The Recycling of ERGIC-53 in the Early Secretory Pathway

ERGIC-53 CARRIES A CYTOSOLIC ENDOPLASMIC RETICULUM-EXIT DETERMINANT INTERACTING WITH COPII

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Further investigation of the targeting of the intracellular membrane lectin endoplasmic reticulum (ER)-Golgi intermediate compartment-53 (ERGIC-53) by site-directed mutagenesis revealed that its luminal and transmembrane domains together confer ER retention. In addition we show that the cytoplasmic domain is required for exit from the ER indicating that ERGIC-53 carries an ER-exit determinant. Two phenylalanines at the C terminus are essential for ER-exit. Thus, ERGIC-53 contains determinants for ER retention as well as anterograde transport which, in conjunction with a dityrosine ER retrieval signal, control the continuous recycling of ERGIC-53 in the early secretory pathway. In vitro binding studies revealed a specific phenylalanine-dependent interaction between an ERGIC-53 cytosolic tail peptide and the COPII coat component Sec23p. These results suggest that the ER-exit of ERGIC-53 is mediated by direct interaction of its cytosolic tail with the Sec23p-Sec24p complex of COPII and that protein sorting at the level of the ER occurs by a mechanism similar to receptor-mediated endocytosis or Golgi to ER retrograde transport.

The early secretory pathway of higher eukaryotes includes the endoplasmic reticulum (ER),1 the ER-Golgi intermediate compartment (ERGIC), and the Golgi apparatus that are connected by vesicle-mediated anterograde and retrograde protein transport pathways (1-7). Vesicle budding is driven by the recruitment of cytosolic coat proteins to the donor membrane resulting in the formation of a coated vesicle. Two different cytosolic coat complexes, COPI and COPII, are involved in the formation of at least two distinct classes of transport vesicles (3). COPII mediates vesicle budding from the ER for anterograde protein transport (8-12), whereas COPI has been implicated in many different traffic pathways including exit from the ER (13, 14), ERGIC to Golgi (15), through the Golgi (16), early to late endosomes (17, 18), and Golgi to ER retrograde transport by direct interaction with dityrosine ER-retrieval signals (19-24).

It is currently unclear which transport pathways directly depend on COPI. Recent in vitro binding studies proposed a bimodal interaction of two COPI subcomplexes with different cytosolic tail sequences of proteins of the p24 family (25). The B-subcomplex (consisting of α-, β1-, and ε-COP) specifically binds to dityrosine ER-retrieval signals (19), whereas binding of the F-subcomplex (consisting of β2-, γ-, and ζ-COP) requires the presence of a critical phenylalanine that may be part of an anterograde transport signal (25). Based on these findings a conformational switch mechanism was proposed by which coatomer may differentiate between anterograde and retrograde transport.

There is growing evidence that exit from the ER is selective rather than by default (26) as indicated by the fact that properly folded secretory (27) and membrane (1, 28-30) proteins are concentrated at this step. In line with this notion, a diacidic signal in the cytoplasmic tail of vesicular stomatitis virus glycoprotein and other membrane proteins was recently shown to be required for selective export from the endoplasmic reticulum (31). It is envisaged now that sorting at the level of the endoplasmic reticulum is accomplished by sorting receptors that bind cargo proteins as well as cytosolic coat complexes (1). A candidate sorting receptor for a subset of proteins in yeast is Emp24p, a major membrane protein of ER-derived COPII vesicles (32). Emp24p belongs to a growing family of related 24-kDa proteins also found in mammalian cells (33-36), but the suggestion that they can transport cargo as cargo receptors remains to be proven.

The ERGIC marker protein ERGIC-53 (p58 in rat, Ref. 37) is another candidate cargo receptor (6). ERGIC-53 is a type I membrane protein (38), carries a dityrosine ER-retrieval signal in its cytoplasmic domain (39), and constitutively recycles between ER, ERGIC, and cis-Golgi (39-41). The protein exhibits properties of a mannoseselective and calcium-dependent lectin due to a lectin domain on its luminal side (42-44). It is conceivable therefore that ERGIC-53 binds newly synthesized glycoproteins in the ER and recruits them into budding vesicles. When the dityrosine signal is inactivated, intracellular transport of ERGIC-53 in COS-1 cells remains slow indicating that a second determinant can also function in intracellular targeting (45). The targeting efficiency of both signals is weakened by two C-terminal phenylalanines. It was suggested that the second targeting determinant is formed by the cytosolic sequence RSQAE adjacent to the transmembrane domain in conjunction with the luminal domain of ERGIC-53 and that it may be responsible for the concentration of ERGIC-53 in the intermediate compartment (45). Thus targeting of ERGIC-53 to the

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§ Supported by a grant from the Sir Jules Thorn Overseas Trust.
1 The abbreviations used are: ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; endo H, endo-β-N-acetyl-glucosaminidase H; mAb, monoclonal antibody.

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ER/ERGIC/cis-Golgi recycling pathway would be the result of retention and retrieval modulated by the C-terminal phenylalanines.

Here we provide evidence that the second determinant for intracellular targeting of ERGIC-53 is constituted by a combination of the luminal and transmembrane domains rather than by RSQSQ. Analysis of ERGIC-53 constructs stably expressed in Lec-1 cells (46) suggests that this second targeting determinant mediates retention of ERGIC-53 in the ER rather than the ERGIC. Most surprisingly, ER-er of ERGIC-53 was dependent on the two C-terminal phenylalanines indicating that the cytosolic tail of ERGIC-53 operates as an ER-exit determinant that is required for efficient transport of ERGIC-53 to the ERGIC. In vitro binding studies revealed a specific and phenylalanine-dependent interaction between the cytosolic tail of ERGIC-53 and Sec23p of the Sec23p complex of COPII. These results suggest that ERGIC-53 is sorted to COPII vesicles by direct interaction with COPII components in a mechanism similar to receptor-mediated endocytosis or dilysine signal-dependent protein retrieval.

EXPERIMENTAL PROCEDURES

Recombinant DNAs—Mutation of ERGIC-53 has been described (45). Briefly, polymerase chain reaction-based splicing and mutagenesis were used to introduce a c-myc epitope tag and an N-glycosylation site into ERGIC-53 cDNA. This construct, termed GM, was directly introduced as pECE expression vector, and additional polymerase chain reaction into ERGIC-53 cDNA. This construct, termed GM, was cloned into a Technolgies, Inc.). COS cells were transfected using the DEAE-dextran method. 15 μg of DNA per 35-mm dish was used. Lec-1 cells (Ref. 46; ATCC CRL 1729) were used at the final concentration of 30 μM methionine, harvested in ice-cold PBS, and subjected to immunoprecipitation with mAb G1/93 against human ERGIC-53 (47). Immunoprecipitates were released from the protein A-Sepharose beads and denatured by boiling for 3 min in 50 μl of endogenous digestion buffer (100 mM citrate, pH 5.5 (NaOH), 0.1% SDS, 0.8% β-mercaptoethanol, and protease inhibitors). After cooling down to room temperature additional 30 μl of digestion buffer were added, and the samples were incubated with 2.5 milliunits of endoglycosidase H (Boehringer Mannheim) for 20 h at 37 °C. Thereafter the samples were boiled in sample buffer and separated on 7–10% gradient SDS-polyacrylamide gels, and the proteins were visualized by fluorography using Kodak X-Omat AR films. Stably transfected Lec-1 cells were pulse-labeled with [35S]methionine, harvested in ice-cold PBS, and subjected to immunoprecipitation with mAb G1/93 against ERGIC-53 (47). Immunoprecipitates were released from the protein A-Sepharose beads and denatured by boiling for 3 min in 50 μl of endo-D digestion buffer (50 mM NaPO4, pH 6.5 (sodium citrate), 5 mM EDTA, 0.1% SDS, 2% Triton X-100, and protease inhibitors). After cooling down to room temperature an additional 35 μl of digestion buffer were added, and the samples were incubated with 2.5 milliunits of endoglycosidase D (Boehringer Mannheim) for 20 h at 37 °C. Thereafter the samples were boiled and subjected to SDS-PAGE.

Cellular Fractionation—The fractionation of Lec-1 cells was based on a procedure developed for HepG2 cells that will be described in detail elsewhere.2 Briefly, Lec-1 cells of four confluent 15-cm plates were homogenized in 3 ml of 120 mM NaCl, 5 mM KCl, 25 mM NaHCO3, pH 7.3 (containing protease inhibitors), by 10 passages through a ball-bearing homogenizer (58) with a clearance of 20 μm. After centrifugation at 750 rpm for 10 min in an SS-34 rotor (Sorvall), the resulting postnuclear supernatant was loaded on a 12-ml linear Nycodenz (Life Technologies, Basel) gradient, 13–29% (w/v) with a 1-ml 35% cushion; all solutions contained 10 mM triethanolamine, pH 7.4, 1 mM EDTA, and protease inhibitors. After centrifugation at 25,500 rpm for 3 h in a TST-28.17 rotor (Kontron), 14 fractions (1.2 ml each) were collected from the bottom of the tube. 80 μl of each fraction were subjected to SDS-PAGE (10%) and transferred to nitrocellulose. The distribution of the ERGIC-53 constructs, p63 (ER-marker), and mannosidase II (Golgi marker) was determined by Western blotting and enhanced chemiluminescence (Amersham Corp.). Films were quantified by densitometry. For immunoprecipitation 50–200 μl of a fraction were diluted with PBS to 3 ml and centrifuged at 100,000 × g for 1 h (TFT 65.13, Centricon) to remove the Nycodenz. The pellet was solubilized in 1% Triton X-100 and subjected to immunoprecipitation with mAb G1/93 against ERGIC-53.

In Vitro Binding Assay—1 mg of peptide (>95% purity, NeoSysterm, Strasbourg; crude peptide, Chiron, Australia) was reduced in 250 μl of 500 mM Tris/HCl, pH 8.0, 6 μ g guanidinium chloride, 25 mM dithiothreitol for 1 h at 37 °C. To remove the dithiothreitol the reduced peptides were loaded onto a PD10 column (Pharmacia Biotech Inc.) that had been equilibrated with coupling buffer (100 mM Tris/HCl, pH 7.5, 0.5 mM NaCl, 1 mM EDTA) and eluted with 3.5 ml of coupling buffer. This peptide solution was added to 250 μl of activated thiol Sepharose 4B beads (Pharmacia) for 1 h at room temperature followed by an overnight incubation at 4 °C. Prior to coupling the beads were washed once with degassed H2O and once with coupling buffer. After coupling the supernatant was removed, and free thiol groups were quenched by incubation in 1.5 ml of 100 mM NH4 acetate, pH 4.0, 0.5 mM NaCl, 9 mM β-mercaptoethanol for 1 h at room temperature. Peptide-coupled beads were washed twice with PBS, 0.1% bovine serum albumin, once with PBS, and stored in PBS, 0.01% sodium azide at 4 °C. Coupling efficiency was between 60 and 90% of reduced peptides, and around 1 μmol of peptide was coupled per ml wet beads, corresponding to the concentration of active thiol groups of 1 μmol per ml wet beads. For standard binding assays a 20,000 × g (20 min) supernatant of HepG2 lysate (50 mM Hepes, pH 7.3, 50 mM KCl, 2.5 mM magnesium acetate, 1% Triton X-100, protease inhibitors) was either preadsorbed to Sepharose 4B beads for 2 h at 4 °C or depleted by incubating with COPI beads by incubating for 2 h with CM1A10 mAb bound to protein A-Sepharose beads (50). The KCl concentration was increased upon COPI-depleted samples was adjusted to 90 mM. Binding with peptide-coupled beads was performed for 2 h at 4 °C. Beads were washed twice with binding buffer (50 mM Hepes, pH 7.3, 90 mM KCl, 2.5 mM magnesium acetate, 1% Triton X-100, protease inhibitors) and once with 50 mM Hepes, pH 7.3. In addition binding

2 M. Fougat, P. Kakpeker and H.-P. Hauri, manuscript in preparation.
The amino acid sequences of the cytosolic tails are indicated in the ERGIC-53 and constructs 3 and 4 the transmembrane domain of CD4. Ref. 45). Constructs 1, 2, and 5 have the transmembrane domain of a n dac—sylation site at position 61 of ERGIC-53 (Chinese hamster ovary, CHO) ceased for immunoblotting (enhanced chemiluminescence detection). From 10 or 20% of the cell lysate used for the corresponding binding and separated on 4–10% gradient SDS-polyacrylamide gels. For cell Bound proteins were eluted by boiling in electrophoresis sample buffer and washed twice with binding buffer and once with 50 mM Hepes, pH 7.3. Preadsorbed to Sepharose 4B beads for 1 h at 4° C. The preadsorbed cell lysates were digested with endo H, separated on a 7–10% gradient SDS-polyacrylamide gel, and visualized by fluorography. glycosylated denotes endo H-resistant form, and deglycosylated denotes endo H-sensitive form.

**RESULTS**

The Transmembrane and the Lumenal Domain Cooperate in Intracellular Retention of ERGIC-53—Our previous interpretation that the pentapeptide RSQQE in the cytosolic domain of ERGIC-53 constitutes the second targeting determination did not take into account that a combination of the luminal and transmembrane domains may be responsible for this retention instead (see “Discussion”). To solve the issue of the second targeting determinant, additional N-glycosylated and myc-tagged cytosolic tail mutants of ERGIC-53 were constructed and analyzed in transiently transfected COS-1 cells by monitoring endoglycosidase H resistance (Fig. 1). Endogenous ERGIC-53 recycles continuously between ER, ERGIC, and cis-Golgi but does not reach the medial Golgi (47, 59, 60). Because endoglycosidase H resistance is a medial-Golgi event, it can denote endo H-resistant form, and there is no indication of transport from ER to Golgi. It is noteworthy that all the constructs were analyzed after a chase of 1 h (Fig. 1, lane 1; Ref. 45). Unexpectedly, a new construct, T53Aα, in which RSQQE was replaced by alanines, also became only 15% endo H-resistant after a chase of 1 h (Fig. 1B, lane 2). These results indicate that the cytosolic sequence RSQQE is not involved in intracellular retention of ERGIC-53. To exclude the possibility that the retention is due to special features of a long alanine sequence, the alanines were replaced by serines in the construct T53Sα. This construct behaved identical to T53α (data not shown). Unlike T53α and T53A, constructs T4A and T4A, in which the transmembrane domain was replaced by that of CD4, had a fast transport rate as indicated by an endo H resistance of about 40% after a 1-h chase (Fig. 1B, lane 1 and 4). Assuming that CD4 is a neutral reporter (61) these results indicate that the lumenal and transmembrane domain of ERGIC-53 in combination influence the rate of transport from ER to Golgi.

In light of the new findings the role of the C-terminal phenylalanines must be reinterpreted. As shown in Fig. 1B, construct T53Aα was transported substantially faster than T53α, suggesting that the cytosolic sequence RSQQEAAAFF counteracts the luminal/transmembrane retention. These findings raise the possibility that this cytosolic sequence is part of an anterograde transport determinant and that the two C-terminal phenylalanines are required for the determinant to function.

*C-terminal Phenylalanines Are Required for Post-ER Localization of ERGIC-53*—Because the above experiments with COS cells cells lose mass of retention of ERGIC-53 from the ER/ERGIC/cis-Golgi recycling pathway, they do not provide information on the precise site where these targeting determinants operate within this pathway. To analyze the role of the two targeting determinants more precisely, two ERGIC-53 constructs (Fig. 2A) were stably expressed in Lec-1 Chinese hamster ovary cells (46) and their trafficking studied by immunofluorescence microscopy, pulse-chase experiments, and subcellular fractionation. Lec-1 cells are deficient in the Golgi enzyme GlcNAc-transferase, and as a consequence N-glycosylated proteins passing through the cis-Golgi become and remain endo-D-sensitive due to trimming by the cis-Golgi enzyme mannosidase I (62). The ERGIC-53 constructs carry an N-glycosylation site and a c-Myc epitope in their lumenal domain. Construct GM has the wild-type cytosolic tail of ERGIC-53. In construct GMAA the two C-terminal phenylalanines were changed to alanines and hence the putative anterograde transport determinant should be inactivated. A representative clone was selected for each construct, and all experiments were performed with these two clones. In pulse-chase experiments the two constructs formed homodimers and homohexamers with kinetics comparable to endogenous ERGIC-53 (data not shown) suggesting that they are correctly folded.

Immunofluorescence microscopy of Lec-1 cells expressing GM exhibited staining in the juxtanuclear area typical for the ERGIC and some reticular ER staining (Fig. 2B, a), whereas GMAA showed prominent staining of the nuclear membrane.
GM gave two peaks (Fig. 3A). A fraction of GM co-distributed with the ER marker reflecting the presence of GM in the ER, whereas the second peak included fractions 8–10 partially overlapping with mannosidase II. The second peak most likely represents intermediate compartment. On a similar gradient prepared with CHO cell membranes, it was previously shown that the intermediate compartment defined by p58, the rat homologue of ERGIC-53 (37), distributes to slightly denser fractions than Golgi membranes (63). In contrast, GMAA (Fig. 3C) entirely co-distributed with ER.

If GM indeed recycles between ER, ERGIC, and cis-Golgi its endo-D sensitivity should be comparable in ER and post-ER fractions at steady state. This was indeed the case, whereas GMAA isolated from the ER fraction as a control was completely endo-D-resistant (Fig. 4). These experiments indicate that GM can recycle via the cis-Golgi defined by mannosidase I while GMAA cannot.

**C-terminal Phenylalanines Are Required for ER Exit of ERGIC-53**—The lack of endo-D sensitivity of the GMAA construct either reflects permanent localization in the ER or efficient retrieval from the ER. To differentiate between these two possibilities we studied the localization of the constructs in AlF₄⁻-treated cells. AlF₄⁻, an activator of trimeric G proteins (65), blocks anterograde transport out of the ERGIC (66, 67). We have recently shown by immunofluorescence microscopy and Nycodenz gradient centrifugation that AlF₄⁻ reversibly blocks ERGIC-53 recycling in the ERGIC (2). Therefore, if GMAA rapidly recycles between ER and ERGIC, it should accumulate in the ERGIC in AlF₄⁻-treated cells very much like endogenous ERGIC-53. After 30 min AlF₄⁻ treatment GM indeed concentrated in a juxtanuclear area, and the reticular ER fluorescence disappeared (Fig. 2B, a and b). The concentration was reversible 60 min after washing out the AlF₄⁻, a dispersed ER pattern was apparent (Fig. 2B, c). In contrast, the distribution of the GMAA construct did not change after AlF₄⁻ treatment (Fig. 2B, d and e).

The effect of AlF₄⁻ was further analyzed by subcellular fractionation using Nycodenz density gradients (Fig. 3). AlF₄⁻ treatment of Lec-1 cells expressing GM or GMAA had no effect on the distribution of p63 or mannosidase II (Fig. 3, B and D). After exposure to AlF₄⁻ the amount of GM in post-ER fractions increased from 30 to 70% (Fig. 3B). In contrast, there was no concentration of GMAA in post-ER fractions by AlF₄⁻ (Fig. 3D). The results of the immunofluorescence and the Nycodenz gradient analyses are in full agreement and strongly argue that the mutant GMAA cannot leave the ER. The permanent retention of GMAA in the ER is most likely due to the second intracellular targeting determinant of ERGIC-53 formed by the luminal/transmembrane domain, because the other intracellular targeting determinant, the dilysine signal, functions in post-ER retrieval (61, 68). Furthermore, we conclude that the C-terminal phenylalanines are required for exit of ERGIC-53 from the ER. Therefore it is likely that they are part of an ER-exit determinant.

**Sec23p Binds to the Cytosolic Tail of ERGIC-53 in a Diphenylalanine-dependent Manner**—Previous in vitro studies showed that cytosolic targeting signals of transmembrane proteins can directly interact with cytosolic coat proteins involved in vesicle budding (19, 25, 69). Therefore we tested whether the cytosolic domain of ERGIC-53 can bind to COPI or COPII coats. Five peptides were synthesized for these experiments, all of which had an N-terminal cysteine used to couple the peptides to thiol-activated Sepharose beads, followed by two spacer serines (Fig. 5A). Peptide 1 corresponds to the wild-type cytosolic tail of ERGIC-53. In peptide 2 the last two phenylalanines are replaced by alanines, a mutation which destroys the ER-
exit determinant (Figs. 2 and 3). In peptide 3 both the dilysine signal and the putative ER-exit determinant are inactivated by changing the last three amino acids from KFF to SAA. In peptides 4 and 5 the dilysine signal was inactivated by replacing the two lysines with alanines, whereas the putative ER-exit determinant was still intact in peptide 4. Binding assays were performed with whole cell lysates under low salt conditions (90 mM KCl, 50 mM Hepes, 2.5 mM magnesium acetate), and bound proteins were separated by SDS-PAGE and immunoblotted with specific antibodies to known cytosolic coat proteins. COPII binding was revealed by blotting with antibodies against Sec23p (a component of the Sec23p-Sec24p subcomplex) and Sec13p (a component of the Sec13p-Sec31p subcomplex). Sec23p showed significant binding to peptide 1 but not peptides 2 and 3 in which the putative ER-exit determinant is nonfunctional (Fig. 5B, lanes 4–6; whole cell lysate, lane 1). In contrast, Sec13p did not significantly bind to any of the three peptides. COPI binding was assessed with antibodies against the coatomer subunits α- and β-COP. Expectedly, α- and β-COP showed a dilysine-dependent interaction; they bound to peptides 1 and 2 but not 3. Interestingly, considerably less coatomer bound to peptide 1 than peptide 2 even though both peptides carry a classical dilysine signal (Fig. 5B, lanes 4–6; whole cell lysate, lane 1). α-Adaptin, a component of plasma membrane-derived clathrin-coated vesicles, and γ-adaptin, a component of trans-Golgi network-derived clathrin-coated vesicles, were unable to bind to any of the three peptides. This excludes the possibility that just any cytosolic coat would bind to peptide 1 and further confirms the specificity of the Sec23p binding to this peptide. To test the possibility that the lack of Sec23p binding to peptide 2 was a result of competition by the strong coatomer binding to this peptide, binding assays were performed with coatomer-depleted whole cell lysate (Fig. 5B, lanes 4–6; whole cell lysate, lane 1). α-Adaptin, a component of plasma membrane-derived clathrin-coated vesicles, and γ-adaptin, a component of trans-Golgi network-derived clathrin-coated vesicles, were unable to bind to any of the three peptides. COPI binding was revealed by blotting with antibodies against Sec23p (a component of the Sec23p-Sec24p subcomplex) and Sec13p (a component of the Sec13p-Sec31p subcomplex). Sec23p showed significant binding to peptide 1 but not peptides 2 and 3 in which the putative ER-exit determinant is nonfunctional (Fig. 5B, lanes 4–6; whole cell lysate, lane 1). In contrast, Sec13p did not significantly bind to any of the three peptides. COPI binding was assessed with antibodies against the coatomer subunits α- and β-COP. 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lysate, lane 3

Fig. 5 shows that the Sec23p binding is not dependent on the dilysine signal but requires the two phenyalalanines. The findings are in line with the in vivo data from COS-1 cells where the two lysines were not required for efficient exit from the ER (Fig. 1). Sec13p showed no significant binding to peptide 4. Thus peptide 4 and 1 have the same specificity in respect to COPII binding.

In summary these in vitro binding experiments demonstrate that the cytosolic tail of ERGIC-53 specifically binds the COPII component Sec23p but not Sec13p. This binding did not require the dilysine signal but was dependent on the two C-terminal phenyalalanines required for the ER exit in vivo. Despite the presence of two phenyalalanines, the cytosolic tail of ERGIC-53 is unable to bind the F-subcomplex of COPI.

**DISCUSSION**

Our previous suggestion that RSQQE may confer intracellular retention was deduced from the combined observations that (a) inactivation of the dilysine signal by replacing the last 4 amino acids with alanines (T53A7) failed to abolish retention, (b) the luminal domain and the transmembrane domain alone were not sufficient to retain ERGIC-53/CD4 hybrids, and (c) the construct L53T4RSQQE3A9, lacking the dilysine signal and in which ERGIC-53’s transmembrane domain was replaced by that of CD4, was largely retained (45). In light of the findings with the new construct T53A10 which is inefficiently transported, we conclude that the dilysine-independent retention of ERGIC-53 is due to a combined action of the luminal and transmembrane domains rather than RSQQE. The fact that T4A displays rapid transport kinetics is further evidence against RSQQE acting as a retention signal. The previously reported retention of L53T4RSQQE3A9 remains unexplained but may be due to a cryptic retention motif recovered by the deletion of the four C-terminal amino acids.

Experiments with stably transfected Lec-1 cells strongly suggest that the site of retention provided by the luminal/transmembrane determinant is the ER. This conclusion is based on a comparison of the GM construct, having a wild-type tail, with the GMAA construct in which the two terminal phenyalalanines required for fast intracellular transport were replaced by alanines. Based on the lack of endo-D sensitivity it can be firmly concluded that GMAA is unable to reach the mannosidase I Golgi compartment. The finding that a cytosolic tail peptide terminating in KKAA is a better substrate than KKKF for coatomer binding in vitro raises the possibility that GMAA may leave the ER but would quantitatively be retrieved from the ERGIC and thereby not acquire endo-D sensitivity. The results of the AlF4 experiments, however, rule out such a pre-Golgi recycling route for GMAA. AlF4− blocks anterograde and retrograde transport in the ERGIC (66, 67). Unlike GM, GMAA was not concentrated in the ERGIC in the presence of AlF4− as assessed by immunofluorescence microscopy and Nycodenz gradient centrifugation. The permanent retention of GMAA in the ER is most likely due to the luminal/transmembrane retention determinant.

The finding of an ER-retention determinant in a dilysine signal-bearing membrane protein is not entirely unexpected. There is evidence that additional ER determinants exist that operate independently of the dilysine retrieval signal (71). For example, removal of the dilysine motif from the ER enzyme UDP-glucuronosyltransferase fails to abolish ER retention (68). It is well known that retention of membrane proteins in the Golgi apparatus is, at least in part, mediated by their transmembrane domain (72, 73). The length rather than the amino acid sequence was shown to be important for this localization (74). Likewise, membrane length is important for tar-
getting the C-terminally anchored protein cytochrome b₆ and the t-SNARE Ufe1p to the ER (75, 76), although in the latter case the presence of polar amino acids clustered on one side of a helical wheel were also important. Replacement of the transmembrane domain of ERGIC-53 by that of CD4 severely affects the intracellular retention of ERGIC-53 constructs. The transmembrane domain of CD4 has 21 amino acids, hence is 3 amino acids longer than that of ERGIC-53. Further experiments are required to determine if ER retention of ERGIC-53 simply depends on the length of its transmembrane domain or requires specific sequence information.

That the cytosolic sequence RSQQE₆₅FF can overcome the luminal/transmembrane retention may be due to two fundamentally different mechanisms. Either RSQQE₆₅FF operates as a transport determinant or it is required for correct folding of ERGIC-53. We consider the latter possibility unlikely because all the constructs we have included in our analysis form disulfide-linked dimers and hexamers with wild-type kinetics. Correct oligomerization is generally considered to reflect correct folding (77).

Experiments in permanently transfected Lec-1 cells revealed that the two C-terminal phenylalanines are strictly required for ER exit. This strongly suggests that the anterograde targeting determinant in ERGIC-53 mediates exit from the ER. We conclude that the cytosolic sequence RSQQΕ₆₅AAFF is part of an ER-exit determinant which is required for the unique dynamic targeting of ERGIC-53 to the intermediate compartment and cis-Golgi. Consistent with this, our in vitro binding experiments revealed significant binding of the COP II coat component Sec23p to cytosolic tail of ERGIC-53 and the cytosolic sequence RSQQΕ₆₅AAFF. In yeast and higher eukaryotes Sec23p forms a stable complex with Sec24p under buffer conditions that can be compared with those we have used in our binding assays (10, 78). No similar binding was observed for Sec13p or α- and γ-adaptin, components of the plasma membrane and trans-Golgi network-adaptor complexes, respectively, suggesting that the cytosolic tail of ERGIC-53 has a specific affinity for Sec23pSec24p subcomplex and does not just bind any kind of cytosolic coat. Interaction with Sec23p was dependent on the two C-terminal phenylalanines that are part of the ER-exit determinant identified by in vitro experiments.

We confirm that the cytosolic tail of ERGIC-53 interacts with COP1 (79). Interestingly, a peptide with a KKAAX dixinsignal showed a much stronger COP1 binding than the wild-type peptide bearing the dixinsignal KKFF. The relatively weak COP1 binding of the cytosolic tail of ERGIC-53 may explain why ERGIC-53 is not completely recycled from the intermediate compartment but can partly escape to and recycle via the cis-Golgi. To exclude the possibility that the lack of Sec23p binding to the KKAAX peptide (i.e. peptide 2) was due to competition by strong COP1 interaction, binding assays were also performed with COP1-depleted cell lysate. COP1 depletion did not modify the efficiency of Sec23p binding arguing against such a competition.

A recent study with cytosolic tail sequences of members of the p24 family of putative cargo receptors suggested a bimodal COP1 interaction. Dixinsignal retrieval signals were reported to bind to the B subcomplex and phenylalanine-dependent forward signals to the F subcomplex (25). At variance with these results, we found no binding of ERGIC-53 to the F subcomplex of COP1 under high salt conditions which are necessary to reveal the bimodal COP1 binding. In these experiments the cytosolic tail of ERGIC-53 bound to the B subcomplex only and hence behaves like a classical dixinsignal.

An antibody directed against the cytosolic tail of ERGIC-53 was recently reported to block the recycling of p58, the rodent homologue of ERGIC-53, in the ERGIC of semi-intact cells (79). The dominant epitope recognized by the antibody includes the C-terminal phenylalanines with weaker contribution of the adjacent lysine residues. The antibody had no effect on ER-exit of p58 which is at variance with our finding that ER-exit is dependent on the two C-terminal phenylalanines. Perhaps the cytosolic tail of ERGIC-53 is masked at the level of the ER and only accessible to the antibody when the protein arrives in the ERGIC (37).

We believe that direct interaction of Sec23p with the cytoplasmic domain collects ERGIC-53 into COP1 vesicles budding from the ER. This notion is in line with the current model of how COP1 vesicles bud from the ER (3); ER-bound Sar1p-GTP first recruits the Sec23pSec24p complex. The binary complex of Sar1p-GTP and Sec23pSec24p can freely diffuse in the plane of the ER membrane, sampling potential cargo proteins by collisional encounter. Favorable interaction with a cargo protein may somehow mark the Sec23p complex for transport. Sec23p-activated cargo may then be clustered and thereby concentrated by multivalent interaction with the Sec13p complex. Accumulation of such coat patches could deform the membrane, creating a bud and ultimately a transport vesicle. ERGIC-53 is perhaps the first cargo protein for which such a sorting mechanism into COP1 vesicles might be suggested. In vitro the cytosolic tail of ERGIC-53 interacts specifically with Sec23p in a diphenylalanine-dependent manner, but the cytosolic tail of ERGIC-53 is probably not sufficient to mediate ER-exit in vivo. CD4 chimeras with the cytosolic tail of ERGIC-53 or the cytosolic tail and transmembrane domain of ERGIC-53 are not targeted to post-ER compartments to the same extent as wild-type ERGIC-53 (45). Similar chimeras with the dimeric reporter protein CD8 and the trimeric reporter VSV-G protein gave identical results, indicating that if Sec23p interacts with the cytosolic tail of these ERGIC-53 chimeric proteins this may not be sufficient to mark the Sec23p complex for transport. It is conceivable that the cytosolic tail of ERGIC-53 must be presented in an optimal way, for instance as a hexamer, to successfully induce Sec23p-mediated clustering.

In conclusion, we show that the intracellular lectin ERGIC-53 has a cytosolic ER-exit determinant that interacts with the Sec23p complex of COP1. These findings support the notion that anterograde transport from the ER is selective and involves active protein recruitment into budding transport vesicles in a way similar to receptor-mediated endocytosis or COP1-dependent retrieval. In addition the presence of an ER-exit determinant in ERGIC-53 provides further evidence that ERGIC-53 may function as a sorting receptor for glycoproteins in the early secretory pathway. Cycling of ERGIC-53 in the early secretory pathway is controlled by a complex interplay of at least three targeting determinants mediating ER-retention, ER-exit, and retrieval from post-ER compartments and of two types of vesicular coats.

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