*, 10297−10303.
40. Lodish, H. F., Hong, N., Snider, M. and Strous, G. J. (1983) *Nature 304*, 80−83.
41. Munro, S. and Pelham, H. R. B. (1986) *Cell 46*, 291−300.
42. Mazzarella, R. A. and Green, M. (1987) *J. Biol. Chem. 262*, 8875−8883.
43. Chen, X., Easton, D., Oh, H. J., Lee-Yoon, D. S., Liu, X. and Subjeck, J. (1996) *FEBS Lett. 380*, 68−72.
44. Glover, J. R. and Lindquist, S. (1998) *Cell 94*, 73−82.
45. Blond-Elguindi, S., Cwirla, S. E., Dower, W. J., Lipshutz, R. J., Sprang, S. R., Sambrook, J. F. and Gething, M. J. (1993) *Cell 75*, 717−728.
46. Swaroop, M., Moussalli, M., Pipe, S. W. and Kaufman, R. J. (1997) *J. Biol. Chem. 272*, 24121−24124.
47. Pilon, M., Schekman, R. and Romisch, K. (1997) *EMBO J. 16*, 4540−4548.
48. Hiller, M. M., Finger, A., Schweiger, M. and Wolf, D. H. (1996) *Science 273*, 1725−1728.
49. Rosenberg, J. B., Foster, P. A., Kaufman, R. J., Vokac, E. A., Moussalli, M., Kroner, P. A. and Montgomery, R. R. (1998) *J. Clin. Invest. 101*, 613−624.
50. Hirschberg, C. B., Robbins, P. W. and Abeijon, C. (1998) *Annu. Rev. Biochem. 67*, 49−69.
51. Dorner, A. J. and Kaufman, R. J. (1994) *Biologicals 22*, 103−112.

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