Mannose-dependent Endoplasmic Reticulum (ER)-Golgi Intermediate Compartment-53-mediated ER to Golgi Trafficking of Coagulation Factors V and VIII*

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The endoplasmic reticulum-Golgi intermediate compartment (ERGIC) is the site of segregation of secretory proteins for anterograde transport, via packaging into COPII-coated transport vesicles. ERGIC-53 is a homohexameric transmembrane lectin localized to the ERGIC that exhibits mannose-selective properties in vitro. Null mutations in ERGIC-53 were recently shown to be responsible for the autosomal recessive bleeding disorder, combined deficiency of coagulation factors V and VIII. We have studied the effect of defective ER to Golgi cycling by ERGIC-53 on the secretion of factors V and VIII. The secretion efficiency of factor V and factor VIII was studied in a tetracycline-inducible HEK293 cell line overexpressing a wild-type ERGIC-53 or a cytosolic tail mutant of ERGIC-53 (KKAA) that is unable to exit the ER due to mutation of two COOH-terminal phenylalanine residues to alanines. The results show that efficient trafficking of factors V and VIII requires a functional ERGIC-53 (14–17). In this report we show that efficient trafficking of ERGIC-53-dependent trafficking is dependent on post-translational modification of a specific cluster of asparagine (N)-linked oligosaccharides to a fully glucose-trimmed, mannose9 structure.

The endoplasmic reticulum-Golgi intermediate compartment (ERGIC) is the site of segregation of secretory proteins for anterograde transport, via packaging into COPII-coated transport vesicles (1–3). However, specific chaperones for the recruitment or concentration of secretory proteins into COPII-coated vesicles have not yet been identified. Although ERGIC-53 was identified in 1988 and is a well defined marker of the ERGIC, its function within the secretory pathway remains an enigma. ERGIC-53 is proposed to operate as a transport receptor for glycoproteins in the early secretory pathway, targeting them to COPII-coated vesicles budding from the ER for trafficking to the Golgi compartment (3–6). Initial reports examining the role of ERGIC-53 suggested that most glycoproteins did not require a functional form of ERGIC-53 for efficient secretion (7). Patients with combined deficiency of coagulation factors V and VIII have between 5 and 30% of normal plasma levels (by antigen and activity). The majority of patients with this autosomal recessive genetic bleeding disorder have null mutations in ERGIC-53. Although this suggests a factor V and factor VIII secretion defect, it is also possible that ERGIC-53 deficiency affects the production and/or stability of factors V and VIII (8).

Coagulation factors V and VIII are plasma glycoproteins of approximately 330 and 280 kDa, respectively, that function as essential cofactors in the blood coagulation cascade. They are both synthesized as single chain polypeptides having the identical domain structure of A1-A2-B-A3-C1-C2 (Fig. 1). Although their respective A and C domains exhibit 40% amino acid sequence identity and provide important structural and functional roles, the B domains have extensively diverged, are dispensable for their activity, and serve an unidentified function (9, 10). B domain-deleted (BDD) forms of factor VIII achieve higher mRNA levels and improved expression into cells in vitro and into plasma in vivo and are utilized in a number of gene therapy strategies for hemophilia A (11). In addition, BDD factor VIII has recently been approved for protein replacement therapy for hemophilia A (12). The B domains are encoded by single large exons and contain 25 and 18 N-linked oligosaccharide (GlcNAc2Man9Glc3) attachment sites for factor V and factor VIII, respectively. Within the early secretory pathway, these asparaginyl (N)-linked oligosaccharides undergo glucose trimming via sequential action of ER glucosidases I and II, and the GlcNAc2Man3Glc3 structures mediate the interactions of factors V and VIII with the ER lectin chaperones calnexin and/or calreticulin (13).

We hypothesized the existence of a specialized pathway for the efficient secretion of a specific subset of heavily glycosylated proteins within higher eukaryotes. The post-translational processing and presentation of the mannose residues of their N-linked oligosaccharides may facilitate the interaction of this subset of glycoproteins with the mannose-binding ERGIC-53 (14–17). In this report we show that efficient trafficking of factors V and VIII is impaired when ERGIC-53 is prevented from cycling between the ER and the Golgi. This impairment requires the B domains of these coagulation factors. In addition, ERGIC-53-dependent trafficking is dependent on post-translational modification of the N-linked oligosaccharides to fully glucose-trimmed, mannose9 structures.

EXPERIMENTAL PROCEDURES
Materials—Rabbit anti-FV polyclonal antibody was purchased from The Binding Site (Birmingham, United Kingdom). Anti-heavy chain factor VIII monoclonal antibody (F-8) conjugated to CL-4B-Sepharose
was a gift from Debra Pittman (Genetics Institute Inc., Cambridge, MA). Castanospermine and deoxymannojirimycin were purchased from Sigma. Fetal bovine serum, alpha modified Eagle’s medium (αMEM), methionine-free MEM, and OptiMEM were purchased from Life Technologies, Inc. Soybean trypsin inhibitor, phenylmethylsulfonylfluoride, and aprotinin were purchased from Roche Molecular Biochemicals (Germany). [35S]methionine (>1000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. En3Hance was purchased from Dupont (Boston, MA).

Cell Culture—HeLa1 cell lines (HeLa) stably expressing a cDNA encoding either wild-type (WT) ERGIC-53 (KKFF) or cytosolic tail mutant ERGIC-53 (KKAA) under control of the tTA promoter were described previously (7). Cells were maintained in 2 μg/ml tetracycline, 500 ng/ml puromycin, and 400 μg/ml geneticin (Life Technologies, Inc.). Cells were transiently transfected with the mammalian expression vector pMT2 (18) containing cDNA’s encoding full-length factors V and VIII and B domain deleted forms. The lipofection method was used according to the manufacturer’s recommendations (LipofectAMINE PLUS, Life Technologies, Inc.). Cells were maintained in the presence of tetracycline throughout the transfection and metabolic labeling unless indicated (+ induction), in which case tetracycline was removed at the time of transfection. For drug treatments with castanospermine (CST) (1 μM) or deoxymannojirimycin (DML) (1 μM), cells were treated for 1 h prior to metabolic labeling, and drug exposure was maintained throughout pulse and chase conditions.

Metabolic Labeling and Immunoprecipitation—Protein synthesis and secretion were analyzed by metabolically labeling cells at 48 h post-transfection for 30 min with [35S]methionine (250 μCi/ml in methionine-free medium), followed by a chase for 3 h in medium containing 100-fold excess unlabeled methionine and 0.02% aprotinin. Cell extracts and cell media were harvested and immunoprecipitations were performed and analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions as described previously (19). Relative radiographic band intensities were determined utilizing NIH Image software (public domain).

RESULTS

Impaired Cycling of ERGIC-53 between the ER and Golgi Reduces Secretion of Coagulation Factors V and VIII—The recycling of ERGIC-53 within the ER is controlled by targeting epitopes at its cytosolic COOH terminus—a dilysine ER retrieval signal followed by a diphenylalanine ER exit determinant (KKFF) (4, 20–23). Previously, in a tetracycline-repressible system, HeLa cells were described that overexpress either WT or a mutant of ERGIC-53 that is unable to exit the ER due to mutation of the diphenylalanine epitope to alanines (KKAA) (7). ERGIC-53 forms homo-hexamers, thus the mutant form of ERGIC-53 should oligomerize with the endogenous ERGIC-53, thus acting as a dominant negative to trap the majority of the ERGIC-53 within the ER (Fig. 2a). Previous immunofluorescence microscopic and density gradient analysis suggested that both the endogenous and mutant ERGIC-53 were quantitatively retained in the ER under inducible conditions (7). Similarly, transfer of the KKAA C-terminal motif onto other proteins also resulted in their permanent ER retention (24).

We transfected expression vectors encoding full-length factors V and VIII into these HeLa cell lines and examined their efficiency of secretion using metabolic pulse-chase labeling with [35S]methionine. Upon removal of tetracycline, an approximately 10-fold induction in the expression of the recombinant ERGIC-53 (both WT and mutant forms) was observed (Fig. 2b), as described previously (7). Factor VIII and factor V were expressed and secreted into the conditioned medium from both the WT and mutant ERGIC-53 cells under tetracycline repression. However, upon induction of the recombinant forms of ERGIC-53, secretion from the WT cells was not significantly affected, demonstrating that the amount of ERGIC-53 expression was not limiting for efficient factors V and VIII secretion. In contrast, upon induction of the mutant ERGIC-53, there was 3-fold reduced secretion of factor V and 5-fold reduced secretion of factor VIII. Time course studies demonstrated that the impaired secretion was maximal at the earliest time points (1 h) and less significant with longer chase times (5 h) suggesting a kinetic effect (data not shown). This is consistent with the finding that ER to Golgi trafficking of the lysosomal enzyme procathepsin C was delayed in this cell system (7). Thus, efficient secretion of factors V and VIII requires ERGIC-53 exit out of the ER. We hypothesize that in this cell system, bulk flow transport through a default, non-ERGIC-53-dependent pathway allows the ultimate secretion of factors V and VIII (25).

The B Domains Within Factors V and VIII Mediate ERGIC-53-dependent Trafficking—We next examined the role of the B domains within factors V and VIII in mediating ERGIC-53-dependent transport (Fig. 3). BDD forms of factors V and VIII were transfected into the HeLa cell lines under identical conditions and their transport examined. Neither BDD factor V nor BDD factor VIII secretion were affected by induction of either WT or mutant forms of ERGIC-53. The minor variability in molecular weight observed for BDD factor V is likely because of differences in glycosylation between the two HeLa cell clones. Taken together, these observations demonstrate that the early secretory pathway is not generally disrupted under conditions of WT and mutant ERGIC-53 overexpression. In addition, it suggests that the clustering of N-linked oligosaccharide structures within the respective B domains of factors V and VIII mediates their interaction with ERGIC-53.

ERGIC-53-dependent Trafficking Is Regulated by Processing of N-linked Oligosaccharides—The role of post-translational modifications of the N-linked oligosaccharides in mediating interaction with ERGIC-53 was studied by inhibiting either
factor V secretion by 3-fold (Fig. 4; lanes 1, 2, 5, and 6). However, when FV was synthesized in the presence of CST, there was a 2-fold increase in the amount of FV rescued to secretion into the cell medium upon induction of the mutant ERGIC-53 (Fig. 4; lanes 5 and 6). Concomitantly, there was less factor V protein retained within the cell extract. The slower mobility within the cell extract of the factor V synthesized in the presence of CST is consistent with the retention of the 3 glucose residues on the multiple N-linked oligosaccharides. We hypothesize a requirement for ERGIC-53 to recognize the proper presentation of the mannose residues on the N-linked oligosaccharides. The factor V polypeptides with the GlcNAc2Man9Glc3 oligosaccharide structures may escape retention by the mutant ERGIC-53 and are instead secreted by a default pathway.

Finally, we tested whether ERGIC-53 recognized a specific mannose structure for efficient interaction (Fig. 4). DMJ inhibits the action of glucosidase I and II, thereby maintaining the GlcNAc2Man9Glc3 structure of the N-linked oligosaccharides. Expression of full-length factor V was examined in the HeLa cells both in the presence and absence of CST. As observed in Fig. 3, induction of WT ERGIC-53 had no effect on the secretion of factor V, whereas induction of the mutant ERGIC-53 reduced factor V secretion by 3-fold (Fig. 4; lanes 1, 2, 5, and 6). The impaired secretion of factor V (3-fold reduction) upon induction of mutant ERGIC-53 correlated with increased retention of factor V within the cell extract (Fig. 4; lanes 5 and 6). However, when FV was synthesized in the presence of CST, there was a 2-fold increase in the amount of FV rescued to secretion into the cell medium upon induction of the mutant ERGIC-53 (Fig. 4; lane 7). Concomitantly, there was less factor V protein retained within the cell extract. The slower mobility within the cell extract of the factor V synthesized in the presence of CST is consistent with the retention of the 3 glucose residues on the multiple N-linked oligosaccharides. We hypothesize a requirement for ERGIC-53 to recognize the proper presentation of the mannose residues on the N-linked oligosaccharides. The factor V polypeptides with the GlcNAc2Man9Glc3 oligosaccharide structures may escape retention by the mutant ERGIC-53 and are instead secreted by a default pathway.

Finally, we tested whether ERGIC-53 recognized a specific mannose structure for efficient interaction (Fig. 4). DMJ inhibits the action of ER mannosidases 1 and 2, thereby inhibiting the removal of the outermost mannose residues of the middle and outer nonglucosylated branches of the GlcNAcMan9Glc3 structure. DMJ had no effect on secretion of factor V in cells that express WT ERGIC-53 (Fig. 4; lane 4). In contrast, secretion of factor V, upon induction of mutant ERGIC-53, was inhibited 7-fold following incubation with DMJ, and this was accompanied by increased retention within the cell extract (Fig. 4; lane 8). This result suggests that the GlcNAcMan9Glc3 oligosaccharide structure is the preferential substrate for interaction and the inability to trim the outer mannose residues leads to sustained retention of the glycoprotein by the mutant ERGIC-53.

Co-immunoprecipitation of ERGIC-53/factor V or ERGIC-53/factor VIII, in the presence or absence of membrane-permeable glucosidase or mannosidase activities (Fig. 4). CST inhibits the action of glucosidases I and II, thereby maintaining the GlcNAc2Man9Glc3 structure of the N-linked oligosaccharides. Expression of full-length factor V was examined in the HeLa cells both in the presence and absence of CST. As observed in Fig. 3, induction of WT ERGIC-53 had no effect on the secretion of factor V, whereas induction of the mutant ERGIC-53 reduced factor V secretion by 3-fold (Fig. 4; lanes 1, 2, 5, and 6). The impaired secretion of factor V (3-fold reduction) upon induction of mutant ERGIC-53 correlated with increased retention of factor V within the cell extract (Fig. 4; lanes 5 and 6). However, when FV was synthesized in the presence of CST, there was a 2-fold increase in the amount of FV rescued to secretion into the cell medium upon induction of the mutant ERGIC-53 (Fig. 4; lane 7). Concomitantly, there was less factor V protein retained within the cell extract. The slower mobility within the cell extract of the factor V synthesized in the presence of CST is consistent with the retention of the 3 glucose residues on the multiple N-linked oligosaccharides. We hypothesize a requirement for ERGIC-53 to recognize the proper presentation of the mannose residues on the N-linked oligosaccharides. The factor V polypeptides with the GlcNAc2Man9Glc3 oligosaccharide structures may escape retention by the mutant ERGIC-53 and are instead secreted by a default pathway.

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ERGIC-53-mediated ER to Golgi Transport

DISCUSSION

These results demonstrate the ability of ERGIC-53 to influence the secretion efficiency of coagulation factors V and VIII, providing mechanistic insight into the molecular defect recently recognized in patients with null expression of ERGIC-53. These data also suggest a model for ERGIC-53 in recruiting factors V and VIII into budding COPII-coated vesicles through the recognition of specific N-linked oligosaccharide structures (Fig. 5). Our results suggest that it is the fully glucose-trimmed mannose9 oligosaccharide core structures, primarily clustered within the B domains of FV and FVIII that are recognized. The de- and re-glucosylation of N-linked oligosaccharide cores, mediated by the sequential actions of glucosidase II and UDP-glucose:glucosyltransferase, occurs during nascent folding of glycoproteins, facilitating retention within the ER prior to achieving the native folded structure (26, 27). Trimming of mannose residues by ER mannosidase I has also been proposed as a mechanism for retention of misfolded nascent glycoproteins. For example, mutant variants of the soluble secretory protein alpha1-antitrypsin are retained within the ER bound to calnexin following mannose trimming of the GlcNAc3Man9Glc1 oligosaccharide core (28). The GlcNAc3Man9Glc1 structure does not undergo further glucose trimming but is rather targeted for degradation by ER-associated degradation machinery. In this manner, it has been proposed that ER mannosidase I acts as a “clock” regulating the disposal of those glycoproteins that are mutant or fold inefficiently (28). Only properly folded glycoproteins, following terminal glucose trimming, are in turn able to begin trafficking to the Golgi. The investigation of the trafficking of factors V and VIII provides insight into the latter phase cross-linking reagents, could not be demonstrated in extracts of cells expressing factor V or factor VIII, including Chinese hamster ovary cells, COS-1 monkey kidney cells, and HeLa cells (data not shown). In vitro pull-down assays using immunoprecipitated ERGIC-53 incubated directly with factor V or factor VIII conditioned medium also failed to demonstrate a direct interaction. These results suggest that this is a low affinity interaction, potentially consistent with a lectin-like function of ERGIC-53. Alternatively, this interaction may be mediated by one or more additional adapter proteins.

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of this ER quality control system, highlighting the importance of the terminal mannose residues. By recognizing the fully glucose-trimmed mannose9 oligosaccharide cores of factors V and VIII, ERGIC-53 can recruit only properly folded native proteins into the secretory vesicles of the ERGIC for trafficking to the Golgi. Further mannose trimming by Golgi mannosidase may ensure unidirectionality for ERGIC-53 chaperone function.

Recent analysis of additional families with combined deficiency of factors V and VIII has identified a significant subset of patients (~26%) that express normal levels of ERGIC-53 antigen and appear to have a genetic defect corresponding to another genetic locus (29). Thus, at least one additional protein appears to be required for the efficient secretion of FV and FVIII. We hypothesize that this gene product functions either upstream or downstream of ERGIC-53 in facilitating the efficient secretion of a limited subset of glycoproteins. Glucosidase II would be a potential upstream candidate, as an inability to remove the terminal glucose residue from the core oligosaccharide structures would impair the proper presentation of the mannose residues and reduce the efficiency of recruitment into secretion vesicles. It is also possible that another gene product functions in a similar manner as ERGIC-53. There is precedence for similar ERGIC chaperones in yeast (30–32). Erv14p (ER-vesicle protein of 14 kDa) is an integral ER membrane protein, localized to the ER and packaged into COPII-coated vesicles (33, 34), Axl2p, a glycoprotein with 16 potential N-linked oligosaccharides, required for an axial budding pattern. Thus Erv14p, localized to the ER and packaged into COPII-coated vesicles, may also function in recruiting a limited set of glycoproteins into these vesicles to facilitate their efficient trafficking. Vesicular-Integral membrane Protein of 36 kDa, or VIP-36, is another ERGIC-53 homologue isolated from MDCK cells (33, 34), which was recently shown to recognize high-mannose type glycans (35) although the specific role for its lectin binding characteristics in sorting within the trans-Golgi network is still not known.

The results reported here suggest that the B domains of factors V and VIII, and potentially similar heavily glycosylated structures, may provide quality control by mediating the retention of misfolded protein variants within the ER and efficient secretion of the properly folded native forms. Our results demonstrate that a functional ERGIC-53 cycling pathway is required to increase the secretion efficiency of factors V and VIII. ERGIC-53 homologues have been identified in yeast (36) and have been conserved from Caenorhabditis elegans to Xenopus, rat and man (37). Therefore, ERGIC-53 appeared in evolution prior to the blood coagulation system and may represent a general transport system to increase the efficiency of ER to Golgi transport for a variety of glycoproteins. Coagulation factors V and VIII have uniquely evolved to usurp this transport system. The existence of ERGIC-53 to facilitate selective glycoprotein transport provides the first example of a chaperone that mediates ER to Golgi transport in higher eukaryotic cells.

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