PROTEIN BIOSYNTHESIS '99
From the ER to the Golgi: Insights from the Study of Combined Factors V and VIII Deficiency

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Once believed to represent a default pathway, the mobilization of cargo from the endoplasmic reticulum (ER) is now well established as a selective event that employs exit signals to sort and concentrate cargo into vesicular carriers (Bannykh et al. 1998). These vesicular carriers and their cargo proteins continue through the secretory pathway to the Golgi apparatus via a network of vesicular-tubular clusters, which are discontinuous with the ER but interconnected with one another. This network has also been referred to as "the ER-Golgi intermediate compartment" (ERGIC) or the "intermediate compartment" (IC). Although several yeast mutants with selective-protein–transport defects have been defined, the recent identification of *ERGIC-53* as the gene responsible for combined deficiency of coagulation factors V and VIII is the first evidence for the existence of a cargo-specific pathway in higher organisms. Here, we summarize the process of vesicle formation and discuss the role of the ERGIC in anterograde and retrograde transport between the ER and the Golgi apparatus. We also review current information about genetic defects in this pathway and their role in human genetic disease.

Exit from the ER

Two types of coat proteins on vesicles are known to participate in the transport between the ER and the Golgi complex: coat proteins (COP) I (Harter 1995; Schekman and Orci 1996; Gaynor and Emr 1997; Gaynor et al. 1998) and COPII (Schekman and Orci 1996; Barlowe 1998; Matsuoka et al. 1998). COPII acts earlier in the pathway, apparently driving the initial budding of vesicles from the ER. COPII consists of the Sar1p GTPase and a pair of protein complexes, Sec23p-Sec24p and Sec13p-Sec31p (the latter protein names reflect the fact that the corresponding genes were first identified in yeast defective in the secretory pathway). Mammalian homologues of Sec23p and Sec13p localize to the ER and to vesicles that derive from it. Because vesicle budding can be reconstituted from mammalian ER membranes, by use of these purified components, and because COPII vesicles can fuse with Golgi membranes, it was concluded that COPII transports cargo from the ER to the Golgi complex.

COPII-coated–vesicle formation begins when Sar1p GTPase is recruited to an active zone of the ER membrane, through a guanine-nucleotide–exchange reaction catalyzed by the integral membrane glycoprotein Sec12p. The membrane-bound activated Sar1p then recruits the Sec23p-24p complex, after which the Sec13p-31p complex binds to the membrane and forms vesicles. This formation of a vesicle by COPII defines the first compartmental boundary of the secretory pathway. Little is known about how vesicle components are recognized and packaged, but it appears that there is considerable specificity in these interactions. These components include "cargo" proteins—soluble or transmembrane proteins that are destined for the cell surface or various intracellular compartments—as well as various intrinsic vesicle proteins that mediate the delivery of the cargo and the recycling of membrane to the ER. In the latter category are the v-SNARE (vesicle-soluble NSF attachment-protein receptor) proteins (Springer and Schekman 1998). v-SNAREs are membrane-bound proteins that interact with their cognate target-SNARE (t-SNARE) proteins in acceptor organelles, such as the Golgi apparatus or the plasma membrane. The specific interaction between v- and t-SNARES ensures that vesicles fuse only with the appropriate organelle.

The selection of cargo within a budding vesicle is dependent on sorting signals—usually discrete peptide domains of 4–25 residues—that are displayed on secretory proteins (Rothman and Wieland 1996). A number of different transport signals have been identified, including some that target proteins for retrieval from the Golgi to the ER (KDEL, KKXX, and XXRR) and others that

Received April 2, 1999; accepted for publication April 19, 1999; electronically published May 5, 1999.

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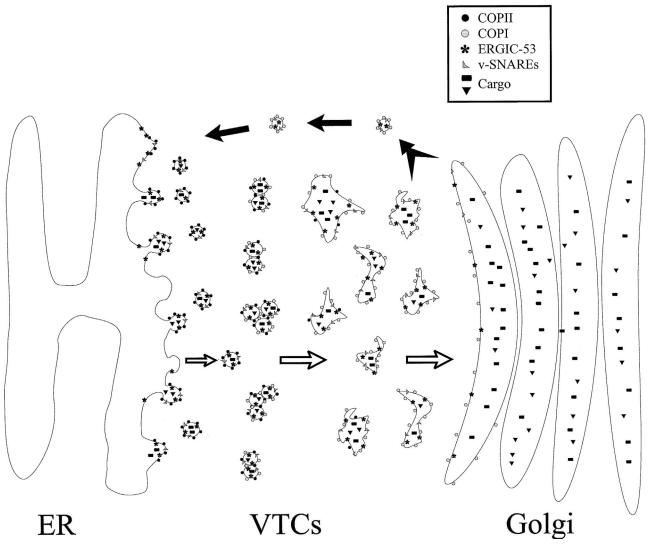

Figure 1 ER to Golgi transport. This drawing depicts events involved in both anterograde and retrograde trafficking, between the ER and the Golgi. COPII-coated vesicles, containing v-SNARES, ERGIC-53, and cargo proteins, bud off from the ER (unblackened arrows). The COPII vesicles shed their COPII coats, which are replaced by COPI coats. Vesicles then fuse to form components of the ERGIC, and ERGIC clusters move in a microtubule-dependent manner toward the Golgi and either fuse, to form the cis-Golgi, or transfer their cargo via COPI-coated vesicles. v-SNARES, ERGIC-53, and other proteins are recycled back to the ER in COPI-coated vesicles, which bud from both the ERGIC and Golgi apparatus (blackened arrows).

signal transport from the Golgi to endosomes or lysosomes (propeptide, mannose 6-phosphate, and tyrosine-rich dileucine motifs). Such transport signals may interact directly or indirectly with COP proteins during packaging (Campbell and Schekman 1997; Aridor et al. 1998; Bannykh and Balch 1998; Kuehn et al. 1998).

Recent studies suggest that the fidelity of cargo recruitment into vesicles may be regulated by a group of transmembrane proteins, the products of the p24 gene family (Stamnes et al. 1995; Fiedler et al. 1996). Yeast and human cells express $\geqslant$16 p24-homologues, some of which carry a dilysine retrograde transport signal. Members of the p24 family may function as links between cargo proteins and the vesicular coat. Emp24p, a member of the p24 gene family from yeast, is a component of the COPII-coated, ER-derived transport vesicles (Schimmöller et al. 1995). A strain of yeast lacking Emp24p fails to transport certain cargo glycoproteins, such as the soluble secreted protein invertase and the plasma-membrane protein Gas1p. Because Emp24-negative cells secrete α-factor, carboxypeptidase Y, or acid phosphatase with normal kinetics, Schimmöller and colleagues provided the first evidence for cargo-protein–specific defects in loading or transporting COPII vesicles.

Transit to the Golgi Apparatus

In the most widely accepted model, once budding of COPII-coated vesicles has occurred the COPII coat is shed, and the vesicles fuse to form the ERGIC (see fig. 1; Presley et al., 1997; Scales et al., 1997; Bannykh et al. 1998; Bannykh and Balch 1998). It is at this point that the other coat-protein complex, COPI, becomes associated with new budding vesicles (Harter 1995; Schekman and Orci 1996; Gaynor and Emr 1997; Gaynor et al. 1998). When COPI was first found, in preparations of in vitro–derived mammalian Golgi vesicles, it was believed to mediate anterograde transport of cargo through the Golgi stack. The COPI complex, consisting of the seven COPs (α, β, β', γ, δ, ε, and ζ), self-assembles in the cytoplasm and binds to membranes en bloc. It has been proposed that GTP-bound ADP-ribosylation factor (ARF), followed by the heptameric COPI complex bind to ERGIC membranes and initiate the budding of COPI-coated vesicles. Genetic analyses on yeast have also identified all of the COPI subunits and ARF; each of these genes is essential for viability except for *SEC28,* which encodes e-COP.

The relative contributions of COPI to anterograde and retrograde transport have been the subject of significant debate in recent years (Harter 1995; Gaynor and Emr 1997; Schekman and Mellman 1997; Gaynor et al. 1998). COPI clearly plays a critical role in Golgi-to-ER retrograde transport (reviewed by Schekman and Orci 1996; Gaynor and Emr 1997; Gaynor et al. 1998), as indicated by both genetic and biochemical experiments. A dilysine (KKXX) motif that permits type I–membrane proteins to be retrieved to the ER (Jackson et al. 1990)

was found to bind specifically to COPI proteins in vitro. Furthermore, a screen for yeast mutants defective for KKXX-mediated retrieval (*ret* mutants) identified three COPI subunits (α, δ, ζ) as products of *RET* genes. All COPI mutants, including some that are designated as "Sec mutants" because they were first identified as a result of their defects in anterograde transport, are lacking in KKXX-mediated retrieval.

Although the direct role of COPI in retrograde (Golgi to ER) transport is widely accepted, the same cannot be said for its role in anterograde (ER to Golgi) transport. Several lines of evidence support a role for COPI in anterograde transport (reviewed by Harter 1995; Gaynor and Emr 1997; Gaynor et al. 1998). In mammalian cells, inhibition of COPI function results in a block in ER-to-Golgi transport. In yeast, the COPI subunits β, β', and γ (products of *sec26, 27,* and *21*) were initially identified through the characterization of mutants defective in anterograde transport. Recently, Gaynor and Emr (1997) generated temperature-sensitive *sec21* mutants in which carboxypeptidase Y and α-factor accumulated in the ER, whereas other cargo proteins (HSP150 and invertase) were secreted normally or sorted properly to the vacuole. These observations demonstrate that COPI is not directly required for anterograde transport of all secreted proteins. Perhaps retrieval of limiting transport factors by COPI is required for the efficient packaging of a select subset of cargo proteins into anterograde ER-derived COPII vesicles.

Recent evidence suggests that COPII and COPI act sequentially in protein transport. Scales et al. (1997) visualized exocytic transport from the ER to the Golgi in living cells, using a chimera of the temperature-sensitive vesicular stomatitis virus glycoprotein and green-fluorescent protein (ts-G-GFP_{ct}). When shifted to permissive temperature, ts-G-GFP_{ct} concentrates into COPII-positive structures close to the ER. These structures build up, to form an intermediate compartment of tubular clusters that contain the KDEL receptor (which mediates the retrieval of resident ER proteins from the ERGIC), as well as the marker protein ERGIC-53. This compartment appears to be the site at which COPII proteins are replaced by COPI. COPI, but not COPII, remains associated with these tubular clusters as they move, in a microtubule-dependent manner, toward the Golgi complex, eventually fusing with each other to form the *cis*-Golgi network. On the basis of these results, Scales and colleagues argue that COPII vesicles form at defined ER-export sites and then fuse to form the ERGIC. The loss of COPII and its replacement with COPI allow vesicles derived from this compartment to interact with the microtubule cytoskeleton and to be transported to the Golgi apparatus. Their data are consistent with either a direct role for COPI in anterograde transport or with the model of Gaynor and Emr (1997), in which COPI mediates the retrieval of vesicles and thus is indirectly necessary for ongoing anterograde membrane traffic between the ER and the Golgi.

Insights from Combined Coagulation Factors V and VIII Deficiency

Coagulation factor V (FV) and factor VIII (FVIII) are both required for the efficient function of the blood coagulation system. Combined factors V and VIII deficiency is a distinct autosomal recessive disorder, first described by Oeri et al. (1954) (for review see Seligsohn 1989). Affected patients demonstrate a moderate bleeding tendency, in association with plasma levels of FV and FVIII (both antigen and activity) in the range of 5%–30% of normal. Since the original report, >89 patients belonging to $\geqslant$64 families have been described (Seligsohn 1989; Nichols et al. 1998, 1999; Neerman-Arbez et al. 1999). Approximately one-half of the families described thus far are of Mediterranean origin, including 15 Italian and nine Israeli families. This disorder appears to be particularly prevalent among Jews of Middle Eastern and Sephardic origin. Among Israeli individuals with those backgrounds the disease occurs in ~1:100,000 (Seligsohn 1989). Inheritance is autosomal recessive and is distinct from coinheritance of both FV deficiency (parahemophilia) and FVIII deficiency (hemophilia A). Because of the high incidence of consanguinity, affected individuals were assumed to be homozygous at a locus that in some manner uniquely regulates the expression of these two specific proteins.

FV is synthesized primarily in megakaryocytes and hepatocytes and is found in the plasma and α-granules of platelets as a 330-kDa single-chain polypeptide (for review of factor V and VIII biosynthesis see Kaufman 1998). FVIII is most likely synthesized in the hepatocyte and reticuloendothelial cells, although the major physiologic source for the in vivo expression of this clotting factor remains unknown. FVIII is proteolytically processed, on secretion from the cell, to form a heterodimer. Both factors circulate in plasma as inactive precursors that are activated through limited proteolysis and subsequently assemble with their substrates (prothrombin and factor X) and enzymes (factor Xa and factor IXa) on a negatively charged phospholipid surface. The natural anticoagulant protein, protein C, functions by proteolytically inactivating FV and FVIII, shutting off further thrombin generation.

FV and FVIII are homologous proteins with a conserved domain organization. The "A" domains of both proteins also demonstrate homology to the plasma copper-binding protein, ceruloplasmin. FV and FVIII undergo similar and extensive posttranslational modifications including formation of conserved disulfide bonds, addition of multiple asparagine- and serine/threonine-

linked oligosaccharides, and sulfation at several tyrosine residues. Despite similarities in structure and function, FV and FVIII differ markedly in plasma concentration, with the quantity of FV exceeding that of FVIII by ~40-fold. These differences appear to be partly because of a higher level of FV gene transcription, as well as greater FV mRNA stability and more efficient protein secretion. The considerably less-efficient secretion of FVIII, relative to that of FV, may be explained in part by FVIII interaction with BiP and other chaperones. BiP, a member of the 70-kDa heat-shock–protein family, exhibits a peptide-dependent ATPase activity. Its expression is induced by the presence of unfolded or unassembled protein subunits within the ER. FVIII release from BiP and transport out of the ER requires high levels of intracellular ATP. In contrast, FV does not associate with BiP and does not require high levels of ATP for secretion.

Two other chaperones, calnexin (CNX) and calreticulin (CRT) (Pipe et al. 1998), also differ in their interactions with the two clotting factors. CNX and CRT are endogenous ER-lectin molecules that bind to nascent glycoproteins. FVIII secretion depends on its interaction with both BiP and CNX. Upon translocation into the lumen of the ER, FVIII immediately binds BiP and is released after ATP hydrolysis. Misfolded FVIII molecules subsequently interact, through their N-linked oligosaccharides, with CNX and CRT (for a review of the ER quality control system see Ashkenas and Byers 1997; Riordan 1999 [in this issue]). Only fully folded FVIII escapes the chaperone-mediated retention mechanism and is finally transported to the Golgi apparatus and out of the cell. FV does not exhibit these extensive interactions with BiP and CNX during its transport through the ER.

ERGIC-53 and the Molecular Basis of Combined Factors V and VIII Deficiency

The molecular basis for combined FV/FVIII deficiency had been a puzzle since its first description in 1954 (for review see Seligsohn 1989). Chance coinheritance of classic hemophilia A (FVIII deficiency) and parahemophilia (FV deficiency) has been reported in four families. However, this explanation could not account for the majority of patients with combined deficiency, for whom the genetic pattern reflects simple autosomal recessive inheritance resulting from a defect in a single gene. As noted above, FV and FVIII are both proteolytically inactivated by activated protein C (APC). Thus, deficiency of protein-C inhibitor, leading to unopposed increased activity of APC, seemed a logical explanation for this disorder when first reported. However, subsequent studies documented normal levels of protein-C inhibitor in these patients and demonstrated that the initial observation of decreased levels was a result of laboratory artifact. The normal half life of exogenous coagulation factors administered to these patients argued against an accelerated clearance mechanism and suggested that this disorder might be due to a common defect in biosynthesis or secretion. However, this latter explanation was difficult to reconcile with the markedly different plasma levels and patterns of expression for these two proteins.

To determine the molecular basis of the disorder, we first localized the gene for combined FV/FVIII deficiency to the long arm of chromosome 18, in nine unrelated Jewish families of Sephardic and Middle Eastern origin, using a homozygosity mapping approach (Nichols et al. 1997). This localization was subsequently confirmed in an independent study of 19 families from Iran, Pakistan, and Algeria (Neerman-Arbez et al. 1997). Recent positional cloning efforts have shown that the disease gene encodes ERGIC-53, a component of the intermediate compartment. Two founder mutations, each predicted to completely inactivate ERGIC-53, accounted for the disease in all nine Israeli families (Nichols et al. 1998). Subsequently, an additional 54 families have been analyzed for ERGIC-53 mutations (Neerman-Arbez et al. 1999; Nichols et al. 1999), and a total of 17 different mutations have been identified in 37 of the 54 families. All but one of the mutations are either nonsense or frameshift alleles whose truncated protein products would be predicted to lack normal ERGIC-53 function. The only missense mutation substitutes a threonine for the initiator methionine. Surprisingly, no mutation could be identified in 17 (~30%) of 64 families analyzed to date. Biochemical and genetic data suggest that a significant subset of combined FV/FVIII deficiency results from mutations in at least one alternative locus.

Schweitzer and coworkers developed a panel of monoclonal antibodies to ERGIC membranes and showed that the ERGIC-53 epitope localizes to this intracellular structure (reviewed by Itin et al. 1996; Kappeler et al. 1997). ERGIC-53 is a type 1–transmembrane protein, which exist as homodimers and homohexamers. ERGIC-53 contains regions of homology to lectins from leguminous plants (Fiedler and Simons 1994) and it binds to glycoproteins in a calcium-dependent, mannose-selective manner (Arar et al. 1995; Itin et al. 1996). Its amino acid sequence contains determinants for anterograde transport and a dilysine ER-retrieval signal, that together are thought to result in constitutive recycling among the ER, ERGIC, and Golgi apparatus (Itin et al. 1996). The protein was identified independently as MR60, a mannose-specific membrane lectin isolated from human monocytes (Arar et al. 1995), and close homologues have been identified in rat (p58), *Xenopus laevis,* and *Caenorhabditis elegans* (GenBank Z81097). The yeast protein Emp47p, which shows significant ho-

mology to ERGIC-53 (Schröder et al. 1995), recycles between the ER and the Golgi apparatus but localizes predominantly to Golgi.

An antibody to the ERGIC-53 cytoplasmic tail blocks the transport of vesicular stomatitis virus glycoprotein, from ER to Golgi, and the recruitment of COPI to the intermediate compartment in permeabilized cells (Tisdale et al. 1997). Thus, ERGIC-53 may be required for the coupled exchange of COPII and COPI coats during segregation of anterograde and retrograde transported proteins. In addition, mutagenesis studies demonstrate that the cytoplasmic tail of ERGIC-53 interacts directly with the COPII coat component Sec23p, which may direct it to vesicles that bud from the ER (Kappeler et al. 1997). This interaction with ERGIC-53 may mark the Sec23p complex for transport, whereupon Sec23p-activated cargo may be concentrated in a patch of membrane by the interaction of the Sec23p complex with the Sec13p complex. Accumulation of such coat patches could deform the membrane, creating a bud and ultimately a transport vesicle. ERGIC-53 may be the first-known example of a cargo protein for which such a sorting mechanism into COPII vesicles occurs. These results suggested that ERGIC-53 is essential for the general transport of glycoproteins through the secretory pathway. However, the limited phenotype of combined FV/FVIII deficiency indicates that ERGIC-53 is only required for the efficient secretion of a specific subset of glycoproteins (Nichols et al. 1998). To date, no other abnormalities have been identified in patients with combined FV/FVIII deficiency, but it seems likely that defects in at least a limited subset of other proteins may also be present.

Vollenweider and colleagues studied a recombinant mutant ERGIC-53, in which AlaAla had replaced the di-phenylalanine motif (Vollenweider et al. 1998). This mutant failed to recycle and it accumulated in the ER, where it exerted a dominant-negative effect on the endogenous normal ERGIC-53. A selective delay in secretion of the lysosomal protein cathepsin C was observed, consistent with the proposed role of ERGIC-53 as a specific sorting receptor. Measurement of cathepsin C levels in patients has not yet been reported. However, the absence of any other apparent disease phenotype in combined FV/FVIII deficiency patients, outside of the bleeding disorder, suggests that the defect in the targeting of cathepsin C or other similar proteins may be too subtle to produce a significant functional abnormality. In addition, the observation of decreased but significant residual FV and FVIII activity in these patients suggests that ERGIC-53 enhances the efficiency of a sorting event during the transport of these proteins, but it is not absolutely required for their secretion.

Conclusions

The unexpected identification of *ERGIC-53* as the gene responsible for combined FV/FVIII deficiency clearly establishes ERGIC-53 as a component of the ER-Golgi transport machinery that is required for the efficient export of coagulation factors V and VIII. This is the first demonstration of a cargo-specific pathway for protein export from the ER in mammalian cells. However, the apparently normal levels observed in these patients for most other plasma proteins demonstrate that ERGIC-53 is not essential for the integrity of the intermediate compartment or the more general process of protein export. The identification of the genetic defect in the subset of patients with FV/VIII deficiency but with intact ERGIC-53 may unmask additional critical components of this unique transport pathway.

Electronic-Database Information

Accession number and URL for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank/index.html (for *Caenorhabditis elegans* [Z81097])

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